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D.Phil Thesis

Selective Release from Carbon Nanocapsules



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Abstract

The optimisation of drug delivery systems is challenging. The approach to drug delivery described here makes use of carbon nanocapsules, including fullerenes and nanotubes. Installation of a targeting moiety to carbon nanocapsules can be achieved *via* sidewall functionalisation. Carbon nanocapsules also have an unique capacity that can be used for the delivery of payloads such as drugs or imaging agents. In addition, selective release following the application of external stimuli can be used to advance the selectivity of the carbon nanocapsule delivery system.

Herein, a photoirradiation reaction triggered reaction that releases a benzoic acid derivative from the carbon surface of fullerenes is described. Fulleropyrazolines were modified with an electron donating aryl group on a nitrogen of the pyrazoline ring. This modification renders the surface functionalisation reversible; the pyrazoline undergoes retro-cycloaddition in the presence of light. Investigations revealed that the method can be used in heterogeneous aqueous solutions.

Cathepsin B is a protease that is overexpressed in inflamed cells, such as tumours and arthritis. The enzyme selectively cleaves the amino acid sequence phenylalanine-valine-arginine. The coupling of an enzymatically cleavable, designed peptide built around this amino acid sequence to a fulleropyrrolidine was realised. Covalent attachment of this fullerene ‘cork’ to the carbon nanotube was investigated and analysed by HRTEM/EDX, XPS, XRF, TGA, Raman, and IR spectroscopy. Carbon nanotubes filled with iodide were able to be corked by these modified fullerenes; preliminary studies towards Cathepsin B mediated cleavage were promising.

In conclusion, advances in using the unique capacity of carbon nanocapsules for drug delivery were made. In the future, the combination of targeted delivery, achieved by sidewall functionalisation with a biomolecule, and the triggered release of payload from corked nanotubes may help to open up a new therapeutic field.

Preface

Drug delivery systems are of increasing interest to enhance the selectivity and targeting of medications. Novel approaches are constantly investigated, in particular the combination of targeting, delivery of more than one drug or imaging agent, and the applicability for both therapy and diagnosis. In 1985 and 1991, respectively, the carbon allotropes fullerene¹ and carbon nanotube² were reported; these have now moved into the focus of research as potential drug delivery systems (DDSs) for diagnosis and therapy. These nanomaterials consist of a unique sp²-carbon system of five- and six-membered rings that form three dimensional ball or tube structures. Their known chemistry has developed rapidly because their emerging properties open up new applications. Exo- as well as endo-hedral modification, such as surface functionalisation and filling of the inner cavity, have led to further possibilities for their application.³

This work focused on the reversibility of functionalisation of both the carbon nanomaterials. The concept is based on the filled and functionalised carbon nanotube system previously studied.⁴ External stimuli such as light or enzyme mediated cleavage were considered to trigger the selective release of small molecules. It was envisaged that the targeted delivery of the payload, which could be a drug or labelling compound, would be achieved.

The general introduction of nanomaterial sciences is followed by the description of the goals of the projects. Detailed background information of the specific subject areas is presented at the beginning of each chapter. The first part of the thesis describes the results of a reversible surface functionalisation of C₆₀ fullerene. Light is used to cleave the fulleropyrazoline to deliver C₆₀ and a benzoic acid derivative, which could potentially be applied in photodynamic therapy. The second project presented herein aims for an enzymatically triggered release of the filling from carbon nanotubes. Here, the synthesis of the specific peptide and its attachment to a C₆₀ fullerene for use as a cork is described. This work is followed in Chapter 3.3 by the discussion and characterisation of the covalent linkage of oxidised carbon nanotubes and the peptidic cork. These constructs show selective end functionalisation. Preliminary studies of filling with payload were undertaken, as well as studies on enzymatically triggered release by cleaving the peptide linker to uncork the carbon nanotubes.

List of Contributors

If not otherwise stated, I undertook the entire work. Nevertheless, the expertise and help of the following people was asked to fulfil a complete study on the functionalisation of carbon nanomaterial.

IR spectroscopy	conducted by Dr. Robert Jacobs, Surface Analysis Facility, Department of Chemistry, University of Oxford.
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TGA	conducted by Magdalena Kierkowicz, Institut de Ciència de Materials de Barcelona.
XPS spectroscopy	conducted by Dr. Ashley Shepherd, Surface Analysis Facility, Department of Chemistry, University of Oxford.
Compounds and laboratory assistance	were contributed by Dr. Sander van Berkel, Dr. Regis Saliba, Dr. Thomas Parsons and Dr. Maria Vallée, Benjamin G. Davis group, Department of Chemistry, University of Oxford and Magdalena Kierkowicz, Markus Martincic, Dr. Laura Cabana, and Dr. Stefania Sandoval, Institut de Ciència de Materials de Barcelona.

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I. Introduction

1.1 Drug Delivery

The efficiency of pharmaceutical medication is based both on the drug and its delivery. Although researchers have often improved the potency of small molecules, the ability to deliver most chemotherapeutics towards the specific site is a major limitation of current medicinal chemistry. Despite an optimised mechanism of action, side reactions during delivery can reduce the effective dose of the drug needed and, concurrently, enhance toxicity. An improved treatment strategy, on that bypasses these side effects, is proposed in the form of novel drug delivery systems. A drug delivery system (DDS) is defined as a formulation or device that combines the effect of a therapeutic drug with a controlled release rate, time, and distribution.⁵⁻⁷ These advantages can make drug delivery systems (DDSs) safe and reproducible in efficacy.^{8,9} Specific targeting of the carrier and release of the cargo drug at a required dosage over a necessary time span can reduce side effects *e.g.* the inherent toxicity of anticancer agents to healthy cells.^{10,11}

To be advantageous, DDSs are required to be multifunctional, biocompatible, and controlled in variables, including size, shape, and functionalisation. Recent advances have resulted in the development of multiple DDSs, including liposomes, micelles, dendrimers, virus vectors, cyclodextrin, nanoparticles, and carbon nanotubes (CNTs). Several DDSs have been established and are currently on the market for therapy.^{5,12}

The most commonly applied DDSs are liposomes, which are used to deliver hydrophilic as well as hydrophobic compounds. This dual capability can be ascribed to their lipid bilayer structure (Figure 1.1), which also enables the delivery of the drugs into cells by endosomal membrane fusion.^{7,13} Micelle structures, also derived from self-assembled amphiphilic molecules, protect their hydrophobic cargo against hydrolysis and enzymatic degradation during delivery.⁷

Cyclodextrin structures were probably the first DDSs to be investigated.⁹ These cylindrical macromolecules are made of cyclic oligosaccharides that can trap hydrophobic guest molecules in their inner cavity, while the outside is relatively hydrophilic in enabling solubility under biological conditions. Recent advances resulted in new materials to overcome the problems of multifunctionality, for example, with nanoparticles, which are polymer-, lipid- or ceramic-based particles, enclosing the drug, imaging agent, or targeting moiety such as metal particles.^{7,8,14,15} These new DDSs have the advantage that the drug and targeting molecules can either be entrapped inside the particle, physically adsorbed, or covalently attached to the particle surface.

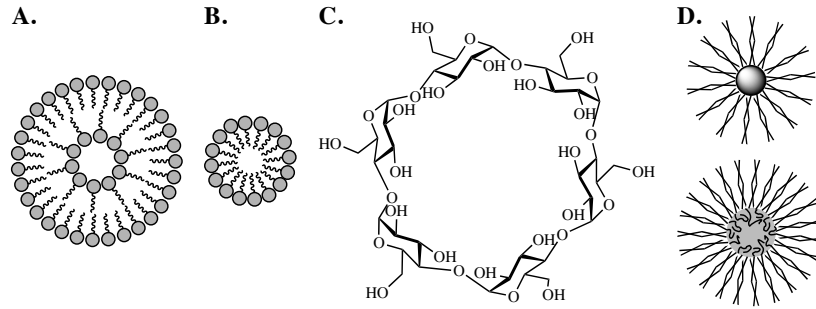


Figure 1.1: Schematic structures of widely applied drug delivery systems (size relation changed for better illustration). **A.** The lipid bilayer structure of liposomes reveals a hydrophilic cavity and outer surface for good aqueous solubility and a lipophilic transmembrane space capable of binding hydrophobic drugs, as well as enabling facile fusion and release into the endosomes; **B.** Micelles can transport hardly soluble, hydrophobic drug molecules in their cores while being soluble under biological conditions; **C.** The shown α -cyclodextrin is composed of six glucopyranose molecules that form the cyclic structure for the DDS; **D.** The nanoparticles are coated with polymer to induce aqueous solubility of their inner core, which could contain a metallic particle or drugs.

Carbon nanotubes are currently the focus of investigations as potential drug delivery systems.^{11,16–20} These systems are proposed to be advantageous in part due to their potential of being selectively modified by many different methods. The inner cavity, which is larger compared to all the above mentioned systems, can be filled and used for the delivery of small molecule drugs, metal complexes, or nanoparticles, as well as radionuclides when the nanocapsules are closed.^{4,6,21,22} Further, drug macromolecules, labelling compounds, and targeting agents can be covalently or non-covalently attached to the outside surface of the carbon nanotube for delivery and increased biocompatibility. Covalent functionalisation might be advantageous for controlled delivery; however, the release of these surface attached compounds has not yet been reported. Their high aspect ratio and surface area makes CNTs as unique as DDSs. Although CNTs have the potential to overcome constraints such as sparse drug solubility, side reaction inhibition, controlled release, and targeted distribution, they are not yet accepted for use as medication, likely due to their potential inherent toxicity. Studies on CNTs have indicated limitations due to inflammatory responses and biodegradation.^{23–26}

1.2 Carbon Nanotubes

Carbon nanotubes can be pictured as long, extremely thin, and hollow fibres resembling convoluted layers of graphene (Figure 1.2). Based on their properties and dimensions, single-walled carbon nanotubes (SWNTs) ($\varnothing = 0.4\text{--}2\text{ nm}$, length $20\text{--}1000\text{ nm}$) and multiple tubes stacked together as so-called multi-walled carbon nanotubes (MWNTs) ($\varnothing = 1.4\text{--}100\text{ nm}$, length $\geq 1\text{ }\mu\text{m}$), can be distinguished.²⁷ Tube chirality and diameter is defined by the roll-up vector $C_h = n \cdot a_1 + m \cdot a_2$, a linear combination of the integers (n,m), and the basis vectors a_1 and a_2 .²⁸ The conjugation and geometry, set by the vector C_h ²⁹ of these carbon constructs determines their electronic

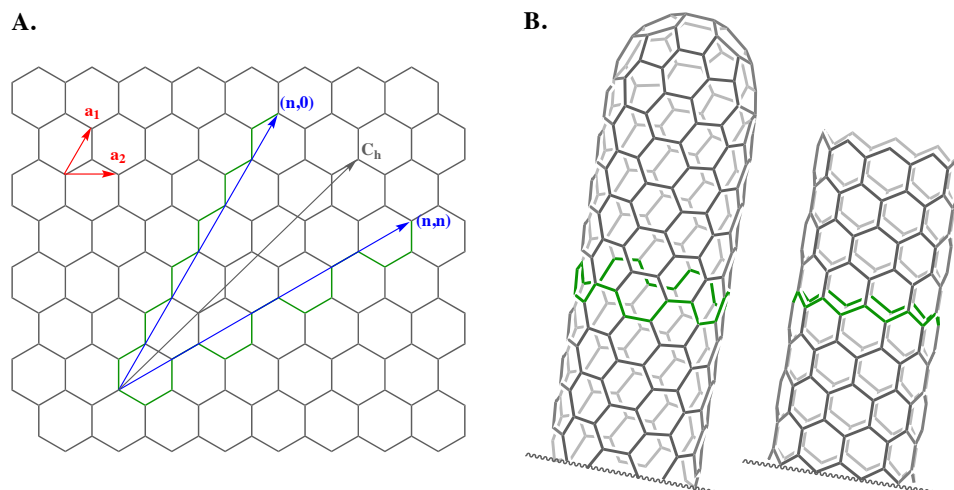


Figure 1.2: Schematic pictures of graphene and carbon nanotubes. **A.** The sp^2 network of a graphene sheet with the lattice vectors a_1 and a_2 and the roll-up vector $C_h = n \cdot a_1 + m \cdot a_2$. Special cases, highlighted in green, are the zigzag $(n,0)$ and armchair vector (n,n) , as depicted in blue. **B.** The structure of a closed CNT with armchair symmetry and an open-ended CNT with a zigzag conformation.

properties, such as conductivity,³⁰ mechanical strength and elasticity,^{31,32} optical properties, and heat conduction efficiency.^{33–36} The diameter and curvature of the tube correlates with the reactivity towards surface functionalisation. The highly strained system becomes more reactive with smaller tube diameters because the s-character of the sp^2 -hybridised carbon atoms increases due to a p_z -orbital expansion.³⁷ With alkene-like reactivity, the potential exists for the chemical functionalisation of the surface for further enhancement of their properties or to use them as biologically relevant application tools.^{38–43}

Carbon nanotubes are manufactured by two main methods: arc-discharge, where under an electrically excited arc, carbon evaporation from two graphite rods is initiated and filamentous carbon wires start condensing; and the second is chemical vapour deposition (CVD), where under high temperatures, a carbon containing volatile precursor, *e.g.* methanol, reacts with the aid of, in most cases, a metallic catalyst to give the hollow fibres. CVD includes thermochemical CVD, high pressure CO disproportionation (HiPCO), and alcohol catalytic CVD (ACCVD).^{3,20}

The potential toxicity of CNTs has affected their use. The asbestos-like pristine fibres have been shown to accumulate in the lungs,^{44,45} depending on their aggregation state⁴⁶ and length^{47,48} and, as a consequence, are carcinogenic. The generation of reactive oxygen species that enhance oxidative stress^{49,50} and pathogenicity^{23–26} in cells has been traced to the presence of small particles of catalyst and is not caused by CNTs.⁵¹ Pristine CNTs are insoluble in most solvents, leading to a higher risk of toxicity. Functionalisation of CNTs can improve dispersibility^{40,52,53} and, therefore, decrease toxicity.^{27,51,54} The carbon material is likely to be transferred into cells due to its hydrophobicity and attractive interaction with lipid membranes. Incorporation into cells may occur *via* endocytosis or penetration, depending on the CNTs surface functionalisation as well as the length and diameter of the tube.^{55–59} The literature indicates that the toxicity of

CNTs can not easily be predicted; therefore, every newly synthesised species should be tested accordingly.

1.3 C₆₀ Fullerenes

A carbon allotrope related to CNTs is fullerene. ‘Fullerene’ is a general term for an entire class of buckyball-shaped molecules with sizes ranging from C₂₀ to greater than 100 carbons. The best-explored species is C₆₀. This highly symmetric, icosahedral molecule consists of twelve pentagons and 20 hexagons to form a unique round shape, $\varnothing = 0.71$ nm.⁶⁰ The double bonds at the shorter 6,6-bond are more reactive compared to the ones at the 5,6-junction^{61,62} because the bonds in C₆₀ are localised, rendering a system with low aromaticity.⁶³ The driving force for chemical

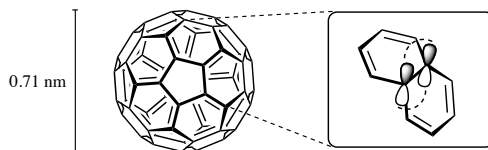


Figure 1.3: C₆₀ consists of 60 sp²-hybridised carbon atoms leading to a football shaped geometry set of hexagon and pentagon structures. Highlighted is the 6,6-bond, which is the most reactive double bond in the system.

functionalisation of C₆₀ is the strain relief of the spherical carbon network.⁶⁴ Functionalisation can increase the sparse solubility in common solvents.⁶⁵ Various means of modifying the alkene system have been reported,⁶¹ *e.g.* introduction of charged groups renders C₆₀ water soluble.^{66–71} Due to the structural similarity of CNTs and C₆₀,⁷² and their similar diameters, the inclusion of fullerenes into nanotubes facilitated by π - π -stacking, hydrophobic, and van der Waals interactions have been reported.^{60,73–80} C₆₀ filled CNTs, so-called peapods, have been made in solution,⁸¹ supercritical fluids,⁸² and the gas phase.^{83–85} Peapod structures show great potential for the synthesis of double-walled carbon nanotubes (DWNTs)⁸⁶ as electronic or optical devices^{73,87} or delivery systems.^{70,88}

1.4 Release from Carbon Nanomaterials

An important specification of drug delivery systems is the reversible release of cargo from the nanomolecular carriers. The manipulation of CNTs can be undertaken chemically with covalent functionalisation of the sidewall^{40,42,52,89} as well as the open ends,^{90,91} non-covalent adsorption onto the surface,^{92,93} including the wrapping of macromolecules around the tubes,⁹⁴ or endohedral filling of the inner cavity.^{87,95–98} A variety of modifications have been shown to be reversible by retro-functionalisation,⁹⁹ heat,^{100–102} solubilisation,^{92,103} or diffusion.¹⁰⁴ Of major interest for this thesis is the filling and subsequent removal of C₆₀ from peapod structures by diffusion or chemical inversion of the attractive interactions.^{70,78,88,105,106}

To date, the reversible functionalisation of C_{60} has been demonstrated in a limited number of examples. Removal of various surface modifications has been investigated using a copper catalyst,^{107,108} as well as electrochemical,¹⁰⁹ thermal,^{110–113} chemical,¹¹⁴ or light-triggered release.^{115–118}

Applied Carbon Nanotubes *in vitro* and *in vivo*

CNTs have been in the focus of investigations as DDSs.^{18,19,119} Therefore, knowledge of their biocompatibility is of major importance. CNT functionalisation renders this unique material highly versatile. Modification of the surface can decrease the toxicity of CNTs by increasing their dispersibility.^{27,120–123} An increased dispersibility implies the potential use of these systems *in vivo*.^{121,123–125} The delivery of functionalised CNTs into the lysosome for drug delivery has been shown.^{59,126}

Functionalisation of the CNT surface with drug molecules has been explored as a DDS.^{17,18} Further, the installation of a targeting molecule provides the possibility of local selectivity of *in vivo* applications.¹⁸ Targeting moieties such as RGD,¹²⁷ folate,¹²⁸ *N*-acetyl-D-glucosamine,⁴ and antibodies^{22,124} have been used. Due to their enormous length, CNTs possess the advantage of multifunctionalisation, such as dual sidewall functionalisation^{11,18,90,129,130} or sidewall modification of filled CNTs.^{4,22,131,132} These dual functionalised CNTs have been explored for the targeted release of drugs from the inner cavity⁹⁸ or the outer surface by covalent or non-covalent attachment.^{11,17,18,133,134} Triggers, which are used to induce the release, are a pH decrease for protonation or hydrolysis and disulfide reduction.

Reversible Functionalisation of Carbon Nanocapsules

Filled and functionalised CNTs can be imagined as combined therapeutic and diagnostic-targeted delivery tools, with the payload inside and a directing biomolecule on the outside. The goal of this work was to develop a reversible covalent surface functionalisation of carbon nanocapsules, which refers to fullerenes and closed CNTs that then could be used as a potential delivery system *in vivo*. It was envisioned that a triggered release of an attached or encapsulated label could be used for diagnosis, whereas a drug^{104,105,135,136} could be utilised in therapy. The focus was on the use of an external trigger, such as light or an enzyme, to selectively initiate the release mechanism.

It was proposed that the introduction of a cleavage site selectively would enable the removal of a payload from the outer surface or inner cavity. This can be realised with a reversible surface functionalisation or re-opening of the capsules. The released moiety could be a targeting agent, label, or drug. An advantage of the reversible CNT sidewall functionalisation is the potential to change its *in vivo* behaviour, such as the cell internalisation mechanism.^{56,57,137}

To conclude the aims of the project, the modification of carbon nanocapsules for the purpose to use them as DDSs are listed below:

- Cleavable surface modification on carbon nanocapsules

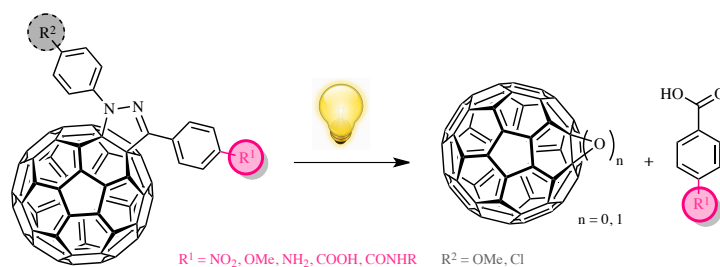
- Release of small molecules, such as labels or drugs for application
- Selectively triggered cleavage under light or in the presence of an enzyme
- Reaction compatible with biological conditions
- Take advantage of C₆₀ surface reactivity and CNT filling capacity.

In the following, the projects of a photoreversible fullerene surface functionalisation and an enzymatically triggered payload release of peptide linked C₆₀-corked carbon nanotubes are described.

1.4.1 Light Triggered Release from C₆₀ Fullerenes

Light has been considered to be a non-interfering source of externally controlled manipulation.¹³⁸ The major drawback for *in vivo* studies is the limited penetration depth of light due to interaction with biological tissue.^{139,140} This problem can be circumvented when near infrared (IR) light is used or when a two-photon process is initiated where excitation occurs by absorption of two photons of double wavelength, a method that interferes less with the biological matter.^{138,141–143} Modified fullerenes have been applied in photosensitive materials and their behaviour investigated.^{72,144–151} These materials demonstrate excellent responses to irradiation, most notably due to the electron accepting abilities of C₆₀,^{89,152} interference with biological matter¹⁵³ or generation of singlet oxygen,¹⁵⁴ and reactive oxygen species (ROS).^{68,71,155} Based on these studies, the goal herein was the photochemically triggered release of C₆₀ fullerenes for their delivery or the liberation of small organic molecules from the carbon surface of C₆₀ or CNTs (Scheme 1.1). Ideally, the system would be applied in cancer cell treatment in which, under light irradiation, the carbon species could further interact with the tissue in a destructive manner.^{120,156} Further, it was envisaged that the liberation of a drug from these carbon surfaces in cells would be investigated.

Scheme 1.1



Reproduced from Rutte *et al.*¹⁵⁷ with permission from the Royal Society of Chemistry.

A bifunctional modification on the C₆₀ was envisaged with the aim of combining the late stage introduction of varying drugs or linkers with the ability to tune the photochemical release. Inspired by light-induced fullerene modifications,^{115–118} [2+2] and [2+3] cycloadditions and their reversibility were approached. A system that assimilated the tunability of the photocleavage reaction and a second handle for functionalisation was found in modified fulleropyrazolines.

1.4.2 Enzymatic Uncorking of Carbon Nanotubes

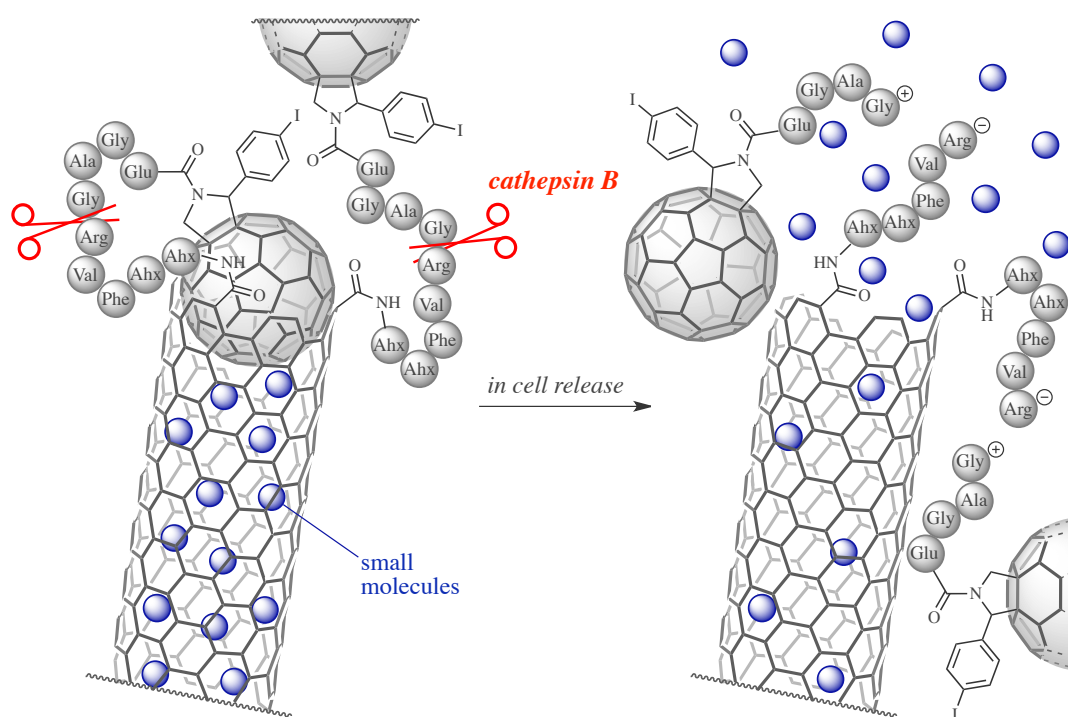
The second project described in this thesis was based on two projects previously undertaken in the B. G. Davis group.

The filling, encapsulating, and functionalisation of CNTs has been studied by Hong.⁴ The conjugation of glycosaccharides (*N*-acetyl-D-glucosamine) on the CNT surface was used to specifically target lung tissue, shown by positron emission tomography (PET) using the Na¹²⁵I encapsulation. These carbon nanocapsules showed no leakage of the radioactive payload.

Further, Cathepsin B, a protease in the lysosome,¹⁵⁸ is upregulated in inflamed cells, such as cancer cells.^{159–162} Cathepsin B recognises a specific sequence to hydrolyse the amide bond of the peptide motif Phe-Val-Arg, cleaving after the arginine residue.^{163–165}

As a result from the above mentioned research, the idea of a reversible filling with a stimulated release mechanism after delivery to distinctive cells evolved. The reversible corking of CNTs with C₆₀ and internalised payload release initiated by an external control was envisaged.⁷⁰ A peptide linker containing the specific amino acid sequence, which can be cleaved in the lysosome, is introduced to link a C₆₀ cork and the CNT. The introduction of additional charges on the fullerene moiety after cleavage should increase the repulsive interaction, as reported,⁷⁰ and cause diffusion from the CNT to free the payload. The aim of the second project worked on in this thesis was the synthesis of the construct depicted in Scheme 1.2.

Scheme 1.2



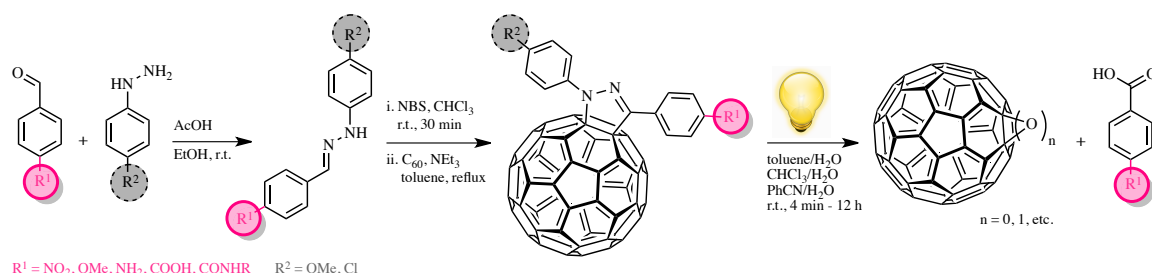
Retrosynthetic analysis of the envisioned drug delivery system revealed a route involving oxidised CNTs, an enzyme cleavable peptide linker, an amine modified C₆₀ fullerene, and a cargo. The

quality and quantity of the functionalisation of the oxidised CNTs was investigated. Further, synthesis of the designed peptides of different lengths and sequences was carried out. Functionalisation of C_{60} *via* the Prato reaction⁸⁹ was undertaken. The linkage, through peptide couplings, of both carbon materials to give the corked hybrid structure was explored.

II. Photoreversible C₆₀ Fullerene Modification

Investigations towards the modulation of C₆₀ fullerene surface functionalisation is described in this chapter. Light as an external trigger is used to induce a novel ‘cycloreversible’ reaction to leave C₆₀ and a benzoic acid derivative. This method of release may find potential applications in a drug delivery system or photodynamic therapy.

Scheme 2.1



Synthesis with facile modification and light triggered cycloreversion on C₆₀ fullerene.¹⁵⁷

2.1 Background

Switchable functionalisation of carbon surfaces, such as carbon nanotubes (CNTs) and C₆₀ fullerenes, is useful to change their inherent properties, such as solubility, conductivity, luminescence, aggregation, and reactivity. Herein, a strategy using photocleavable organic modifications to C₆₀ fullerene (C₆₀) is described. Light irradiation was used due to the possibility of independence with respect to specific cellular conditions.

2.1.1 Photochemistry of C₆₀

Photochemic reactions occur in the presence of photons interacting with the absorbing material. The excited molecules can exchange electrons, energy (two electrons, back and forth), be quenched, isomerise, dissociate, or emit light *via* luminescence or phosphorescence.¹⁶⁶ The concentration,^{166,167} as well as the presence of halogen atoms^{167–169} or amines,¹⁷⁰ can influence

photoreactions due to their energy and electron transfer abilities. The efficiency of photochemical reactions is given by the quantum yield, representing the number of reacted molecules per absorbed photon of induced light.¹⁷¹

Analysis of the excited states of C₆₀ has revealed the energy levels and lifetimes that are shown in Figure 2.1.^{172–174} The excited singlet state ¹C₆₀* (*S*₁) decays relatively fast into the highly reactive triplet state ³C₆₀* (*T*₁) with 100% conversion. Due to the long lifetime, ³C₆₀* will be rapidly quenched by triplet oxygen ($k_q(O_2) = 2 \cdot 10^9 \text{ M}^{-1}\text{s}^{-1}$).^{172,174} Therefore, photochemical modifications of fullerenes should be undertaken in oxygen-free conditions. On the other hand, ³C₆₀* is an excellent singlet oxygen generator and could be used as such when a release of C₆₀ from targeting moieties by photoirradiation is desired. The change of reduction potential in molecules

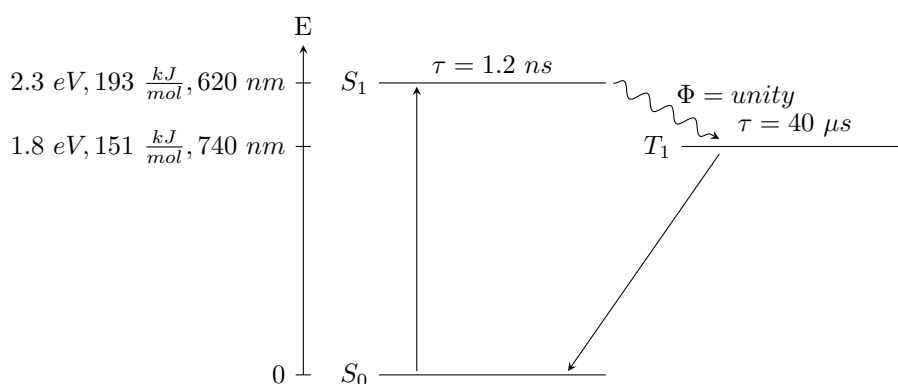


Figure 2.1: Simplified Jablonski-diagram of C₆₀ with the important energies, lifetimes τ and quantum yield Φ for the intersystem crossing.

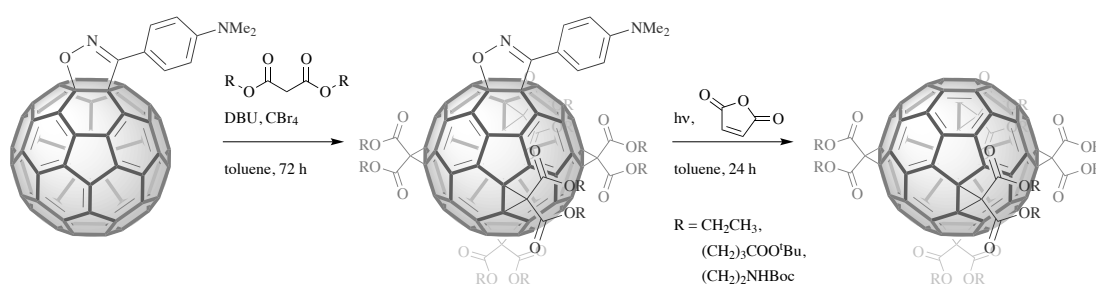
gives insights into their photochemical behaviour and reactivity, which is based on their highest occupied molecular orbital (HOMO) and lowest occupied molecular orbital (LUMO) energies. Generally, the functionalisation of C₆₀ raises the LUMO level due to saturation of a double bond, which decreases the electron acceptor ability of the C₆₀.

2.1.2 Cycloaddition and Cycloreversion on C₆₀

C₆₀ undergoes cycloaddition functionalisation *via* reaction of its electron-deficient 6,6-bonds.^{175–178} Based on this reactivity, many cycloadditions, a class of pericyclic reactions,¹⁷⁹ have been reported as a surface functionalisation of C₆₀.^{61,89,112,114,180} Commonly known cycloreversible reactions of alkenes include the Diels–Alder reaction^{181–184} and the 1,3-dipolar cycloadditions.¹⁸⁵ The thermal cycloreversion from C₆₀ fullerene has been shown with cyclopentadiene¹⁸⁶ and derivatised furans.^{187,188} Fulleropyrrolidine retrocycloaddition occur under thermal conditions,^{99,189} upon microwave irradiation in ionic liquids,¹⁹⁰ and under Cu^{II} catalysis.¹⁹¹ Isoxazoles on fullerenes are cleaved thermally¹⁹² and in the presence of a Mo catalyst.¹⁹³ Thermal cycloreversion of isomünchnone modified C₆₀ has been reported to further occur under acidic conditions.¹¹¹ Furthermore, C₆₀ is known to undergo photochemical [2+2] cycloaddition reactions with themselves^{144,194–197} or various alkenes.^{116–118} The introduction of light also impells the cleavage of

the attached molecules.¹¹² The lack of control in the reaction progression is disadvantageous. Either the cycloaddition reversion is concurrent to the formation of the cycloadduct with the incident wavelength, hence the product equilibrium could not be controlled, or the mechanism of the retrocycloaddition differs from the forward reaction and leads to new, oxidised products. The reported cycloreversibility of electron deficient alkenes¹⁹⁸ has led to the recently published photocleavable cycloreversion on fullerene systems by Hörmann and co-workers (Scheme 2.2).¹¹⁵ An electron-rich substituent on the isoxazoline modification induces cleavage that is based on an electron transfer, which is followed by recombination to conclude the homolytic separation.

Scheme 2.2



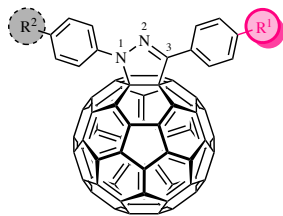
The fullereneisoxazoline, developed by Hörmann and co-workers, undergoes cycloreversion under irradiation of light.¹¹⁵

2.1.3 Design of a Cycloreversible System

The design of a reversible reaction on C_{60} is challenging because of the many functions to be considered. Cycloaddition with an electron rich, preferentially π -donor diene is a preferred reaction of C_{60} . Such a reaction could be promoted by lowering the free energy, reducing the strain in the system, forming an aromatic moiety, or a combination of these. Functions promoting the modification reaction need to be considered carefully because it must be reversed. The cleavage reaction of a cycloadduct is favoured by electron rich substituents on the small molecule adduct, *e.g.* amines or cyclopropanes that stabilise the transition state for the reversed reaction by donating electron density.

Further, dual modification of C_{60} can be envisaged. It is desirable to combine the late-stage introduction of various drugs or linkers with the ability to tune photochemical release.

Inspired by previously reported light-induced fullerene modifications,^{115–118} many [2+2] cycloadditions and their reversibility were investigated. A system that assimilated all requirements was found in modified fulleropyrazolines (Scheme 1.1, Figure 2.2). The tunability of the photochemical cleavage reaction can be achieved by modifying the LUMO energy level *i.e.* the reduction potential. The introduction of a functional group on the fulleropyrazoline consisting of either an electron-donating or an electron-withdrawing group can influence the cycloreversion.¹⁵¹ Electron-rich substituents influenced the least the excellent electron affinity of C_{60} due to a low lying LUMO level.¹⁹⁹ In particular, the pyrazoline moiety itself was found to mediate the charge separation.²⁰⁰ Modifications could be made at the *N*-1^{201–204} or *C*-3^{149,151,200,205} position of the pyrazoline, affecting their charge and energy transfer tendency, respectively.

Figure 2.2: Fulleropyrazoline with modifications on *N*-1 and *C*-3.

To summarise the requirements on a reversible cycloaddition of C₆₀, that were aimed to achieve:

- Light triggered cycloreversion on C₆₀
- Simple functionalisation with the possibility to easily change modifications
- Facile modulation for tunability
- Modification needs handle for installation of drug or targeting moiety
- Biocompatible reaction, reversibility of reaction under aqueous conditions, and without necessary additives.

2.2 Results and Discussion

[2+2] Cycloaddition reactions on C₆₀ with electron-rich reactants, such as styrol^{116,117} and tetraethoxyalkene¹¹² as well as electron-poor alkenes, such as cycloenones,^{175,206} have previously been reported. The formation of cyclobutanes is considered to be reversible under photoirradiation, as predicted by the Woodward–Hoffmann rules.²⁰⁷

The first approaches for a [2+2] cycloaddition to C₆₀ with the electron-poor, conjugated systems of coumarin, maleic anhydride, cyclohexenone, phenylketene, and styrylamine²⁰⁸ were undertaken herein. Various photolytic reaction conditions with varying wavelengths, powers, solvents, compound ratios, concentration and catalyst additions, and thermal conditions did not show any reactivity towards the electron-deficient nature of the C₆₀ surface.^{175,206} Both reaction partners were considered to react as dienophiles,¹¹³ and so the observed absence of reactivity was not surprising.¹⁰⁸

Nevertheless, [3+2] cycloaddition reactions with C₆₀ were tried and successfully reverted by photoirradiation, as described below.

2.2.1 Photocleavable Fulleropyrazolines

Many studies have been made on the electro- and photochemical properties of pyrazoline functionalised C₆₀.^{151,203,209} Mainly due to their electron and energy transfer ability, pyrazolines are widely applied in photovoltaics. Pyrazoline substitution patterns and their influence on photolytic behaviour, such as the tunability of the HOMO and LUMO energies,^{185,209} have been

explored, as well as the thermal stability of pyrazolines.¹⁰⁷ Collectively, this knowledge led to the hypothesis that a simple manipulation of pyrazoline chemistry could yield a photosensitive fullerene adduct.

2.2.1.1 Fulleropyrazoline Synthesis

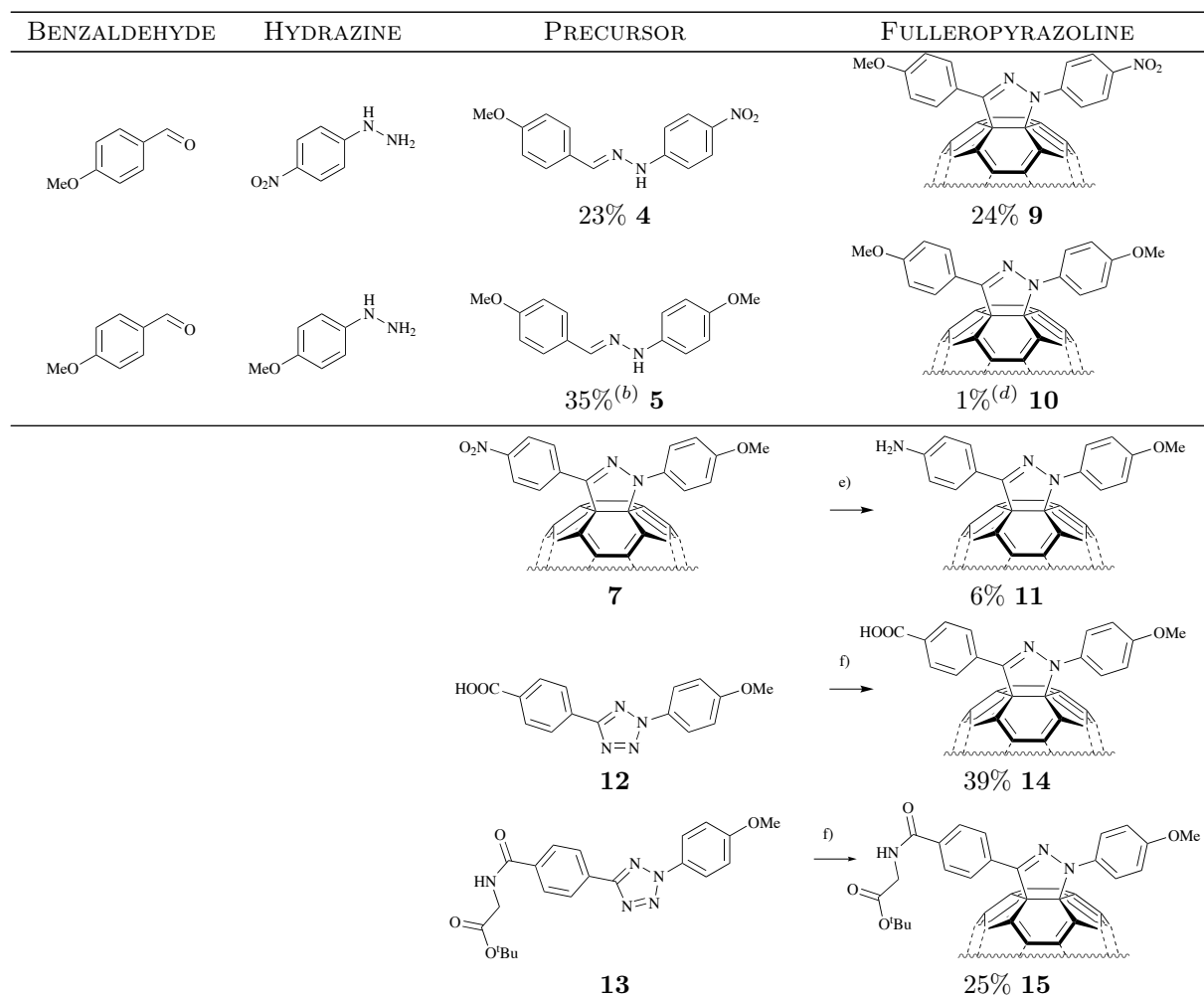
Fulleropyrazolines combine the dual functionality of tunability and further modification. The synthesis of the precursor hydrazone (**1-5**) as well as tetrazole (**12** and **13**) allowed the installation of electron-donating and electron-withdrawing groups (Table 2.1). The 1,3-dipole nitrile imine was generated *in situ* via reaction of *N*-bromosuccinimide (NBS) and triethylamine,^{185,201} or by photoirradiation^{210,211} undergoing addition to the dipolarophilic 6,6-bond of C₆₀.²¹² The range of fulleropyrazolines that may be accessed by the route described was somewhat limited. An attempted synthesis of hydrazones bearing a simple alkyl chain or benzyl moiety was unsuccessful because the product could not be formed or was unstable and decomposed rapidly. For example, heptanal formed an acetal, which could not be condensed to give the required hydrazone. The reaction between benzaldehyde and *para*-methoxyphenylhydrazine gave an unstable hydrazone²¹³ that could not be used in the following fulleropyrazoline synthesis.

Table 2.1: Synthesis of hydrazones and monofunctionalised fulleropyrazolines.

$R^1 = \text{NO}_2, \text{OMe}$
 $R^2 = \text{NO}_2, \text{OMe}, \text{Cl}$

BENZALDEHYDE	HYDRAZINE	PRECURSOR	FULLEROPYRAZOLINE
		 13% 1	 15% 6
		 83% 2	 40% 7
		 92% 3	 51% 8

II. Photoreversible C₆₀ Fullerene Modification



Reagents and conditions: a) aldehyde, hydrazine, AcOH, EtOH, 78 °C;²¹⁴ b) aldehyde, hydrazine, NaOH, silica, r.t., 40 min;²¹⁵ c) hydrazone, NBS, CHCl₃, r.t., 30 min; then C₆₀, NEt₃, toluene, microwave;^{212,216} d) hydrazone, (diacetoxy-iodo)benzene, C₆₀, toluene, 40 °C, 3.5 h;²⁰⁴ e) Sn, HCl_{aq}, CHCl₃, reflux, 6 h, 6%;^{203,217} f) tetrazole, C₆₀, toluene, medium pressure Hg-lamp, 3.5 h.^{210,211}

The pyrazoline formation from the hydrazones was successful, yielding monoaddition products under microwave irradiation to accelerate the reaction rate.^{199,200,214} The major products formed, as observed by thin layer chromatography (TLC), were the monoaddition products. The assignment was confirmed by means of matrix-assisted laser desorption/ionisation (MALDI) and nuclear magnetic resonance (NMR) (Figure 2.3). Minor quantities of disubstituted products, which were observed by MALDI, were separated by column chromatography. Unwanted side reactions occurred during the synthesis of pyrazolines with electron-donating substituted hydrazones, such as **5**, as monobromination *ortho* to the methoxy group took place. For the synthesis of **10**, the mild halogenating agent (diacetoxyiodo)benzene²⁰⁴ was used to avoid formation of a similar byproduct. Furthermore, reduction of **7** delivered the amino-functionalised, extremely light sensitive fulleropyrazoline **11** in poor yield.

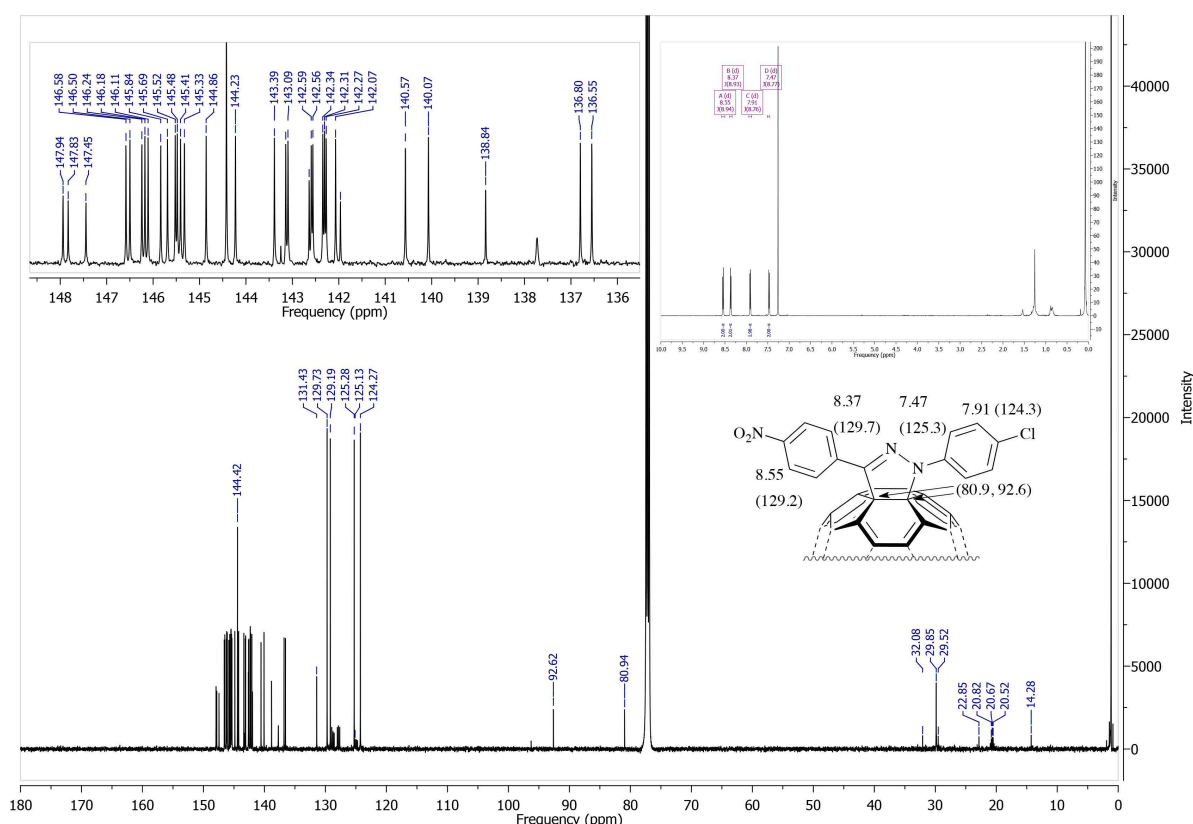


Figure 2.3: ^1H - and ^{13}C -NMR spectra of fulleropyrazoline **8** show the monoaddition product.

A wider substrate applicability was realised by the introduction of a functional handle, such as a carboxylic acid group on the fulleropyrazoline. Compounds **14** and **15** were synthesised *via* the 1,3-dipolar cycloaddition of the light-activated corresponding tetrazole,^{210,211,218,219} which was kindly donated by Dr. Sander van Berkel.

2.2.1.2 Pyrazoline Photocleavage

As shown in Table 2.2, the initial photocleavage experiments were undertaken in a 1 mM solution of degassed toluene in a quartz vessel and irradiated using a water-cooled medium pressure mercury lamp (500 W). An excess of maleic anhydride (30 eq.) was initially included as a trapping agent to prevent the re-addition of the released 1,3-dipole with the free C_{60} .^{107,115} As expected for the literature-known photovoltaic compounds, the irradiation of nitrophenyl-fulleropyrazolines **6** and **9** showed no cleavage.¹⁵¹ The introduction of an electron-donating group at *N*-1, such as the methoxyphenyl derivatised fulleropyrazolines **7**, **10**, **11**, **14**, and **15** enabled photocleavage to give an inseparable mixture of C_{60} (**16**) and oxidised fullerene C_{60}O (**17**). Speculations pointed towards the strong positive mesomeric effect of the methoxy group,²²⁰ which stabilises the development of the ensuing radical cation during the transition state of the 1,3-dipolar cycloreversion (shown in Scheme 2.5). This was confirmed by fulleropyrazoline **8** that bears a,

II. Photoreversible C₆₀ Fullerene Modification

when compared to the methoxy group, less electron-donating *para*-chloro substituent, which was cleaved, but far more slowly than the methoxy analogue. The importance of the substituent at *N*-1 confirmed a key role of this atomic centre, perhaps through the lone pair of the sp³-nitrogen, in charge transfer processes.²⁰⁴ The benzyl moiety on *C*-3 can bear any substitution for the photocleavage because the substitution pattern did not have any effect on the reactivity of light irradiation; all examples had comparable reaction rates. The reaction rate was determined by measuring the time-dependent formation of C₆₀ products in the crude reaction mixtures, which were analysed and compared with high performance liquid chromatography (HPLC).

Table 2.2: Cleavage under light irradiation of fulleropyrazolines with different substitution patterns on *N*-1 and *C*-3.

R ₁	R ₂	FULLEROPYRAZOLINE	RESULT
NO ₂	NO ₂	6	no reaction
NO ₂	OMe	7	cleavage
NO ₂	Cl	8	cleavage
OMe	NO ₂	9	no reaction
OMe	OMe	10	cleavage
NH ₂	OMe	11	cleavage
COOH	OMe	14	cleavage
CONH-Gly-O ^t Bu	OMe	15	cleavage

Reaction conditions: fulleropyrazoline, maleic anhydride (20 eq.), toluene or chloroform [1 mM], $h\nu$ (medium pressure mercury lamp), 4 min–12 h.

As a control, fulleropyrazolines **6**, **7**, and **8** showed great stability under reaction conditions without light or under heating. Reaction progress was monitored by TLC, NMR, MALDI, and HPLC (example in Figure 2.4). This novel photo-induced cleavage reaction was investigated for its dependance on the incident wavelength, influence of present alkene, solvent compatibility, and the product formation. If not stated otherwise, fulleropyrazoline **7** was used for screening the reactions due to its simple synthesis route and possibility of large-scale synthesis.

Reverse-Phase HPLC The reaction progress was monitored by reverse-phase HPLC using a TSKgel ODS 80TM column (150 · 4.5 mm, 5 μ m) and a gradient of hexane in 2-isopropanol 20%→50% over 10 minutes.^{186,221,222} The retention times were as follows: C₆₀ t_R = 9.1 min; C₆₀O monooxidised fullerene (C₆₀O) t_R = 8.7 min; presumed C₆₀O₂ t_R = 8.3 min; presumed C₆₀O₃ t_R = 8.0 min; fulleropyrazolines $t_R \approx 6.0$ min (*e.g.* **7** has a retention time of $t = 6.2$ min). The trapping alkenes present in the reaction mixture, *e.g.* maleic anhydride, and formed cleavage

fragments could not be detected because their retention time was found to be similar to the injection peak ($t_R = 2.0$ min). HPLC chromatograms were extracted at $\lambda = 254$ nm and integration values of the peaks used for the quantification (Figure 2.4).

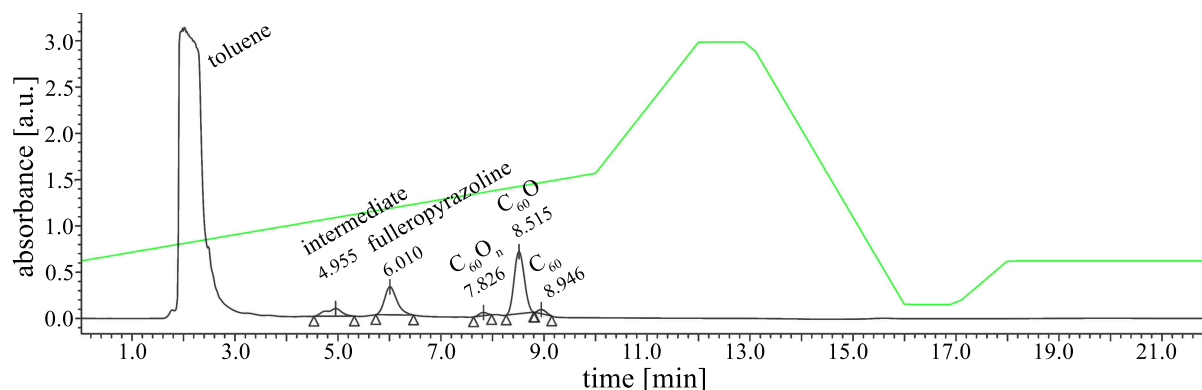


Figure 2.4: This is the HPLC trace of a reaction control of **7** (1 eq.), maleic anhydride (30 eq.), toluene [1 mM], medium pressure Hg-lamp, 20 min. Integration of the peaks was undertaken manually. Retention times: $t_R(\text{toluene}) \sim 2$ min, $t_R(\text{intermediate}) \sim 5.0$ min, $t_R(\mathbf{7}) = 6.0$ min, $t_R(\mathbf{17}) = 8.5$ min, $t_R(\mathbf{16}) = 8.9$ min. Green line – solvent gradient.

Reaction Progress The photocleavage of fulleropyrazoline **7** with maleic anhydride (20 and 5 eq.) and toluene [1 mM] was followed by HPLC (Figure 2.5 and 2.6, respectively). The variations of the product formation observed could not be easily correlated to external influences such as light, temperature, or solvent. The amount of substances were correlated with their integration value of the HPLC UV detectable peaks and, due to the clearness of the graphs, only the major products were listed. The product of fulleropyrazoline, intermediate, C_{60} , and $C_{60}O$ gives a total of 100%.

II. Photoreversible C₆₀ Fullerene Modification

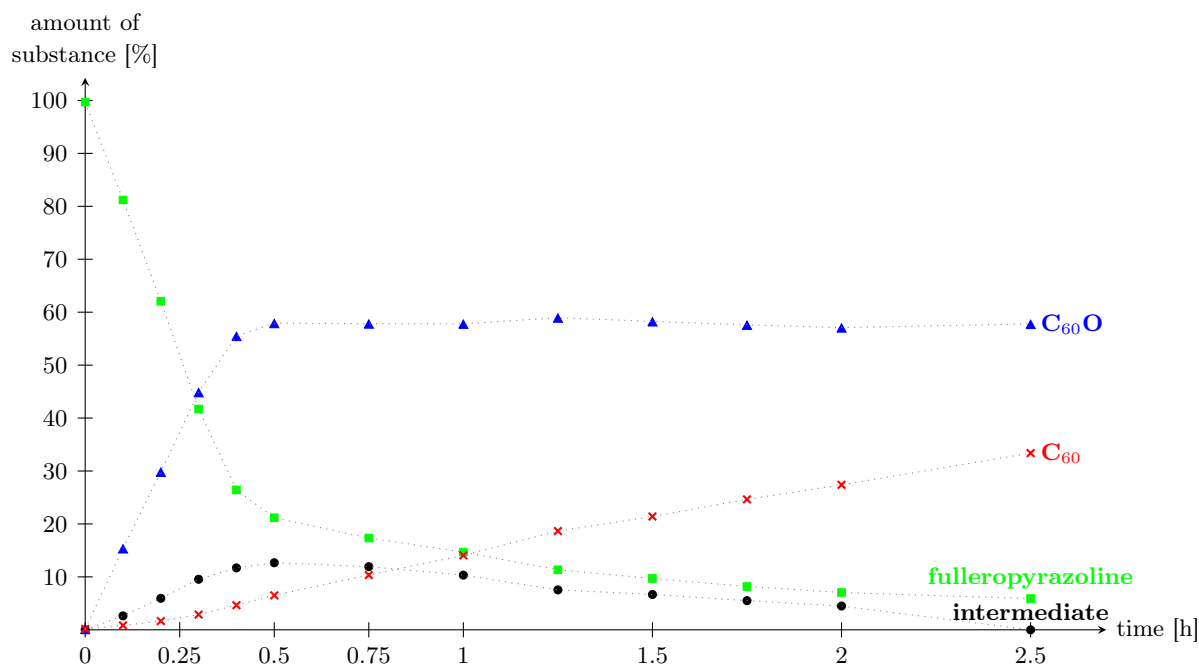


Figure 2.5: Photocleavage of **7** monitored by HPLC. Reagents and conditions: maleic anhydride (20 eq.), toluene [1 mM], med. pressure Hg-lamp, 2.5 h. Black dot: intermediate, green square: fulleropyrazoline **7**; red cross: C₆₀; blue triangle: C₆₀O.

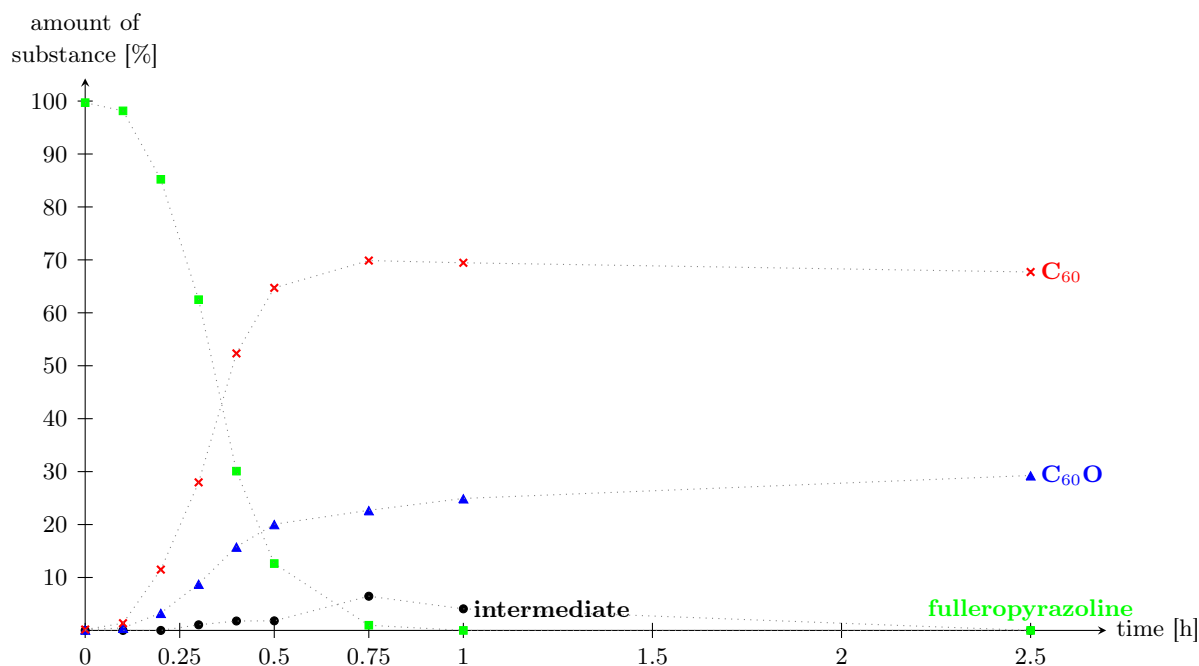


Figure 2.6: Photocleavage of **7** monitored by HPLC. Reagents and conditions: maleic anhydride (5 eq.), toluene [1 mM], med. pressure Hg-lamp, 2.5 h. Black dot: intermediate, green square: fulleropyrazoline **7**; red cross: C₆₀; blue triangle: C₆₀O.

Table 2.3 summarises the results from all photocleavage reactions obtained by HPLC analysis. The overall yields of the reactions were calculated by combining the yields of C_{60} and $C_{60}O$. The proportions of $C_{60}O_n$ with $n \geq 3$ obtained did not show any simple correlation with the reaction conditions. As mentioned, the irradiation of C_{60} can cause the oxidation thereof.²²³

Table 2.3: The photocleavage of fulleropyrazolines (**7** if not stated otherwise) resulted in a mixture of C_{60} and $C_{60}O$. The ratios $C_{60}O/C_{60}$, where C_{60} is normalised to one, were calculated from the substance amount observed in the HPLC chromatograms after 2 h irradiation of the reaction mixture with a medium pressure Hg-lamp (unless otherwise stated).

	ALKENE	EQ.	SOLVENT	REMARKS	$C_{60}O/C_{60}$
1	maleic anhydride	5	toluene	$\lambda = 313nm$ irradiation	15.50
2	maleic anhydride	5	toluene	$\lambda = 350nm$ irradiation	21.00
3	maleic anhydride	5	toluene	$\lambda = 254nm$ irradiation	3.00
4	maleic anhydride	5	toluene	$\lambda = 365nm$ irradiation	31.00
5	maleic anhydride	5	toluene	halogen lamp	24.67
6	coumarin	5	toluene		1.75
7	allylamine	5	toluene		0.00
8	allylbromide	5	toluene		0.00
9	dimethylallylbromide	5	toluene		0.00
10	dimethylallylbromide	5	toluene		0.08
11	dimethylallylbromide	5	$CHCl_3$		0.41
12	allylchloride	5	toluene		1.06
13	chlorostyrene	5	toluene		0.19
14	maleic anhydride	30	toluene	degassed solvent	9.50
15	maleic anhydride	30	$CHCl_3$	degassed solvent	0.98
16	maleic anhydride	30	toluene		9.57
17	maleic anhydride	30	toluene		1.85
18	maleic anhydride	20	toluene		4.19
19	maleic anhydride	10	toluene		0.66
20	maleic anhydride	5	toluene		0.43
21	maleic anhydride	5	toluene		0.00
22	maleic anhydride	1	toluene		0.47
23	maleic anhydride	1	toluene		8.29
24	–		toluene		0.44
25	–		toluene		2.23
26	maleic anhydride	5	toluene	anhydrous solvent	24.67
27	–		toluene	anhydrous solvent	3.00
28	maleic anhydride	5	toluene	1-phenylnaphthalene (10 eq.)	5.18
29	maleic anhydride	5	toluene	1-phenylnaph. (10 eq.), anhyd.	4.92
30	maleic anhydride	5	toluene	2-methylbut-2-ene (10 eq.)	8.13
31	maleic anhydride	5	toluene	DABCO (10 eq.)	–
32	maleic anhydride	5	$CHCl_3$	photocleavage of compound 8	0.43
33	maleic anhydride	5	$CHCl_3$	photocleavage of compound 8	0.69
34	–		$CHCl_3$	photocleavage of compound 8	0.37
35	–		$CHCl_3$	photocleavage of compound 8	0.72

II. Photoreversible C₆₀ Fullerene Modification

	ALKENE	EQ.	SOLVENT	REMARKS	C ₆₀ O/C ₆₀
36	maleic anhydride	5	toluene	photocleavage of compound 8	1.40
37	maleic anhydride	5	toluene	degassed solvent, compound 10	3.14
38	–		CHCl ₃	photocleavage of compound 11	0.50
39	–		CHCl ₃	photocleavage of compound 14	1.44
40	–		CHCl ₃	photocleavage of compound 14	1.38
41	–		CHCl ₃	photocleavage of compound 15	4.54
42	–		PhCN/H ₂ O 1:1	photocleavage of compound 15	2.36
43	–		CHCl ₃		1.56
44	–		CHCl ₃		1.50
45	–		CHCl ₃		1.33
46	maleic anhydride	5	DMSO		0.07
47	–		DMSO		–
48	maleic anhydride	5	DMSO/H ₂ O 1:1		–
49	maleic anhydride	5	DMSO/toluene 1:1		0.14
50	maleic anhydride	5	PhCN		2.45
51	–		PhCN/H ₂ O 1:1		0.68
52	–		PhCN/H ₂ O 1:1	gentisic acid (10 eq.)	1.69
53	–		PhCN/H ₂ O 1:1	ascorbic acid (10 eq.)	0.65
54	–		PhCN/H ₂ O 1:19		1.17
55	–		CDCl ₃	gentisic acid (10 eq.)	0.04
56	–		CDCl ₃	acrylamide (10 eq.)	0.05

Dependancy on the Wavelength Various light sources were investigated for the photocleavage of fulleropyrazoline **7** (λ_{max} = 256 & 315 nm) under standard conditions. Comparable efficiencies in product formation (60–75% conversion after 1.5 h) were observed when using a handheld UV (λ = 365 nm, 8 W), filtered (λ = 313 nm, 70 W)^{224,225} source, with the full spectral medium pressure mercury (λ > 280 nm, 125 W), Rayonet (λ = 350 nm, 8x120 W), or commercial halogen (λ > 300 nm, 500 W) lamps. Smaller wavelengths, such as the handheld UV lamp (λ = 254 nm), showed reduced efficiency, with only 22% conversion after 3.5 h. Because of practical reasons, the medium pressure Hg-lamp was used for all subsequent experiments.

Dependancy on Alkenes for Trapping A dipolarophile was added to the photoreaction mixture to prevent the generated 1,3-dipole from the cycloreversion from undergoing re-addition to C₆₀.¹¹⁵ Initially, an excess of maleic anhydride was introduced; this caused large quantities of precipitation that were presumably due to the polymerisation of maleic anhydride.^{226,227} The higher quantities of maleic anhydride were, the faster the initial reaction rates were, but this gave an incomplete conversion. Lowering the equivalents of maleic anhydride from 30 to 1 led to complete photocleavage after 2.5 h (Figure 2.7).

Other alkene additives were tested to trap the 1,3-dipolar intermediate for characterisation and to find the influence of trapping on the reaction time (Table 2.4). No new cycloaddition products were found. Nevertheless, the rate of the photoreaction was accelerated in the presence of

3,3-dimethylallylbromide or *para*-chlorostyrene. The cycloreversion also progressed efficiently in the absence of alkenes. This finding opens up the possibility to introduce the photocleavage system to biological applications and deliveries, which would be impractical with a reaction dependent on two components.

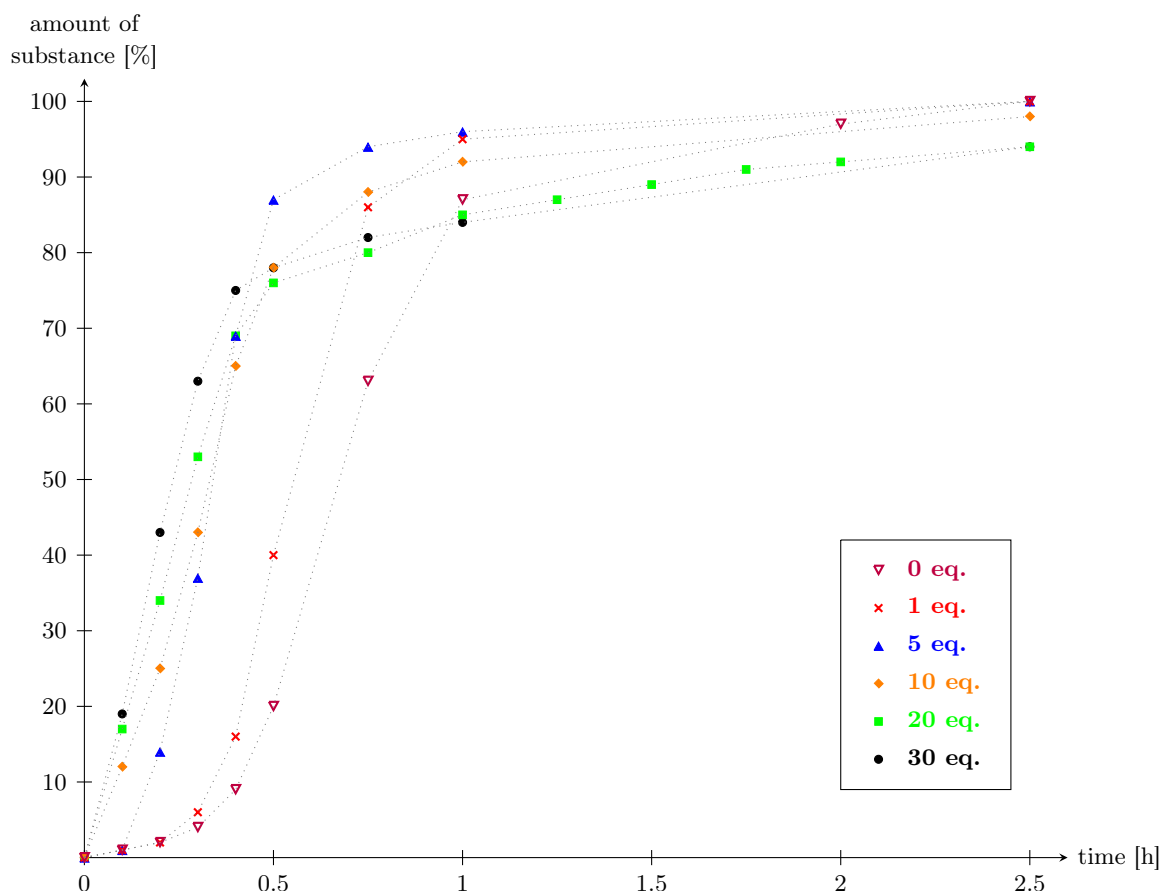


Figure 2.7: The time dependant photocleavage of fulleropyrazoline in the presence of varying concentrations of maleic anhydride as a trapping agent. Black dot: 30 eq., green square: 20 eq., orange diamond: 10 eq., blue triangle: 5 eq., red cross: 1 eq., purple hollow triangle: 0 eq. The amount of substance includes both the yields of C_{60} and $C_{60}O$ as observed in the HPLC chromatograms.

Table 2.4: Reversible cleavage of fulleropyrazoline **7** in toluene [1 mM] under irradiation of a medium pressure Hg-lamp was achieved with the following alkenes (20 eq.). The yield reflects the extent of the reaction measured by HPLC and was calculated as $y = \frac{\text{amount of } C_{60} \text{ and } C_{60}O}{\text{amount of all observed compounds}}$.

ENTRY	ALKENE	REACTION TIME	YIELD
1	maleic anhydride	2.5 h	94%
2	3,3-dimethylallylbromide	45 min	97%
3	<i>p</i> -chlorostyrene	45 min	90%
4	allylbromide	2 h	97%
5	allylchloride	3 h	75%
6	coumarin	4 h	67%
7	allylamine	10 h	58%
8	—	2 h	97%

Influence of the Solvent The solvent compatibility of the photoreaction was tested. To start with, degassed toluene was used to minimise the possible interference of excited oxygen species to the reaction. Surprisingly, when an oxygen-containing solvent was used, the reaction time was decreased by approximately threefold but did not show an influence on the C₆₀/C₆₀O ratio (Figure 2.8). The appearance of the oxygen atom in the product could not be ascribed to the presence of water because comparable product mixtures were obtained under anhydrous reaction conditions (Table 2.3).

As a consequence of changing the solvent to chloroform, the fulleropyrazoline solubility was increased and the photoreaction time significantly decreased (Line 4, Table 2.5). This enhancement could be reasoned to the presence of heavy atoms, such as chloride, accelerating the S₁→T₁ intersystem crossing by induction of a spin-orbit coupling,^{169,228} the increased polarity to support and stabilise emerging ionised species during the cleavage by dipole-dipole interaction, or the introduced acidity (*vide infra*).

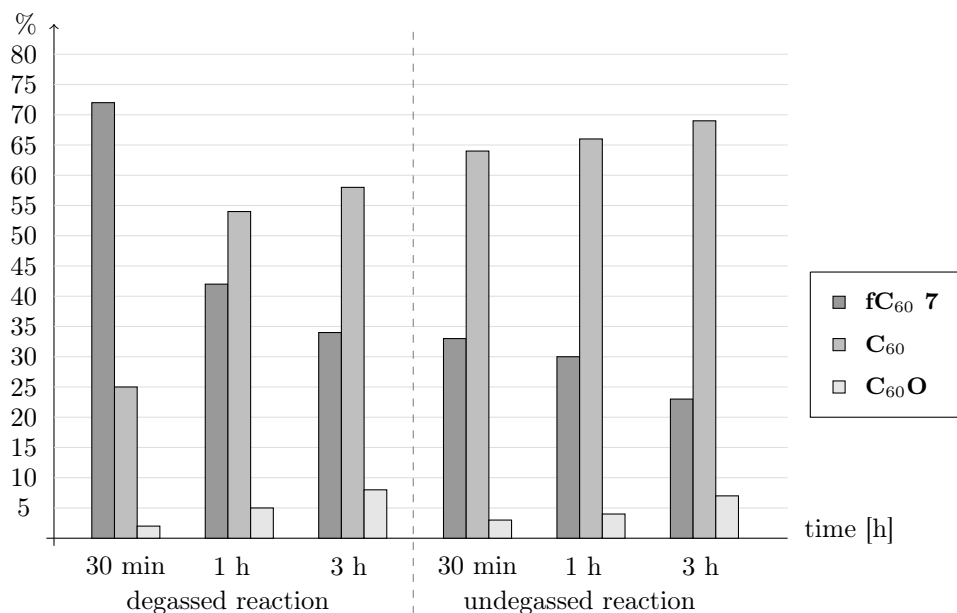


Figure 2.8: Comparison of the photocleavage reaction of fulleropyrazoline **7** and the products C₆₀ and C₆₀O in degassed and undegassed toluene over 3 h with maleic anhydride (30 eq.) as a trapping agent.

Table 2.5: Reversible cleavage of fulleropyrazoline **7** under irradiation with a medium pressure Hg-lamp was achieved without a trapping alkene in various undegassed solvents [1 mM].

	SOLVENT	DIPOL MO- MENT ²²⁹ [CM]	REACTION TIME ^{a)}	REMARKS
1	toluene	0.37	3 h	
2	toluene/H ₂ O 1:1		>1.5 h	
3	toluene/MeOH 1:1		30 min	
4	CHCl ₃	1.04	10-45 min	as received, or alumina neutralised
5	CHCl ₃ /H ₂ O 1:4		30 min	
6	PhCN	4.18	1 h	

	SOLVENT	DIPOL MO- MENT ²²⁹ [CM]	REACTION TIME ^{a)}	REMARKS
7	PhCN/H ₂ O 1:1		45 min	
8	PhCN/H ₂ O 1:19		20 min	
9	DMF	3.82	3 h	rapid decomposition ^{b)}
10	DMF/H ₂ O 1:1		5 h	no reaction
11	DMSO	3.96	3 h	no reaction
12	DMSO/H ₂ O 1:1		3 h	no reaction
13	DMSO/toluene 1:1		2 h	66% cleavage
14	pyridine	2.21	5 h	no reaction
15	1,1,2,2-Tetrachloroethane	1.32	20 min	
16	Tetrachloromethane	0	>2 h	cleavage very slow
17	H ₂ O	1.85	11 h	not soluble, no reaction

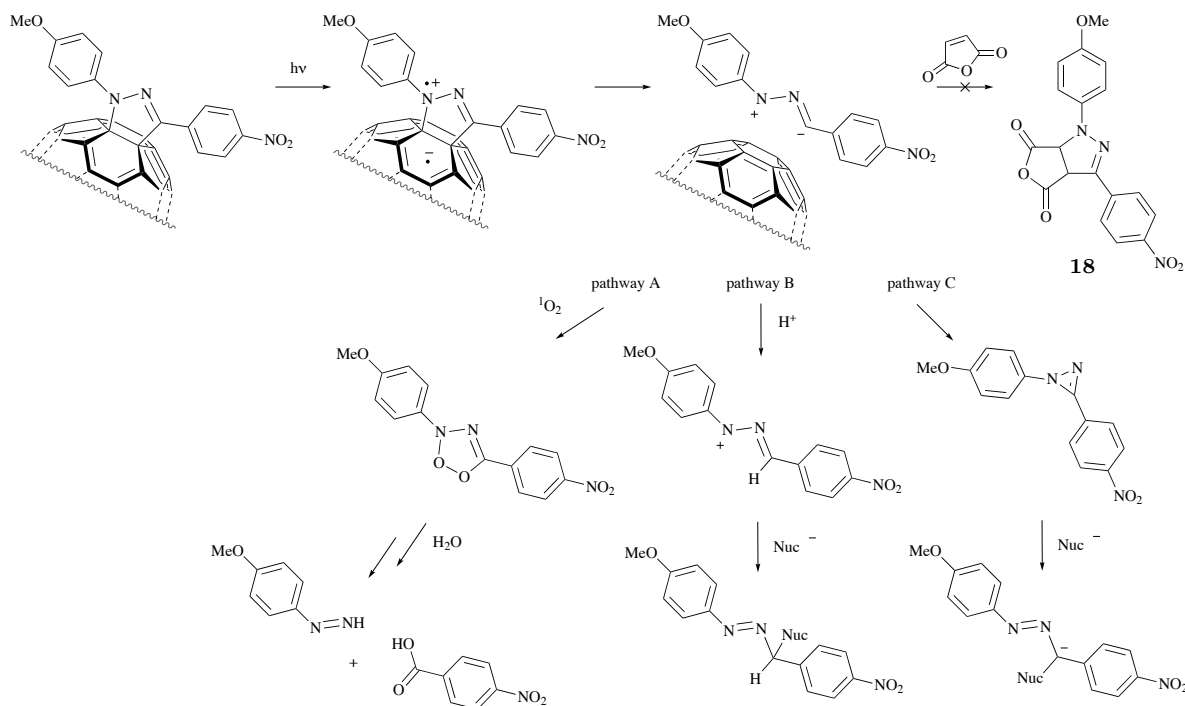
a) given is the time until starting material **7** was fully consumed; b) starting fulleropyrazoline was consumed in 30 min, but no C₆₀ product was formed.

The results implied that the limiting factor for the photocleavage reaction was the solubility of fC₆₀ in most hydrophilic solvents, such as water or methanol. Mixtures with toluene, chloroform, or benzonitrile in various ratios demonstrated their utility for accelerating the photocleavage reaction compared to single-solvent reactions (Table 2.5). The decrease of reaction time of the successful photocleavage with increasing water or methanol content indicated either stabilisation of the intermediate by H-bonding or trapping of the intermediate because of its nucleophilicity. The reaction rate was additionally influenced by the presence of a protic solvent and acids (*vide infra*). These observations suggested the formation of a charge-separated diradical species and zwitterionic intermediate that are better stabilised under polar conditions (Scheme 2.3). Furthermore, electron transfer from the pyrazoline moiety to the C₆₀ sphere is enhanced by the basic character of the sp³-nitrogen of the pyrazoline due to the stabilising effect of the electron-donating *para*-methoxyphenyl group.²⁰⁴ Increased solvent polarity promoted the photocleavage reaction by the presumed formation of a charged intermediate (Scheme 2.3).

Scavengers As mentioned, the presence of oxygen accelerated the photoinduced cleavage reaction of fulleropyrazolines. The suppression of excited oxygen species should reveal their impact on the cleavage mechanism. Reported singlet oxygen quenchers, such as 1-phenylnaphthalene, 2-methylbutene, and 1,4-diazabicyclo[2.2.2]octane (DABCO) were tested.^{230–232} DABCO suppressed the photocleavage completely, whereas the other two compounds slowed the reaction only marginally.

As well as DABCO, pyridine suppressed the reaction completely. In the case of triethylamine, no photocleavage occurred. This led to the conclusion that amine bases inhibit the cycloreversion due to their propensity to undergo electron transfer with the present C₆₀ and quench the putative pyrazoline radical.^{170,233} A charge-separated diradical species of fulleropyrazolines formed under photoirradiation,^{115,149,205,214,234} as shown in the proposed mechanism (Scheme 2.3), results from this observation.

Scheme 2.3



Potential breakdown mechanism of the photocleavage of fulleropyrazoline **7** via a zwitterionic diradical forming a reactive 1,3-dipole that is stabilised in a polar solvent. Breakdown pathway A might occur because in undegassed solvent systems, the photocleavage rate was increased. Pathway B appears more likely given the large increase in rate in the presence of proton donors. The reactive intermediate formed might undergo hydrolysis or radical cleavage. Pathway C can result in the same reaction products as in B. Product **18** originates from the trapping with an alkene (*e.g.* maleic anhydride) of *in situ* generated dipole.

To support the theory that a diradical species is formed, gentisic acid, sodium ascorbate, or acrylamide were added as potential radical scavengers^{235,236} to the photocleavage reaction in either a chloroform or benzonitrile/water 1:1 mixture. In the biphasic system, these additives had no effect on the reaction rate. The presence of 2,5-dihydroxybenzoic acid or acrylamide in chloroform led to an acceleration in the reaction rate. This result could be solely owed to the fact that these additives possess a protonating nature that can quench the fast evolving zwitterionic intermediate (pathway B).

Reaction Products To shed light on the cleavage products, the photocycloreversion was conducted in a deuterated solvent in NMR tubes under irradiation using the medium pressure Hg-lamp and analysed by means of NMR, mass spectrometry (MS), and infrared (IR) spectroscopy. According to the reverse reaction, the small molecule product of the photocleavage would be the 1,3-dipolar cycloadduct with the trapping alkene (Scheme 2.3, maleic anhydride forms product **18**). However, the formation of this new alkene-pyrazoline, the original hydrazone **2** or the brominated hydrazone intermediate, were not observed. As a control, the synthesis of the methoxyphenyl nitrobenzyl pyrazoline with dimethyl allylbromide or maleic anhydride were undertaken. No reaction was observed due to presumed low reactivity of the *in situ* gen-

erated 1,3-dipole towards these dipolarophiles. This finding supported the noted absence of a newly formed pyrazoline product in the photocleavage reaction mixture.

The photocleavage reaction of **7** was completed (1 h in CDCl₃, 5 h in toluene-d₈, and 2 h in toluene-d₈/CS₂ 10:1), and ¹H-NMR were taken (Figure 2.9-2.11). Various *para*-substituted aromatic species were formed under the reaction conditions, as expected from the starting fulleropyrazoline. Depending on the solvent and the irradiation time, different product distributions were discovered.

In CDCl₃, three major aryl compounds and two methoxy substituents were observed, whereas in toluene-d₈, a mixture of seven *para*-substituted aryl products were found, of which only two were identical with the ones found in CDCl₃. Comparison with the previously reported chemical shifts^{237–240} (Table 2.6) suggested that the products shown in Figure 2.9 were formed during the reaction. The photocleavage in toluene-d₈/carbon disulfide 9:1 showed an acceleration of the reaction rate and decrease in the number of products to two major products that appeared to be similar to those formed in CDCl₃. These observations suggested that different solvent-dependent mechanisms may occur.

Table 2.6: NMR shifts of disubstituted (R¹, R²) benzylic products of the photoirradiation of fulleropyrazoline **7** in CDCl₃.

R ¹	R ²	¹ H SHIFTS [PPM]		¹³ C SHIFTS [PPM]	
		FOUND	REPORTED	FOUND	REPORTED
NO ₂	COCl ₃	8.34, 8.37	8.31, 8.40 ^{a)}	124.1, 131.9, 137.1, 151.1, 179.0	95.2, 123.8, 129.7, 142.8, 152.3, 176.5 ^{a)}
NO ₂	COCl				123.9, 132.1, 137.9, 151.5, 161.8 ²³⁷
NO ₂	CN				116.7, 118.3, 124.2, 133.3, 150.0 ²³⁷
CF ₃	COCl ₃		7.77, 8.38 ²³⁸		94.5, 123.4(q), 125.3, 131.9, 132.5, 135.1, 180.6 ²³⁸
OMe	COCl ₃		6.96, 8.28 ²³⁸		55.8, 95.8, 113.8, 121.3, 134.0, 164.2, 179.8 ²³⁸
NO ₂	OMe	3.95, 7.07, 8.06	3.9, 6.95, 8.19 ²³⁹	56.1, 114.9, 127.0, 147.0, 165.3	56.1, 114.1, 126.0, 141.6, 164.7 ²³⁹
NO ₂	NHOH	3.81, 6.91, 7.29	3.80, 6.60, 6.63 ^{a)}	55.3, 114.0, 129.5, 158.6	55.8, 114.5, 126.1, 145.8, 158.0 ^{a)}
NO ₂	OH		5.72, 6.92, 8.18 ²⁴⁰		115.9, 126.5, 141.7, 161.58 ²⁴⁰

a) calculated shifts by ChemDraw Professional 15.0.

II. Photoreversible C₆₀ Fullerene Modification

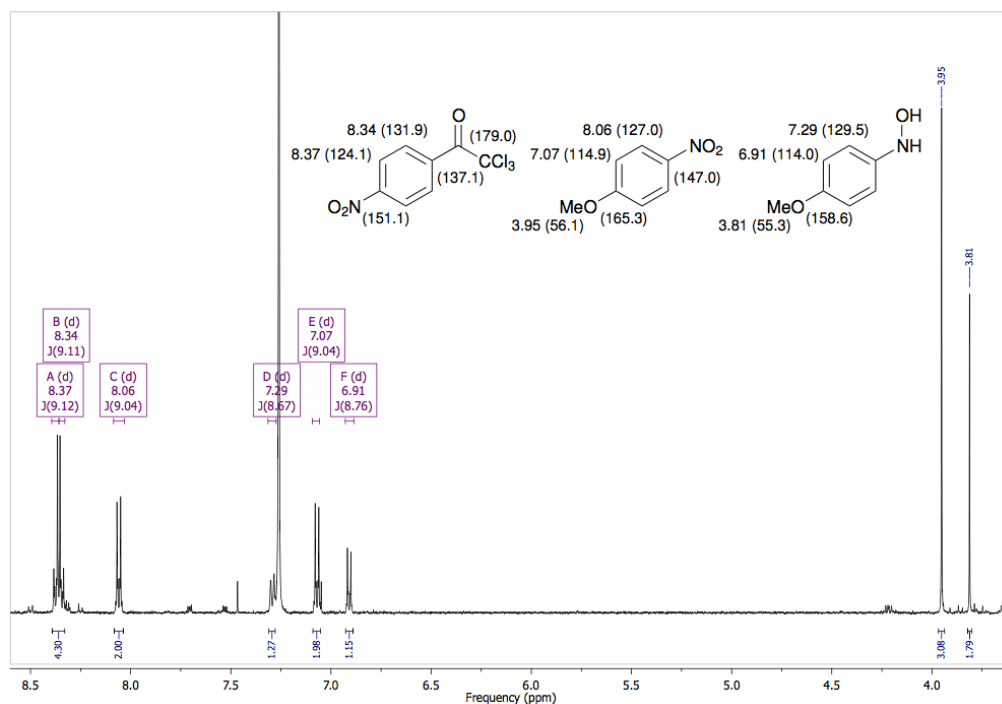


Figure 2.9: ¹H-NMR spectrum of irradiated fulleropyrazoline **7** in CDCl₃ [1 mM] with a medium pressure Hg-lamp for 1 h and the possible products indicated.

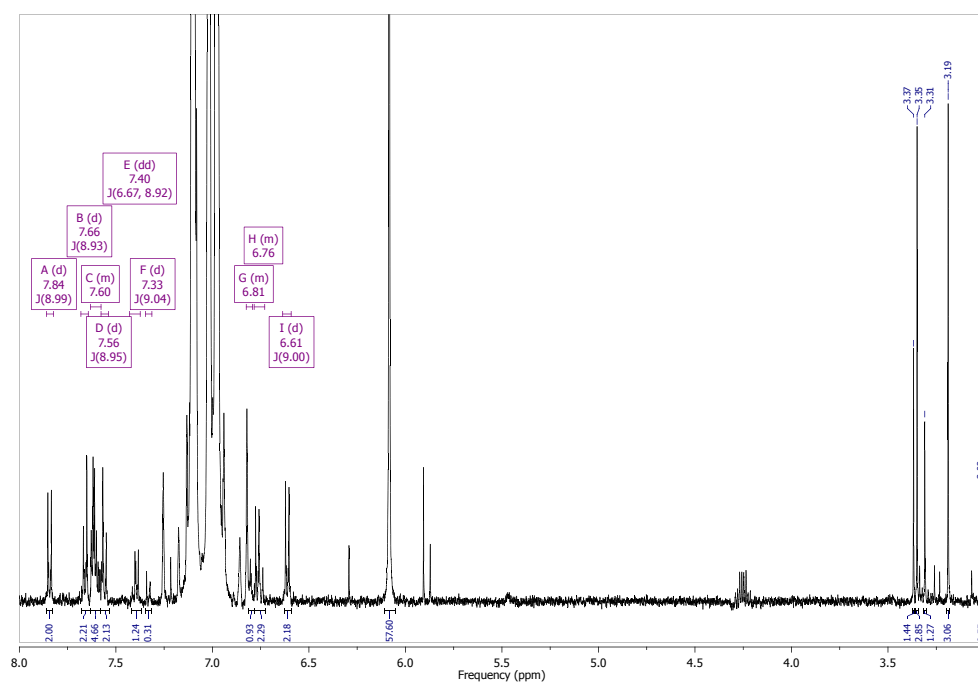


Figure 2.10: ¹H-NMR spectrum of irradiated fulleropyrazoline **7** in toluene-d₈ [1 mM] with a medium pressure Hg-lamp for 5 h.

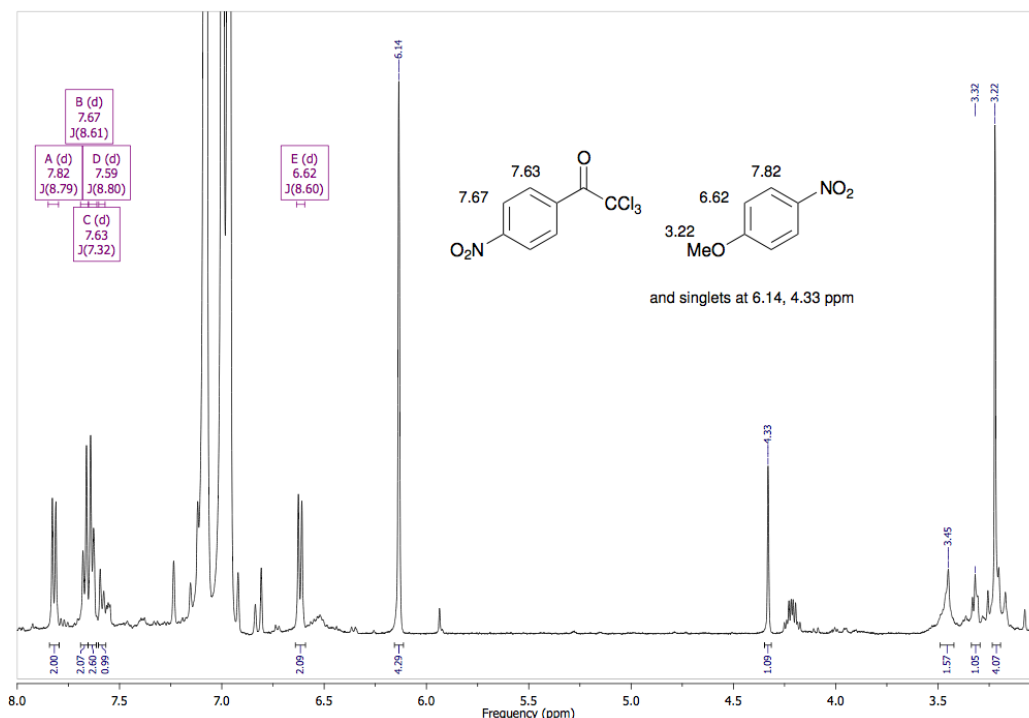


Figure 2.11: ^1H -NMR spectrum of irradiated fulleropyrazoline **7** in toluene- d_8 / CS_2 10:1 [1 mM] with a medium pressure Hg-lamp for 2 h with the possible products indicated.

High resolution mass spectrometry of the crude chloroform mixture revealed the putative hydrolysed products *para*-nitrobenzoate and *para*-nitrophenol. The IR spectrum showed peaks at 1704 ($\text{C}_{\text{aryl}}\text{C}=\text{OO}$), 1601, 1528 (NO_2), 1421, 1345 (NO_2), 1259, 1106, and 800 cm^{-1} , which are characteristic for *para*-nitrobenzoic acid.^{241,242} These findings support the proposed mechanism of oxidised hydrazone intermediates to yield the benzoic acid species.

The product mixture arising from the photocleavage of **7** in CHCl_3 [1 mM] was subjected to flash column chromatography ($\text{CHCl}_3/\text{MeOH}$ 4:1). The collected fractions contained mixtures of up to seven *para*-substituted aromatic products, of which three were previously observed in the crude reaction mixture. This result indicates the formation of reactive intermediates that underwent further manipulations during work-up and purification. One new substrate was tentatively assigned a *para*-nitrobenzoic ethyl ester (**19**) by ^1H -, ^{13}C -, COSY, HSQC, and HMBC NMR, and MS (Figure 2.12).²⁴³ Esterification suggested the presence of a reactive intermediate, such as an acyl chloride or benzoylhydrazine, in the crude.

It has been reported that hydrazones can either undergo autoxidation in the presence of oxygen, followed by a breakdown *via* a radical mechanism,^{213,244} or directly cleave under UV irradiation to give the aminyl and iminyl radical species (Scheme 2.4).²⁴⁵ Assuming a retrocycloaddition, a breakdown of the fulleropyrazoline to a diradical hydrazone species could lead to similar breakdown pathways. However, the products formed in the photocleavage under oxygen-free reaction conditions were identical to those formed in undegassed CDCl_3 . From this finding, it was concluded that the breakdown of the species were not likely due to autoxidation and cleavage of

II. Photoreversible C₆₀ Fullerene Modification

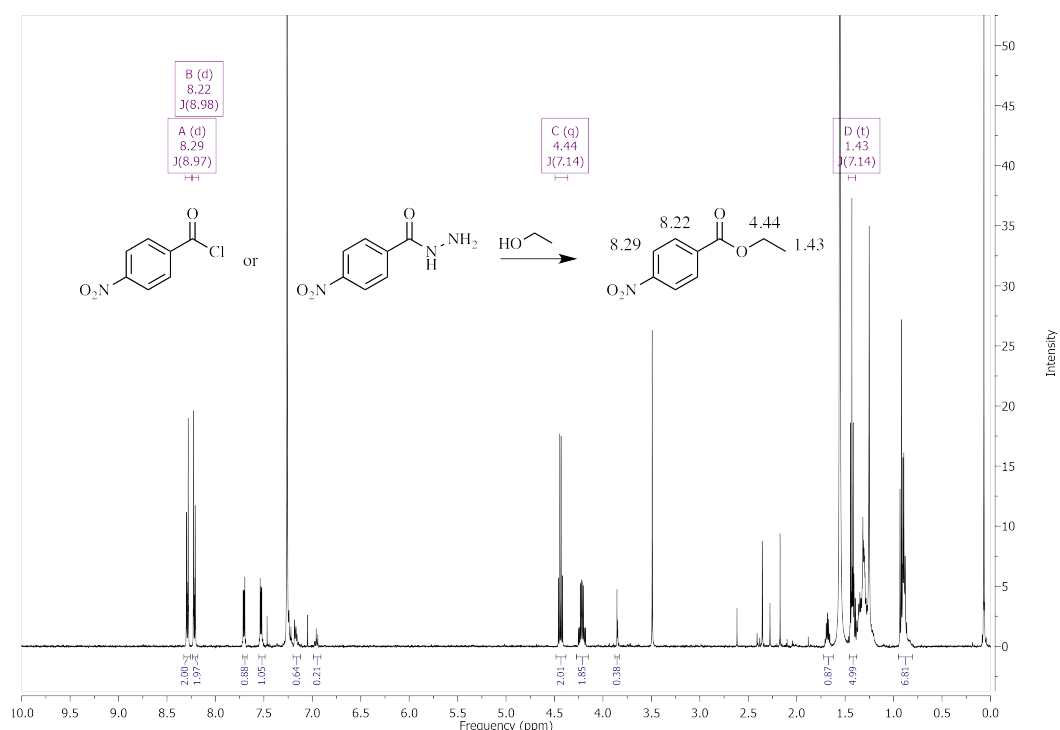
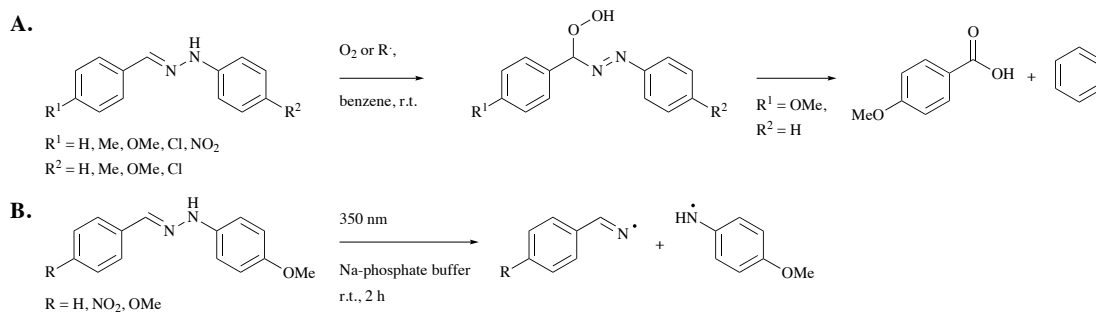


Figure 2.12: ¹H-NMR spectrum of the mixture of products isolated by column chromatography from the photocleavage reaction of fulleropyrazoline **7** in CHCl₃ [1 mM] with a medium pressure Hg-lamp. The major product was identified as the *para*-nitrobenzoic ethyl ester **19**.

the hydrazone intermediate.^{213,244} Nevertheless, hydrazone **2** was subjected to irradiation in CDCl₃. Complete consumption was observed after 1 h, and numerous unidentifiable products were formed. Amongst these, one was observed that corresponded to the photocleavage product of fulleropyrazoline **7** (Figure 2.13). The mechanism of the emergence is not known but could be due to oxidation of the hydrazone intermediate.

Scheme 2.4



A. In the presence of triplet oxygen or radical starters, aryl hydrazones undergo autoxidation.²¹³ The hydroperoxide decomposes to give the corresponding benzoic acid;²⁴⁴ **B.** The hydrazones bearing an electron-donating group on the phenyl group undergo photolytic cleavage to give an iminyl ($\text{R}_2\text{C}=\text{N}^\bullet$) and aminyl ($\text{R}_2\text{N}^\bullet$) radical that are used to promote deoxyribonucleic acid (DNA) fission.²⁴⁵

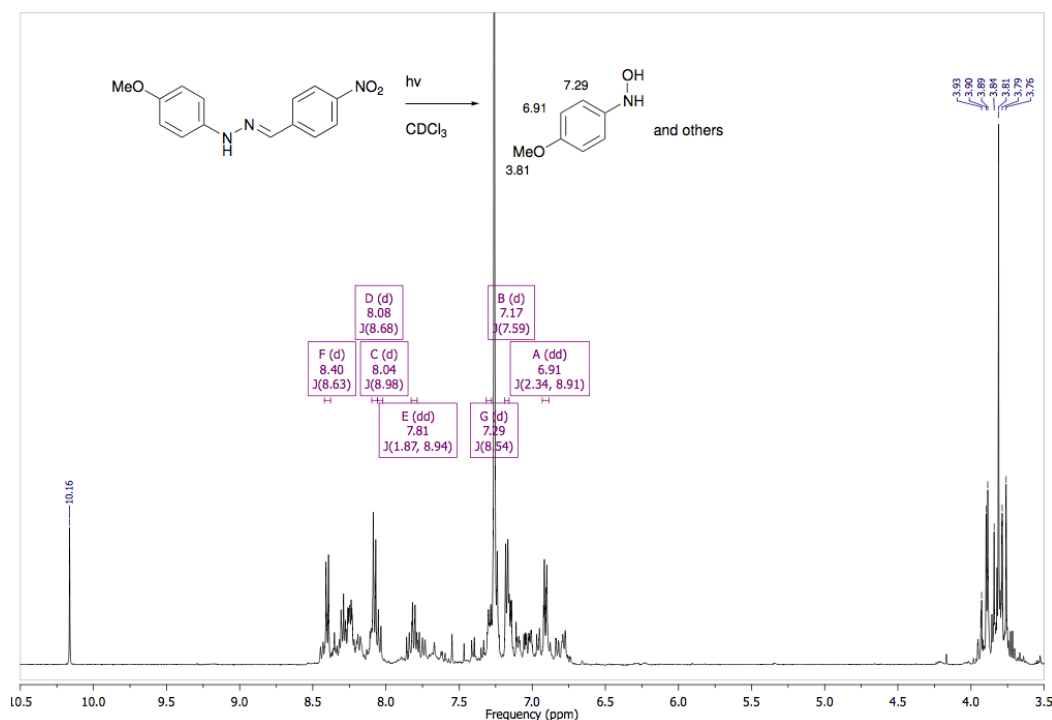


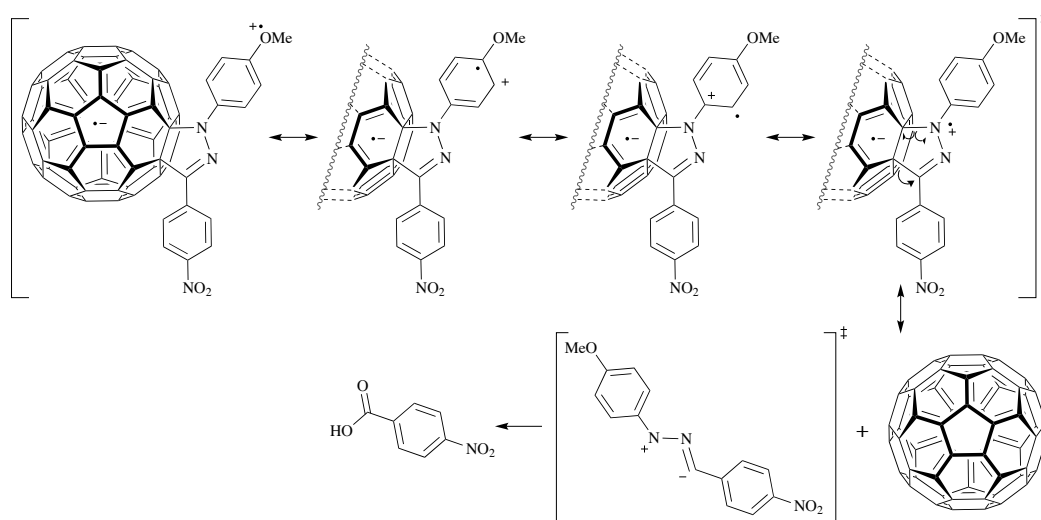
Figure 2.13: ^1H -NMR spectrum of irradiated hydrazone **2** in CDCl_3 [1 mM] with a medium pressure Hg-lamp for 1 h.

Mechanistic Discussion Based on the literature precedent,²³⁴ substrate compatibility, and the observations (*vide supra*), the initial step in the photocleavage was believed to be charge separation (Scheme 2.5). Various reaction pathways may be envisaged for this step. Photocleavage *via* the breakdown of the triple state of the fulleropyrazoline ($\text{f-}^3\text{C}_{60}^*$) could be excluded due to the ineffectiveness of oxygen as a quencher.¹⁷² The fact that the trapping alkene was not essential indicated that the cleavage occurs without participation of such species. The acceleration of the reaction rate in a polar solvent suggests a polar intermediate (Scheme 2.3).

Furthermore, the electron-donating group of the pyrazoline moiety at *N*-1 could have a stabilising effect that reinforces the basic character of the sp^3 -nitrogen, which plays a key role in the charge transfer process.²³⁴ This was supported by the fact that bases (*e.g.* pyridine, DABCO, and triethylamine (NEt_3)) inhibit the process, presumably by facile electron transfer^{170,233} to the present electron acceptor C_{60} .²⁴⁶ In the presence of a base, the fulleropyrazoline might not be able to form the charged diradical intermediate leading to the surmise that this step may be essential for the photocleavage reaction. The presence of acidic protons accelerated product formation, which indicated that they may be directly involved in the mechanism, *e.g.* by trapping intermediates.

A breakdown of the corresponding hydrazone **2** under UV irradiation resulted in formation of various cleavage products and suggested the presence of a similar intermediate during the photocleavage reaction. Apart from these findings, the results of the photocleavage in the presences of radical scavengers suggests a radical breakdown pathway.

Scheme 2.5



Potential breakdown mechanism under photocleavage of fulleropyrazoline **7**. Under induced light, a diradical charge species evolves, which is stabilised by mesomeric structures. Cycloreversion will give C₆₀ and potentially the 1,3-dipolar species, which undergoes oxidation and hydrolysis to yield *para*-nitrobenzoic acid.

2.3 Outlook

The goal of finding an applicable C₆₀ fullerene-based photocleavage system was successfully accomplished. Depending on the introduced substituent, the [2+3] cycloaddition of nitrile imines on C₆₀ showed reversibility under light irradiation. The synthetic scope of substituents on the *N*-1 and *C*-3 position was examined. The presence of an electron-donating residue on the phenyl residue was pivotal in the light-driven releasing mechanism. Dual functionality was given by the second substituent, which can be modified for delivery of prodrugs or targeting agents. Herein, the use of a simple amino acid was demonstrated.

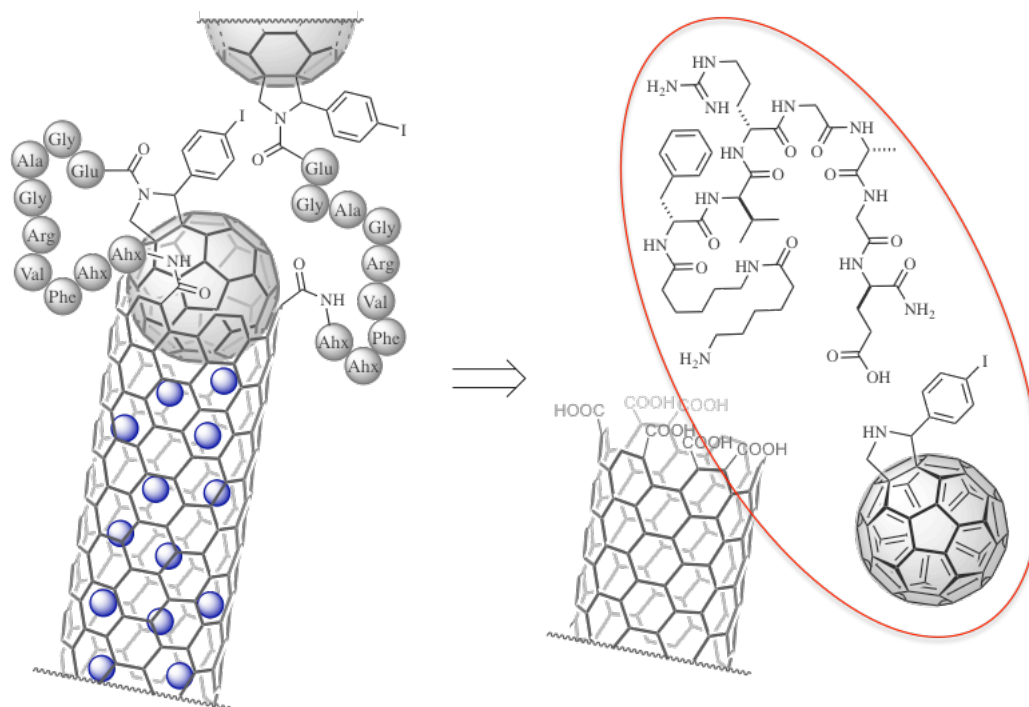
The reaction was investigated towards its reactivity on different wavelengths, the presence of a side product trapping alkene, and solvent compatibility. The analysis of the product formation, as well as the reaction fate in presence of quenching additives, shed light on the cleavage mechanism. A bifunctional system for tuning and delivery was found, which can be externally stimulated by light to release both a functional group and C₆₀.

To conclude, the essential points of this novel delivery system were explored; its application could be further considered. The compatibility with aqueous solvent systems suggests the photocleavage of fulleropyrazoline should be investigated in detail *in vitro*. The photolytic triggered release delivers a benzoic acid and C₆₀ derivative that has a potential application in photodynamic therapy. Furthermore, the reaction could be transferred onto carbon nanotubes and used as reversible sidewall functionalisation for controlled release in drug delivery. In principle, drugs could be released at a specified location or time by light application.

III. Functionalisation of Fullerenes with Peptides

The second project aimed for the filling of drugs or labelling compounds of carbon nanotubes (CNTs) and the triggered release of these from the inner cavity, then, would aid in therapy and diagnosis. Hence, efforts were made to investigate the reversible sealing of CNTs. The idea was to use modified C_{60} fullerene (C_{60}) molecules as corks, which are kept in place by an enzymatically cleavable peptide sequence. Retrosynthetic analysis of such ‘ C_{60} -corked’ CNTs revealed the synthesis of compounds, such as oxidised CNTs, a peptide linker and modified C_{60} (Scheme 3.1). In this chapter, the synthesis and coupling of the latter two are described.

Scheme 3.1



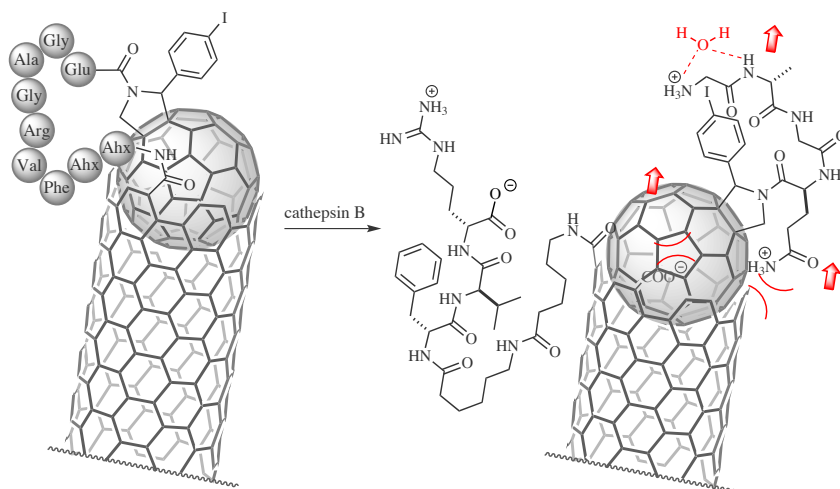
A retrosynthetic approach to functionalised carbon nanotubes with an enzymatically cleavable peptide linked C_{60} as a drug delivery system (DDS). In this chapter, a focus is laid on the peptide synthesis and functionalisation of the C_{60} (red circle).

3.1 Background

A key goal of the thesis was the design of an enzymatically triggered release from a sealed carbon nanocapsule, which is a closed carbon nanotube. The interaction between C_{60} fullerenes and CNTs is strong and is mediated *via* π - π stacking and van der Waals interaction.⁷⁵ Fullerenes are easily incorporated into CNTs and form peapod structures.^{79,87} This has led to investigations where C_{60} has been applied as a stopper at the end of filled tubes. These corks have been liberated with the use of organic solvents^{88,105} or acidic solutions, causing an increase in solubility of modified fullerenes and hydrophilic-hydrophobic repulsion between the quaternary amine on the fullerene and the carbon nanotube (CNT) wall; therefore, there has been decreased corking ability.⁷⁰ The introduction of charged groups on the surface has been used for the synthesis of many other water-soluble C_{60} derivatives for various purposes.^{66–71}

This led to the proposal that the assembly of a C_{60} and CNT with a peptide linker could form closed capsules. After enzymatic cleavage, a potential increase of solubility of the peptide modified fullerene could drive the release of the cork (Scheme 3.2).

Scheme 3.2



The uncorking procedure could potentially be favoured by enhanced solubility of the functionalised C_{60} and hydrophilic-hydrophobic repulsions, both caused by an increased number of the charged groups of the peptide fragment.

3.1.1 Exohedral Fullerene Modification

The surface modification of C_{60} is essential for introducing the functionality of fullerenes for further applications. C_{60} fullerenes react as electron-deficient dienophiles or dipolarophiles. To date, many thermally or photochemically induced additions to fullerenes have been investigated, such as [2+1], [2+2], [2+3], [2+4], and ‘higher’ cycloadditions (Figure 3.1).^{61,247–249} Important representative reactions are the Bingel,²⁵⁰ Prato,^{62,89} and Diels–Alder reaction.^{113,114,175,178,186,188,251,252} In addition to the pyrrolidine functionality, fulleropyrazolines^{149,151,212,253,254} and fulleropyrazolines^{115,254,255} have been synthesised *via* 1,3-dipolar cycloaddition. The introduction of

isomünchnones on the C_{60} surface¹¹¹ and a Pauson–Khand²⁵⁶ or Morita–Baylis–Hillman reaction²⁵⁷ have been successful.

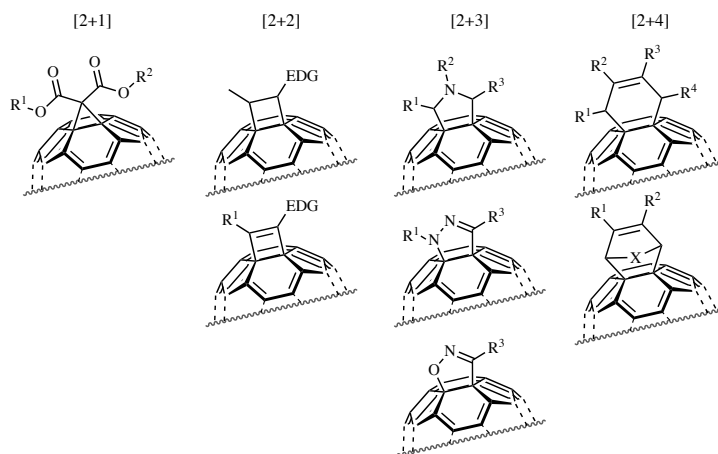


Figure 3.1: A schematic overview of the surface functionalisation at C_{60} *via* cycloaddition.^{61,247–249}

The Prato reaction has been widely applied, including a stereoselective version for C_{60} modification.²⁵⁸ Further modification of the so-formed fulleropyrrolidine can be achieved by palladium-catalysed Heck coupling.²⁵⁹ The 1,3-dipolar cycloaddition forming a pyrrolidine allows the introduction of a variety of functional groups.

The Prato reaction opens the possibility of introducing an amine, which could be linked to the C-terminus of the peptide. Instead of synthesising an α -amino acid with a protected amine,⁴ the direct use of glycine in the reaction was envisioned. Due to the reaction mechanism, this would give a free amine on the C_{60} surface for further functionalisation in just one step. The syntheses of both fulleropyrrolidines are described below, as well as investigations on their reactivity towards peptide coupling.

3.1.2 Peptide Synthesis

The cleavable peptide sequence was designed according to previous results found by other Benjamin G. Davis group members. The amino acid sequence Phe-Val-Arg is specifically recognised by the protease cathepsin B,^{163–165} which is upregulated in inflamed cells.^{159–162} Work by the Davis group revealed that the sequence FmocNH-Ahx-Ahx-Phe-Val-Arg-Gly-Ala-Gly-Glu-NH₂ (XXFVRGAGE), with Ahx being the unnatural amino acid 6-aminohexanoic acid, is hydrolysed by cathepsin B.²⁶⁰ The introduction of the corresponding D-amino acid FmocNH-Ahx-Ahx-Phe-Val-Arg^D-Gly-Ala-Gly-Glu-NH₂ (XXFVR*GAGE), or a variation of the specific motif to FmocNH-Ahx-Ahx-Phe-Val-Ile-Gly-Ala-Gly-Glu-NH₂ (XXFVIGAGE), prevents enzymatic cleavage. Based on these results, the peptide XXFVRGAGE was envisioned to function as a cleavable linker with XXFVIGAGE as a non-cleavable analogue between the C_{60} and CNT (Figure 3.2).

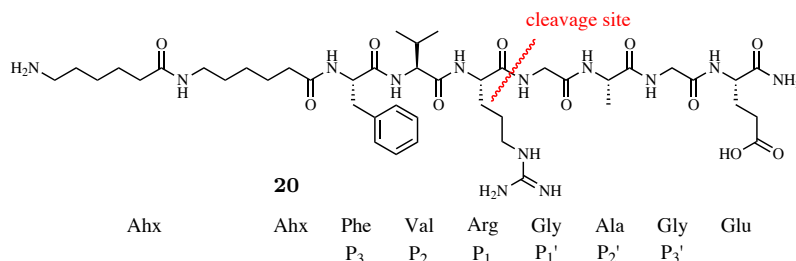


Figure 3.2: The peptide sequence of unprotected XXFVRGAGE.

The peptide contains three essential elements. The recognition motif, which fits into the active site due to the interaction of the specific amino acid residues (Phe-Val-Arg), is in the middle of the sequence and flanked by hydrophobic amino acids to increase the accessibility to the active site of the enzyme. On the N-terminus, a selectively deprotectable amine functionality is necessary for coupling to the carboxylic acid groups on the oxidised CNTs species. On the C-terminus, the carboxylic acid group is required for the coupling to functionalised fC₆₀ to occur. The length of the peptide can be varied to investigate the dependence of the corking efficiency on the distance C₆₀↔CNT and the accessibility of the enzyme for the cleavage.

Herein, peptide synthesis was conducted on a peptide synthesiser using a resin as a solid support and standard Fmoc-conditions.²⁶¹ The rink amide resin was initially used because the synthesis route was already elaborated.^{164,165,260,262} The synthesis route delivered an amide functionality on the C-terminus. Therefore, glutamic acid was introduced as the first amino acid to achieve the introduction of a coupling partner for the fC₆₀. Various other resins with different cleavage methods inducing modification to the C-terminus were investigated. The choice of resin and the design of the final peptide were suited to the needs of the functional linker.

3.1.3 Design of a Cleavable Cork

The general aim was to demonstrate the enzymatically triggered release of a payload from closed CNTs. The requirements to achieve this goal are addressed in the following chapters. Here, the design of the enzymatic cleavable linker bound to a C₆₀ fullerene, which functions as a cork to seal the nanotube ends, is investigated. The specifications of this compound are listed below:

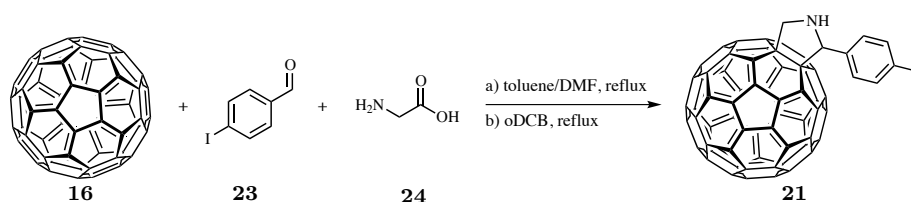
- An amine functionality on the C₆₀ fullerene surface
- A peptide with the cleavable recognition sequence Phe-Val-Arg, a carboxylic acid group for the coupling to the C₆₀ cork, and an amine functionality, protected with an orthogonal protecting group
- Variations in length of the peptide for optimised corking, cleavage, and release
- Facile coupling of both these substrates and subsequent deprotection.

3.2 Results and Discussion

3.2.1 Functionalising C₆₀ fullerene *via* Prato Reaction

Maggini *et al.* described the functionalisation of C₆₀ with an α -amino acid and aldehyde.⁸⁹ The azomethine ylide is generated *in situ*, which undergoes a 1,3-dipolar cycloaddition to form a pyrrolidine. The reported reaction conditions were applied using both glycine (Scheme 3.3) and a protected diethylenamine glycol linker⁴ (Scheme 3.4). Both fullero-amines were synthesised to compare their reactivity in the subsequent peptide coupling. **21** was more accessible but proved lower yielding compared to **22**. The secondary amine of fulleropyrrolidine **21** might be more reactive but sterically hindered for further modifications.

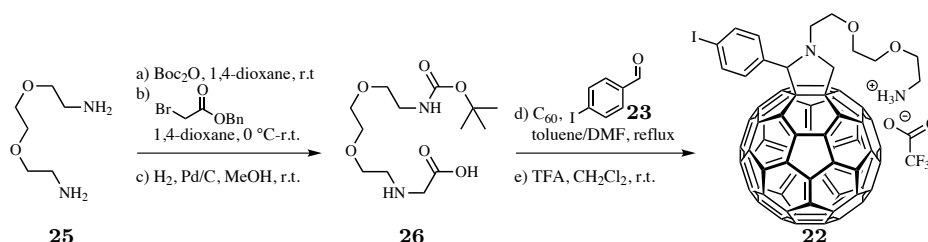
Scheme 3.3



Reagents and conditions: a) **23** (1 eq.), **24** (1 eq.), C₆₀ (1 eq.), toluene/DMF 9:1 [3 mM], reflux, 6–21 h, 13–38% (47–53% on recov. C₆₀); b) **23** (1 eq.), **24** (2 eq.), C₆₀ (1 eq.), oDCB [3.6 mM], reflux, 6–28 h, 19–45%.

The reactivity of the Prato reaction was enhanced by adding a small amount of *N,N*-dimethylformamide (DMF), likely due to the improved solubility of glycine (**24**), inducing better imine formation and stabilisation of the reactive 1,3-dipole. Further optimisation led to the use of *ortho*-dichlorobenzene (oDCB), which possesses the before-mentioned advantages in addition to a high boiling temperature for an accelerated decarboxylation.

Scheme 3.4



Reagents and conditions: a) **25** (6 eq.), Boc₂O (1 eq.), 1,4-dioxane [1.2 M], r.t., 25–67 h, 85–93%; b) R-NH₂ (3 eq.), benzyl 2-bromoacetate (1 eq.), 1,4-dioxane [0.27 M], 0 °C→r.t., 15–19 h, 79–99%; c) R-NHBn (1 eq.), H₂ (excess), Pd/C (0.1 mmol/mg), MeOH [0.5 M], r.t., 15 h, 59%; d) **23** (1 eq.), **26** (1 eq.), C₆₀ (1 eq.), toluene/DMF 9:1 [3 mM], reflux, 3 h, 26–39% (44–49% on recov. C₆₀); e) R-NHBoc (1 eq.), trifluoroacetic acid [7.5 mM], DCM [15 mM], r.t., 0.5–1.5 h, 90–99%.

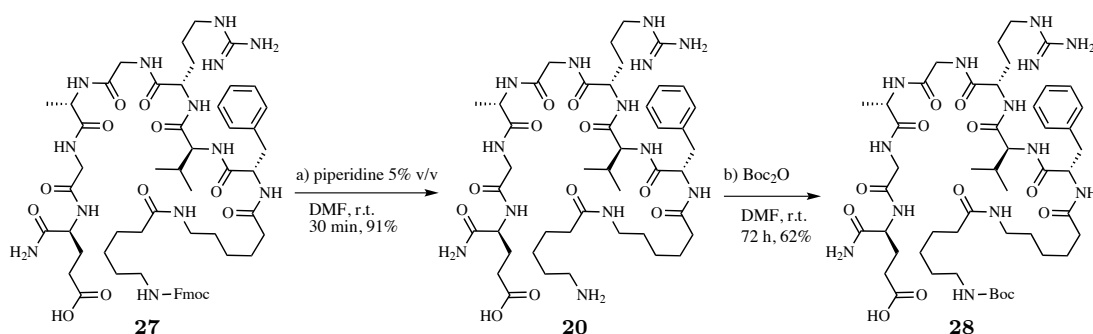
3.2.2 Synthesis of the Peptide Linker

Preparation of peptide XXFVRGAGE The synthesis of the designed peptide **27** on the rink amide resin was undertaken. Problems arose during *N*-(9-fluorenylmethoxycarbonyl) (Fmoc) deprotection on the C₆₀ coupled compound (Section 3.2.3.3).

III. Functionalisation of Fullerenes with Peptides

To circumvent this late-stage, basic deprotection, the peptide was subjected to a Fmoc-deprotection Boc-reprotection procedure (56% overall yield, Scheme 3.5). The successful acidic deprotection of the peptide- C_{60} species led to investigations of new peptide syntheses to avoid additional de- and re-protection steps.

Scheme 3.5

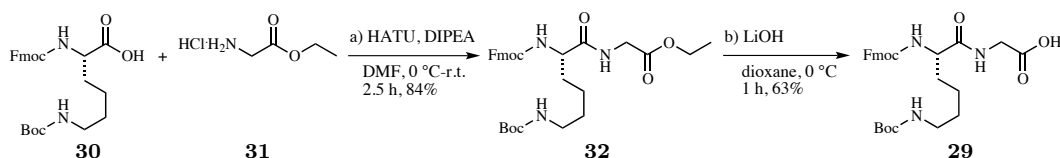


Reagents and conditions: a) peptide **27** (1 eq.), piperidine (5% v/v), DMF [2 mM], r.t., 30 min, 91%; b) **20** (1 eq.), Boc_2O (1 eq.), DMF [1 mM], r.t., 72 h, 62%.

3.2.2.1 Peptide Sequence Re-Design

Preparation of peptide KG 29 The peptide functionalised fullerene could profit from both orthogonal protecting groups, Fmoc and *tert*-butyloxycarbonyl (Boc). The Fmoc group was used as a chromophore for quantification, whereas the Boc group functioned as an acid cleavable protection group in further coupling steps. The protecting groups of arginine (pbf or mtr), which were incorporated in the peptide sequence XXFVRGAGE, did not show sufficient and reliable UV/vis signals to be considered useful for quantification. The dipeptide Fmoc-Lys(Boc)-Gly-OH possessed these features and was synthesised as a model for the evaluation of CNT corking yield (Scheme 3.6).

Scheme 3.6



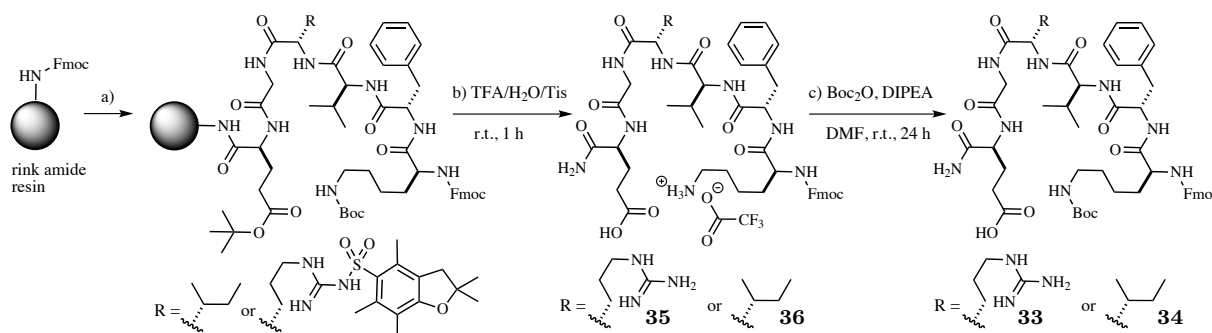
Reagents and conditions: a) **30** (1 eq.), **31** (1.3 eq.), HATU (1.5 eq.), DIPEA (2.5 eq.), DMF [63 mM], 0 °C→r.t., 2.5 h, 95%; b) **32** (1 eq.), $LiOH_{aq}$ (2.2 eq.), 1,4-dioxane, 0 °C, 1 h, 63%.

Lysine has the same sidechain length as the unnatural amino acid 6-aminohexanoic acid (Ahx) in peptide **20**, but the introduction of a Fmoc-protecting group additionally provides a chromophore that makes it an ideal substitute.

Preparation of peptide KFVRGE 33 and KFVIGE 34 The variation of the peptide linker length was investigated. The potentially enzymatically active sequence, such as KFVRGE **33**

and the corresponding inactive peptide KFVIGE **34**, were synthesised on the rink amide resin, followed by in-solution Boc protection (Scheme 3.7).

Scheme 3.7



Reagents and conditions: a) peptide synthesiser: rink amide resin (1 eq.), amino acids (4.4 eq.), HBTU (20 eq.), DIPEA (120 eq.), HOBT (12–80 eq.), DMF, microwave (μ wave), then piperidine 20% v/v in DMF; b) resin, TFA/Tis/H₂O 95:2.5:2.5 [1 mL/mg], r.t., 1 h; c) crude peptide **35** or **36** (1 eq.), Boc₂O (10 eq.), DMF (10 mg/mL), r.t., 18 h, then DIPEA (2 eq.), r.t., 5 h; overall yield 40% (**33**), 85% (**34**).

The problems in later coupling steps (see Section 3.2.3.1) were observed with the peptides synthesised on rink amide resin. Furthermore, the acidic cleavage conditions did not allow for installation of the Boc protecting group on the solid support, which made the introduction of an additional step necessary and, therefore, reduced the yield. To circumvent these problems, the 4-hydroxymethylbenzoic acid aminomethyl polystyrene (HMBA-AM) resin was used, which afforded the change of the original peptide sequence XXFVRGAGE to XXFVRGAGQ **37**. The basic cleavage conditions left the amino acid side chains protected; hence, they did not interfere with subsequent couplings.

In addition, the introduction of an element, such as sulfur in the peptide sequence, has been considered for unambiguous identification of the successful coupling to the CNT. Cysteine undergoes nucleophilic ester formation and has the advantage of a preinstalled chromophore (Fmoc protecting group) to be used for quantification. The peptide sequence CXFVRGAGQ was designed for this purpose (**38**, Figure 3.3). The syntheses are described in the following sections.

3.2.2.2 Base-Cleavable Resin for Peptide Synthesis

The change of the resin and according cleavage procedures that introduced a carboxylic acid at the C-terminus and Boc protecting group at the N-terminus was investigated to synthesise the peptide sequences.

Preparation of peptide XXFVRGAGQ **37** Synthesis of the potentially enzymatic cleavable peptide sequence XXFVRGAGQ was undertaken. The preloading of the HMBA-AM resin with Gln(trt)-OH with *N,N'*-dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino) pyridine (DMAP) and end capping with Ac₂O was essential for a good yield.²⁶³ After Boc protection, the peptide was cleaved from the resin under basic conditions with 1 M NaOH_{aq}. The yield varied between 5–39%, depending on the preloading efficiency and the time the loaded resin was

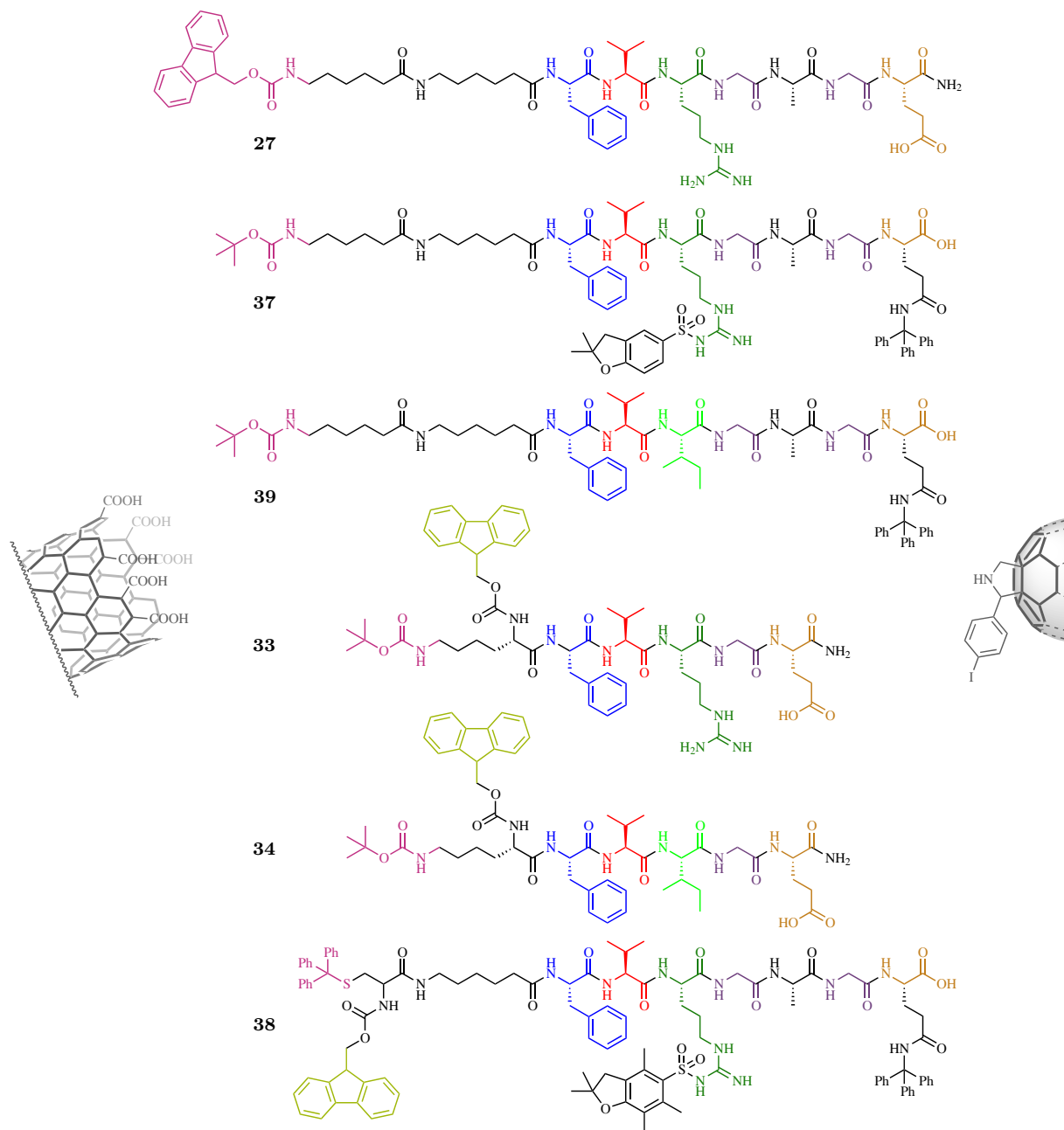
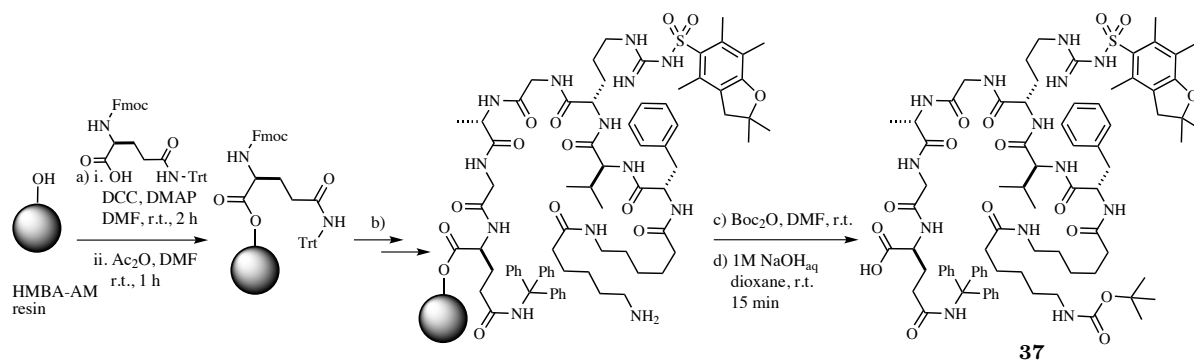


Figure 3.3: An overview of the enzymatically cleavable peptide linkers and negative controls: The initially synthesised peptide XXFVRGAGE **27**; Fully protected peptide XXFVRGAGQ **37** made on the HMBA-AM resin and the enzymatic cleavage resistant analogue **39** with an isoleucine residue; A shorter peptide sequence (**33**) bearing the enzymatic cleavage site as well as a chromophore for quantification and its protease inactive analogue (**34**); the cysteine containing peptide sequence **38** for alternative coupling methods to the CNT *via* the sulfide.

presented to *N,N*-diisopropylethylamine (DIPEA) in reaction step c (Scheme 3.8). Furthermore, the attained purity was not good, yielding two stereoisomer in a 1:1.7 ratio.

Scheme 3.8



Reagents and conditions: a) i. HMBA-AM resin (1 eq.), Fmoc-Gln(trt)-OH (3 eq.), DCC (4 eq.), DMAP (0.1 eq.), DMF [10 mg/mL], r.t., 2 h; ii. Ac₂O (6 eq.), DMAP (0.1 eq.), DMF [10 mg/mL], r.t., 1 h; b) peptide synthesiser: amino acids (4.4 eq.), HBTU (20 eq.), DIPEA (120 eq.), HOBt (12–80 eq.), DMF, μ wave, then piperidine 20% v/v in DMF; c) resin (1 eq.), Boc₂O (10 eq.), DMF [10 mg/mL], r.t., 15–19 h; d) icecold NaOH_{aq} [1 M, 6 mL/g], 1,4-dioxane [20 mL/g], r.t., 15 min; overall yield 5–39%.

Preparation of peptide XXFVIGAGQ 39 The concept of using the HMBA-AM resin was applied to synthesise the inactive peptide sequence XXFVIGAGQ **39**. The obtained yields in several attempts were found to be marginal, suggesting that either the cleavage procedure was not efficient, even though repetitive cleavage procedures did not increase the yield, or a bad coupling efficiency for the first and all of the following amino acids of this hydrophobic peptide occurred. Therefore, potential agglomeration during synthesis reduced the accessibility. To circumvent these problems, the choice of resin was reconsidered.

3.2.2.3 Acid-Cleavable Resin for Peptide Synthesis

Preparation of peptide XXFVIGAGQ 39 The poor yielding syntheses of peptide XXFVIGAGQ led to the investigation of a new strategy. As described before, three main features of the peptide had to be addressed, such as the introduction of the functionality of a carboxylic acid, an amine group and the enzymatically recognised amino acid sequence. A carboxylic acid functionality at the C-terminus can be introduced by several resins, such as the HMBA-AM, Wang, or chlorotrityl resins (see Figure 3.4). Peptide synthesis of XXFVIGAGQ using the Wang resin was undertaken, which did not improve the yield compared to the HMBA-AM resin attempts.

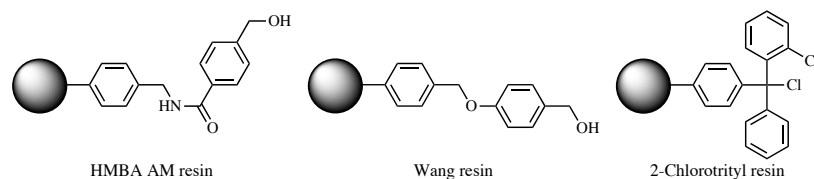


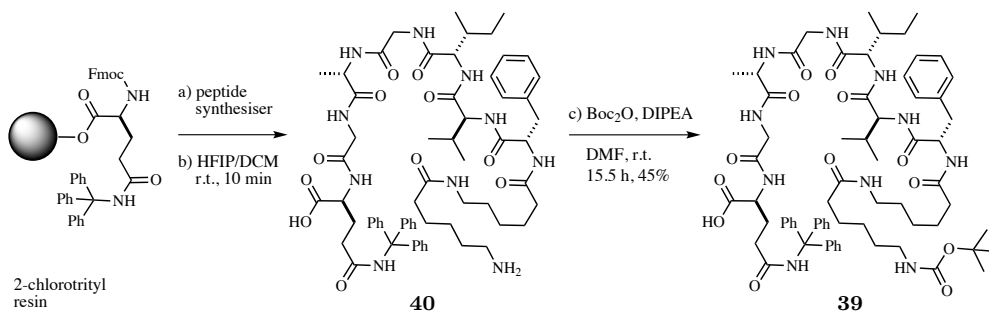
Figure 3.4: The different resins used for peptide synthesis prior to loading.

The peptide synthesis was successful when using commercial, Gln(trt)-preloaded 2-chlorotrityl resin. Mild cleavage conditions (HFIP/DCM 1:4)²⁶⁴ resulted in the triphenylmethyl (trt)-

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protected peptide XXFVIGAGQ **40** (Scheme 3.9). The additional step of the liquid phase Boc-protection was disadvantageous. Nevertheless, the peptide was received in good purity and sufficient quantity.

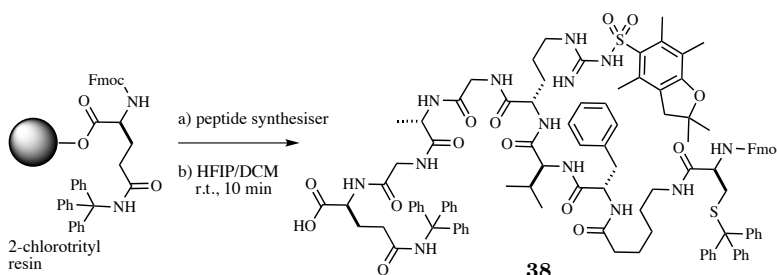
Scheme 3.9



Reagents and conditions: a) peptide synthesiser: amino acids (4.4 eq.), HBTU (20 eq.), DIPEA (120 eq.), HOBt (12–80 eq.), DMF, μ wave, then piperidine 20% v/v in DMF; b) resin (1 eq.), HFIP/DCM 1:4 [27 mL/mg], r.t., 10 min, crude 39%; c) **40** (1 eq.), Boc₂O (10 eq.), DMF [13 mM], r.t., 14.5 h, then DIPEA (5 eq.), r.t., 1 h, 45%.

Preparation of peptide CXFVRGAGQ **38** The previous results encouraged the synthesis of the peptide CXFVRGAGQ. The cysteine residue was introduced to aid in the validation of the new bond formation during the corking experiments (Section 4.2.2.6). The peptide was successfully synthesised using the preloaded 2-chlorotrityl resin. Mild deprotection conditions gave the fully protected peptide **38** (Scheme 3.10).

Scheme 3.10



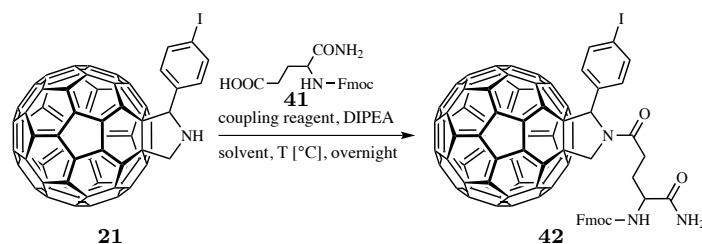
Reagents and conditions: a) peptide synthesiser: amino acids (4.4 eq.), HBTU (20 eq.), DIPEA (120 eq.), HOBt (12–80 eq.), DMF, μ wave, then piperidine 20% v/v in DMF; b) resin (1 eq.), HFIP/DCM 1:4 [27 mL/mg], r.t., 10 min, overall 19%.

3.2.3 Peptide Coupling on fC₆₀

Having various peptides at hand, the coupling of these linkers onto modified C₆₀ to give the ‘corks’ was approached. First, the coupling procedure was investigated with a simple glutamic acid derivative **41**, kindly donated by Dr. Regis Saliba (Table 3.1). Different coupling reagents were used, and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium-3-oxid hexafluorophosphate (HATU) gave the best yield. Optimisation led to explore a solvent mixture for increased substrate solubility combined with an elevated temperature (Entry 4). When using

HATU as the coupling reagent, a 30 min preactivation time of the acid, followed by the addition of the amine, was found to be necessary for good results.

Table 3.1: Peptide coupling on a modified C₆₀ fullerene.

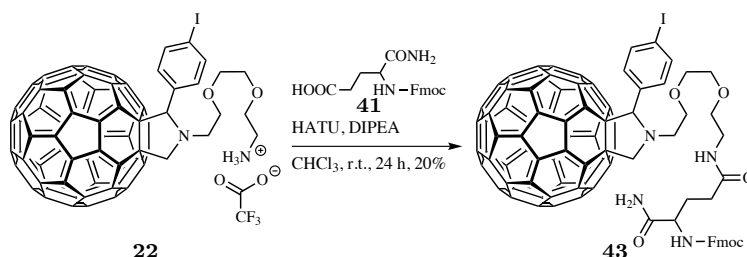


ENTRY	SOLVENT	DCC	EDC, DMAP	HATU
1	DCM	0% ^{a)}	4% (18% on recov. 21)	
2	DCM/DMF 2:1	0% ^{a)}		
3	CHCl ₃			19% (39% on recov. 21)
4	CHCl ₃ /DMF ^{b)} 19:1			40% (78% on recov. 21)

a) also at reflux; b) at 60 °C. Reagents and conditions: **21** (1 eq.), **41** (1.03–1.2 eq.), coupling reagent (1.15–1.5 eq.), DIPEA (1 eq.), anhydrous solvent [3 mM], r.t., overnight.

Further, the diethylene glycol functionalised C₆₀ analogue was synthesised to compare the reactivity in the peptide coupling step. As shown in Scheme 3.11, the yield was comparable with the result obtained for the synthesis of **42**. Considering the synthesis efficiencies of the starting materials **21** and **22** (Scheme 3.3 and 3.4), the glycine derived fC₆₀ was used in all further modifications.

Scheme 3.11

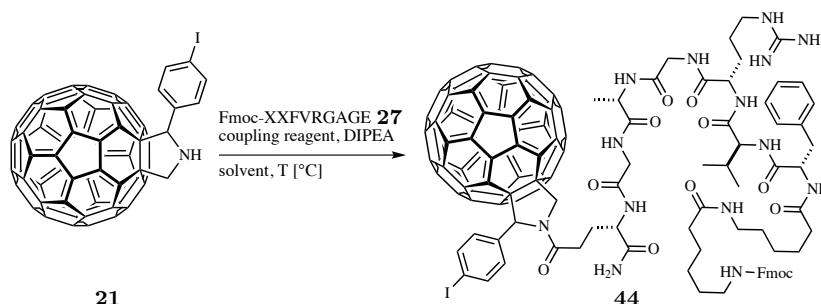


Reagents and conditions: **22** (1 eq.), **41** (1.03 eq.), HATU (1.15 eq.), DIPEA (10⁻³ eq.), anhydrous CHCl₃ [3 mM], r.t., 24 h, 20%.

3.2.3.1 Optimising the Peptide Coupling to fC₆₀

Coupling of peptide XXFVRGAGE Having found good conditions for the peptide coupling of a simple amino acid to the fulleropyrrolidine, all synthesised peptides underwent the procedure. The coupling of the peptide Fmoc-XXFVRGAGE **27** with both *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) and HATU was successful and gave product **44** (Scheme 3.12).

Scheme 3.12

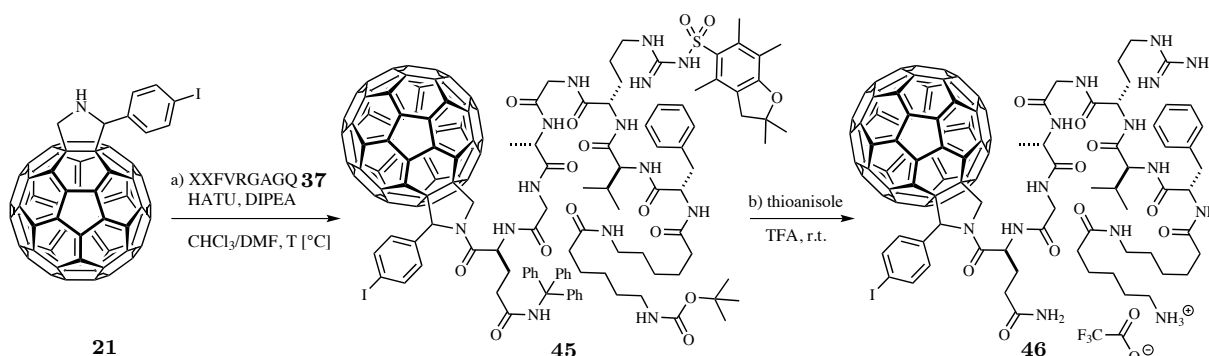


Reagents and conditions: a) **21** (1 eq.), **27** (1.2 eq.), EDC (1.5 eq.), DMAP (1 eq.), DIPEA (2.5 eq.), anhydrous DMF [3 mM], 0 °C → r.t., 22 h, 16% (51% on recovered **21**); b) **21** (1 eq.), **27** (3.6 eq.), HATU (6 eq.), DIPEA (11 eq.), anhydrous DMF/CHCl₃ 1:4 [3 mM], r.t., 47 h, 24%; c) **21** (1 eq.), **27** (1.2 eq.), HATU (4 eq.), DIPEA (4 eq.), anhydrous DMF/CHCl₃ 1:9 [3 mM], r.t., 27 h, 10%.

The presence of a base during Fmoc-deprotection on the peptide modified fullerene caused stability problems (Section 3.2.3.3). Therefore, the Boc-protected peptide sequence XXFVRGAGE **28** was also introduced to the coupling reaction to **21** using the optimised HATU coupling protocol. Unfortunately, the desired product was not formed; however, the peptide starting material was completely consumed during the preactivation step. The formed products could not be identified; the suggested cyclic peptide or polymer structures, formed by the coupling of the preactivated carboxylic acid and unprotected guanidine, were not found. As a consequence of these results, the substrate sequence and synthesis method were varied.

Coupling of peptide XXFVRGAGQ The optimised peptide synthesis delivered the fully protected peptide XXFVRGAGQ **37**. Coupling was achieved using optimised conditions, as shown in Scheme 3.13. Elevating the temperature to 60 °C gave the best yields, whereas increasing the temperatures to 70–80 °C over hours (18–24 h) resulted in less product isolation. The successful coupling of the peptide chain onto the fulleropyrrolidine was demonstrated by nuclear Overhauser effect spectroscopy (NOESY) (Figure 3.5). Deprotection was accomplished successfully, as described further in Section 3.2.3.3.

Scheme 3.13



Reagents and conditions: a) **21** (1 eq.), **37** (1.1 eq.), HATU (1.5 eq.), DIPEA (1.5 eq.), anhydrous DMF/CHCl₃ 1:9 [3 mM], r.t. → 60–80 °C, 3.5–24 h, 17–43% (33–81% on recovered **21**); b) **45** (1 eq.), PhSMe [10 mM], TFA [1 mM], r.t., 30–40 min, 83–94%.

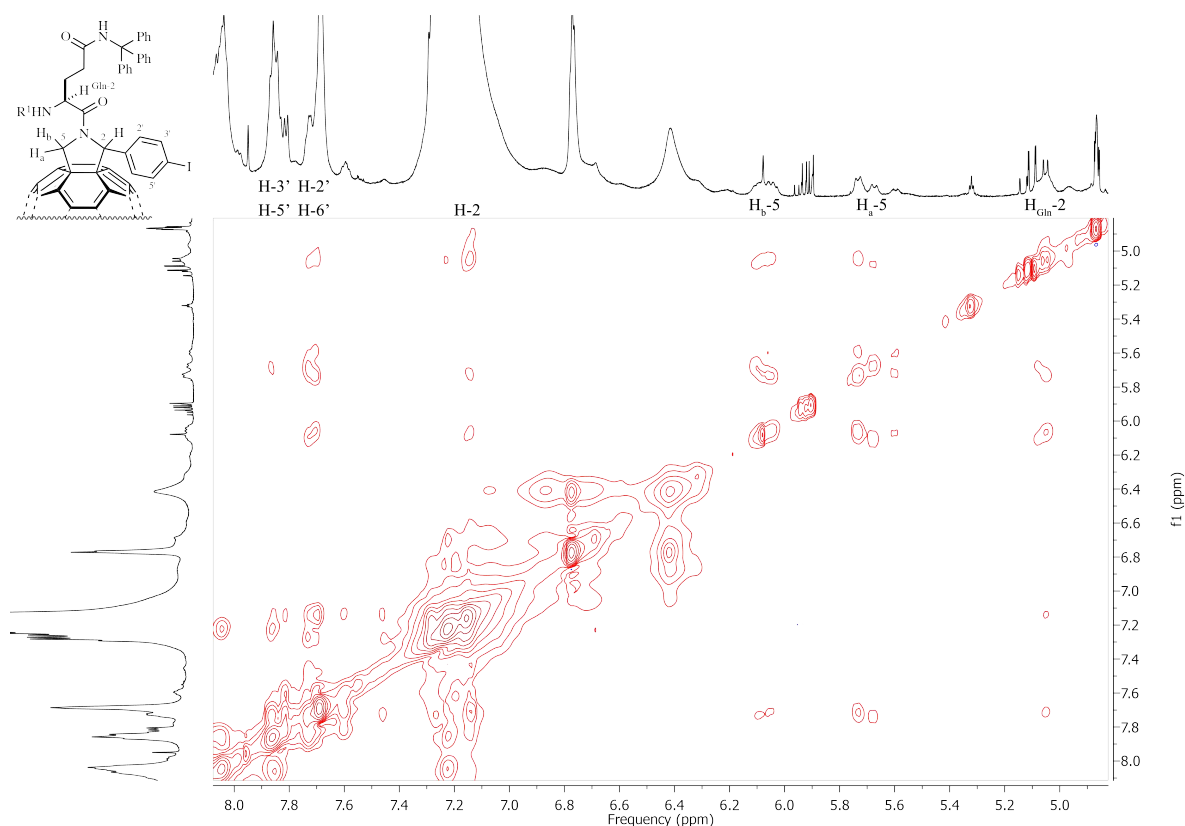
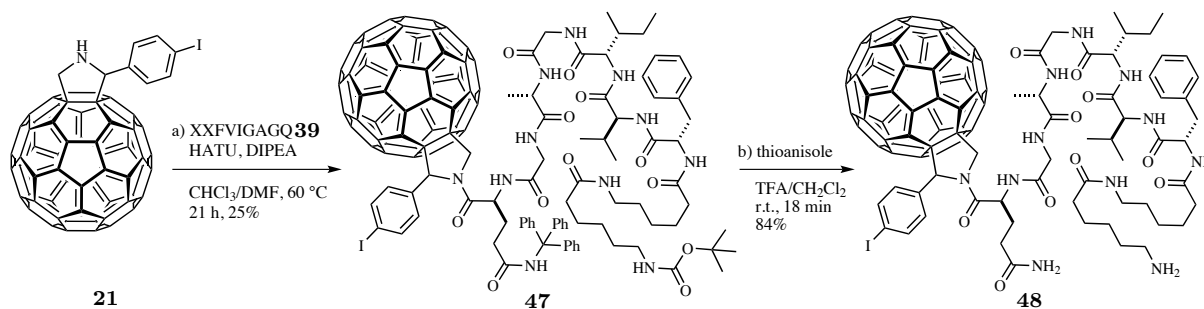


Figure 3.5: The NOESY spectrum of **45** shows clear evidence of the proximity of the glutamic acid proton (H_{Gln-2}) and the pyrrolidine ring protons ($H-5$ and $H-2$). The three doublet peaks for H_a-5 correspond to the two diastereomers and, potentially, a rotameric form as the rest (R^1) is bulky and sterically hinders the free rotation.

Coupling of peptide XXFVIGAGQ Using the optimised reaction conditions, the peptide analogue, which showed inactivity towards the enzymatic cleavage, was successfully coupled to give **47** (Scheme 3.14).

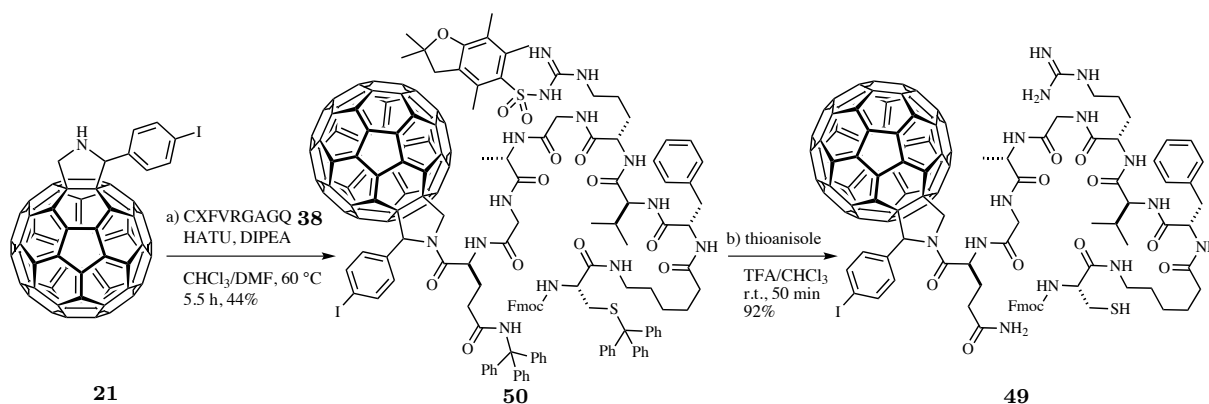
Scheme 3.14



Reagents and conditions: a) **21** (1 eq.), **39** (1 eq.), HATU (3 eq.), DIPEA (3 eq.), anhydrous DMF/ $CHCl_3$ 1:9 [3 mM], 60 °C, 21 h, 25% (33% on recovered **21**); b) **47** (1 eq.), PhSMe [10 mM], TFA/ CH_2Cl_2 1:1 [1 mM], r.t., 18 min, 84%.

Coupling of peptide CFVRGAGQ Without problems, the cysteine-bearing fullerene cork **49** was synthesised following the established protocol (Scheme 3.15). CHCl_3 was added to the deprotection reaction to circumvent aggregation. The presence of free sulfide groups without any disulfide formation was supported by matrix-assisted laser desorption/ionisation (MALDI).

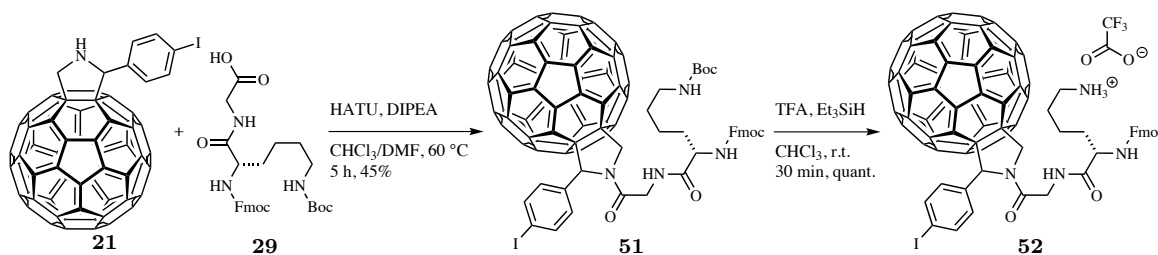
Scheme 3.15



Reagents and conditions: a) **21** (1 eq.), **38** (1.2 eq.), HATU (2 eq.), DIPEA (2 eq.), anhydrous DMF/ CHCl_3 1:9 [3 mM], 60 °C, 5.5 h, 44% (51% on recovered **21**); b) **50** (1 eq.), PhSMe [20 mM], TFA/ CHCl_3 3:1 [1.5 mM], r.t., 50 min, 92%.

Coupling of peptide GK Peptides of various lengths were synthesised to be used as shorter linkers between the CNT and C_{60} cork. The short peptide sequence glycine-lysine, which was further used for quantification of the CNT corking efficiency, was coupled successfully to fulleropyrrolidine **21** (Scheme 3.16). Residual acetic acid completely oppresses the synthesis of **51**.

Scheme 3.16



Reagents and conditions: a. **21** (1 eq.), **29** (1.5 eq.), HATU (2 eq.), DIPEA (2 eq.), anhydrous DMF/ CHCl_3 1:19 [3 mM], 60 °C, 5 h, 45% (53% on recovered **21**); b. **51** (1 eq.), TFA [20 mM], Et_3SiH [0.1 M], CHCl_3 [10 mM], r.t., 30 min, quantitative.

Coupling of peptide KFVIGE The coupling to the fulleropyrrolidine of six amino acids containing peptide sequence KFVRGE **33** and the potentially enzymatic resistant analogue KFVIGE **34** was investigated and showed to be unsuccessful under previously established conditions. Many solvents, temperatures, and times were tested (Table 3.2).

Changing the reagents and re-purification of the starting material did not affect the reaction outcome. These results, combined with the observed poor solubility of the very apolar peptide **34**, were found not to be promising enough to continue optimisation thereof.

Table 3.2: Various reaction conditions for the coupling of peptide KFVRGE **33** or KFVIGE **34** to fulleropyrrolidine **21**.

Reaction scheme: Fulleropyrrolidine **21** (a C₆₀ fullerene cage with a pyrrolidine ring) reacts with peptide **33** or **34** in the presence of HATU and DIPEA in a solvent at temperature T for time t to form the coupled product **53** or **54**. The peptide structures show various side chains (R) and protecting groups (Boc, Fmoc).

	SOLVENT	T [°C]	TIME	OBSERVATION
1	CHCl ₃ /DMF 9:1	70	19 h	gel-like solution, preactivation, no couplig
2	CHCl ₃	r.t.	2 h	no preactivation
3 ^{a)}	CHCl ₃ /DMF 9:1	60→80	4+3 h	purified 34 , gel-like, preactivation, precipitation, no coupling
	then NMP	80	18 h	traces of coupling product by MALDI
4	CHCl ₃ /NMP 9:1	50→80	2+14 h	preactivation, precipitates, no couplig
5 ^{b),c)}	CHCl ₃ /NMP 9:1	60→75	15+6 h	preactivation, no coupling
6 ^{b)}	NMP	r.t.	20 min	preactivation decolourised
7	CHCl ₃ /DMSO 25:1	r.t.	30 min	no preactivation
8 ^{a)}	CHCl ₃ ^{d)} /DMSO 1:1	r.t.→60→80	2+2+14 h	purified 34 , preactivation decolourised, precipitation, no coupling
9 ^{e)}	CHCl ₃ /DMSO 2:1	r.t.→60→80	15 h	traces of product 53 by MALDI

a) a fresh bottle of DIPEA was used; b) NEt₃ was used as a base; c) HBTU was used as a coupling reagent; d) neutralised with neutral alumina; e) peptide KFVRGE **33** was used.

3.2.3.2 Solid Phase Peptide Synthesis with Fullerenes

Modified fullerenes have been previously used as unnatural amino acids in solid phase peptide synthesis (SPPS).²⁶⁵ Here, the idea was that the peptide linker could be synthesised on the fC₆₀ cork directly when the fulleropyrrolidine was used as the solid support. An advantage of this system would be the partial solubility of fC₆₀ in organic solvents.⁶⁵ The homogeneous coupling reaction could be combined with the facilitated ‘washing and filtration’ purification of the precipitated product.

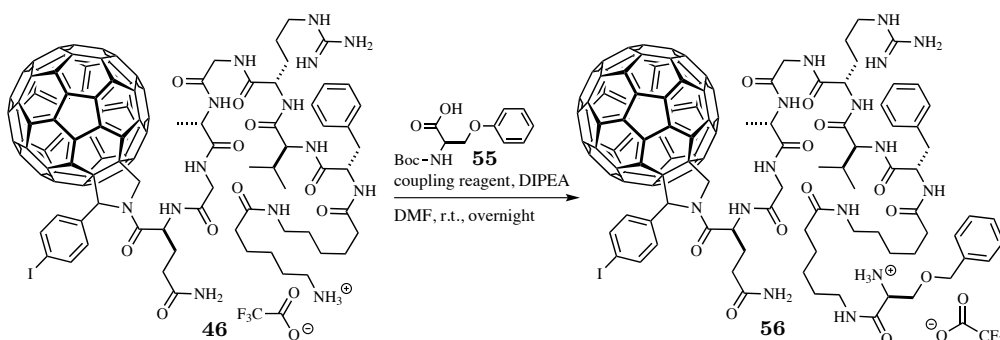
As shown in Table 3.1, the first amino acid was successfully coupled to the fulleropyrrolidine. The subsequent Fmoc-deprotection of **42** was undertaken with 20% v/v piperidine in DMF.⁴ The starting material was consumed completely. Black precipitate was formed that could not be characterised by nuclear magnetic resonance (NMR) and MALDI.²⁶⁵ Changing the conditions

to 5% v/v piperidine or tetra-*N*-butylammonium fluoride (TBAF)²⁶⁶ in DMF did not lead to either product formation or starting material recovery.

Nevertheless, an addition of bases revealed the formation of cleaved dibenzofulvene by thin layer chromatography (TLC) and NMR, which suggested that the free amine on the fC₆₀ rendered the material insoluble due to strong polarity differences. A subsequent coupling to a second amino acid was assumed to improve the solubility for characterisation by TLC, NMR, and MALDI, but no product formation was observed. In conclusion, the basic deprotection methods of **42** made the substrate unreactive towards further modification.²⁶⁵

This problem was overcome by installing a Boc-group on the N-terminus, as shown above by the successful acidic cleavage of various peptide modified fC₆₀. One of these functionalised fullerenes, **46**, underwent a subsequent coupling procedure to extend the amino acid sequence by one (**55**, Scheme 3.17). The coupling reagents EDC and HATU were compared, and they both gave product **56** in marginal to good yields. Nevertheless, residual trifluoroacetic acid (TFA)

Scheme 3.17



Reagents and conditions: a) **46** (1 eq.), **55** (1 eq.), EDC (1.5 eq.), DIPEA (1.5 eq.), anhydrous DMF [1.6 mM], r.t., 20 h, 21%; b) **46** (1 eq.), **55** (1 eq.), HATU (1.5 eq.), DIPEA (1.5 eq.), anhydrous DMF [1.6 mM], r.t., 14 h, 73%.

from the starting amine resulted in *in situ* Boc-cleavage of the desired product and a mixture of uncontrollable coupling side products. This could potentially be circumvented by an anion exchange of the starting material.

In summary, various deprotection- and peptide-coupling procedures on both Fmoc- and Boc-protected peptide functionalised fulleropyrrolidines were tried; none of these showed promising results to be further investigated for C₆₀ supported SPPS. Each reaction step gave low yields and revealed the need of column chromatography purification. The fC₆₀ system proved to not be comparable to the existing SPPS²⁶¹ and applicable for the peptide-linked cork synthesis.

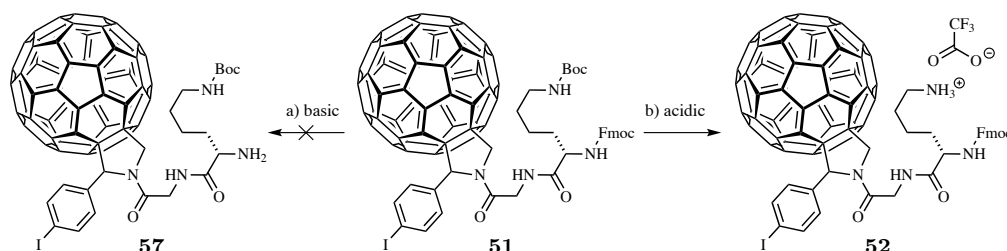
3.2.3.3 Deprotection of Functionalised Fullerenes

Basic deprotection Fmoc-protected XXFVRGAGE functionalised fulleropyrrolidine **44** was synthesised to be used as an enzymatic cleavable cork on CNTs (Section 3.2.3.1). Therefore, the terminal amine was deprotected using piperidine 5% v/v or TBAF in DMF or DBU/DMSO/DMF 1:25:25. No product could be identified in the crude reaction mixtures by TLC, NMR in different

solvent systems, mass spectrometry, MALDI with *trans*-2-[3-4-*tert*-butylphenyl]-2-methyl-2-propenylidene]malononitrile (DCTB), α -cyano-4-hydroxycinnamic acid (CHCA) and without matrix in positive and negative ionisation mode, and high performance liquid chromatography (HPLC) (TSKgel ODS 80TM, *i*PrOH/hexane 90:10, $t_{21} = 4.6 \text{ min}$, $t_{44} = 10.6 \text{ min}$). The complete consumption of the starting material, with concurrent formations of dibenzofulvene and an insoluble, dark brown precipitate, suggested product decomposition under basic conditions or multiple successive additions of the base.²⁶⁵

Stability of fulleropyrrolidines The decomposition of peptide-modified fullerene in the presence of a base was investigated to confirm its inability to be used in the cork synthesis. A shorter peptide sequence with orthogonal protecting groups was used to shed light on this problem (Scheme 3.18). Under acidic conditions, the Boc-group of fulleropyrrolidine **51** was successfully cleaved to give **52**. Under basic conditions, the solution turned black instantaneously with the addition of the first drop, and neither product nor starting material could be identified by TLC and HPLC. The insoluble precipitate could not be analysed by NMR. Peptide-modified C₆₀ was shown not to be stable under basic deprotection conditions.

Scheme 3.18



Reagents and conditions: a) i. **51** (1 eq.), piperidine (2 eq.), DMSO [0.7 mM]; ii. **51** (1 eq.), piperidine 5% v/v, DMSO [0.5 mM]; iii. **51** (1 eq.), piperidine 5% v/v, DMF [2 mM]; b) i. **51** (1 eq.), TFA/CHCl₃ 1:1 [5 mM], r.t., 27 min; ii. **51** (1 eq.), TFA [20 mM], Et₃SiH [0.1 M], CHCl₃ [10 mM], r.t., 30 min, quantitative.

The question whether the peptide modification or the functionalised fullerene itself caused this result was addressed. Fulleropyrrolidines **51** and **21** were found to be stable in solvents such as DMF, dimethyl sulfoxide (DMSO), and chloroform (CHCl₃) for at least 74 h. The addition of bases such as piperidine (5% v/v), TBAF [0.05 M], and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (20% v/v) showed fulleropyrrolidine decomposition in 72 h, 4 h, and 1 min, respectively. In conclusion, the pyrrolidine moiety proved to be not stable under these basic conditions, and the Fmoc-protecting group in the designed cork system had to be avoided (Section 3.2.2.1).

Acidic deprotection The previous results led to the installation of N-terminal Boc protection. These fully protected peptides coupled on fC₆₀ underwent the deprotection in dilute TFA/CHCl₃. The Boc and trt-groups were successfully removed, but the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (pbf) group was found to be untouched. An extension of the reaction time overnight led to fulleropyrrolidine decomposition. An increased concentration using pure acid gave fully deprotected products. To circumvent any side reaction of these harsh conditions,

scavengers such as triethylsilane or thioanisole were added. The mixture TFA/PhSMe 10:1 showed good results in only 30–40 minutes (see Schemes 3.13, 3.14, and 3.15). The addition of accelerators, such as TMSOTf,²⁶⁷ TMSBr, or HBF₄, did not advance the deprotection.

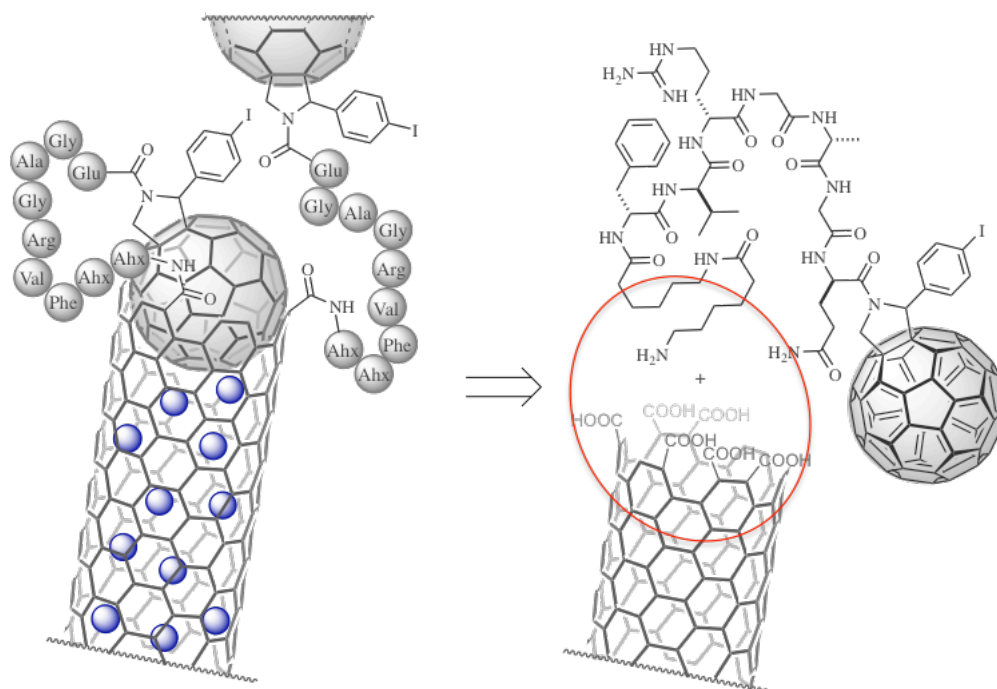
3.3 Outlook

In this chapter, the successful synthesis of the peptide linked C₆₀ fullerenes, to be used as cork for CNTs, was described. First, the modification of the C₆₀ to introduce an amine was approached. Various peptides of different amino acid sequences, lengths, and reactive coupling motives were designed, synthesised, and characterised. Optimisation of the amide coupling of the aforementioned peptides to fC₆₀ was undertaken. Problems regarding the final deprotection and inherent fC₆₀ stability were circumvented by changing the peptide design. The resultant constructs possessed a fullerene ‘cork’, a heavy element tag such as an iodide atom for identification purposes *via* scanning transmission electron microscopy (STEM), and a recognisable peptide sequence for the enzymatic cleavage. Further, a reactive nucleophilic group for further coupling onto the CNTs was present.

IV. Characterisation of Corked Carbon Nanotubes

In the second project, the reversible sealing of filled carbon nanotubes (CNTs) and their potential use as a drug delivery system (DDS) was investigated. The approach for an enzymatically triggered release of the payload was to use C_{60} fullerene (C_{60}) corked carbon nanotube systems made of oxidised CNTs, a peptide linker, and modified C_{60} (Scheme 4.1). The enzymatically cleavable peptide sequence was successfully attached onto the functionalised C_{60} surface, as described in Chapter III. In the following, the functionalisation of CNTs with these fullerene corks is described.

Scheme 4.1



A retrosynthetic approach to functionalised carbon nanotubes with an enzymatically cleavable peptide linked C_{60} as a DDS. In this chapter, focus is laid on the covalent functionalisation of CNTs and the characterisation of these products.

4.1 Background

The functionalisation of CNTs changes their properties *e.g.* conductivity and dispersibility and their potential utility in targeted delivery.^{4,22} Therefore, various reactions to modify CNTs have been established.

4.1.1 Covalent Sidewall Functionalisation of Carbon Nanotubes

Besides the covalent sidewall modification of CNTs, there are also various established methods to non-covalently attach molecules to their outsides to ameliorate their dispersibility.⁴¹ Covalent modification is advantageous for the specific release under *in vivo* conditions, which follows the cleavage of the permanent link between the carbon nanotube (CNT) and its functionalisation. Sidewall functionalisation reactions range from the use of radicals to cycloadditions, as described by many groups.^{39–43,91} Similar to the C₆₀ modification, the Bingel,²⁶⁸ Prato,^{4,269–271} and Diels–Alder^{272–274} reactions have been established on CNTs. Furthermore, various other [2+1], [2+3], and [2+4] cycloadditions have shown their utility, as well as carbene and nitrene undergoing addition to form cyclopropane and aziridine derivatives, respectively.²⁷⁵ Cyclopentenone, pyrrolidine, pyrazoline²⁰⁹ and isoxazole have been formed by 1,3-dipolar cycloaddition. Highly functionalised CNTs are formed from the Tour reaction, introducing alkyne and aryl groups *via* diazonium salt chemistry.^{276,277} Besides the nucleophilic addition of *e.g.* lithium alkynylides,²⁷⁸ the electrophilic Friedel–Crafts acylation has also been carried out. Further methods to form C–C bonds on the surface of CNTs, including radical reactions with reduced CNTs and diacyl peroxide derivatives²⁷⁹ or photolysed diazo compounds,²⁸⁰ have been accomplished. Fluorination of the CNT sidewalls has been successful, which could be further modified by nucleophilic substitution.^{100,101,281}

The introduction of chemically bonded groups onto the surface of CNTs modifies their properties, including solubility and functionality, and enhances their photovoltaic features.⁷² CNTs for biological application with targeting linkers have been approached.^{4,90}

4.1.2 Functionalisation of the Ends of Oxidised Carbon Nanotubes

Besides the above-mentioned surface functionalisation, the oxidation of CNTs is used to introduce reactive groups such as carboxylic acids, which can undergo further modifications.^{41,91} Initially, the oxidation was applied for purification and free commercial CNTs from carbon impurities and residual catalyst materials.^{282–286} The carboxylation procedure opens both tube ends and oxidises them. Defect sites on the sidewall are introduced by oxidation; the overall process shortens the tube lengths.²⁸⁷ Conditions to perform the selective end functionalisation while lowering sidewall destruction have been investigated using mixtures of oxidising acids and related techniques.^{288–291}

Besides enabling the purification of CNTs, the carboxylic acid groups have been subjected to amide and ester couplings^{39,41,43,91} to improve the CNT solubility^{52,93,292} and functionality. Mo-

dification of both the carboxy groups and the sidewall functionalisation have been reported to increase the dispersibility and potential of CNTs applications.^{40,42,271}

4.1.3 Synthesis of Fullerene-Carbon Nanotube Constructs

In 2007, Delgado *et al.* showed the first covalent modification of single-walled carbon nanotubes (SWNTs) with C_{60} and the subsequent cleavage of the newly formed amide bond under acidic conditions.²⁹³ The presence of C_{60} coupled *via* an amide bond ameliorates the electronic features inherent to CNT (Figure 4.1),^{294,295} whereas covalent attachment *via* a Tour reaction on the surface improved the optical properties of both materials.²⁹⁶

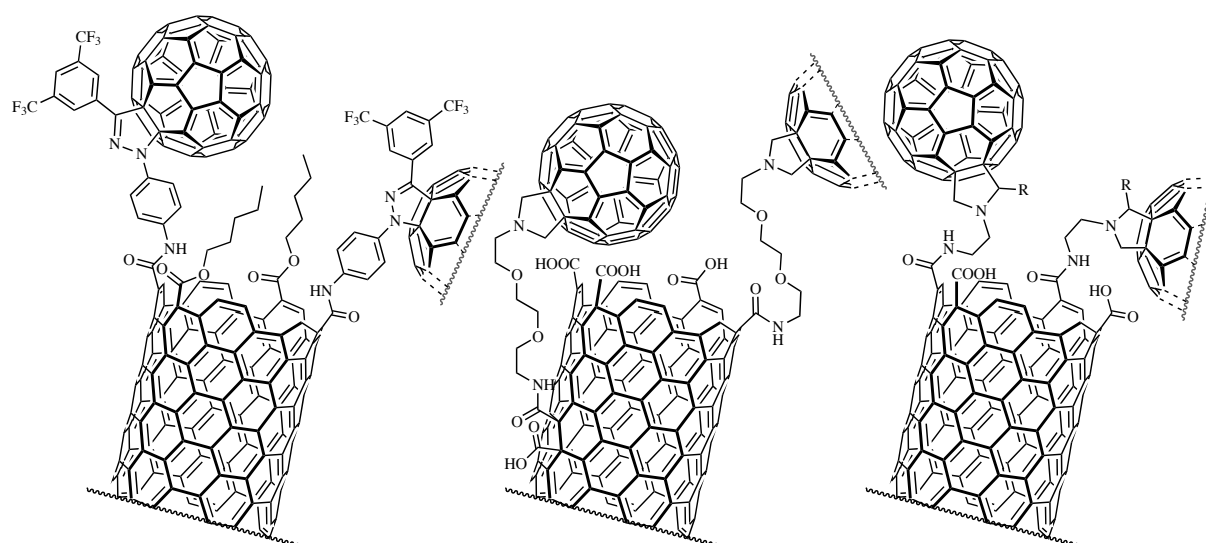


Figure 4.1: An overview of the CNT- C_{60} hybrid structures that have been made by amide coupling of oxidised CNTs.^{293–295} The R stands for various molecules, such as ferrocene, porphyrin, or DMAP.

4.1.4 Characterisation Methods Used for Carbon Nanotubes

The characterisation of the functionalised CNTs can give valuable information about reaction success and purity, which is especially critical when using them for biological applications. Problems arise due to the samples' inhomogeneity. Besides the wide range of tube lengths, the material tends to agglomerate because of π - π -stacking and van der Waals interactions forming bundles, which are difficult to separate. The CNTs vary in diameter, helicity, and, therewith, conductivity. Further, the defects in the sidewall structure are not predictable. The reactivity of each single tube can differ depending on these factors.

The diversity in each CNT sample requires complementary methods to achieve characterisation.^{91,119,283,297} The most frequently used techniques are described below.

IR spectroscopy Infrared spectroscopy is commonly used to characterise functionalised CNTs. Various techniques, such as attenuated total reflection Fourier transformed infrared

(ATR-FTIR), ZnSe supported, or KBr pellets, are standard techniques. The carbonyl group and aromatic C-C bond give distinctive peaks. The fingerprint region is less informative because this is masked by the strong reflection of the CNTs.²⁹⁸ In this thesis, most infrared (IR) spectra were recorded on a diamond ATR-FTIR crystal. Due to the similar refracting indices of diamond and CNTs, the recorded reflectance spectra showed weak signals.

Raman spectroscopy The characteristic features of a Raman spectrum are the radial breathing mode (RBM) ($150\text{--}300\text{ cm}^{-1}$), D-band (at $\sim 1320\text{ cm}^{-1}$), G-band (at $\sim 1575\text{ cm}^{-1}$), and G'-band (at $\sim 2610\text{ cm}^{-1}$). The wavenumbers may vary depending on the induced laser energy. The RBMs give information about the single-walled carbon nanotube (SWNT) and double-walled carbon nanotube (DWNT) diameters.^{86,299} The D-band shows defects in the tubular nanotube sidewall structure: The stronger the peak, the more porous and holey the CNT sample is. The G-band reflects the longitudinal stretch vibration of sp^2 -hybridised carbons, and according to the shape, the samples' inherited conductivity can be described.^{283,300–302} The G'-band is a second overtone of the D-band.³⁰³ Raman spectroscopy is commonly used to determine the level of sidewall destruction during modification procedures *via* the I_D/I_G band intensity or A_D/A_G band area ratios. The values are relative. A comparison between the starting material and product reveals information about the destructiveness of the reaction to the sidewall by defect sites (holes, epoxides, hydroxy groups, and additions). In the following, the term A_D/A_G is given, and the smaller the obtained value, the better the value is because no sidewall functionalisation was undertaken.

HRTEM CNTs can be visualised by high resolution transmission electron microscopy (HRTEM), which is the only method used herein to localise the introduced functionality. The low nanometer resolution allows for an analysis of the C_{60} , as well as to distinguish between SWNTs and multi-walled carbon nanotubes (MWNTs). For a good image, it is crucial to find the right focal plane that enables unambiguous conclusions about the constructs. The grayscale images of CNTs are two-dimensional, meaning that close to the Scherzer-focus, the tube is visualised as two parallel black lines. A single layer of carbon atoms cannot be viewed; only the thickest part, both rims, show enough diffraction for the electron beam to give a contrast to the background. The sample is prepared on a copper grid with a holey carbon film as support; only the tubes sticking out from the bundles in the vacuum atmosphere of the instrument can be viewed. Due to the 300 kV high voltage electron beam utilised to give high resolution images, the CNT sample is rapidly destroyed, and it is hard to focus on the tube ends because these twist and wriggle under the beam. The use of solvents during the functionalisation process of CNTs introduces carbon impurities, which makes the interpretation of the images more difficult, as previously observed by Khlobystov *et al.*⁸²

STEM imaging This technique is applicable if the CNTs are filled or functionalised with heavy elements. The high-angle annular dark field (HAADF) mode can easily show atoms with a high

Z-number; they appear as bright spots in contrast to the light carbon element present in CNTs. For example, the introduction of an iodine onto the fC₆₀ cork was based on the unambiguous evidence for its CNT functionalisation by scanning transmission electron microscopy (STEM).³⁰⁴ Depending on the electron microscope and its resolution, individual atoms may be identifiable.³⁰⁵

EDX and XRF Inherent to the transmission electron microscope is a chemical characterisation tool: the energy dispersive X-ray spectroscopy (EDX). The electron beam is used to generate element-dependent X-rays that are detected and indicate the elemental composition of the sample. In combination with STEM, whole images can be mapped and analysed. The design of the fullerene corks involved the functionalisation with an iodine atom, which can be used as a tracer. Detection thereof unambiguously confirms its presence in a C₆₀-CNT hybrid. Further, besides a signal for carbon and oxygen, copper from the grid and iron from the catalyst used in CNT manufacturing are expected for the synthesised samples.

X-ray fluorescence spectroscopy (XRF) gives similar results but is not used *in vacuo* conditions and is, therefore, not as sensitive. Here, a synchrotron electron beam was used for characterisation.³⁰⁶

XPS This semi-quantitative procedure uses X-rays to analyse the elemental composition of samples by detection of the ejected electrons, which are element specific. Due to the high energy used in this technique, it is regarded as a surface sensitive method for characterisation. The measured energies reveal information about the atom, as well as its bonds, *e.g.* the carbonyl C=O bond has a different energy than a C-O bond. Quantification is dependent on the vacuum conditions as background absorption of carbon and oxygen occurs, as well as on the sample thickness and, therewith, the signal-to-noise ratio. The measured data herein had to be considered with caution because the ratios of iodine per carbon and nitrogen per oxygen gave inconsistent numbers of functionalisations.

TGA This method is utilised to evaluate the purity and composition, including functionalisation and filling of CNT samples.^{78,91} The weight loss of the sample is measured under heating in either an inert gas or air atmosphere and is associated with the combustion of the material. Functionalisation of CNTs can be quantified under inert gas combustion.²⁹⁵ In air, the determination of a thermogravimetric analysis (TGA) gives information about the number of different species present in the sample and the amount of residual heavy metal catalyst;³⁰⁷ although, the latter is not of great importance for the characterisation of the CNT-C₆₀ constructs. However, the assignment of mass losses can be ambiguous because the combustion of the materials depends on their local environment. This instrumental method gives variations that exceeds the precision and reproducibility due to the inhomogeneous nature of the CNT samples. However, this problem can be circumvented by a series of repetitive experiments.⁹¹

4.1.5 Fullerene-Carbon Nanotube Hybrids

Investigations of the covalent, regioselective end functionalisation of oxidised CNTs with peptide functionalised C_{60} fullerene corks are described in this chapter. Special attention is given to the characterisation of the newly formed amide bond between these two carbon materials. To confirm the formation of the hybrid structure, a combination of the above-mentioned analysis methods is applied. The project's aims are summarised in the following:

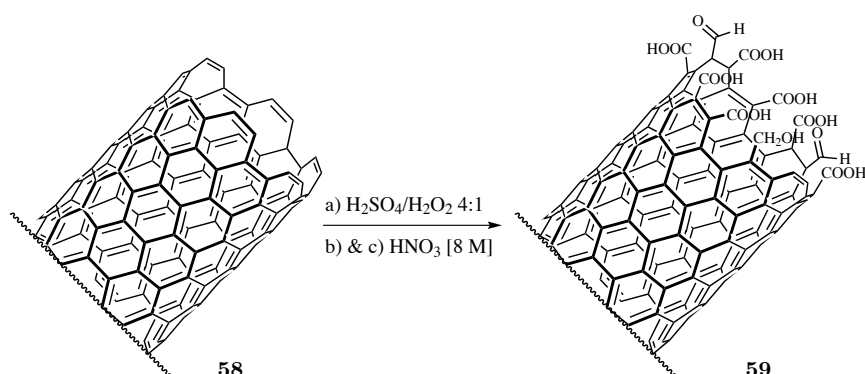
- Oxidation of CNTs with low sidewall destruction
- Highly, specifically end-functionalised CNTs
- Reveal the newly formed amide bond by IR spectroscopy
- Show the presence of fC_{60} in the hybrid structure with Raman spectroscopy, HRTEM, and STEM
- Quantification of corks in hybrid sample by XPS and TGA.

4.2 Results and Discussion

4.2.1 Oxidising Carbon Nanotubes

The carbon material had to be processed to undergo further functionalisation. The CNTs used in this work were manufactured by chemical vapour deposition (CVD) using iron as the catalyst.^{308,309} The crude material was steam purified^{310,311} by collaborators at the Institut de Ciència de Materials de Barcelona (ICMAB) to remove amorphous carbon, graphitic shells, and catalyst particles and to open the tube ends and shorten their lengths to an average of $L = 300$ nm. The carboxylic acid functionality was specifically introduced to the tube ends.^{284,288,289,312} Treatments with piranha (sulphuric acid and hydrogen peroxide 4:1) and nitric acid solutions were compared to circumvent unwanted side reactions, *e.g.* the introduction

Scheme 4.2



Reactions and conditions: a) SWNT, H_2SO_4/H_2O_2 4:1 (1 mL/mg), r.t., 1 h; b) SWNT, HNO_3 [8 M] (0.5 mL/mg), sonication, 40 °C, 30 min; c) SWNT, HNO_3 [8 M] (0.5 mL/mg), sonication, 40 °C, 1.5 h.

of sidewall defects (Scheme 4.2). Using sonication rather than heat with HNO_3 caused less sidewall destruction.²⁸⁹ The washing procedures using $\text{H}_2\text{O}_{dest}$ (Method a and b) and NaOH_{aq} (Method c) were compared.

Characterisation using ATR-FTIR, Raman, HRTEM, and X-ray photoelectron spectroscopy (XPS) was undertaken (Appendix Table 8.1). The IR spectra of oxidised CNTs showed the $1593\text{--}1597\text{ cm}^{-1}$ C=C stretch, as well as the C=O vibration at $1740\text{--}1749\text{ cm}^{-1}$ and C-O stretch at $1203\text{--}1223\text{ cm}^{-1}$; both of these bands were assigned to the carboxylic acid groups.^{295,301,310,313} With the help of Raman spectroscopy, the A_D/A_G band ratios of oxidised CNTs were calculated and compared to pristine CNTs. A slight increase of A_D/A_G between 6% and 8% was observed when oxidising CNTs with Method a and b. More effective were the NaOH washes (Method c) to remove oxidised carbon debris,^{285,290} which caused a decrease of the A_D/A_G ratio due to solubilisation and the removal of oxidised graphitic carbon (Figure 4.2).²⁸⁴

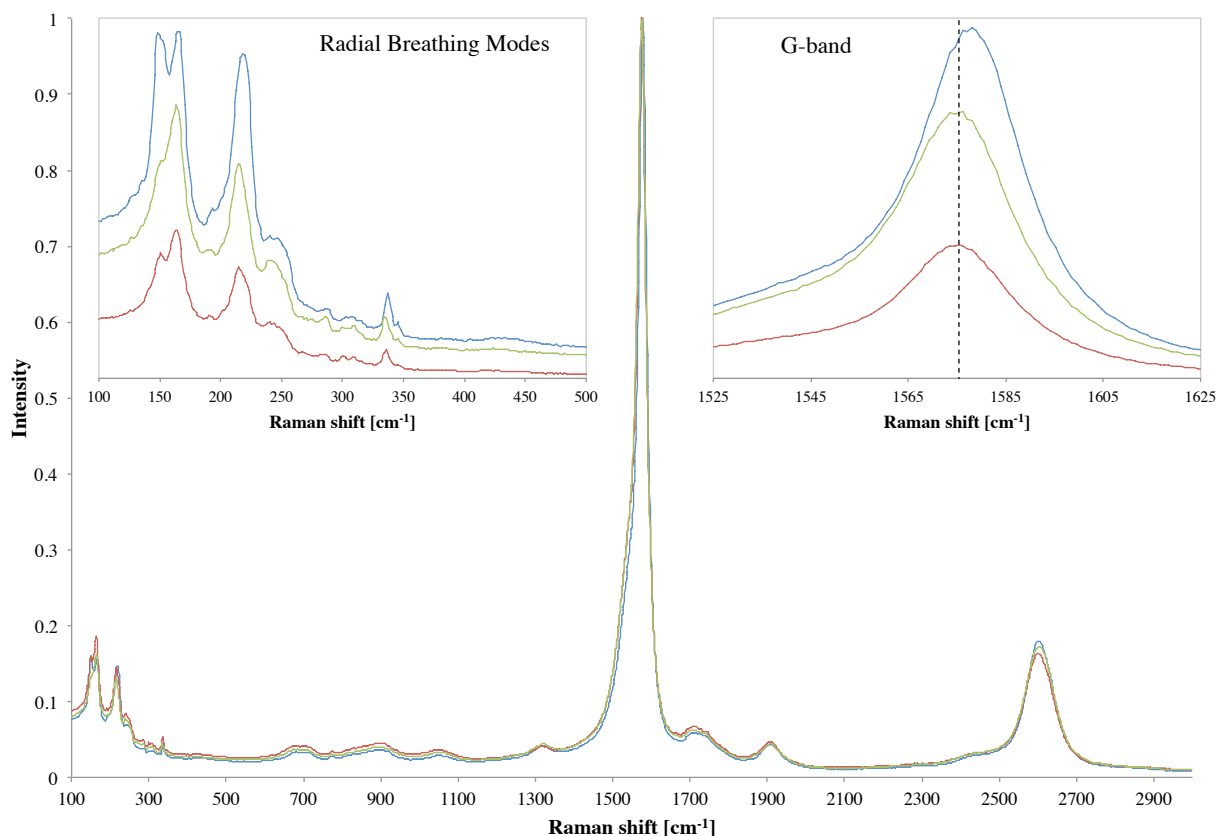


Figure 4.2: Three Raman spectra recorded on three different positions of oxidised carbon nanotubes' surfaces made in $\text{HNO}_3(aq)$ for 1.5 h (Method c) are normalised to one for comparison at their G-band maximum. The variation in the RBM peaks (left insert) show the different distributions of SWNTs and DWNTs for each scanned spot. The G-band shifts up to 8 cm^{-1} (right insertions), which can not be assigned to the presence of C_{60} .²⁹³

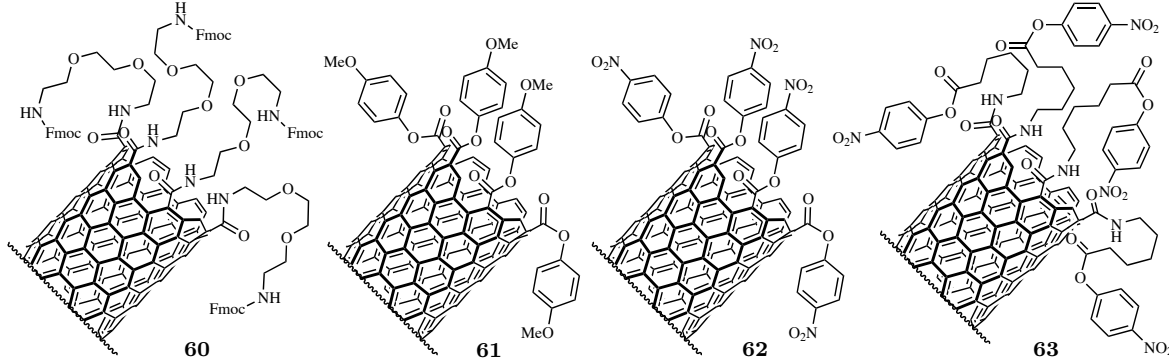
Piranha-solution-treated CNTs delivered nanotubes contaminated with amorphous carbon, as shown by HRTEM. The quantity of these impurities decreased upon treatment with nitric acid

and with longer reaction times. Semi-quantitative analysis by XPS revealed a carbon-to-oxygen ratio of 22.7 *versus* 17.5 for the piranha and nitric acid (1.5 h) solution, respectively. Additionally, the TGA of the oxidised CNTs (Method c) showed one combustion temperature at $T = 602.5 \pm 3.6$ °C,^{4,290,307} confirming the CNT-COOH purity. The selective oxidation of CNT ends was successful, and the material was subjected to further modification.

4.2.1.1 Quantification of Carboxylic Acid Groups

Functionalisation yields of fCNTs have been based on the number of introduced carboxylic acid groups. In two previous reports, the quantification of these has been verified by titration and have shown 6 mol% and 3 mol% (2.4 $\mu\text{mol}/\text{mg}$) oxidation.^{314,315} Here, the acid-base titration of two different CNT-COOH samples made with a piranha solution indicated the introduction of 56 mol% and 28 mol% of oxygen. These values were too high to be theoretically possible, so this evaluation method was discarded. The accuracy might be affected by the low test amount of only ~ 1 mg compared to 100–170 mg found in the literature.

Table 4.1: The quantification of CNT-COOH by functionalisation and subsequent cleavage of various groups. Repetitions were undertaken for some methods, revealing inconsistent results.



	METHOD	CONDITIONS	N [NMOL/MG CNT]	MOL% COOH ^{a)}
1	titration	0.1 M NaOH _{aq} , 23 h	46.3·10 ³ , 23.3·10 ³	56%, 28%
2	Fmoc 60	piperidine 20%, CHCl ₃ , 25 h	372.9	0.4%
		piperidine 5%, DMF, 150 h	408.8	0.5%
3	Fmoc 60 , <i>via</i> Boc	piperidine 20%, CHCl ₃ , 23 h	685.0	0.9%
		piperidine 5%, DMF, 150 h	454.9	0.5%
		control CHCl ₃ , 23 h	871.6	1.0%
4	benzoquinone 61	5 M NaOH _{aq} , CHCl ₃ , 25 h	1778.2, 4467.9 ^{b)}	2.1%, 5.3% ^{b)}
5	nitrophenolate 62	5 M NaOH _{aq} , CHCl ₃ , 25 h	57.6	0.1%
6	nitrophenolate 63	5 M NaOH _{aq} , CHCl ₃ , 24 h	189.1	0.2%
7	XPS of 59	carbon-to-oxygen ratio		5.5%, 7.1%
8	Fmoc of 66	piperidine 5%, DMF, 6 h	183.9	0.3%

a) For the calculation the approximation that the sample consists of 100% carbon was assumed; b) Coupling was promoted with DIPEA (1 eq.).

The following described methods relied on the reactivity of SOCl_2 preactivated CNTs^{52,284,304,315} with small organic molecules. The products are depicted in Table 4.1. The addition of a base released UV/vis-absorbing molecules that are useful for quantification.

Both the *N*-(9-fluorenylmethoxycarbonyl) (Fmoc) protected diamine (Entry 2) and the on the nanomaterial *tert*-butyloxycarbonyl (Boc) deprotected and Fmoc re-protected analogue⁴ (Entry 3) showed low functionalisation rates. The control reaction without the addition of a cleaving base showed similar results. All UV/vis absorptions from this so-called Fmoc-numbering could be referred to the background absorption.

Besides the Fmoc-numbering, various other UV/vis markers were tested for their usability for the quantification of introduced COOH groups. Using benzoquinone as the reporter moiety revealed a high functionalisation rate (Entry 4), which was further increased by the addition of a base to drive the ester formation.

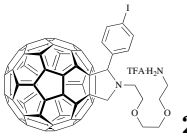
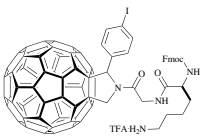
Labelling the carboxylic acids with a nitrophenol ester directly (**62**) or *via* a 6-aminohexanoic acid (Ahx) linker (**63**) revealed very low functionalisation that could possibly be attributed to a decreased reactivity or unwanted hydrolysis of the ester prior to measurement.

In conclusion, quantification of the functionisable carboxylic acid groups present at the CNT ends did not lead to unambiguous and reproducible results. The problem could have arisen from small test amounts, sample inhomogeneity, or falsification due to π -stacking of the aromatic labelling molecules onto the CNT surface. In contrast, the quantification results by XPS were found to be in good agreement with previous results but have to be considered with caution because this is a preparation-related surface sensitive, semi-quantitative characterisation method. For all following reactions, the amine was used in excess at 0.5–1 mass equivalent.

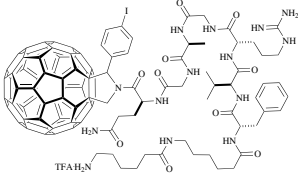
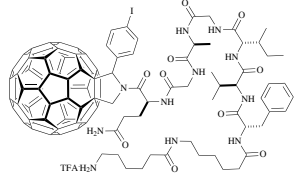
4.2.2 Coupling Peptide-Linked Fullerenes to Carbon Nanotubes

The corking of SWNTs with functionalised fullerenes was investigated using different linkers, solvent systems, and coupling reagents, as reported in Table 4.2.

Table 4.2: The table shows the conditions used to couple the trifluoroacetic acid salts of the various functionalised fullerenes to the carboxylic acid groups on the CNTs.

	CNT-COOH 59
 22	A) i. SOCl_2 , 70 °C, 24 h; ii. fC_{60} , DIPEA, CHCl_3 , r.t., 40 h
 52	B) i. SOCl_2 , 70 °C, 65 h; ii. fC_{60} , DIPEA, CHCl_3 , r.t., 41 h

IV. Characterisation of Corked Carbon Nanotubes

	CNT-COOH 59
 46	C) i. SOCl ₂ , 70 °C, 15 h; ii. fC ₆₀ , DIPEA, CHCl ₃ , r.t., 87 h D) i. SOCl ₂ , 70 °C, 15 h; ii. fC ₆₀ , DIPEA, CHCl ₃ , 70 °C, 87 h E) i. SOCl ₂ , 70 °C, 15 h; ii. fC ₆₀ , DIPEA, CHCl ₃ /MeOH, r.t., 87 h F) i. SOCl ₂ , 70 °C, 28 h; ii. fC ₆₀ , DIPEA, DMF, r.t., 40 h G) i. SOCl ₂ , 70 °C, 28 h; ii. fC ₆₀ , DIPEA, DMF, r.t., 86 h H) EDC, DIPEA, DMF, r.t., 40 h I) grinding, neat, r.t., 10 min
 48	J) i. SOCl ₂ , 70 °C, 25 h; ii. fC ₆₀ , DIPEA, CHCl ₃ , r.t., 70 h K) EDC, DIPEA, DMF, r.t., 39 h L) EDC, NHS, DIPEA, DMF, r.t., 39 h M) HATU, DIPEA, DMF, r.t., 39 h

Note: **46** was successfully coupled to Boc-serine-OH with the conditions N) EDC, DIPEA, DMF, r.t., 20 h, 21% and O) HATU, DIPEA, DMF, r.t., 14 h, 72% (see Scheme 3.17 in Section 3.2.3.2).

4.2.2.1 Corking of CNTs with the Diethylene Glycol-Linked C₆₀ Fullerene

First, a simple diethylene glycol-linked fullerene corking system was designed and synthesised using standard CNT coupling conditions (Condition A, Table 4.2).^{293,294} Characterisation by IR spectroscopy showed a new signal at 1686 cm⁻¹; this carbonyl vibration corresponds to an amide bond (Figure 4.3).^{294,295} Non-covalent salt bridges of functionalised CNTs such as CNT-COO⁻ NRH₂⁺ show a bond vibration peak that is red-shifted by approximately 57 cm⁻¹ in comparison to the covalent amide bond.²⁹² Their presence could not be excluded herein because the expected region was overlaid by the strong C=C vibration. Nevertheless, the coupling method used had previously shown excellent amide-bond formation.^{292-294,316}

The Raman spectra showed no new additional peaks compared to the oxidised CNTs, which could be due to low functionalisation.²⁹⁵ The previously reported red-shift of the peaks of approximately 5 cm⁻¹ of a C₆₀-linked nanotube could not be confirmed.²⁹³ Peapod structures cause a CNT diameter dependent on the up- or down-shift of the G-band.⁸⁵ However, after recording at least three spectra for each sample, shifts of up to 10 cm⁻¹ from one to the other were noticed (see also G-band insert in Figure 4.2). Each spectrum reflected the sample composition of the microenvironment at the recorded spot.^{79,88,317}

Analysis by HRTEM showed highly functionalised ends of the CNTs (15 functionalised out of 18 observed open tube ends). The number of fullerenes on each tube end varied and could not be accurately assigned. Furthermore, peapod structures of end-functionalised CNTs were observed. In order to fully remove all unbound fullerenes, the purification method might be improved, as discussed further in Section 4.2.2.3.

The EDX data showed an iodine signal, confirming its presence in the sample. Besides the expected Cu, Fe, C, and O content, Si (glass), Al, Ca, Na, S, and Cl were also detected. This could be due to contamination during the purification process, as well as residual thionyl chloride from the preactivation or detector dependent. The XRF revealed the presence of low levels of chlorine,

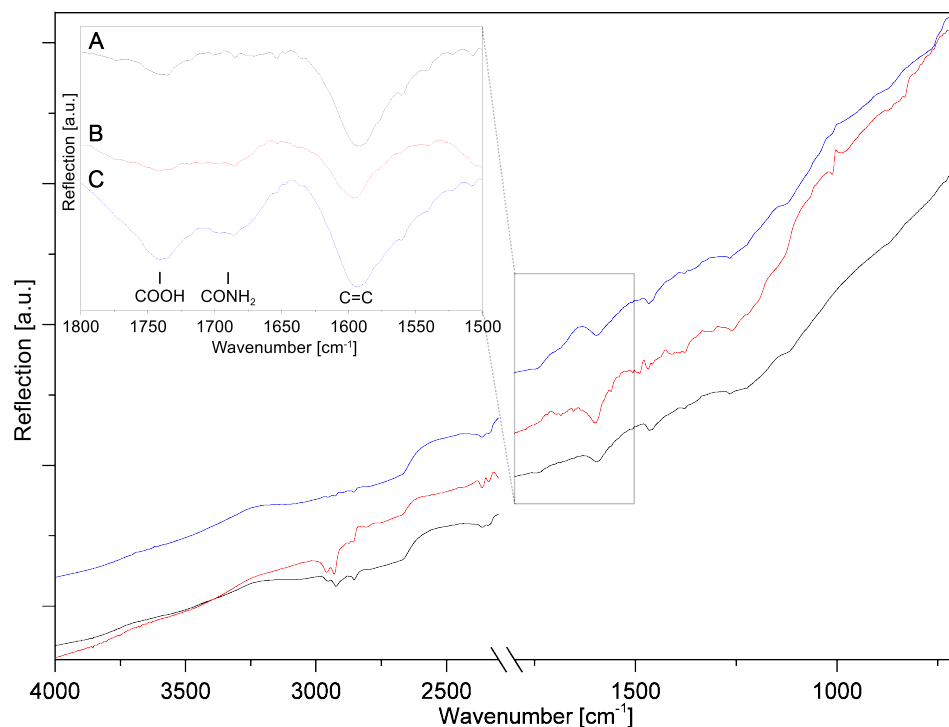


Figure 4.3: IR spectra of CNTs with characteristic bands at $\sim 1900\text{ cm}^{-1}$ for CH vibrations, 1595 cm^{-1} for C=C stretch,³⁰¹ as well as the C=O vibration at $\sim 1745\text{ cm}^{-1}$ for carboxylic acids,^{285,301,313} and $\sim 1690\text{ cm}^{-1}$ for amide bonds.^{294,295} For better comparability, the spectra in the insertion were baseline-corrected. **A**: The IR of oxidised CNT **59** revealed the presence of carboxylic acid functionalities. **B**: The spectrum of diethylene glycol-linked functionalised CNT **65** showed a second carbonyl vibration band coming from the amide bond formation due to successful coupling. **C**: The IR spectrum of GK dipeptide-linked fullerene functionalised CNT **66** revealed two distinctive C=O bands, which are characteristic for carboxylic acids and amides. The spectra were recorded by Dr. Robert Jacobs from the Surface Analysis Facility, Department of Chemistry, University of Oxford.

calcium, chromium, and nickel and large amounts of argon, iodine, and iron in sample **65**. The elements Cl, Ar, Ca, Cr, Fe, and Ni were also found in the background measurement, so they must come from the steel plate and tape holding the sample, and the surrounding air and can be neglected for the characterisation. The detection of low-energy atoms, such as oxygen, nitrogen, and sulfur, was not possible using this apparatus.

The XPS spectrum did not show the presence of other atoms besides carbon, nitrogen, oxygen, and iodine (Figure 4.4). This supports the hypothesis that the various other elements found by EDX and XRF were artefacts arising from the techniques and equipment and should not be overestimated for interpretation. Quantification displayed a ratio of one iodine per 117 carbon and two nitrogen atoms per nine oxygens; this corresponds to every fourth carboxylic acid group being functionalised.

In summary, there was evidence of a successful functionalisation of the carbon nanotubes with C₆₀ fullerene *via* a simple diethylene glycol-linker. Even though the analysis by Raman spectroscopy did not show the new functionalisation with the linked fullerene, as has been previously stated for CNT-C₆₀ hybrid structures,^{293,294} the elemental detection of iodine with various methods

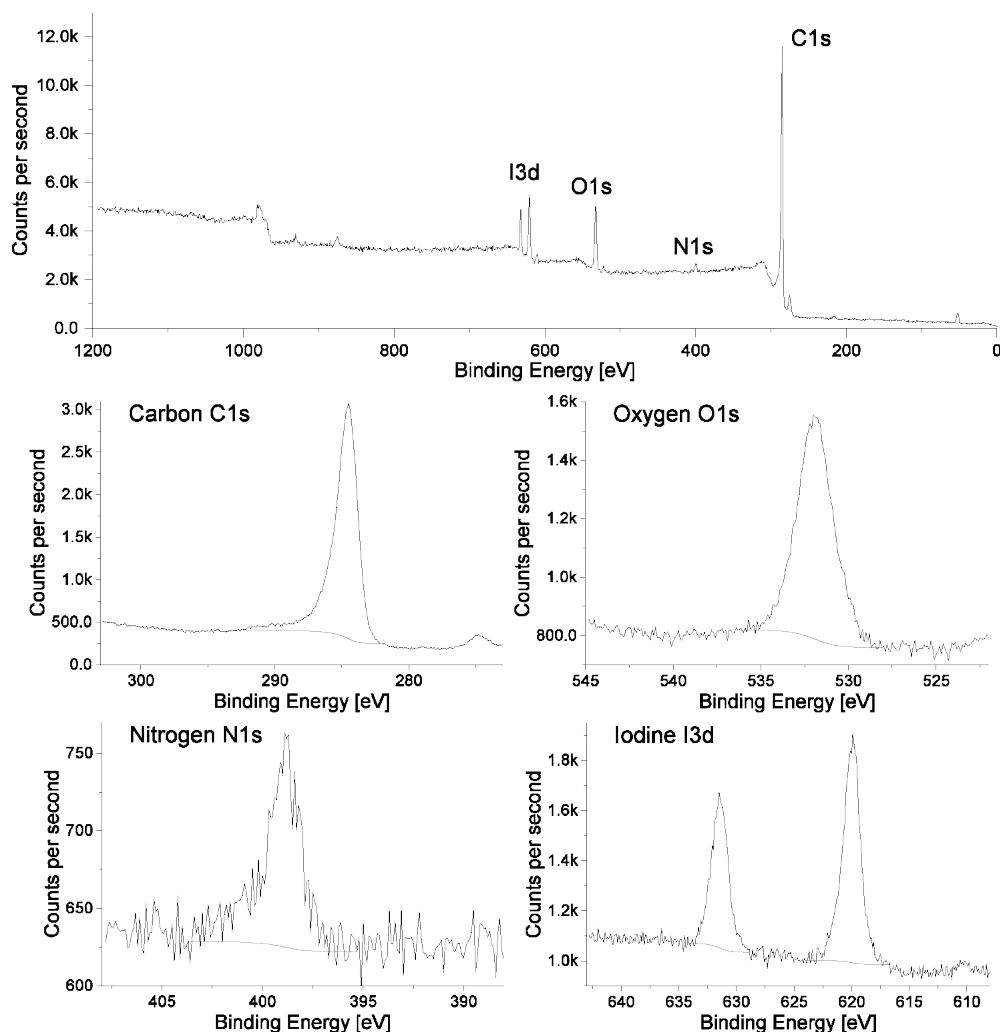


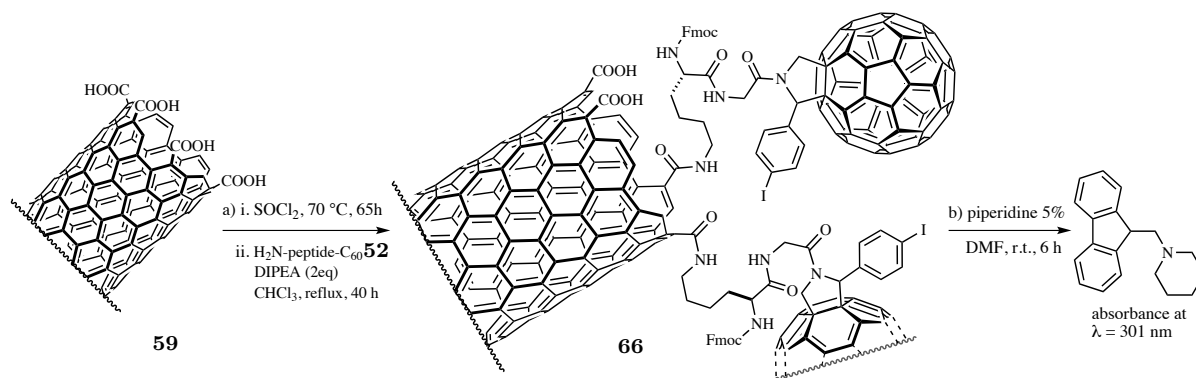
Figure 4.4: The XPS spectrum of functionalised CNT with diethyl glycol-linked fullerenes (**65**, Condition A) revealed a large amount of iodine. The spectrum was recorded by Dr. Ashley Shepherd from the Surface Analysis Facility, Department of Chemistry, University of Oxford.

and the visualisation by transmission electron microscopy (TEM) confirmed that the coupling to the CNTs ends was successful. The signal in IR revealed the formation of a new amide bond that confirmed covalent attachment of fC_{60} onto the CNTs and, therewith, the viability of the coupling method.

4.2.2.2 Corking CNTs with a GK Peptide-Linked C_{60} Fullerene

The successful conditions as used for the diethylene glycol-linked corked CNTs were applied using the peptide-functionalised fullerene **52** to give the CNT- C_{60} hybrid **66** (Condition B in Table 4.2, Scheme 4.3).

Scheme 4.3



Reagents and conditions: a) i. **59** (1 eq.), SOCl_2 [2 mg/mL], 70°C , 65 h; ii. **52** (1.4 mass eq.), DIPEA (2 eq.), CHCl_3 [0.2 mg/mL], r.t., 40 h; b) **66** (1 mg), piperidine (5% v/v), DMF [1 mg/mL], r.t., 6 h, quantitative.

The presence of the amide bonds was verified by IR spectra. Besides the peaks at 1597 cm^{-1} (consistent with a conjugated carbon bond) and 1751 cm^{-1} (consistent with a carboxylic acid carbonyl vibration), a new peak at 1686 cm^{-1} indicated the presence of amide carbonyl vibrations (Figure 4.3.C). This result did not assign unambiguously the new covalent bond formed during the coupling because amide bonds were already present in the peptide-functionalised C_{60} ; however, it can be assumed due to previous evidence observed under the same reaction conditions for the diethylene glycol-linked hybrid. Again, Raman spectroscopy did not show any particular features or significant changes that would support the presence of modified fullerenes in the sample. The A_D/A_G ratio was increased from 0.0998 to 0.1505 (51%) from the corresponding oxidised CNT. This value indicated an unexpected increase of destruction of the CNT sidewall structure by the non-invasive coupling reaction and may be due to, dependent on the microenvironment, observation of more contamination, such as graphitic carbon particles. The XPS spectrum confirmed the introduction of iodine, oxygen, and nitrogen into the CNT sample. The carbon-to-oxygen ratio decreased to 8.0 from 17.5 in the starting material, implying the introduction of functionalised C_{60} . The oxygen-to-nitrogen ratio revealed that every tenth carboxylic acid group was modified.

Introduction of a Fmoc group in the peptide sequence enabled the use of a complementary method of quantification⁴ of the coupling reaction. Analysis of the cleavage of the Fmoc group under basic conditions, as depicted in Scheme 4.3, indicated, by UV/vis absorption, a functionalisation of 183.9 nmol **52**/mg CNT. Calculations of a functionalisation rate of $f\text{C}_{60}$ per carbon atom were undertaken with equations 4.1 and 4.2 in which the molarity $M_{f\text{CNT}} = M_{\textbf{66}} = 12\text{ g/mol}$ was approximated.

$$n_{\text{Fmoc}} = n_{f\text{C}_{60}} = \frac{m_{f\text{CNT}}}{M_{f\text{CNT}} + x \cdot M_{f\text{C}_{60}}} \cdot x \quad (4.1)$$

$$x = \frac{M_{f\text{CNT}} \cdot n_{f\text{C}_{60}}}{m_{f\text{CNT}} - M_{f\text{C}_{60}} \cdot n_{f\text{C}_{60}}} \quad (4.2)$$

$x = 2.95 \cdot 10^{-3}$ equals a functionalisation of every 338th carbon atom with one fC₆₀ **52** (Table 4.1). This outcome was not in agreement with the observed ratio by means of XPS and could reflect incomplete Fmoc cleavage due to the formation of fC₆₀ peapod structures or π -stacking of the dibenzofulvene onto the nanotube walls.

In conclusion, the characterisation results were comparable to the outcome of the diethylene glycol-functionalised CNTs, except that the level of functionalisation as calculated from the XPS analysis appeared to be lower by 2.5-fold. These results indicated the modification and coupling of peptide-functionalised fullerenes to CNTs was achieved.

4.2.2.3 Purification of Functionalised CNTs

The purification of covalently functionalised CNTs is of essential importance to remove contaminating fullerenes and iodine that falsify the interpretation of the data. Several washing procedures, along with the optimisation of the coupling procedure, were explored.

The diethylene glycol-linked fCNT **65** was purified by washing and filtering the black solid multiple times using various solvents chosen based upon their fC₆₀ and reagent solubility, such as *N,N*-dimethylformamide (DMF), H₂O and chloroform (CHCl₃). After repetitive washing, uncoupled fC₆₀ was still found as peapods (17%) by HRTEM (Figure 4.5). This result led to a change in the purification protocol from ‘washing and filtration’^{4,70,82,99} to a ‘sonication and decanting’ procedure.^{105,314,318}

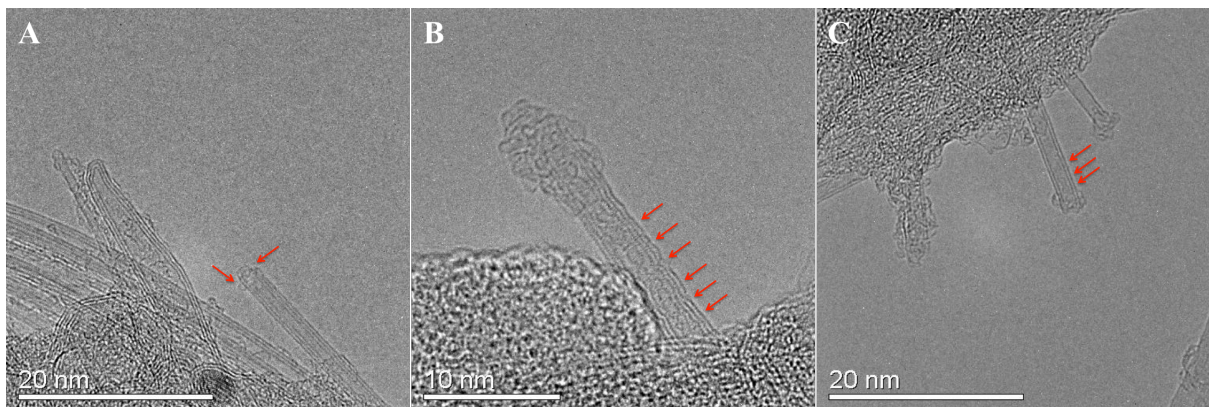


Figure 4.5: HRTEM images of the diethylene glycol-linked CNT-C₆₀ hybrid **65**. **A**. Fullerenes, indicated with the arrows, were solely found at the nanotube end and not inside; **B**. The DWNT was end functionalised and filled with non-covalently bound C₆₀ molecules to form so-called peapods. The C₆₀ are highlighted by arrows, and their round structures appear damaged by the electron beam; **C**. Two end-functionalised SWNTs, of which one was filled with C₆₀ fullerene, as marked by arrows.

First, the activated CNTs have to be quenched, which was normally carried out using water. Using dimethyl sulfoxide (DMSO) as the first washing solvent caused a nucleophilic functionalisation of the pre-activated carboxylic acid groups (compare Swern oxidation mechanism)³¹⁹. This was confirmed by TGA ($T_{combust.} = 274.8 \pm 4.2$ °C) and the presence of sulfur in the EDX and XPS of the corresponding samples. Even after avoiding sulfur-containing solvents for purifi-

cation, its presence by EDX was observed; although, the TGA traces did not show the oxidation of covalently bound solvent molecules as before. The sulfur appearance might result from a cross-contamination by other solvents or containers and not from residual thionyl chloride, as hypothesised before, because it was also detected in samples in which other coupling agents were used.

Different washing solvents used were DMSO, DMF, and benzonitrile (PhCN), which solubilised modified fullerenes and thereby removed residual, unbound fC₆₀.⁸⁸ The screening of those did not lead to improvement regarding the solvent efficiency in fC₆₀ removal. Five different coupling reactions were compared using the same washing procedure H₂O (3x) → DMF (3x) → MeCN (3x) → Et₂O (3x) and impurities, as well as peapod structures in unrelated quantities of 0%, 11%, 13%, 15%, and 24% were found by HRTEM. The number of washing steps should be controlled by the complete removal and disappearance of the fC₆₀ signal by ultraviolet/visible light spectroscopy or thin layer chromatography (TLC). The elimination of peapod structures might not be trivial because in those cases, the van der Waals interaction C₆₀↔CNT might have reached its energetic minimum.^{60,74,80} An analysis of all the peapod structures found by HRTEM revealed an average inner tube diameter of 1.61±0.41 nm (Figure 4.6), which was in agreement with the reported optimal fit of 1.3–1.5 nm with respect to the additional peptide modification. Complete removal of C₆₀ from these peapod structures, as reported, has not been successful.^{78,79,88} Furthermore, the carboxylic acid groups at the CNT ends not only hinder the C₆₀ from diffusing inside, which could be the reason why only a small amount of peapods formed,^{82,320} but might also be responsible for their capture.

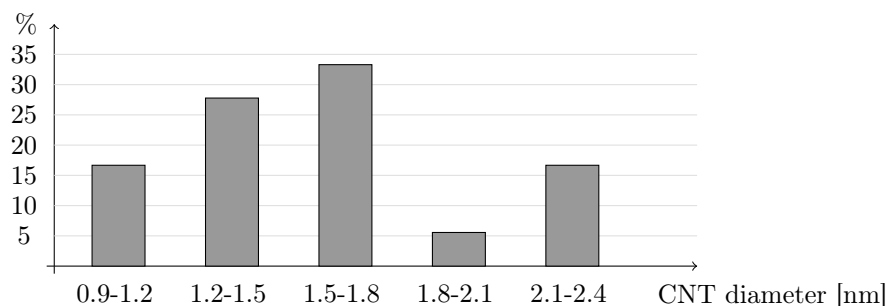


Figure 4.6: The percentage of peapods that were found after purification of CNT-C₆₀ hybrids by HRTEM was dependent on the CNT diameter.

To remove the barely volatile solvents, the washing procedure was finished with solvents such as CHCl₃, dichloromethane (DCM), and Et₂O. DCM should be avoided because large amounts of carbon impurities were observed by HRTEM, making the analysis difficult. Further, the detection of chlorine by EDX and XRF let the chlorinated solvent be avoided because it might not be volatile enough or the chemisorption too strong to be removed during the drying process. However, this was disproved because even when not using these solvents, a not negligible amount of chlorine was measured in the samples. This confirms the potential cross-contamination from other solvents or containers or could be due to an artefact in the measurements.

Analysis of Uncoupled Control Reactions As a reference for all the characterisation methods, the coupling reaction of CNT-COOH and peptide-functionalised iodo-fullerene **46** without any coupling reagent was investigated. Purification included sonication and decantation with (a) DMSO, H₂O, acetonitrile (MeCN), and DCM; (b) DMSO, PhCN, toluene, and DCM; (c) H₂O, DMF, MeCN, and diethyl ether (Et₂O), and (d) no solvents as a control. Method b and c were performed by Dr. Maria Vallée, which further tested the common applicability of this technique. Analysis by IR spectroscopy revealed carbonyl vibration bands characteristic for amide bonds in all four samples, indicating the contamination by the peptide-modified fullerene (Figure 4.7). In reference to the positive control (Method d) a slight decrease of the amide band intensity was solely found when using Method a, which could be due either to the choice of the first solvent for fC₆₀ removal and not initial quenching or the different preparation methods used by two people.

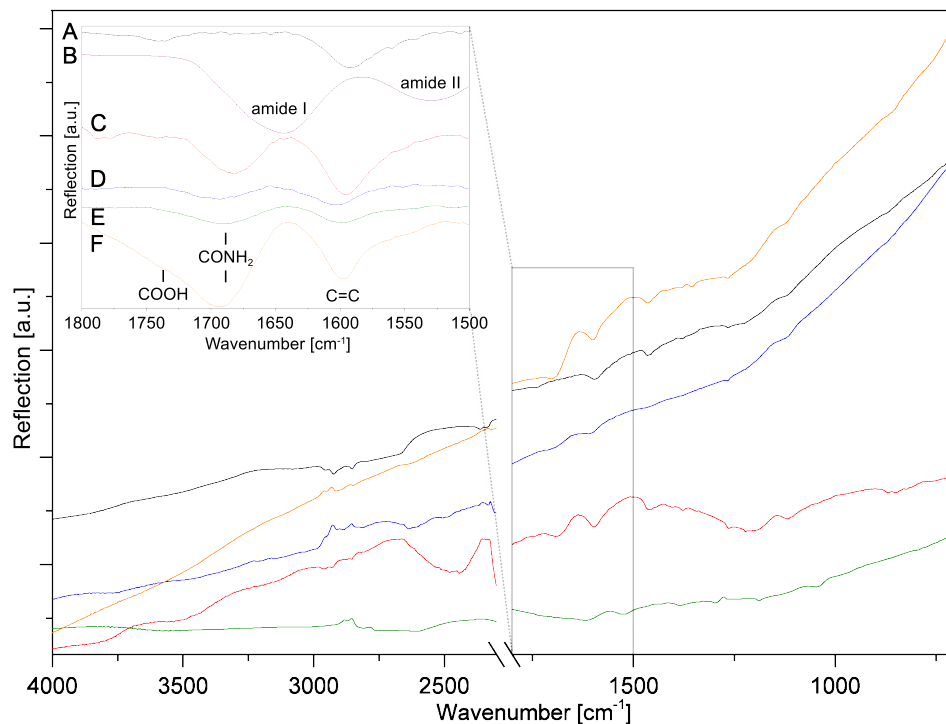


Figure 4.7: IR spectra of non-functionalised CNTs with XXFVRGAGQ-linked C₆₀ **46** (conditions: CNT, fC₆₀, DIPEA, CHCl₃, r.t., 91 h) with characteristic bands at $\sim 1900\text{ cm}^{-1}$ for CH vibrations, 1595 cm^{-1} for C=C stretch³⁰¹, the C=O vibration at 1745 cm^{-1} for carboxylic acids,^{285,301,313} and 1690 cm^{-1} for amide bonds.^{294,295} For better comparability, the spectra in the insertion were baseline-corrected. **A**: For comparison, the starting material CNT-COOH is depicted. **B**: Peptide-modified fC₆₀ with the amide I and II band at 1643 and 1530 cm^{-1} , respectively. **C**: The spectrum of the positive control without further purification showed characteristic vibrational bands for the CNT and peptide-modified fC₆₀. The amide peak is blue-shifted to higher wavenumbers compared to the free fC₆₀ due to the proximity of the amide bonds to the electron withdrawing and energy absorbing CNT. **D**: The sample was purified *via* Method a, leaving residual fC₆₀ **46** in the product. **E**: The sample was purified *via* Method b, showing fC₆₀ in the product. **F**: Sample purification *via* Method c left large amounts of fC₆₀ present in the product mixture, as revealed by the distinctive amide band. The spectra were recorded by Dr. Robert Jacobs from the Surface Analysis Facility, Department of Chemistry, University of Oxford.

Raman analysis of the two samples (positive control, Method d, and purification Method a) revealed increases of the A_D/A_G ratio. Interestingly, this was higher for the positive control (26%) than after the purification (17%), revealing that exposure to sonication for 70 min did not damage the tube walls. The rate increase might be related to the presence of functionalised C_{60} because this bears a high aspect ratio of defect sites (1:30). Furthermore, the remarkable fullerene peaks could not be observed due to the presumed degradation caused by the laser irradiation.

Analysis of the samples by HRTEM was informative, showing the presence of fC_{60} , which was associated with the inner tube sidewall and the ends. Only the open tube ends (50–59% in all samples) were considered for the following characterisation. Whereas the positive control sample revealed half of the CNTs to be filled with fC_{60} , the washing procedure decreased this portion to a quarter for Methods a and c and to 37% for Method b. Interestingly, only in the purified controls were the characteristic round shapes of C_{60} were found to be agglomerated at the tube ends. The numbers vary with each purification method (a: 25%, b: 84%, and c: 50%). Half of them were sparsely modified, with only one or two fC_{60} present at each CNT end compared to flower-like agglomerations. This finding suggested a very strong ionic interaction between the carboxylic acid groups on the carbon nanotubes and the amine and guanidine residue of the modified C_{60} , as predicted by calculations^{321,322} and reported as functionalisation methods.^{292,316} By EDX, carbon, oxygen, iodine, iron, aluminium, silicon, sulphur, and chlorine were observed in all tested samples. The finding of these elements in the control sample without purification (Method d) further supported the hypothesis that these contaminations derived from the storage container or electron microscope (EM) sample preparation. These artefacts were not related to the CNT constructs and can, therefore, be neglected.

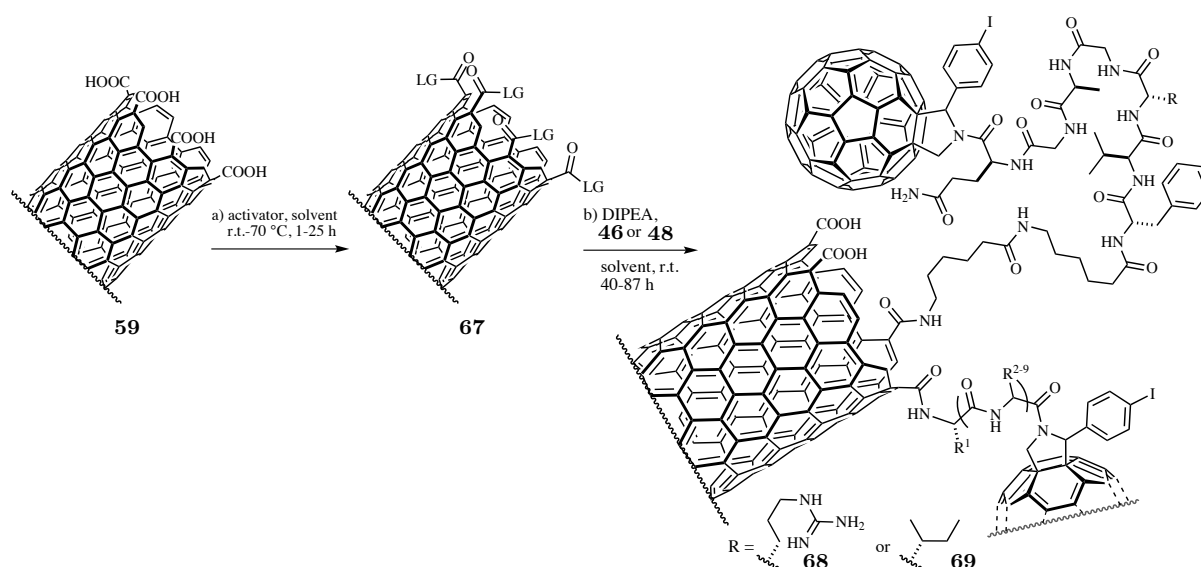
In summary, characterisation by means of IR, HRTEM, and EDX of all control samples revealed the presence of modified fullerenes. As judged by IR and HRTEM findings, the cleaning procedure undertaken by another person (Method b and c) left more fC_{60} impurities in the samples. This could either be ascribed to the implied solvent systems or indicating that the process was not universally applicable. The latter is more likely because in the following experiments with the same purification methods, less contamination was observed. No final conclusion on the optimal solvent composition during the washing process could be drawn. The purification method needs further improvement. Conclusively, simple washing procedures can not remove impurities because the interaction between CNT and C_{60} , as well as the carboxylic acid groups and the peptide, are too strong. In all following reactions, three potential modifications of CNT-COOH, such as covalent and ionic end functionalisation and non-covalent filling, were assumed to be present.

4.2.2.4 Corking of CNTs with the Active XXFVRGAGQ Peptide-Linked C_{60}

The previously mentioned successful functionalisation conditions for nanotubes were used for the attachment of the XXFVRGAGQ peptide-linked fullerenes (Condition C in Table 4.2,

Scheme 4.4). Low functionalisation using the standard SOCl_2 preactivation method was observed, which initiated the analysis of different reaction solvents (CHCl_3 , $\text{CHCl}_3/\text{MeOH}$, DMF, and neat), temperatures (r.t. and 70°C), and times (40 h and ~ 85 h). An increase in functionalisation was inherent with the improvement of functionalised fullerene solubility; DMF provided the best results, leading to the highest modification yield. The use of a chloroform/methanol mixture, though improving the fullerene solubility, showed low functionalisation – possibly due to nucleophilic competition. In the following paragraphs, the characterisation of those samples is described.

Scheme 4.4



Reagents and conditions: a) i. CNT-COOH **59** (1 eq.), SOCl_2 [1 ml/mg], 70°C , 25 h; a) ii. CNT-COOH **59** (1 eq.), EDC or HATU (2xmass), DMF [0.5 ml/mg], r.t., 1 h; b) **67** (1 eq.), **46** or **48** (0.4 mass eq.), DIPEA (2 eq.), CHCl_3 or DMF, r.t., 40–87 h.

Analysis by IR All samples displayed characteristic peaks for CH, C=C and carbonyl vibrations (Figure 4.8). A second, blue-shifted carbonyl band uncovered the presence of amide bonds, suggesting the successful introduction of the peptide derivative.

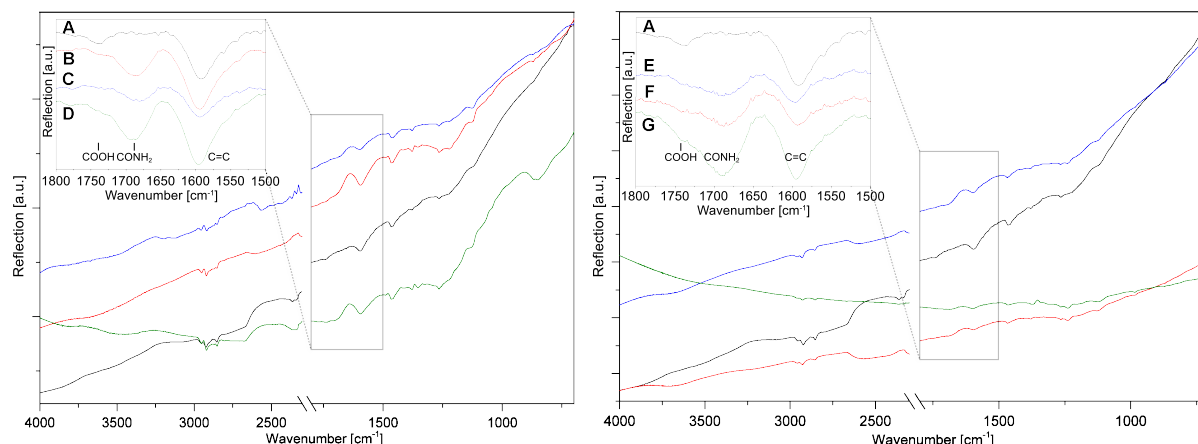


Figure 4.8: IR spectra of functionalised CNTs (**68**) with XXFVRGAGQ-linked C_{60} . Characteristic bands were found at $\sim 1900\text{ cm}^{-1}$ for CH vibrations, 1595 cm^{-1} for C=C stretch,³⁰¹ the C=O vibration at 1745 cm^{-1} for carboxylic acids,^{285,301,313} and $1688\text{--}1697\text{ cm}^{-1}$ for amide bonds.^{294,295} For better comparability, the spectra in the insertion were baseline-corrected. **A**: The CNT-COOH is depicted as a unmodified reference. **B**: The hybrid structure synthesised *via* Condition C. **C**: The CNT- C_{60} construct synthesised *via* Condition D. **D**: Synthesis Conditions E revealed the presence of amide, as well as carboxy bond vibrations. **E**: The IR spectrum of the hybrid structure synthesised *via* Condition F. **F**: Synthesis *via* Condition G. **G**: IR spectrum of fCNT made with Condition H showed distinctive presence of amide bonds. The spectra were recorded by Dr. Robert Jacobs from the Surface Analysis Facility, Department of Chemistry, University of Oxford.

Analysis by Raman spectroscopy SWNTs and DWNTs of different diameters were observed in the RBM region. The increase of the A_D/A_G ratios for all reactions besides two of 5–39% compared to oxidised CNTs could be ascribed to the presence of carbon debris and functionalised fullerenes. A decrease of 3% was discovered when methanol was present in the reaction mixture. Potential methyl ester formation might have facilitated the removal of oxidised graphitic carbon, which would thereby reduce the D-band intensity. Notably, when grinding the sample (Condition I), the A_D/A_G ratio increased by 184%. This could be attributed to the destruction of the conjugated carbon nanotube structure.

To summarise, the Raman analysis neither confirmed nor dismissed the functionalisation of CNTs with fullerenes. Although, examination of the measured compound surface through the optical microscope available in the Raman equipment revealed the presence of brown-golden fC_{60} particles incorporated into the black carbon material for sample ‘Condition G’, as it was observed for the diethylene glycol-linked fCNT **65**. The visibility and high coverage suggested good functionalisation; nevertheless, no spectrum of these spots corresponding to the functionalised C_{60} could be recorded, presumably due to their sensitivity and rapid degradation.

Analysis by HRTEM The presence of MWNTs, including DWNTs as well as SWNTs, was confirmed by HRTEM analysis. Closed tubes, unstructured carbon materials associated with the ends or clustered spherical carbon ($\varnothing = 7.0 \pm 1.2\text{ \AA}$) manifesting as floral shapes, as shown in Figure 4.9 and 4.10, were observed. The latter structures could be assigned to fullerene functionalised nanotubes^{293,294} with varying yields from 42–89% (see Table 4.3 and 8.4) The number

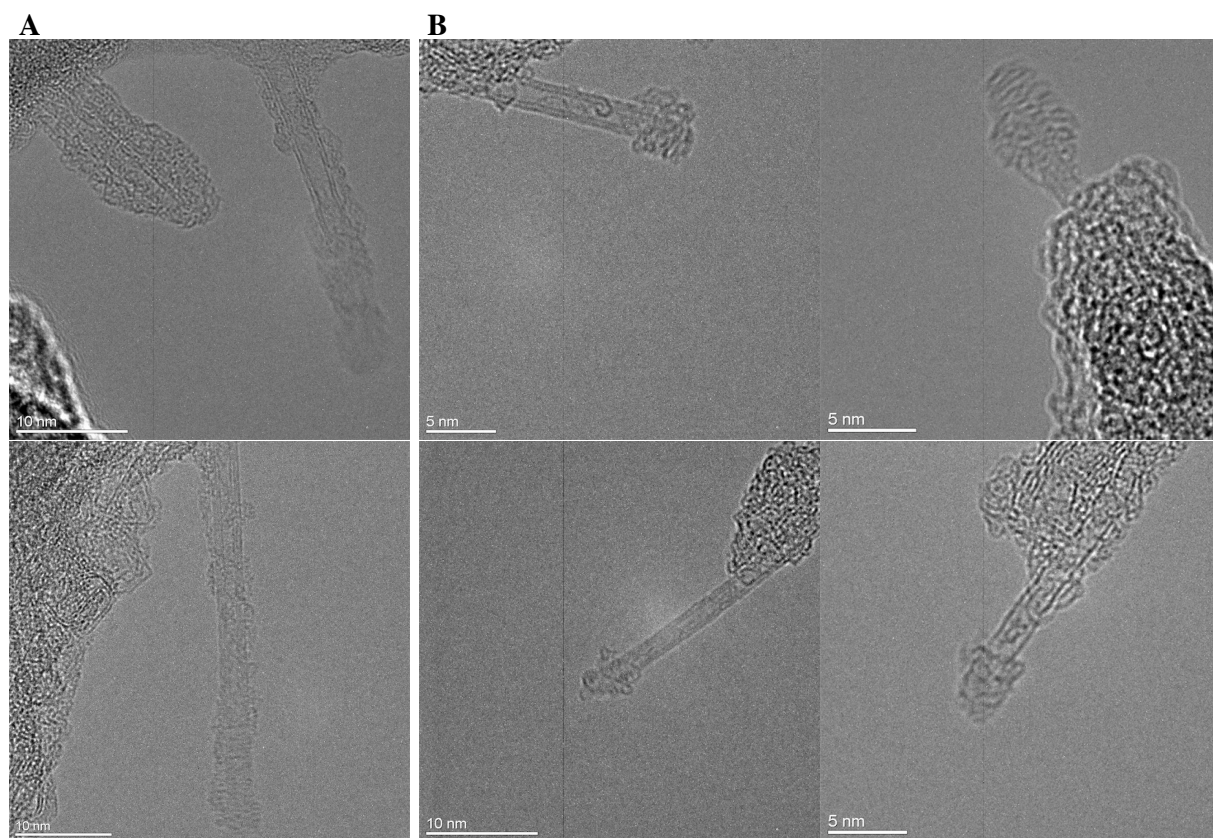


Figure 4.9: A selection of HRTEM images of fCNT **68** made with Condition E (in CHCl₃/MeOH). **A**: The carbon impurity and its association around the CNTs complicated the analysis. **B**: The CNT ends were functionalised with round shaped C₆₀ to give these floral shapes.

increased with the solubility of the modified fullerene and reaction time and can compete in the best case with the functionalisation yield obtained by HRTEM for the simple diethylene glycol-linked C₆₀ (Conditions A in Table 4.2). C₆₀ was not only attached to the ends, but also inside, forming peapods or, rarely, at the outer nanotube walls at defect sites. Nevertheless, HRTEM gave clear evidence of the successful tip-functionalisation, showing C₆₀ molecules clustering at the CNT ends to give flower- or mushroom-shaped structures.

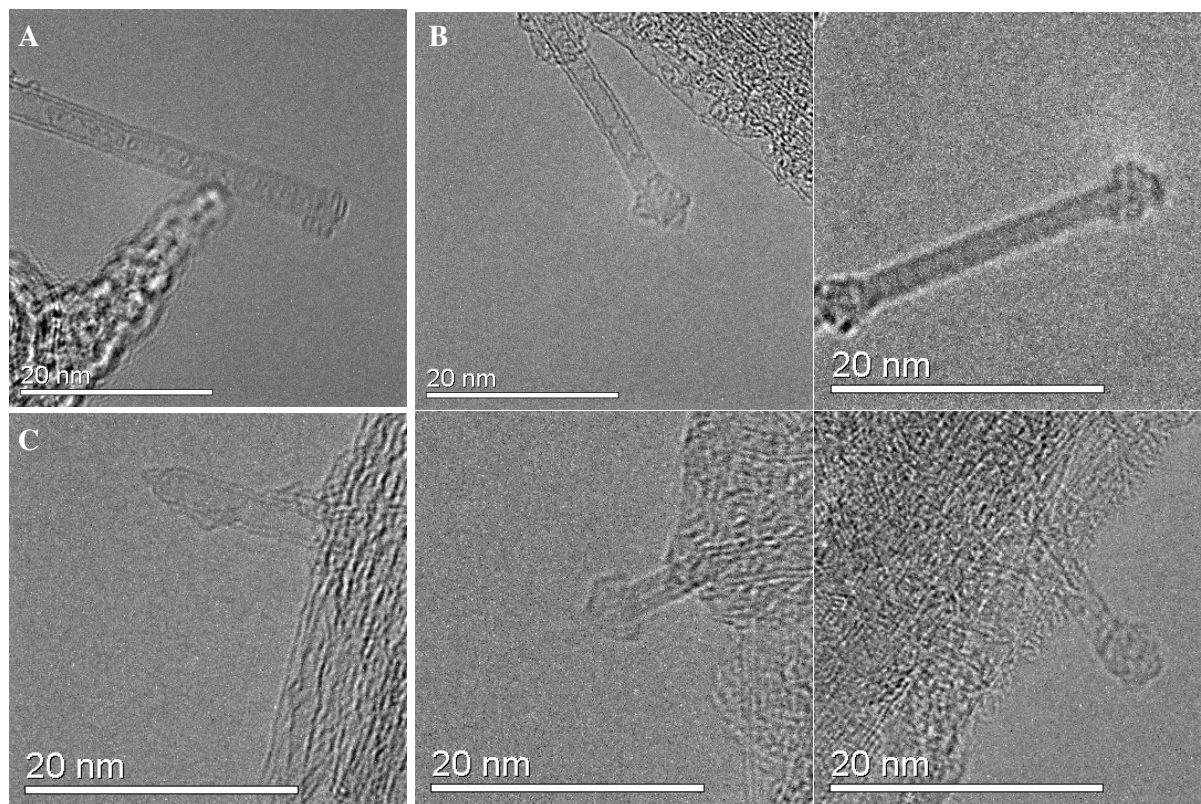


Figure 4.10: A selection of HRTEM images of fCNT **68** made with Condition F (DMF, 40 h). **A**: Depicted is a fullerene-filled and -corked DWNT. **B**: The ends were functionalised with C_{60} to give floral shapes of corked CNTs. **C**: A closed CNT sticking out of the bulk material.

Table 4.3: Comparison of the functionalisation yield $y = \frac{\text{number of } C_{60} \text{ end-functionalised CNTs}}{\text{total number of open CNT ends}}$, as observed by HRTEM for fCNT **68** using the different reaction Conditions C to I.

ENTRY	PREPARATION METHOD	CONDITIONS	FUNCTIONALISATION Y [%]
1	Condition C	CHCl_3 , r.t.	75%
2	Condition D	CHCl_3 , 70 °C	70%
3	Condition E	$\text{CHCl}_3/\text{MeOH}$, r.t.	69%
4	Condition F	DMF, 40 h	76%
5	Condition G	DMF, 86 h	89%
6	Condition H	DMF, 40 h ^{a)}	69%
7	Condition I	neat, grinding	42%

a) Carboxylic acid groups were preactivated with *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC).

Analysis by STEM One of the samples (fCNT **68** made with Condition E) was subjected to a ptychography supported-STEM microscope. Previous STEM attempts did not reveal a good enough resolution to identify the iodine atom tag and assign its localisation. Recent innovations enabled the simultaneous recording of the Z-contrast information by annular dark field (ADF) and the bright field electrons with a fast detector to solve the structure of fCNT at atomic-

resolution (Figure 4.11). Data analyses allowed *via* phase reconstruction from the scattered radiation, so called ptychography, for an image that were recorded by Dr. Yang at the Department of Materials.³⁰⁵

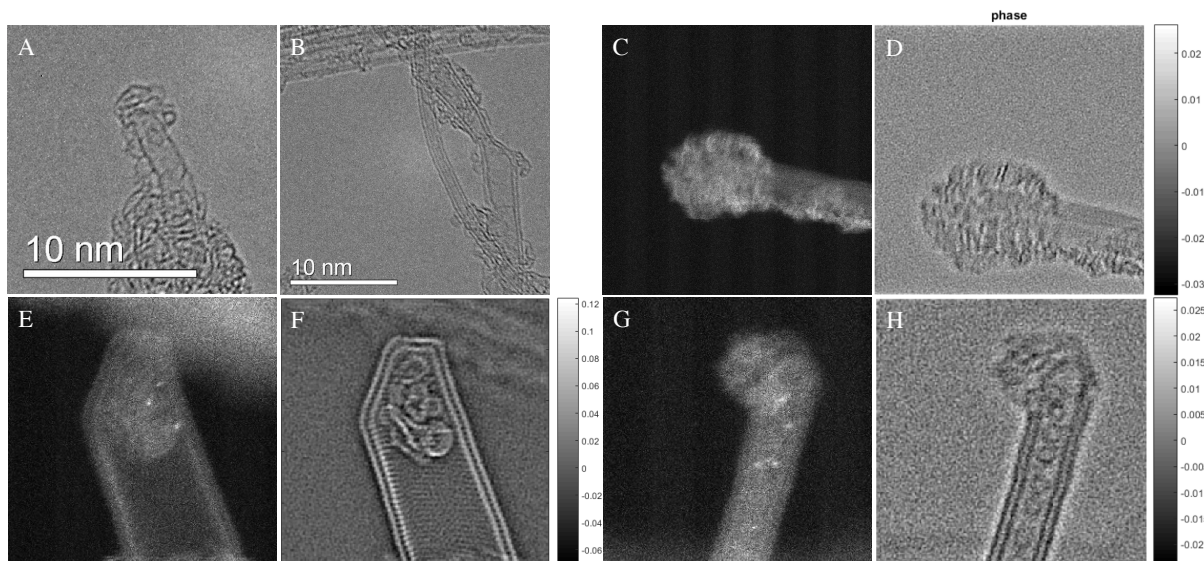


Figure 4.11: A selection of EM images of fCNT **68** made with Condition E (in $\text{CHCl}_3/\text{MeOH}$). **A**: The HRTEM shows at least two round shaped C_{60} molecules associated at the end of a functionalised SWNT. One is kept inside and corks the tube. **B**: The HRTEM revealed the functionalisation outside a DWNT on a potential defect site. **C**: A STEM image of a CNT with carbon agglomeration at the end. The brighter areas were the result of higher Z-contrast density reflecting overlaying carbon material. No iodine atoms were detected because those were not in the focal plane. **D**: The reconstructed phase of image C confirmed the carbon impurity. Because the image was not in focus, a conclusion if fC_{60} was present is difficult to make. **E**: The STEM image shows the end of a CNT with carbon materials inside. Due to Z-contrast imaging using an ADF detector, single iodine atoms could be detected as bright spots. **F**: The phase reconstruction of the image shown in C displayed a closed DWNT with fullerene contamination inside. **G**: The STEM image revealed iodine atoms inside the carbon nanotube. **H**: The phase reconstruction of image E disclosed a DWNT filled with fC_{60} as a peapod structure. Each bright iodine spot could be associated with a fullerene. The spectra were recorded by Dr. Hao Yang from the Department of Materials, University of Oxford.

The phase images, coupled with STEM, resolved single iodine atoms that could be associated to the appearance of fullerene molecules in the sample. This new high resolution technique led to the assignment of filled CNTs and the formation of peapod structures during the reaction, revealing non-covalent functionalisation. Because those structures were not found before, all previous HRTEM results were revisited for re-imaging and interpretation to show similar occurrences that are now reported herein. The formation of peapod structures is dependent on the CNT diameter,^{60,74,80} and if the optimal van der Waals radius was exceeded, the fC_{60} filled the tubes without a regular structure. Unfortunately, due to the difficult circumstances to take an image of a nanotube end in focus, not every structure could be unambiguously determined (see Figure 4.11.C and D). In conclusion, the phase reconstruction coupled STEM imaging can be used for further analysis of modified CNT samples and support the characterisation.

Analysis by EDX EDX coupled to the TEM was used to confirm the presence of the iodine-tagged fullerenes. For all samples, iodine was detected, even though they were in low quantities. Further, sulfur, chlorine, silicon, and aluminium were detected along with the expected copper, iron, carbon, and oxygen. These elements could be ascribed to sample contamination from the purification procedure or EM sample preparation. At the synchrotron (Diamond Light Source at the Harwell Science and Innovation Campus), XRF measurements revealed the presence of the element iodine in most samples. For only one example (sample preparation Condition E), no iodine was detected. The background measurement the elements Cl, Ar, Ca, Cr, Ni, and Fe were found.

Analysis by XPS Semi-quantification of the functionalisation yield and evaluation of the success of the different coupling methods was undertaken by XPS. As expected, the carbon-to-oxygen ratio decreased in all samples with the introduction of peptide-functionalised fullerenes compared to CNT-COOH (Table 8.4). According to the iodine-to-carbon ratio, the highest yield of modification was achieved in chloroform at 70 °C (Condition D) with one iodine every 321 carbons. This outcome stood in contrast with previous observations where the strongest signals (IR and EDX) and highest functionalisation yields (HRTEM) were found in other samples (Conditions E-G). It is likely that this measurement reflected the variability and unpredictable influence of the carbon in the background. Further, the nitrogen-to-oxygen ratio was calculated to give the best result for the Condition G (DMF, 86 h), revealing a functionalisation of every tenth carboxylic acid. For both results, significant discrepancies for the iodine-to-nitrogen correlation (1:12 and 1:20, respectively, ideally 1:14 from compound **46**) were observed. Nevertheless, the conclusion was that none of the samples showed a comparable functionalisation yield to the simple diethylene glycol-linked C₆₀-CNT hybrid.

Analysis by TGA The TGA measurements in the inert gas atmosphere of oxidised CNTs (**59**) showed a weight loss of only 3.5% for 100–470 °C, whereas functionalised nanotubes **68** (Condition G) lost 18.8% of the material. The increased value could be attributed to the residual organic molecules present in the sample that were introduced during the functionalisation reaction. The obtained curves were weak and of low accuracy; the peak resolution was improved dramatically when the TGA was undertaken in air, as reported in the following.

In the TGA measurements in air, three peaks below the temperature 580 °C were frequently observed. The first oxidation occurred at 274.8±4.2 °C and was solely found in samples with methanol in the reaction mixture or DMSO, as this was the first solvent used in the purification method. This peak might reflect the combustion of potentially formed esters.

The second oxidation temperature at 339.7±7.4 °C was found in all samples, as well as in a control sample of dry-mixed fullerene **46** and CNT-COOH (336.1 °C). Furthermore, the control sample and the samples of the reactions run in CHCl₃/MeOH (Condition E) or DMF (Conditions F and H) showed combustion of a species at T=529.0±38.7 °C. The assignment of both observed combustion temperatures was not unambiguous; they could be referred to the oxidation

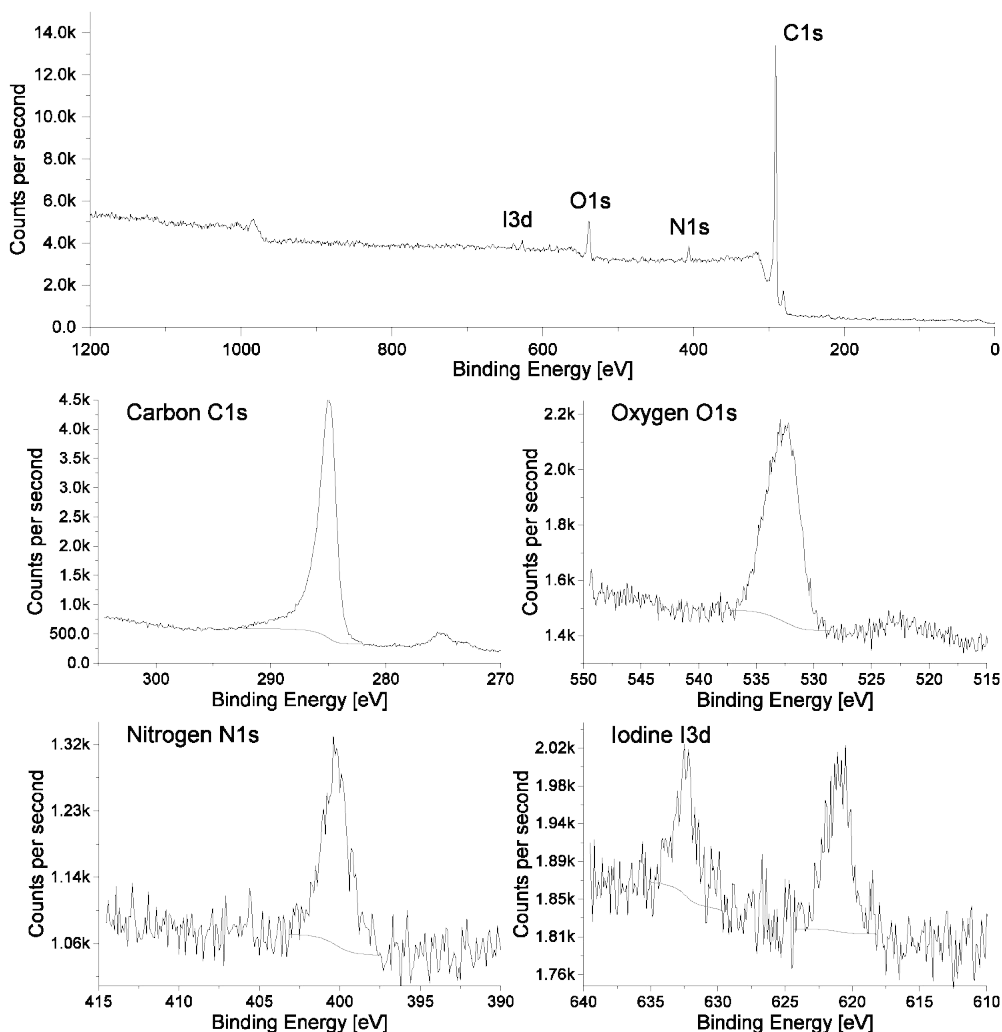


Figure 4.12: A representative recorded XPS for the modified CNT **68** with Condition F. Multiple carbon species (C-C/C-H, C-O, and C=O), two oxygen (C=O and O=C-O), one nitrogen, and iodine species were found. The spectra were recorded by Dr. Ashley Shepherd from the Surface Analysis Facility, Department of Chemistry, University of Oxford.

of both the fullerene and the peptide linker. According to Fan *et al.*, C₆₀ combusts between 300 and 540 °C.⁷⁸

In all samples, the majority of the material oxidised at 612.2±13.7 °C, which was in agreement to the combustion of CNT-COOH (609.7±8.9 °C).

All of the information gained from the analysis by TGA are (i.) the number of molecular contaminants in the sample and (ii.) the relative amounts of these, but even this output should be reviewed critically.⁹¹

Conclusions

The synthesis of peptide-linked CNT-C₆₀ constructs was likely achieved, as implied by the characterisation results. The presence of amide bonds in the modified nanotube samples was shown by IR spectroscopy and supported by the detection of nitrogen *via* XPS. Semi-quantification indicated a low degree of functionalisation of the CNTs. The covalent bond formation could not be measured; nevertheless HRTEM revealed the presence of C₆₀, located mostly at the nanotube ends, consistent with the covalent attachment to the carboxylate groups. Elemental techniques such as EDX, XRF, and XPS further supported the hypothesis of fC₆₀ introduction with the appearance of the characteristic iodine signal. The heavy atom was introduced as a tag on the C₆₀ cork, which could be further uniquely detected by ptychography-supported STEM. This novel technique revealed the presence of peapod structures of functionalised fullerenes in CNTs, confirming a strong non-covalent interaction. Only Raman spectroscopy did not give conclusive data to support the functionalisation. The conditions of the method proved to be too destructive for fullerenes and fullerene-containing samples.

The reaction studies revealed a strong dependency on the solubility of at least one of the starting materials (fullerenes). Solvents such as DMF improved the reactivity and functionalisation yield of the hybrid structure.

The novel C₆₀ corked CNT constructs were analysed, and according to various methods, it is likely that the indicated structures were synthesised. Nevertheless, further modifications, such as ionic interaction or peapod formation due to van der Waals forces, could also be present in the samples (Figure 4.13). It is proposed that the introduction of the long peptide linker made the functionalisation and corking more difficult compared to the simple diethylene glycol- or dipeptide-linked C₆₀. This peptide, XXFVRGAGQ, may be too long, less accessible, or reactive, which could impair the functionalisation yield.

4.2.2.5 Corking of CNTs with the Inactive XXFVIGAGQ Peptide-Linked C₆₀

A control sequence for the enzymatic cleavage and release of the corked carbon nanotubes was designed. Approaches to couple XXFVIGAGQ-modified fC₆₀ **48** to CNT were undertaken (Scheme 4.4). For comparability reasons, the standard reaction conditions in chloroform were applied, yielding low functionalisation rates. To improve the synthesis, various coupling reagents were screened and the functionalisation was compared, as summarised in Table 4.2 and 8.5. These coupling reagents were chosen because they had previously shown good reactivity on small molecules, as well as in literature reports.^{55,124,132,295,304,318,323} Analyses of the results are described in the following.

Analysis by IR IR analysis revealed the presence of amide bonds for all reaction conditions (Figure 4.14). Bands at 1603 cm⁻¹ for the C=C vibration, ~1692 cm⁻¹ for the amide C=O vibration, and ~1765 cm⁻¹ for the acidic analogue were clearly visible in the baseline-corrected

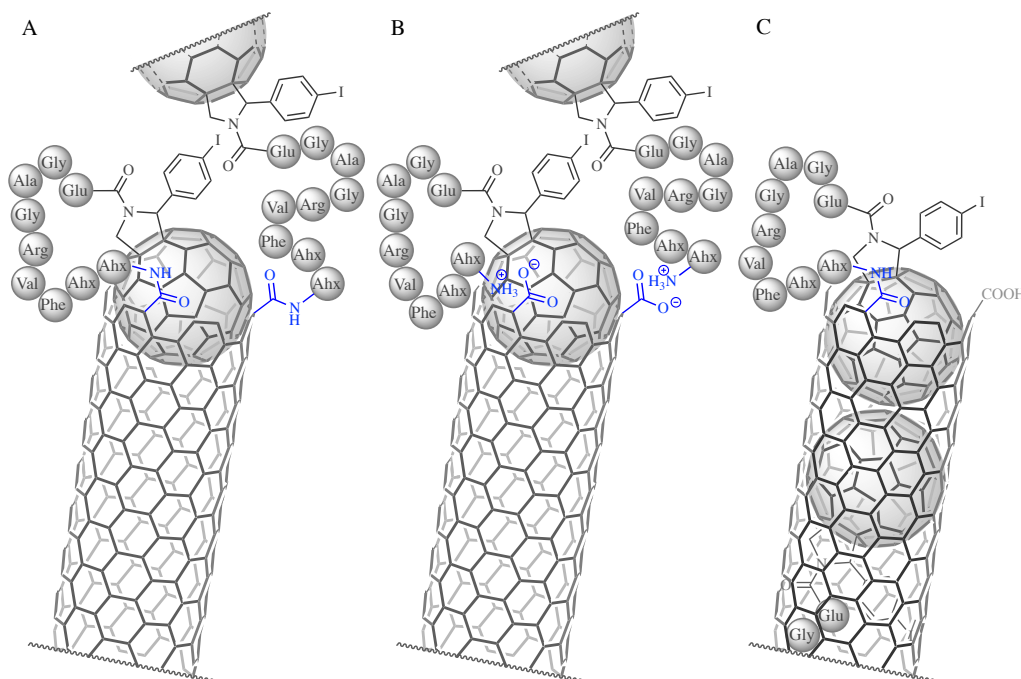


Figure 4.13: Three potential functionalisations of carbon nanotubes that explain the analysed presence of fC₆₀. **A:** The designed structure of C₆₀ corked CNTs **68** supported by a covalent amide bond. **B:** Strong ionic interactions retaining the peptide-linked fullerenes at the functionalised CNT ends are improbable because preactivation and coupling were used successfully.^{293–295} **C:** Aggregations and peapod formation due to van der Waals forces of the carbon material presumably complicated the analysis of C₆₀ corked carbon nanotubes, which was revealed by ptychography coupled STEM.

spectra. A significant improvement on the amide band intensity was made when changing the solvent system to DMF, suggesting a higher functionalisation for those constructs.

Analysis by Raman spectroscopy Raman spectroscopy was exclusively used to evaluate the A_D/A_G ratios. An increase of 1–11% was found in all of the samples besides the thionyl chloride activation (Condition I), which decreased by 11%. Because the sample purification of the latter differed from the others, it could be assumed that the utilisation of benzonitrile removed residual graphitic carbon because the centrifuged solution was still dark green, containing solubilised modified carbon debris.^{275,324}

Analysis by HRTEM For the four different reaction conditions, flower or mushroom look-alikes supporting a successful functionalisation of CNTs with fC₆₀ were observed by HRTEM. Whereas only 20% product under the standard SOCl₂ conditions were found, the functionalisation rate increased dramatically to 85% and 89% for the EDC and 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium-3-oxid hexafluorophosphate (HATU) coupling in DMF, respectively. Surprisingly, the addition of *N*-hydroxysuccinimide (NHS) to the EDC reaction mixture decreased the end-functionalisation (33%). In contrast, an increased amount

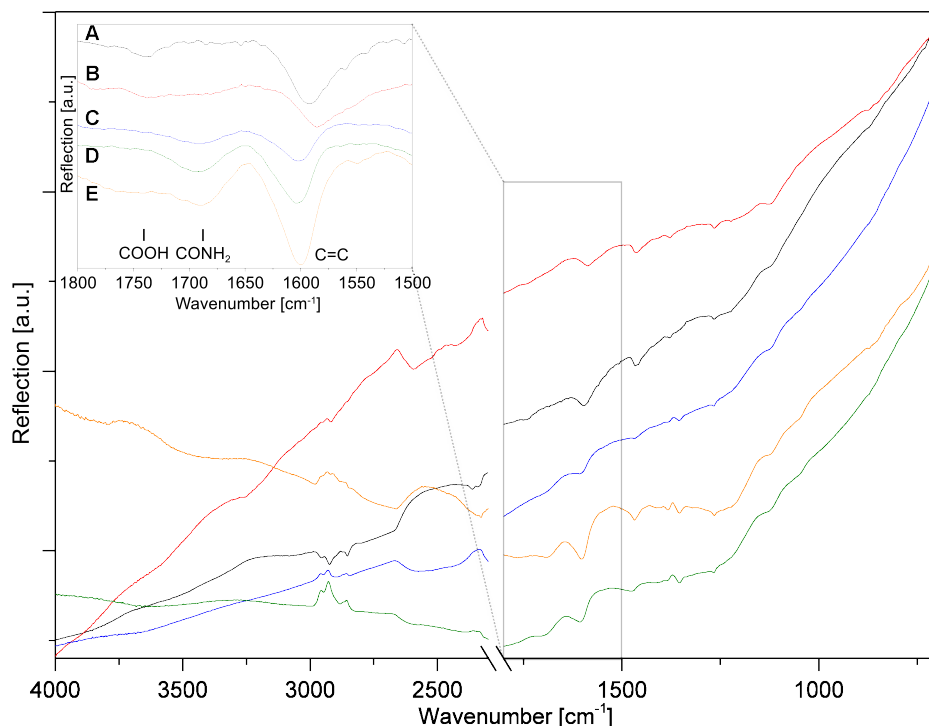


Figure 4.14: IR spectra of functionalised CNTs (**69**) with XXFVIGAGQ-linked C_{60} . Characteristic bands were found at $\sim 1900\text{ cm}^{-1}$ for CH vibrations, 1595 cm^{-1} for C=C stretch³⁰¹, the C=O vibration at $\sim 1745\text{ cm}^{-1}$ for carboxylic acids,^{285,301,313} and $\sim 1690\text{ cm}^{-1}$ for amide bonds.^{294,295} For better comparability, the spectra in the insertion were baseline-corrected. **A**: The CNT-COOH is depicted as a unmodified reference. **B**: A weak amide bond vibration band indicated a sparsely functionalised CNT- C_{60} hybrid made with Condition J. **C**: Synthesis Conditions K improved the modification. **D**: The spectrum of CNT- C_{60} synthesised *via* Conditions L revealed a strong amide band. **E**: Using Conditions M introduced the peptide-linked f C_{60} successfully. The spectra were recorded by Dr. Robert Jacobs from the Surface Analysis Facility, Department of Chemistry, University of Oxford.

of carbon debris, such as amorphous carbon wrapped around the nanotubes, was observed that made the analysis and the coupling reaction more difficult. Besides closed CNT ends, filled tubes and peapod structures were found in all samples. As discussed in Section 4.2.2.4 (page 65), this might result from the strong host-guest interaction of the inner tube wall and C_{60} molecules, and it can be difficult to remove the material using simple washing procedures.

Analysis by EDX The elemental composition measured with EDX revealed the presence of iodine in all four samples. These findings were confirmed by XRF and XPS. Calculations from the latter method gave a functionalisation yield for the different coupling reagents SOCl_2 (every 33rd COOH was functionalised), HATU (22nd), EDC (16th), and EDC/NHS (13th). For the last-mentioned, an increased iodine-to-nitrogen ratio 1:15 of original 1:11 was found, whereas the other examples were in good agreement with 1:11. The error implied an incorrect value for the functionalisation yield (nitrogen-to-oxygen ratio) and originated from the high signal-to-noise ratio and a possible background activity. In general, the observations showed that the functionalisation improved with the solubilisation of f C_{60} , as described above. In contrast to the

small molecule experiments, HATU-promoted coupling was not as effective. This might result from a relatively short preactivation time combined with a worse coupling efficacy of HATU in the complete reaction mixture, as experienced in the peptide modification of fulleropyrrolidine **21**.

Analysis by TGA As described above, the combustion temperature of esters derived from small organic nucleophiles used in the washing procedure was found at 262.0 °C in the appropriate sample. In all four samples, the $T_{\text{combust.}} = 344.1 \pm 17.1$ °C for presumably the C₆₀ fullerene, as well as $T_{\text{combust.}} = 602.8 \pm 5.9$ °C for oxidised CNTs, were observed. The combustion temperature assumed to belong to the peptide-linked fC₆₀ oxidation was not detected in the two samples when EDC was used and was difficult to detect for the other two. Furthermore, an additional

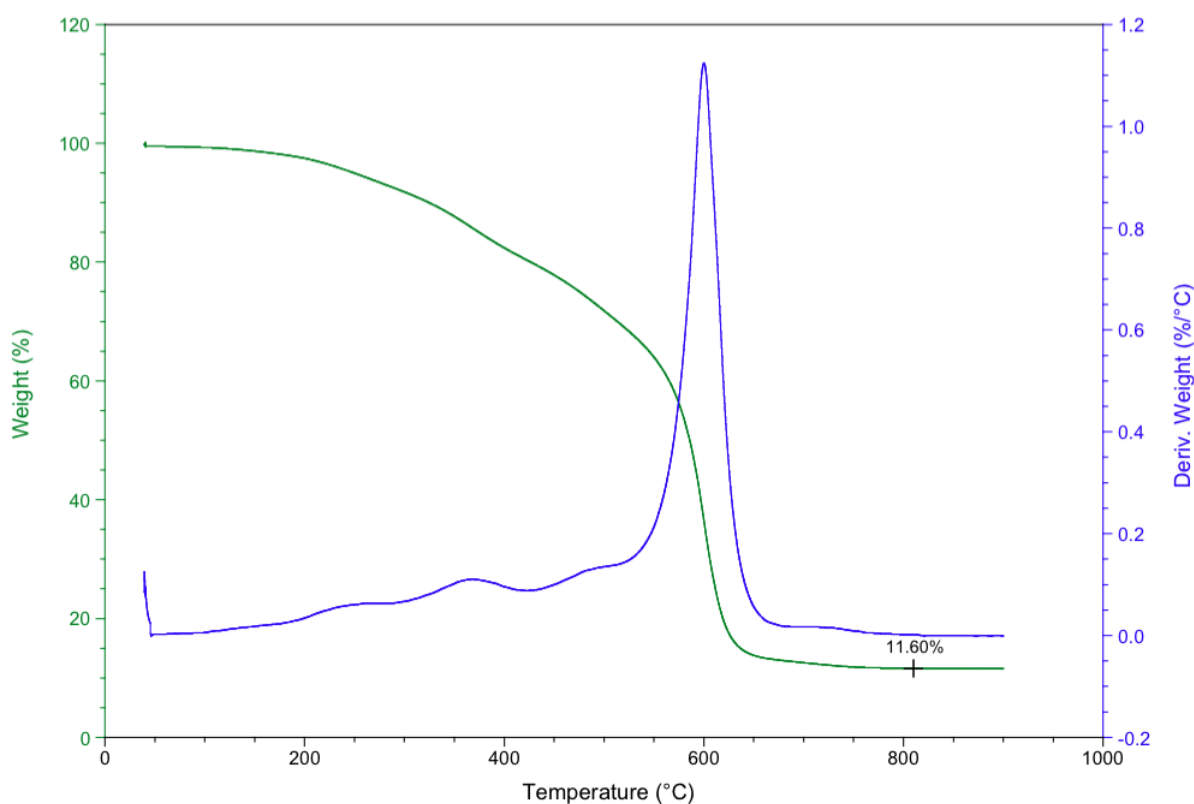


Figure 4.15: The TGA data and its derivative of XXFVIGAGE-linked C₆₀ carbon nanotubes construct **69** synthesised with SOCl₂ in chloroform. The combustion temperatures were 262.0 °C (DMSO ester), 367.9 °C and 488 °C (C₆₀ and peptide linker), and 600.1 °C for CNT-COOH. The oxidised metal content of 11.6% was higher than what could be iron from the CNT manufacturing and indicated the sample contamination with non-organic molecules such as Si, Al, and Na, as suggested by elemental analyses. The TGA data were measured by Magdalena Kierkowicz at the ICMAB.

signal at $T = 629$ °C for the EDC-coupled product was observed. The combustion temperature was higher than for the CNT-COOH, which suggested the oxidation of any inorganic material contaminating the sample, *e.g.* small amounts of a broken glass pipette.

Conclusions

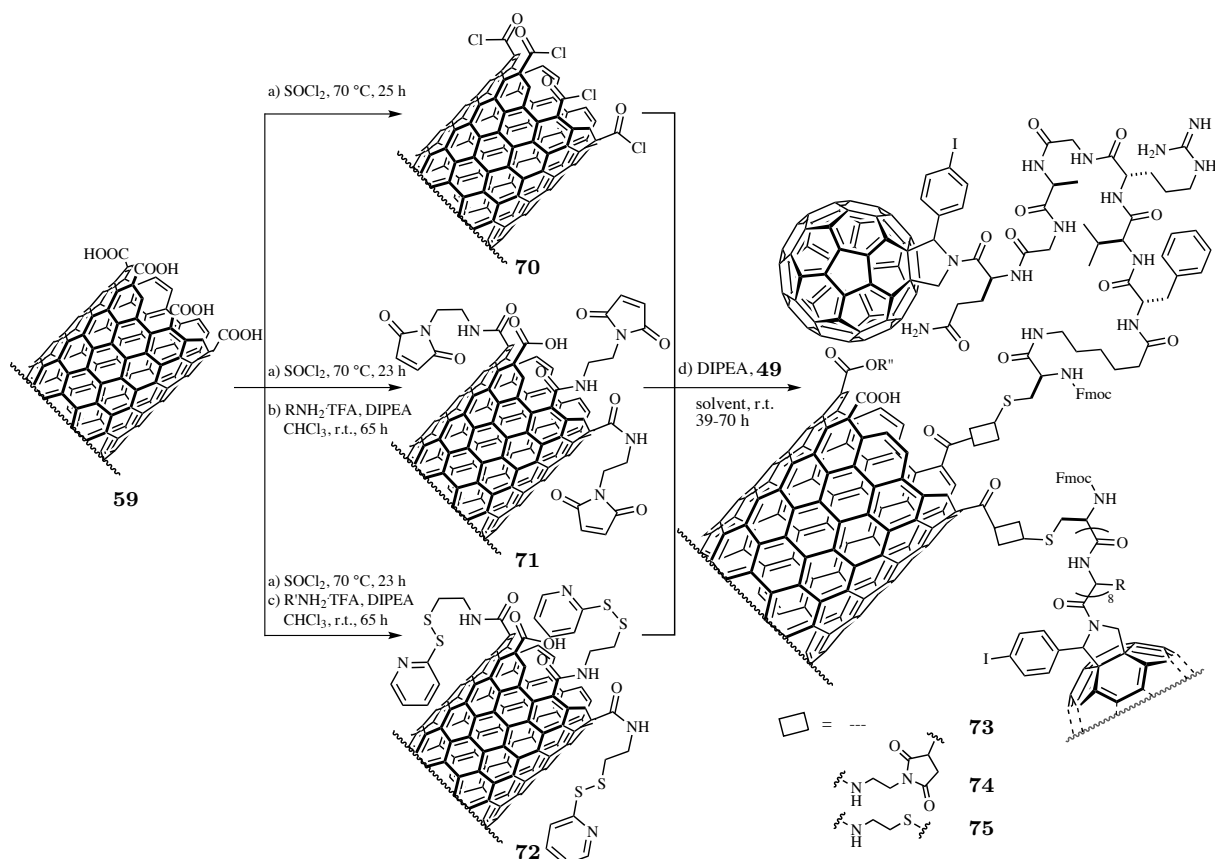
In this section, previous results indicating the solvent dependency on the reactivity were confirmed, and the study was further expanded to the coupling reagent. The initially limiting coupling method with SOCl_2 in chloroform yielding low functionalisation of CNTs could only marginally be enhanced by the use of EDC, EDC/NHS, or HATU in DMF. Surprisingly, the coupling undertaken with HATU did not reveal the expected results from the small molecule coupling on fC_{60} (Scheme 3.17, page 46). The results indicated that these syntheses methods were less successful than those mentioned before (Section 4.2.2.4) and confirmed the hypothesis that the long peptide sequence of nine amino acid residues made the coupling procedure extremely difficult.

The characterisation of the XXFVIGAGQ-linked C_{60} corks on CNTs suggested the successful modification of the nanomaterial. The IR spectra indicated the presence of the amide bonds in the hybrids. This was supported by the detection of nitrogen and iodine by elemental analyses and TGA displaying the presence of fC_{60} in the constructs. Observations by HRTEM revealed the predominant localisation of fullerenes at the tube ends where the coupling to the carboxylic acid groups is likely. Unfortunately, covalent bond formation could not be proved. The proposed composition of the constructs is a combination of covalent and non-covalent interactions that keep the functionalised fullerenes in close proximity to the CNT material.

4.2.2.6 Corking CNTs with the Active CXFVRGAGQ Peptide-Linked C_{60} Fullerene Linking CNT and C_{60} *via* a Thioester Bond

The unambiguous evidence of the formation of the amide bond to covalently link the CNT and peptide-linked C_{60} could not be supplied by using the analytical methods as described before. Instead of using the characterisation data to prove this linkage, it was decided to change the chemistry of the newly formed bond to one with a distinct characterisation pattern for definite identification. Therefore, the peptide sequence was redesigned, and a new element that could undergo covalent bond formation with the carboxylic acid group on the CNT was introduced (Scheme 4.5). This element should have a distinctive characterisation signature due to the environmental and chemical change during bond formation. A thiol group bears all of these advantages. The presence of sulfur is unique in the construct, and its attachment could be visualised by IR, EDX, XPS, and in combination with the Ellman's reagent, unambiguously verifying the covalent bond formation. The analysis results are summarised in the Appendix (Table 8.6). Analysis by IR did not show a distinctive new thioester bond. According to the literature, the specific carbonyl vibration is at $\sim 1675\text{ cm}^{-1}$ and overlaps with the carbonyl amide vibration.³²⁵ Further, free thiol could not be assigned due to the overlap of OH and NH vibration coming from the peptide linker. Nevertheless, a weak carbonyl amide peak was observed (Figure 4.16.B). Raman analysis was not conclusive, as described above. The A_D/A_G ratio decreased by 10% compared to oxidised CNT-COOH. The use of PhCN during the purification washes removed

Scheme 4.5



uncoupled fC₆₀ and potentially functionalised graphitic and amorphous carbon debris. Analysis by HRTEM revealed the functionalisation of 54% of all observed, open carbon nanotube ends. This result led to the hypothesis that the formed thioester bond was less stable than the amide bond and rapidly hydrolysed during the EM grid preparation in ethanol (EtOH). Low presence of iodine was measured by EDX and XRF, supporting the hypothesis of fC₆₀ **49** hydrolysis, or, as discussed before, detection of sulfur and chlorine from contaminations or artefacts occurred and could not be considered as a characteristic evidence. Semi-quantification by XPS unveiled the presence of sulfur in an iodine-to-sulfur ratio of 1:4 instead of the expected 1:1, which might be referred to the presence of DMSO used during purification. The functionalisation yield was calculated from the oxygen-to-nitrogen ratio to give every 46th carboxylic acid group as being functionalised. TGA analysis showed combustion at $T_{\text{combust.}} = 279.2, 366.6, 515.1,$ and 600.9 °C, as previously discussed for attached DMSO, C₆₀, peptide linker, and CNT-COOH, respectively.

The advantages of this new system were the control possibilities *via* Fmoc-numbering,⁴ where the Fmoc-protecting group is cleaved and used for quantification of the introduced fC₆₀

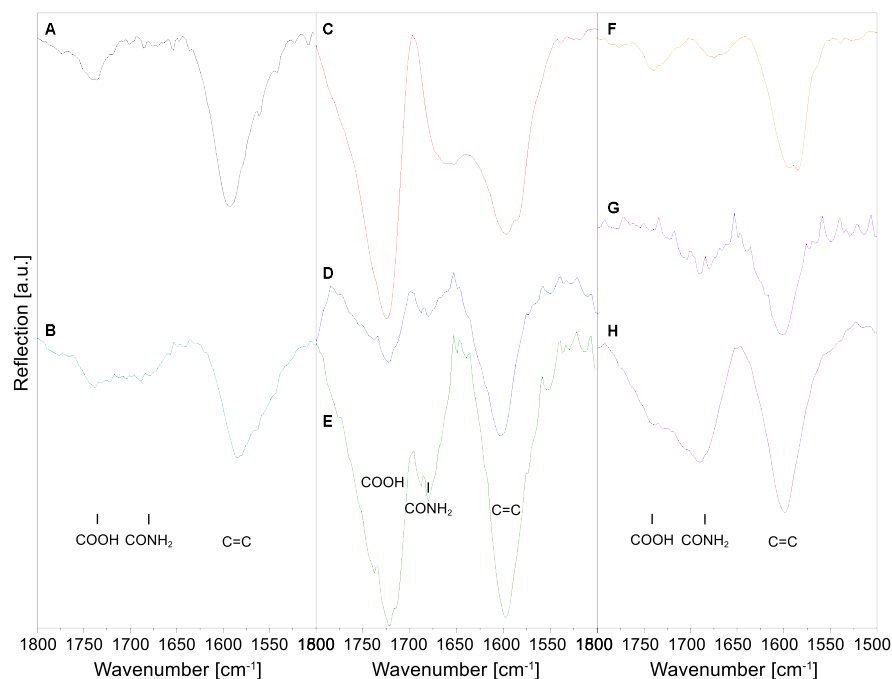


Figure 4.16: The IR spectra of functionalised CNTs with a CXFVRGAGQ-linked C_{60} were baseline-corrected extracts, which shows the characteristic C=C stretch (1595 cm^{-1})³⁰¹ and the C=O vibration at $\sim 1745\text{ cm}^{-1}$ for carboxylic acids^{285,301,313} and $\sim 1690\text{ cm}^{-1}$ for amide bonds.^{294,295} **A**: Oxidised carbon nanotubes only revealed C=C and carboxylic acid bands. **B**: Functionalisation *via* a thioester bond (**73**) displayed the presence of amide bonds in the product. **C**: The IR spectrum of maleiamide linker modified CNTs **71** revealed the introduction of amide bonds. **D**: The addition of f C_{60} shifted the amide band to higher wavenumbers. **E**: The presence of NaI during the synthesis of **74** did not affect the amide bond formation compared to spectrum D. **F**: The IR spectrum of a disulfide functionalised CNTs (**72**) confirmed the formation of an amide bond. **G**: Synthesis of fCNT **75** increased the ratio of amide-to-carboxy band intensity. The introduction of peptide shifted the amide bond vibration towards lower energies. **H**: In presence of NaI, the introduction of f C_{60} to the nanotube sample was successful. The spectra were recorded by Dr. Robert Jacobs from the Surface Analysis Facility, Department of Chemistry, University of Oxford.

molecules, and Ellman's reagent to countercheck the presence of uncoupled thiol impurities from non-covalently bound f C_{60} .^{326–328} The solutions, after treating the functionalised CNTs with pyridine or Ellman's reagent, were analysed by ultraviolet/visible light spectroscopy (UV/vis spectroscopy). The corresponding absorption peaks could not be observed for the dibenzofulvene, cleaved Fmoc-group, and carboxynitrophenolthiolate released from corresponding disulfide. Not being able to detect the presence of peptide-functionalised C_{60} by UV/vis spectroscopy *via* this sensitive reactions validated the very low functionalisation of CNTs. In conclusion, the reaction using the cysteine thiol as the nucleophile to form a thioester bond in the CNT- C_{60} construct was not successful. This might be because of the lowered reactivity of the reaction conditions, as perviously discussed, or due to the instability of the formed thioester and rapid hydrolysis thereof.

Introduction of a Thiophilic Connector

The low functionalisation of CNT-COOH with fC₆₀ **49** could either be caused by a low accessibility of reactive groups on the CNT ends or by a low reactivity of the peptide-linked C₆₀. A new strategy including a short linker introduced to the CNT-COOH might shed light on this. Installation of a thiophilic moiety on the CNT, which reacts with cysteine containing fC₆₀ instantaneously, potentially increases the accessibility by decreasing the crowded environment at the tube ends. Further, the presence of the sulfur would give all of the characterisation advantages described above.

Small spacer molecules bearing functional groups, such as a maleimide or protected thiol, were kindly donated by Dr. Thomas B. Parsons. These were attached to the preactivated carbon nanotubes to give **71** and **72**, as shown in Scheme 4.5 and analysed by IR, Raman, XRF, and XPS. The IR spectra showed in both samples the carbonyl amide vibration at 1653 cm⁻¹ for **71** and 1682 cm⁻¹ for **72**. The red-shift of 29 cm⁻¹ in the former sample was explained by the presence of the maleimide species. Raman spectroscopy confirmed the coupling to CNTs proceeded without affecting the sidewall structure. XRF was inconclusive because the light elements such as carbon, oxygen, and nitrogen were difficult to record, though the presence of sulfur in **72** was observed. From the XPS results, the functionalisation yield was calculated and found to be high: All carboxylic acid groups were functionalised with the maleimide (**71**), and every tenth carboxylic acid group was modified with the protected sulfide (**72**).

These intermediates underwent the addition of CXFVRGAGQ-modified fullerene **49** in DMF/H₂O. The solvent combination was chosen due to the comparability to the investigated filling experiments. The aqueous phase was used to solve sodium iodide and fill the nanotubes *via* capillary wetting with the salt. The characterisation of these NaI-filled samples is described here, and the discussion of the filling analysis in Section 5.2.1, page 86. The iodide salt was used as a placeholder to investigate the filling procedure and chosen because of its great aqueous solubility and good contrast as a heavy element for STEM characterisation of the constructs.

The IR spectra of the products of the thia-Michael addition to the maleimide sample to give fCNT **74** uncovered a characteristic carbonyl amide vibration at 1688 and 1690 cm⁻¹ in the empty samples and the ones filled with NaI, respectively (Figure 4.16.D and E). Raman spectroscopy did not show additional peaks or peak shifts^{105,329,330} for the corked neither in the filled or corked CNTs.^{135,136} The HRTEM data revealed the successful functionalisation of 78–86% C₆₀ functionalised CNT ends for both species (Figure 4.17). The salt filling could not be identified by TEM, and the observations thereof by STEM are discussed later. Analysis by EDX and XRF clearly showed the presence of iodine, which was quantitatively increased when measuring the NaI-filled constructs. Unfortunately, not enough material to semi-quantify the functionalisation and filling yield by XPS or TGA was available.

In Figure 4.16.G and H, the IR spectra of the nucleophilic substitution to form the disulfide-linked CNT-C₆₀ hybrid **75** with and without NaI revealed an increase of the distinctive carbonyl amide vibration. Raman spectroscopy revealed for both samples a decrease of the A_D/A_G ratio

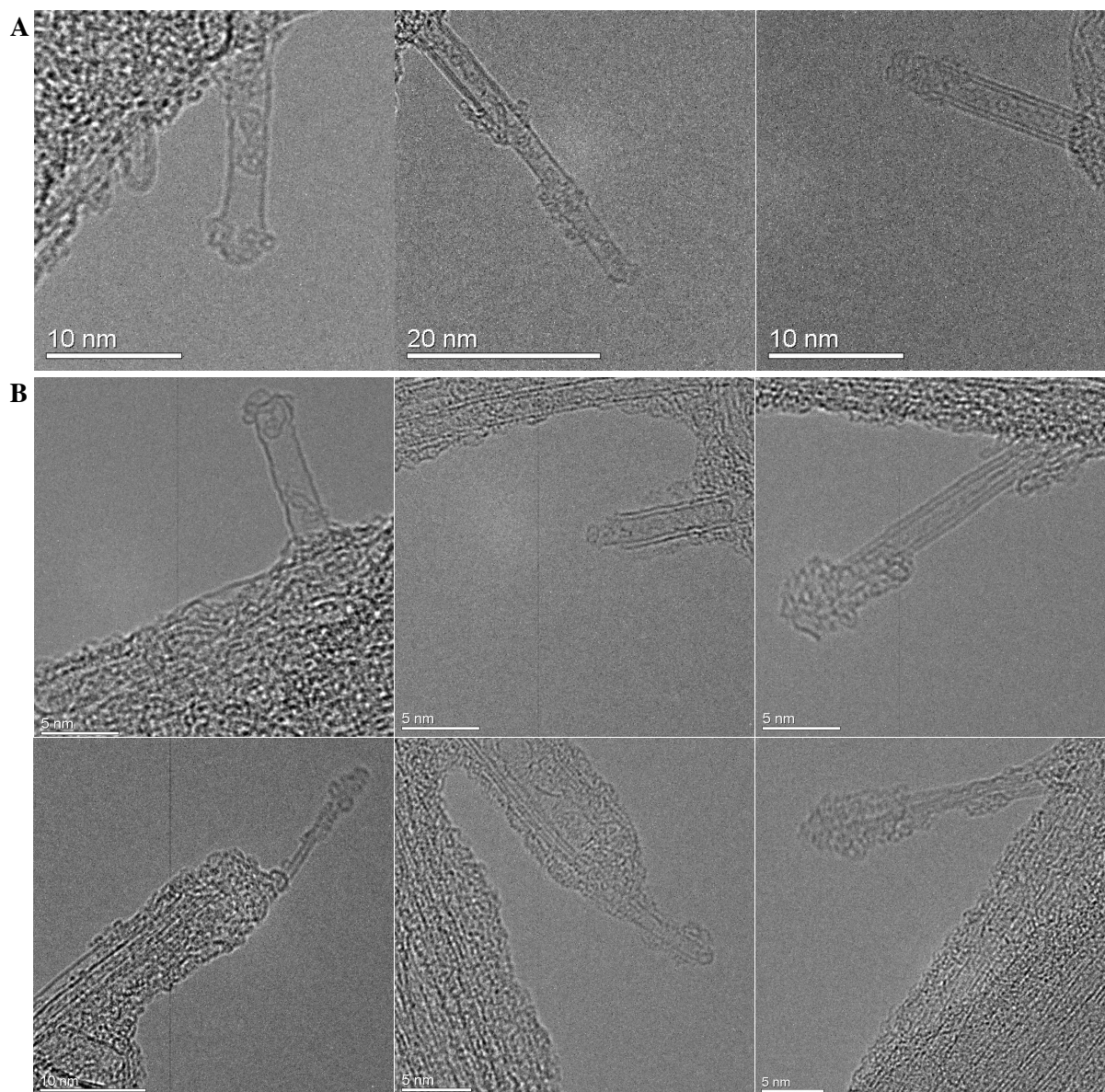


Figure 4.17: The HRTEM images of modified carbon nanotubes with cysteine bearing peptide-linked C_{60} fullerene *via* the maleimide coupling **A**: without and **B**: in the presence of NaI as filling material in the reaction mixture. End functionalisation, as well as filling of the hollow tubes and peapod structures, were observed.

of 23–24% compared to the starting material fCNT **72**. This suggested the removal of potentially functionalised carbon debris, which became soluble due to the introduction of f C_{60} . Functionalisation of the sample might result in a lower functionalisation yield of CNTs ends. This was confirmed by HRTEM analysis; the functionalisation yield was 50–71%. Additionally, the iodine signal by EDX and XRF were weak but characteristic.

In conclusion, the functionalisation forming a disulfide bond to link CNT and f C_{60} was not as successful as using a maleimide linkage. The latter clearly showed evidence of CNT functionali-

sation in good yields for all characterisation methods, whereas **75** revealed low functionalisation. Unfortunately, a quantitative comparison using XPS or TGA to show the effectiveness of these corking methods could not be made due to the low amount of prepared samples. The stepwise insertion of first a reactive spacer and second the addition of the cork proved to be advantageous for the filling procedure, as discussed later.

4.3 Outlook

Functionalisation of CNTs with peptide-functionalised C_{60} fullerenes to give corked species was achieved. Control couplings introducing C_{60} with simpler linkers such as diethylene glycolamine or the GK dipeptide showed promising results and good reactivity towards the oxidised CNTs. Analysis of the constructs by IR, Raman, HRTEM including EDX, and, if informative, also STEM, XRF, XPS, and TGA were made. The observation of all the analysed techniques gave evidence of the specific CNT-end functionalisation. The characterisation revealed a poor presence of the nine amino acid long peptide-coupled fullerene corks **68** and **69**. This could be led back to a lowered accessibility of the coupling partners *e.g.* (i.) the rigid, more crowded and already blocked by coupled fC_{60} CNT ends, (ii.) low reactivity of the peptide-linked C_{60} caused by formation of secondary structures and coiling of the peptide sequence, or (iii.) structural and chemical inhomogeneity of the nanomaterial and the heterogeneous reaction conditions. The choice of the N-terminal amino acid based on low sterical hindrance of the unnatural 6-amino-hexanoic acid and its therewith improved accessibility was made to circumvent at least one of those problems.

Various reaction conditions, such as coupling reagents, solvents, reaction time, and coupling mechanisms, were compared. In general, the functionalisation improved with an increased solubility of the coupling partners, such as the modified fullerene. Thionyl chloride proved to be the most effective preactivation reagent of the carboxylic acid groups. Changing the nucleophile for the coupling from the N-terminal amine to a side chain thiol introduced more possibilities of characterisation and functionality but also decreased the stability of the newly formed covalent bond. The development of a thiophilic spacer located at the reactive CNT ends improved the reactivity and stability of the CNT- C_{60} construct. The spacer reduced the bulkiness at the CNT ends for coupling partners and made the system more flexible and the reactive ends more accessible for the fC_{60} . In the future, a corresponding spacer could be introduced between the carboxylic acid and the amine of the peptide-functionalised C_{60} , and the functionalisation yields could be compared.

The characterisation of poorly functionalised CNTs proved to be challenging. The use of multiple orthogonal analysis methods gave evidence of the formation of $fCNT$. IR, HRTEM, and XPS showed to be the most informative of those. Investigation into the characterisation of the constructs by atomic force microscopy (AFM), matrix-assisted laser desorption/ionisation (MALDI), and solid state nuclear magnetic resonance (NMR) were initiated and found not to be elucidative. An improvement for the unambiguous CNT characterisation was found in ptychography-

supported STEM.³⁰⁵ In the future, more methods, such as solid state UV/vis spectroscopy, elemental CHN analysis by sample combustion, and inductively coupled plasma mass spectrometry (ICP-MS), could be examined.³³¹ Furthermore, the enzymatic cleavage could shed light on the quantification of coupled fullerenes, which is discussed in the next chapter.

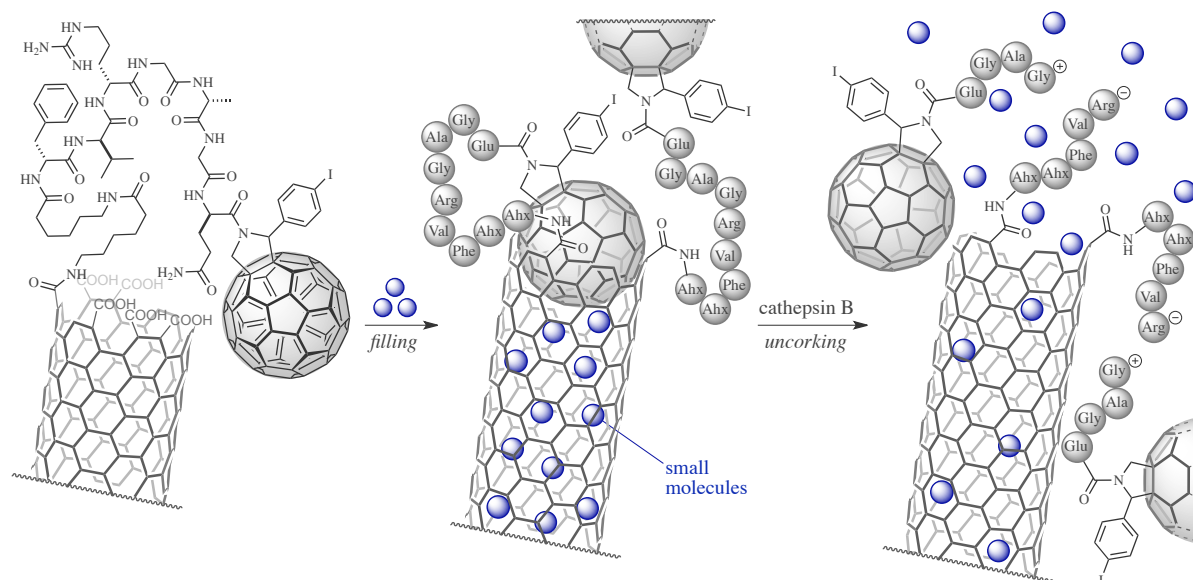
Nevertheless, all of the above-mentioned reasons leading to a sparsely functionalised carbon nanotube did contribute to achieving the overall goal of the project. The aim was to fill the tubes with a payload, cork and seal the containers with modified fullerenes, and, after delivery, uncork and release the stopper and content (Scheme 1.2, page 7). The less the corks are present at the end of every tube, the more this purpose is served. The enzymatic cleavage will potentially be more successful due to less hindrance by a bulky substrate, and the removal and diffusion of the cork would be supported because less attractive, non-covalent interactions will keep them in place.

Despite the disadvantage of those systems showing sample inhomogeneity, characterisation with many different analysis methods supported the successful, but low yielding, functionalisation. The covalent bond formation could not be unambiguously proven for these systems but assumed due to the results achieved with simpler linkers.

V. Filling and Uncorking of Functionalised Carbon Nanotubes

In this project, the development of a drug delivery system based on the functionalisation of carbon nanotubes (CNTs) was envisaged. The investigations led to the successful synthesis and characterisation of C_{60} fullerene (C_{60})-corked CNTs. As described below, these tubes are used to demonstrate their filling potential with salts and the ability of enzymatically triggered uncorking and release of fC_{60} and the payload (Scheme 5.1).

Scheme 5.1



A schematic overview of the filling of C_{60} functionalised CNTs and the enzymatic release of these fC_{60} corks, as discussed in this chapter.

5.1 Background

The controlled filling and release of a biologically active payload from the functionalised carbon nanocapsules needs to be demonstrated for drug delivery purposes.

5.1.1 Filling of Carbon Nanotubes

The cavity of CNTs has attracted many researchers to investigate carbon nanotube (CNT) filling,^{3,73,96,97,332,333} as theoretically predicted in the mid-1990s.^{334,335} The introduction of C₆₀ molecules into the tubular structure of CNTs is well established.^{60,73–85} These ‘peapods’ are stable due to π - π -stacking, hydrophobic, and van der Waals interaction. Extending these preliminary studies, there are reports of filling CNTs with functionalised C₆₀.^{70,77} Fullerenes have also been used as corks to enclose small organic molecules¹⁰⁵ and salts in CNTs.^{106,313} Additionally, although the interaction between the carbon materials is strong, C₆₀ can diffuse out of the CNT.^{70,78,88,105,106}

Besides the encapsulation of C₆₀, many applications of the CNTs have been explored.¹⁷ The CNTs have been filled with therapeutic related imaging agents,⁴ dyes^{125,329,336} and drugs;^{18,105,128,133,135,337,338} the associated release mechanisms have been studied.³³⁹

The two strategies used for filling CNTs with inorganic materials are liquid phase and molten phase filling.^{41,96} Molten phase filling, as the name suggests, is carried out at temperatures above the substrate melting point.^{4,307,313,330,340} Alternatively, soluble inorganic salts may be used for liquid phase-filling methods.^{41,70,95,106,332,341,342} Here, liquid phase filling was envisaged to be more suitable because the fullerene corks would not sustain elevated temperatures, as verified by thermogravimetric analysis (TGA). NaI and KI were chosen as payloads. They exhibit good solubility and small surface tensions in aqueous media.^{334,335,343,344} Water was proposed as the solvent of choice for the filling procedure because the CNT content is to be released *in vivo*, where residual organic solvents could be hazardous. Further, an iodide payload could be advantageous for the characterisation by scanning transmission electron microscopy (STEM), energy dispersive X-ray spectroscopy (EDX), X-ray photoelectron spectroscopy (XPS), or by a quantitative analysis with starch.

5.1.2 Proteases and Enzymatic Cleavage of Peptides

Proteases cleave the backbone of specific peptide and protein sequences.³⁴⁵ The cathepsins are a subclass of proteins that are responsible for the nucleophilic cysteine-catalysed hydrolysis of amide bonds. Cathepsin B is a cysteine protease with endopeptidase (*i.e.* cleaving a peptide bond in the middle of a protein) and C-terminal dipeptidylpeptidase (*i.e.* cleaving the amide bond at the C-terminus) activities. This well-studied protease,^{161,162,345–347} generally found in the lysosome,¹⁵⁸ is upregulated in cells targeted by inflammatory responses, such as cancer or arthritis, and involved in the inflammatory process.^{159–162,346} Structural details^{159,346} have revealed insights into the mechanism and specificity of the recognition motif.^{163–165,348,349} Cathepsin B recognises the specific peptide motif Phe (P3), Val (P2), and Arg (P1) for hydrolysis.²⁶⁰ Herein, a peptide that contained this amino acid recognition sequence was utilised to link C₆₀ and CNT. A cathepsin B-triggered cleavage of these nanocapsules enhances the potential applicability as a drug delivery system by a selectively controlled release mechanism.

5.1.3 Filling and Uncorking of C₆₀-CNT Hybrid Structures

For the application of the synthesised C₆₀-peptide-CNT nanocapsules as a drug delivery system (DDS), the filling and subsequent release mechanism was then evaluated. The ultimate payload would be a drug that could be readily delivered to the lysosome of inflamed cells, for example, hexamethylmelamine,¹⁰⁵ taxol,³³⁹ *cis*-platin,¹³⁶ and carboplatin.^{104,135,350} The filling of characterised CNT-C₆₀ corked constructs was initially performed with heavy, non-natural occurring atoms, such as iodine, platinum, silver, or nickel, in water soluble salts or complexes. Scanning transmission electron microscopy (STEM), EDX, XPS, and TGA were used in order to easily assess this crucial process. After proof of concept, the investigation of filling potential, mechanism optimisation, and release kinetics will be performed in future studies.

Enzymatic cleavage conditions for the peptide linker were previously demonstrated²⁶⁰ but needed further optimisation because of the hydrophobic, bulky environment of functionalised CNTs.

This chapter has a focus on the following aspects that had to be investigated:

- Universal applicable liquid phase filling, demonstrated with salt and potential drug cargo
- Quantification of the payload and diffusion kinetics
- Enzymatic cleavage and uncorking of C₆₀-CNT construct under physiological conditions.

5.2 Results and Discussion

5.2.1 Towards the Filling of Corked Nanotubes with Inorganic Salts

The initial experiments were designed to investigate the filling procedure of fullerene functionalised CNTs. Three different strategies were investigated, as schematically illustrated in Figure 5.1.

Procedure A was designed to vary the payload at a late stage. Functionalised CNTs (**65**) were used to demonstrate the KI_{aq} filling *via* this route. After excessive washing, the filling was evaluated by STEM and EDX. Increased amounts of iodine were observed by EDX. STEM analysis revealed that the salt only aggregated on the outside of the CNT bundles, with not a single filled tube. Assuming that the presence of water caused the hydrophobic carbon nanotubes and C₆₀ fullerenes to agglomerate, minimising the surface area and forcing the corks inside the tube ends led to the conclusion that the CNTs were blocked from filling with the salt under these conditions.^{81,106,313}

To circumvent this problem, the CNT-C₆₀ constructs were first dispersed in benzonitrile (PhCN); this solvent has excellent solubility for fC₆₀ and would not cause as much harm *in vivo* as other solvents such as DMF. Then, KI_{aq} was added to the greenish dispersion. No filled tubes were observed by high resolution transmission electron microscopy (HRTEM), STEM, and EDX, only aggregated potassium iodide outside the tubes was revealed. Although, organic solvent was in-

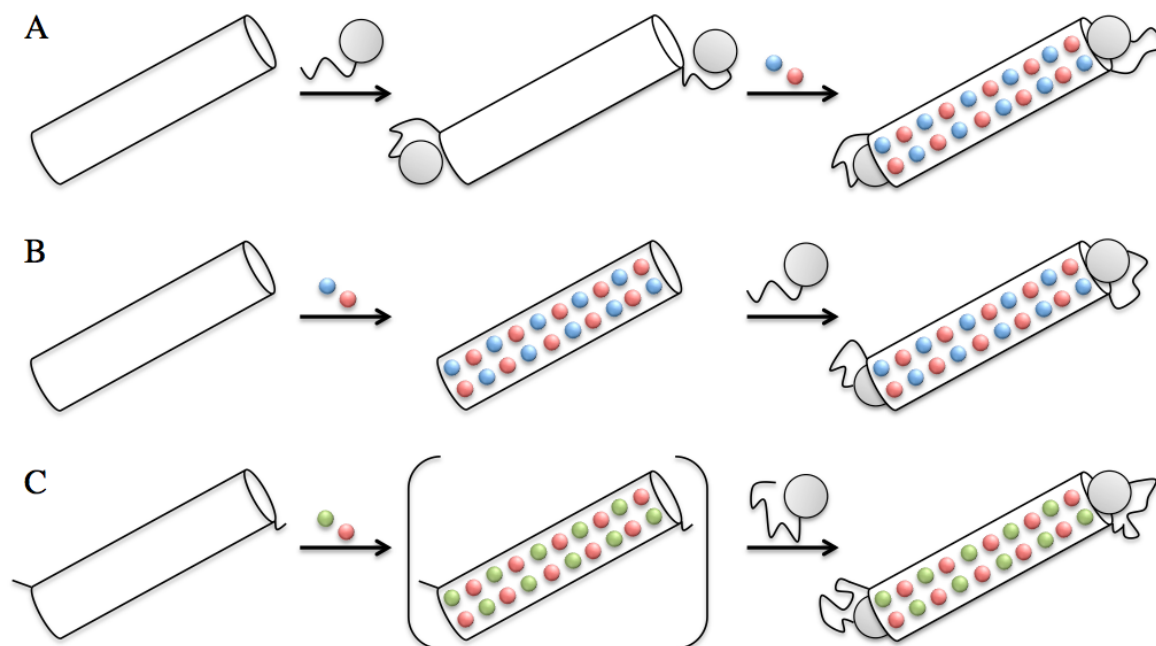


Figure 5.1: A schematic representation of the three approaches to liquid phase filling of CNTs. **A.** The oxidised CNTs were functionalised with fC₆₀ before the purified constructs underwent filling with inorganic salts in water or aqueous solvent mixtures; **B.** CNTs were filled with iodide salt in H₂O (and not in the molten phase) to leave the ends open for further functionalisation, purified and undertaken peptide coupling with functionalised C₆₀ to cork the tube ends; **C.** The thiophilic spacer-functionalised CNTs underwent a one-pot stepwise filling procedure: filling in water, then adding the peptide-functionalised C₆₀ in DMF to cork the substrates. The blue dots indicate potassium ions, the green are sodium, and the red ones are iodide atoms.

troduced to facilitate the potential temporarily uncorking; the method might be optimised by investigating the optimal ratio of applied solvents.

In parallel, filling approach B was examined. The sequential order was inverted to overcome the possible blocking of the CNTs by the presence of the C₆₀ corks. The oxidised and open CNTs were dispersed in saturated KI_{aq} prior to an aqueous wash and functionalisation with fC₆₀ **22** under standard conditions (SOCl₂, then fC₆₀, DIPEA in CHCl₃). The analysis by HRTEM and EDX of the filled intermediate KI@CNT revealed the absence of iodine inside the tubular structures, as well as decontamination. It can be assumed that the washing procedure was too successful and removed the salt filling.³¹³ A future experiment will include the direct coupling of the fC₆₀ cork to unprocessed material.

The third procedure (Figure 5.1.C) was investigated using a more concentrated sodium iodide solution due to its increased solubility compared to the potassium salt. As mentioned in Section 4.2.2.6, the introduction of a small spacer possessing a thiophilic group, such as maleimide or disulfide, which reacts with the cysteine installed on the peptide linked C₆₀, opened the possibility to conduct the corking reaction under aqueous conditions. This led to a one-pot filling and corking procedure, diminishing the potential removal of the filling during work-up. The

analyses by infrared (IR), Raman, HRTEM, EDX, and X-ray fluorescence spectroscopy (XRF) have been discussed for both filled and unfilled substrates of fCNTs **74** and **75** (Scheme 4.5 on page 78). Characterisation of the filled substrates revealed an increased presence of iodine only for the maleimide-linked construct (**74**). The disulfide containing system (**75**) revealed a lower functionalisation rate by means of HRTEM, which was in agreement with less corking and tube sealing. The comprehensive study of the NaI-filled sample **74** by STEM showed only a small fraction of filled CNTs represented by the continuous bright lines enclosed in carbonous material (Figure 5.2). A ratio of filled-to-empty tubes could not be given because the identification of single CNTs was not possible. Single iodine atoms from the attached functionalised C₆₀, as shown by IR and HRTEM, could not be detected by STEM due to the instrument sensitivity. The filled CNTs revealed the presence of fullerene corks at the ends, which closed and sealed the tubes. The extensive washing procedure did not remove the salt, as shown for the uncorked material.^{70,106,313} The filling yield might be improved by utilisation of a saturated NaI_{aq} solution and extended reaction time.

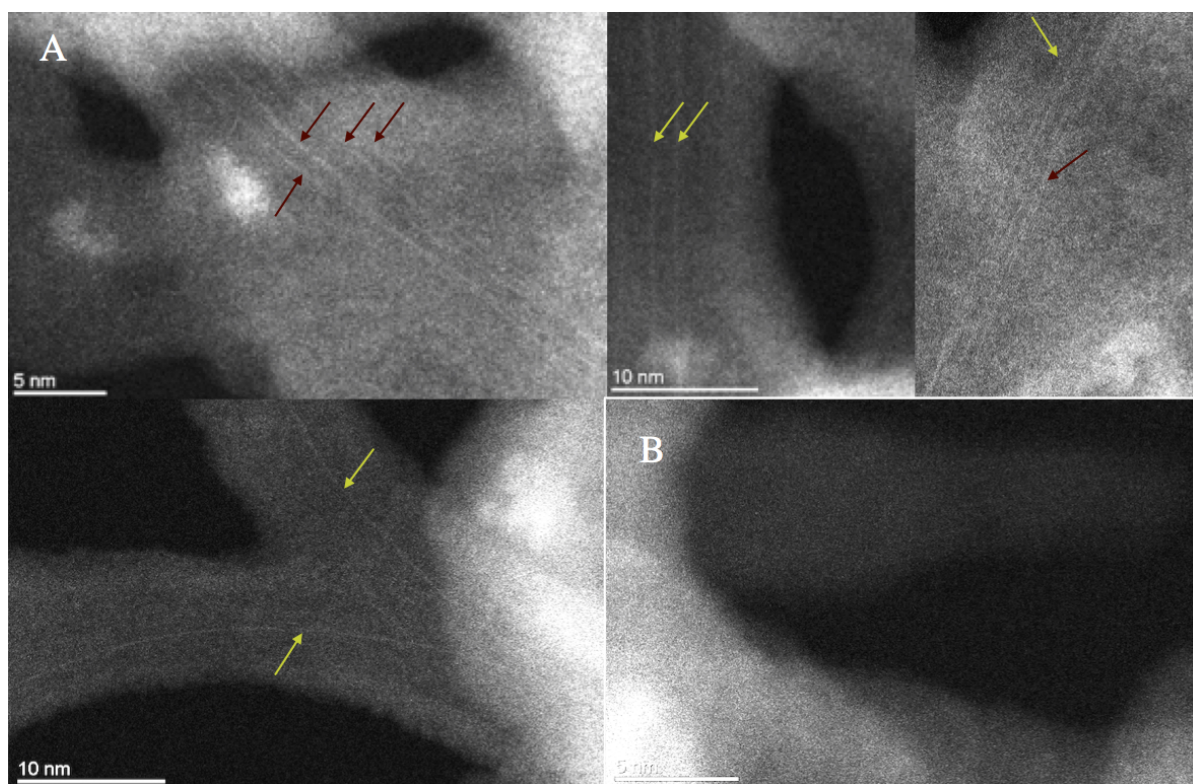


Figure 5.2: The STEM images of NaI-filled and corked CNTs. The bright dots and lines correspond to the presence of an element with a high atomic number, *i.e.* are consistent with iodine, whereas the brighter big patches indicate sample thickness of overlapping carbon material. **A.** The lines, indicated by the arrows, represent the NaI-filled carbon nanotubes; **B.** The end of a single CNT did not show the presence of iodine atoms from the modified fullerene cork due to the low sensitivity of the JEOL-3000F instrument or the non-functionalisation of this particular tube end.

In conclusion, not all of the approached filling procedures were efficient. The choice of filling solvents to promote a temporary uncorking and either the purification method or the coupling in presence of the filling salt have to be addressed further. One positive finding was that the introduction of a small spacer molecule indicated that the filling and coupling could be conducted in one pot. In the future, this approach might also forward the corking with the amine functionalised C₆₀. The use of AgNO₃⁹⁷ or *cis*-/ or carbo-platin^{104,135,136} might be considered for the filling to introduce materials with good and distinctive visibility by STEM and EDX, as well as potential application for quantification or medical treatment.

5.2.2 Towards the Enzymatic Cleavage and Release of Corked Carbon Nanotubes

Commercial cathepsin B was tested in preliminary studies to indicate the prospective application potential of the project. The peptide sequence Fmoc-XXFVRGAGE has been previously used for enzymatic cleavage.²⁶⁰ Herein, investigation into the usability of this protease for the sequences XXFVRGAGQ and XXFVRGAGQ-C₆₀ were undertaken.

5.2.2.1 Testing the Enzymatic Activity of Cathepsin B

The activity of the enzyme was verified using the Cbz-Lys-OC₆H₄NO₂ nitrophenol ester to show a specific activity of 21.0 units/mL. The unprotected peptide XXFVRGAGQ **76** was exposed to the enzyme for 24 h, and the resulting mixture was analysed by matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry, high performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC/MS). MALDI did not show peaks corresponding to the cleavage products. HPLC revealed a new peak at $t_R = 6.25$ min compared to $t_R = 9.02$ min for the starting peptide sequence. The use of LC/MS uncovered the unambiguous success of the peptide cleavage into smaller fragments (Figure 5.3). Although the peptide was varied by one amino acid from prior studies, cathepsin B still recognised the specific cleavage sequence Phe-Val-Arg. The positive result encouraged to the continuation with the fC₆₀ modified substrates.

5.2.2.2 Enzymatic Cleavage of fC₆₀ and fCNT

The enzymatic cleavage of the peptide attached to the modified C₆₀ was undertaken. Analysis of the crude reaction mixture after 24 h by MALDI mass spectrometry led to the detection of only unreacted **46**. HPLC analysis revealed the presence of a new peak at $R_t = 6.40$ min. Because the modified fullerene products were not injected due to solvent and column incompatibilities, the peak could only result from peptide fragments. LC/MS confirmed this finding because the same cleavage products obtained with the pure peptide sequence were detected. This result indicates that the peptide-coupled C₆₀ can be cleaved by cathepsin B.

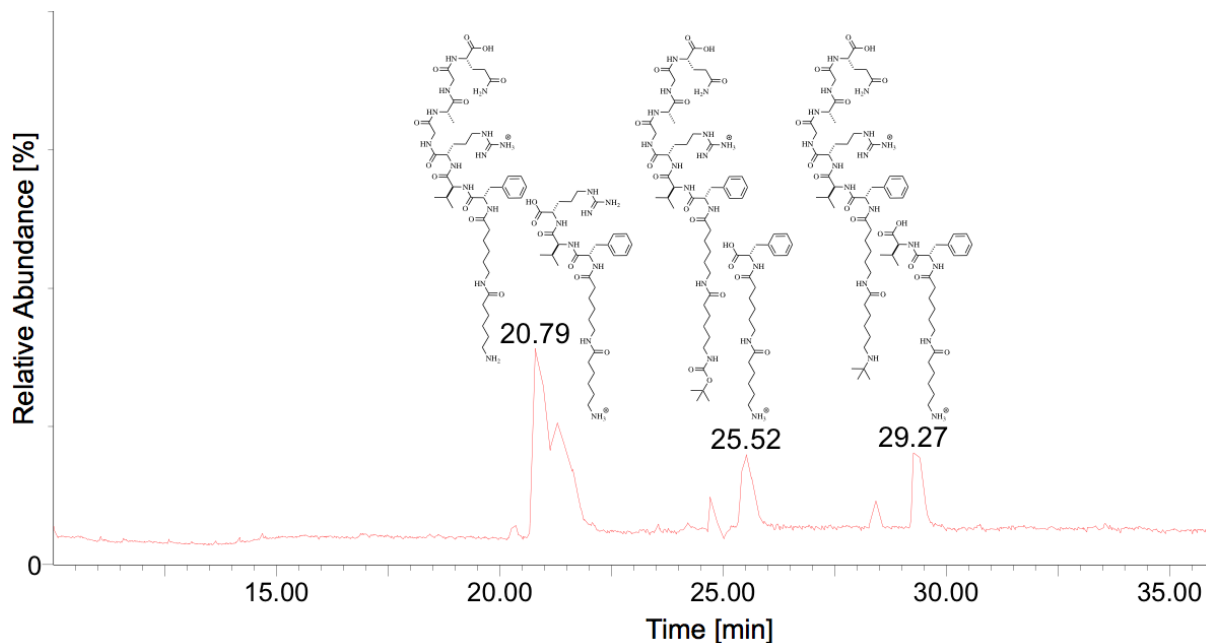


Figure 5.3: The LC/MS chromatogram of the XXFVRGAGQ (**76**) cleavage reaction mixture after 24 h in the presence of cathepsin B.

The CNT-peptide- C_{60} construct was subjected to the enzymatic cleavage in aqueous conditions. Using MALDI, HPLC, and LC/MS, no peptide or modified fullerene segments were observed in the crude material. After separation by repeated washes in DMF, the fC_{60} fraction was analysed by nuclear magnetic resonance (NMR) and MALDI. For both analyses, no C_{60} fragment could be identified, potentially due to the extremely small amount of the isolated sample. The residual $fCNT$ was characterised by HRTEM and EDX. Elemental composition revealed the presence of iodine in the sample. The signal potentially arose from non-cleaved corks and peapod structures. Analysis by HRTEM uncovered a reduction of functionalised CNT ends compared to the starting material (Table 5.1). Although the number of observations was small but representative, the quantity of C_{60} molecules on each CNT end seemed to have decreased, suggesting potential partial cleavage of coupled fC_{60} (Figure 5.4).

Table 5.1: Results of the analysis by HRTEM of construct CNT-peptide- C_{60} **68** (condition G) before and after subjection to cathepsin B catalysed cleavage for 24 h.

OBSERVED CNT	BEFORE CLEAVAGE	AFTER CLEAVAGE
total	26	32
open ends	18 69%	17 53%
functionalised	16 89%	10 ^{a)} 59%
peapods	6 33%	3 18%

a) Two of these tube ends (12%) showed the previously described flower-like appearance, and the residual eight (47%) showed a reduced presence of solely two to three C_{60} molecules per end (Figure 5.4).

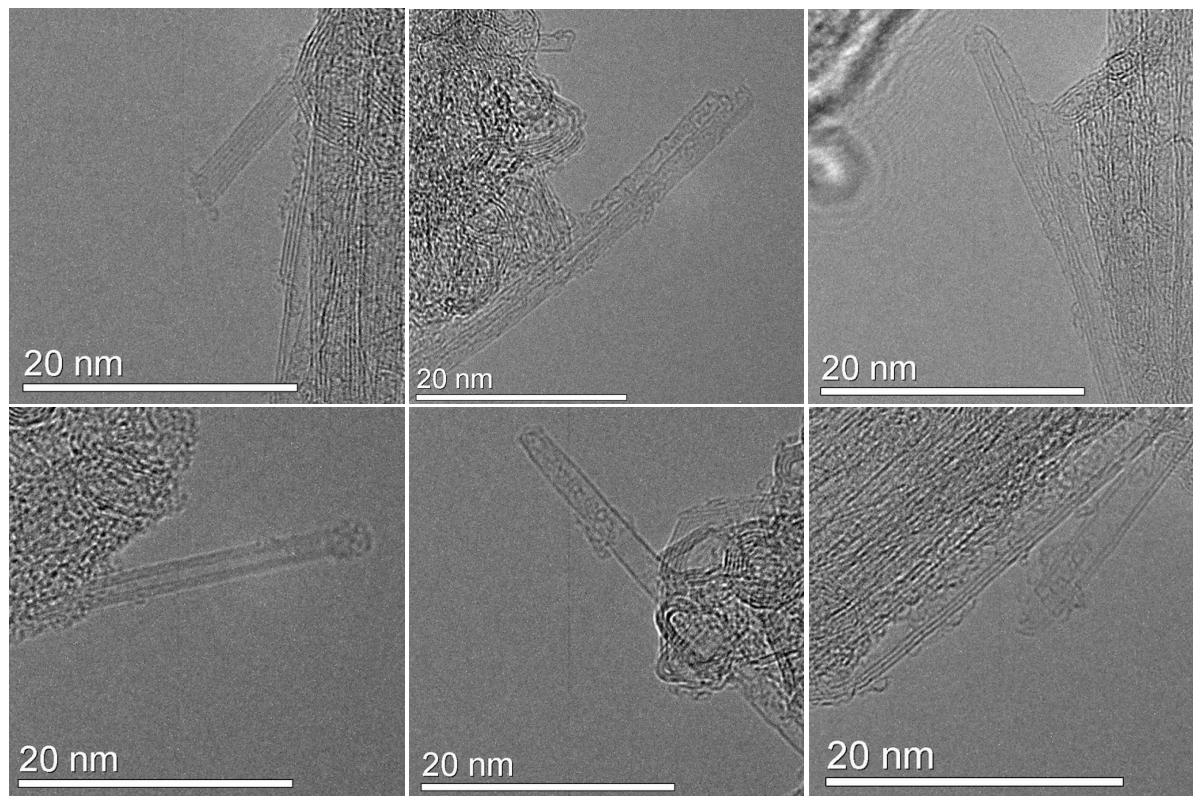


Figure 5.4: The HRTEM images of cathepsin B-treated CNT-C₆₀ construct **68** revealed sparsely functionalised ends and peapod structures.

5.3 Outlook

The filling, as well as cathepsin B catalysed cleavage, were investigated with modified CNTs, even though the characterisation (Chapter 3.3) indicated the presence of non-covalently bound peptide-C₆₀, such as peapod structures. NaI-filled CNT-C₆₀ constructs were successfully synthesised in a one-pot reaction that used a water compatible thia-Michael-addition with a maleimide group. In comparison, the stepwise amide coupling and filling procedure and *vice versa* did not yield loaded and corked CNT constructs. The successful filling of modified CNTs showed the reversibility of fC₆₀ corked tube ends; the filled salt was prevented from leaking out.

The cathepsin B catalysed cleavage of the peptide alone and the peptide attached to the fullerene showed good results. Further optimisation to drive the cleavage to completion should be undertaken. Cathepsin B showed efficiency towards the uncorking of modified CNTs-C₆₀ constructs, as shown by HRTEM. Nevertheless, complete removal of fC₆₀ was not observed. The identification of both cleavage products needs further work to unambiguously prove the cleavage. These results gave the proof of principle that the designed system can be used as a potential DDS.

VI. Summary and Future Work

This thesis was a disclosure of the functionalisation of C_{60} fullerene (C_{60}) and carbon nanotubes (CNTs) in work towards drug delivery. The goal was the development of fullerene and nanotube systems that allow payloads attached to the surface or within the carbon nanocapsules to be released in the presence of external stimuli.

C_{60} was modified in order to enable the controlled release of surface-bound small molecules by light. Fulleropyrazolines are well explored as photovoltaic compounds. Knowledge of their electron and energy transfer properties, led to the proposal that the installation of an electron-donating group at the $N-1$ position may allow reverse addition of the pyrazoline and cause the release of a hydrazone moiety in response to light. Reaction conditions for the reversed reaction were optimised, and its applicability in aqueous solutions was verified. A second substitution site enabled the installation of a handle, which could be used for the introduction of drugs or targeting molecules. In the future, this cycloreversion reaction could be applied in photodynamic therapy. Further, the reaction is anticipated to be applicable to modify the properties of CNTs, *e.g.* to improve their solubility, install releasable substrates, and enable to use this as a drug delivery system (DDS).

Further, the inner cavity of CNTs, which can be filled with a payload, was taken advantage of an approach to the application of carbon nanomaterials as potential DDS. It had previously been shown that C_{60} was capable of corking carbon nanotubes, but the reported reversible closure was not responsive to external stimuli, which are applicable under biological conditions.⁷⁰ To develop an improved DDS system, a modified C_{60} ‘cork’ was attached to the carbon nanotube (CNT) using an enzymatically cleavable peptide linker. The peptide sequence was chosen as a target of the enzyme cathepsin B, which is over-expressed in inflamed tissue like cancer. Peptides with various lengths and different amino acids, which could undergo coupling reactions, were synthesised and successfully attached onto modified C_{60} .

The coupling conditions to allow covalent attachment of the peptide-conjugated fC_{60} to the CNT were optimised. The presence of fC_{60} in the constructs was shown by IR, HRTEM/EDX, XPS, and TGA. Raman spectroscopy was found to not give reliable characterisation of the CNT- C_{60} hybrid. The newly formed amide bond could not be assigned unambiguously by any tested technique and can only be inferred based on the control synthesis of a simpler hybrid construct. STEM analysis revealed the formation of peapod structures. In conclusion, the evidence suggests that both covalently and non-covalently bound fC_{60} were present in the CNT- C_{60} constructs.

This detailed analyses revealed that there is a high level of heterogeneity inherent in the nano-materials and their products, and it revealed the challenges of unambiguous characterisation of modified carbon allotropes. Further efforts should be made on the removal of unbound fC₆₀ corks by investigating the purification protocol, *i.e.* washing the conjugates with mixtures of organic solvents to remove the non-covalently incorporated fC₆₀ would provide a means for improved characterisation and clearer interpretation of downstream experimental results.

First, filling and enzymatic cleavage experiments suggest the potential of this system to be used as DDS. One-pot filling and corking was successful when using sodium iodide. Additional evaluation and optimisation of conditions have to be undertaken. The filling yield has to be increased by varying solvent conditions and procedures, quantified, and then the payload expanded towards therapeutic molecules. Furthermore, the cathepsin B-induced cleavage of the synthesised peptide, as well as the C₆₀- and CNT-bound peptide species was achieved, but needs future optimisation. The cleavage and release mechanism of the C₆₀-CNT hybrid structure have to be investigated in more detail. The influence of co-solvents and the pH on the reaction progress has to be analysed. Moreover, the enzymatic activity and release kinetics should be studied to give a better estimation of the potential of this novel DDS. Another question that can be addressed by cell-based assays would be the verification of the assumed location of delivery and the release of the payload in the lysosome.

VII. Experimental

7.1 General

EDX An Oxford Instruments EDS detector equipped with an ultra-thin polymer window and controlled by Oxford Instruments Isis software INCA was used for the acquisition on the JEOL JEM-3000F.

HPLC was performed on a Waters[®] 2795 Separations Module, AllianceHT with a reversed phase column TSKgel ODS 80TM (150x4.5 mm, 5 μ m) from Tosho and a gradient of *i*PrOH/hexane 80:20 for 10 min at a flow rate of 1 mg/ml.^{186,221,222} Substrates were detected at $\lambda = 254$ nm with a Waters[®] 2996 Photodiode Array Detector. The data was processed with the software Empower Pro, copyright[©] 2002 Waters Corporation. Furthermore, a Phenomenex Jupiter 4u Proteo 90A (250x4.6 mm, 0.9 mL/min) and Synergi 4u Hydro-RP 80A (100x4.6 mm, 4 micron, 1 mL/min and 100x21.2 mm, 4 micron, 15 mL/min) were used for peptide work. The solvent system was mixed appropriately from H₂O (MilliQ purity) and MeCN, 0.1% formic acid (FA) each.

HRMS data was determined under positive ion conditions using a Micromass Q-Tof mirco (resolution = 4×10^{-3} D) using H₃PO₄-clusters as a lock-mass in positive ion mode.

HRTEM SWNT samples were dispersed in EtOH [10 μ g/mL], sonicated for 30 to 60 min, placed dropwise (4 x 6 μ L) onto a Lacey carbon copper support grid, and dried by evaporation. The JEOL JEM-3000F with a field-emission gun operated at 300 kV. The microscope was equipped with a 1k Gatan 794 MultiScan camera. The data were analysed with DigitalMicrograph.

LC/MS was performed on a Waters SYNAPT HDMS with a NanoAcquity UPLC inlet and time of flight acquisition. The data were processed with MassLynx.

Infra-red spectra for small organic molecules were recorded on a Bruker Vector 22 IR spectrometer, with the sample being pressed into KBr pellets. The IR of CNT samples were recorded using attenuated total reflection Fourier transformed infrared (ATR-FTIR) on a Varian FTS-7000 spectrometer and a DuraSamplIR II diamond crystal by Dr. Robert Jacobs in the Surface Analysis Facility, Chemistry Research Laboratory. Data were processed with Vario Resolution Pro 6.0.

IUPAC names and atom numbering of the compounds described in the experimental section were given using the program CS ChemDraw Ultra 12.0.3, 14.0, and 15.0.

MALDI-ToF spectra were recorded on a Waters[®] Micromass[®] MALDI micro MX[™] Mass

Spectrometer in the positive reflectron mode. The data were analysed with MassLynx V4.1.

^1H NMR spectra were recorded at room temperature on the following spectrometers: 200 MHz: Bruker DPX200, 300 MHz: Bruker DPX300, 400 MHz: Bruker DQX400 and AVIII HD 400, 500 MHz: Bruker AV500. Unless reported otherwise, spectra were recorded in CDCl_3 solution. Chemical shifts are reported in δ units relative to CHCl_3 ($\delta_{\text{H}} = 7.26$). The following abbreviations were used throughout: s = singlet, d = doublet, dd = doublet of doublet, dt = doublet of triplet, t = triplet, tt = triplet of triplet, q = quartet, quin = quintett, m = multiplet.

^{13}C NMR spectra were recorded at room temperature on the following spectrometers: 75 MHz: Bruker DPX300, 100 MHz: Bruker DQX400, 125 MHz: Bruker DRX500. Unless reported otherwise, spectra were recorded in CDCl_3 solution. Chemical shifts are reported in δ units relative to CDCl_3 ($\delta_{\text{C}} = 77.23$ {central line of triplet}).

^{19}F NMR spectra were recorded at room temperature a 376 MHz: Bruker AVIII HD 400. Unless reported otherwise, spectra were recorded in CDCl_3 solution. Chemical shifts were reported in δ units.

Raman spectra were recorded on a Jobin Yvon spectrometer (Horiba) equipped with a microscope through a 50-fold magnification objective (Olympus Co.) by adding three sets of spectra together. A 20 mW He-Ne laser (632 nm) was used. The 1800 L/mm grating provides a resolution starting from 1.0 cm^{-1} at 100 cm^{-1} up to 0.5 cm^{-1} at 3000 cm^{-1} . The abscissa was calibrated with a silicon standard, and the sharp Raman shifts were accurate to $\pm 2\text{ cm}^{-1}$. Raman spectra were directly taken from the solid.

TGA SWNT samples were measured in synthetic air flow (20 mL/min) using the model STA 449 F1 Jupiter[®] from Netzsch. The temperature was kept isothermal for 15 min, and then ramped at $10\text{ }^\circ\text{C}/\text{min}$ to $900\text{ }^\circ\text{C}$.

UV/vis was performed on a NanoDrop[®] ND-1000 spectrophotometer with a full-spectrum 220–750 nm or a Waters[®] 2996 Photodiode Array Detector with a spectrum range of 220–400 nm. The sample contained $1\text{ }\mu\text{L}$, and the transmission length was stated as $L = 0.1\text{ cm}$. 96 Well plate and cuvette measurements were recorded with a CCD detector at a λ 220–1000 nm using a SPECTROstar *Nano* from BMD LABTECH; data were processed with MARS Data Analysis Software and Microsoft Excel.

XPS data were collected under high vacuum (approx. 10^9 torr) on a VD Escalab XPS Spectrometer by Ashley Shepherd in the Surface Analysis Facility, Chemistry Research Laboratory. Analysis was undertaken with CasaXPS software.

7.1.1 Reagents and Solvents

All reagents and solvents were obtained from Sigma-Aldrich, Alfa Aesar, Carbosynth, Fluka, and Frontier Scientific and were used directly as supplied, unless otherwise reported. Anhydrous solvents were from Merck and Sigma-Aldrich. All non-aqueous reactions were performed in oven- or flame-dried apparatus under argon or nitrogen atmosphere using anhydrous solvents. Carbon nanotubes were supplied by Thomas Swan (produced using iron catalyst *via* chemical vapour

deposition)^{308,309} and steam-purified by Dr. Gerard Tobias, Institut de Ciència de Materials de Barcelona (ICMAB).^{310,311}

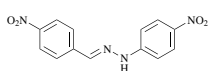
7.1.2 Techniques

Reactions were monitored by thin layer chromatography on pre-coated aluminium-backed plates (Merck Kieselgel 60 with fluorescent indicator UV₂₅₄). Spots were visualised by quenching of UV fluorescence and by staining with potassium permanganate, iodine, ninhydrin, *p*-anisaldehyde, or vanillin.

Flash column chromatography was performed according to the method described by Still, Kahn, and Mitra³⁵¹ with silica gel 60 (0.040 - 0.063 mm; Geduran[®] Si 60) applying head pressure by means of manual flushing.

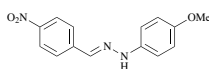
7.2 Hydrazone Syntheses

7.2.1 (*E*)-1-(4-Nitrobenzylidene)-2-(4'-nitrophenyl)hydrazone²¹⁴ (**1**)



To a stirred solution of *p*-nitrobenzaldehyde (197.4 mg, 1.3 mmol, 1 eq.) in EtOH (10 mL, 0.13 M) at 78 °C was added *p*-nitrobenzyl hydrazine (200 mg, 1.3 mmol, 1 eq.). The solution was heated to reflux for 30 min until t.l.c. analysis indicated complete consumption of the starting material. The reaction mixture was cooled to room temperature, and the solvent was removed under reduced pressure. The crude product was purified by recrystallisation from EtOH, followed by flash column chromatography on silica gel (petrol. ether/CH₂Cl₂ 11:9 with 2% of 7 N NH₃ in MeOH) to give purified hydrazone **1** as a red solid (48.1 mg, 13%). **R_f** 0.25 (petrol. ether/EtOAc 1:1); ¹H NMR (500 MHz, acetone-d₆) δ_H 7.38 (d, ³J_{2',3'} = 9.0 Hz, 2H, H-2', H-6'), 8.02 (d, ³J_{2,3} = 8.8 Hz, 2H, H-2, H-6), 8.17 (s, 1H, CH=NNH), 8.20 (d, ³J_{3',2'} = 9.3 Hz, 2H, H-3', H-5'), 8.29 (d, ³J_{3,2} = 8.9 Hz, 2H, H-3, H-5), 10.65 (s, 1H, CH=NNH); ¹³C NMR (500 MHz, acetone-d₆) δ_C 113.0 (C-2', C-6'), 124.8 (C-3, C-5), 126.7 (C-2, C-6), 128.0 (C-3', C-5'), 139.7 (CH=NNH), 141.4 (C-1'), 142.4 (C-1), 148.6 (C-4), 150.9 (C-4'). Data were consistent with the literature values.²¹⁴

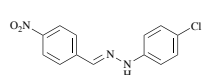
7.2.2 (*E*)-1-(4'-Methoxyphenyl)-2-(4-nitrobenzylidene)hydrazone²¹⁴ (**2**)



To a stirred solution of *p*-methoxyphenyl hydrazine hydrochloride (200 mg, 1.15 mmol, 1 eq.) and NEt₃ (0.16 mL, 1.15 mmol, 1 eq.) in EtOH (1.6 mL, 0.3 M) at 78 °C was added *p*-nitrobenzaldehyde (173.1 mg, 1.2 mmol, 1 eq.) and one drop of acetic acid. The dark red solution was refluxed for 65 min with 0.4 nm molecular sieve until t.l.c. analysis indicated complete consumption of the starting material. The reaction mixture was cooled to room temperature, and the resultant precipitate was filtered. The crude product was purified by recrystallisation from EtOH to give purified hydrazone **2** as red crystals (177.1 mg, 82%). **R_f** 0.35 (petrol. ether/EtOAc 3:1); ¹H NMR (400 MHz, acetone-d₆) δ_H 3.75

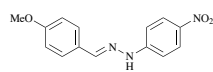
(s, 3H, OCH₃), 6.88 (d, $^3J_{2',3'} = 9.0$ Hz, 2H, H-2', H-6'), 7.18 (d, $^3J_{3',2'} = 9.0$ Hz, 2H, H-3', H-5'), 7.85 (d, $^3J_{2,3} = 8.9$ Hz, 2H, H-2, H-6), 7.93 (s, 1H, CH=NNH), 8.20 (d, $^3J_{3,2} = 8.9$ Hz, 2H, H-3, H-5), 10.03 (s, 1H, CH=NNH); ^{13}C NMR (400 MHz, acetone-d₆) δ_{C} 55.8 (OCH₃), 114.8 (C-3', C-5'), 115.5 (C-2', C-6'), 124.8 (C-3, C-5), 126.6 (C-2, C-6), 133.4 (C-1'), 139.5 (CH=NNH), 144.1 (C-1), 147.3 (C-4), 155.2 (C-4'); UV/vis $\lambda_{\text{max}} = 430$ nm; 260 nm. Data were consistent with the literature values.^{107,245,352}

7.2.3 (*E*)-1-(4'-Chlorophenyl)-2-(4-nitrobenzylidene)hydrazone²¹⁴ (**3**)



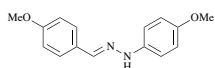
To a stirred solution of *p*-chlorophenyl hydrazine hydrochloride (200 mg, 1.12 mmol, 1 eq.) and NEt₃ (0.14 mL, 1.12 mmol, 1 eq.) in EtOH (1.7 mL, 0.65 M) at 78 °C was added *p*-nitrobenzaldehyde (168.8 mg, 1.12 mmol, 1 eq.) and one drop of acetic acid. The solution turned dark red immediately and was heated at reflux for 20 min until t.l.c. analysis indicated complete consumption of the starting material. The reaction mixture was cooled to room temperature, and the resultant precipitate was filtered. The crude product was purified by recrystallisation from EtOH to give purified hydrazone **3** as red crystals (284.0 mg, 92%). **R_f** 0.40 (toluene); **mp** 161.0-162.2 °C; UV/vis λ_{max} (MeOH) [nm] = 413 (ϵ [dm³mol⁻¹cm⁻¹] 42300), 285sh (19400) and 261 (25200); IR (film) ν_{max} [cm⁻¹] 3303 (C=C-H), 2856, 15962 (C=C_{arom}), 1565, 1514, 1487, 1410 (CH₂), 1336, 1288, 1259, 1151, 1108, 1090 (C_{aryl}-Cl), 907, 856, 823, 750, 693, 674, 658. 639, 611; ^1H NMR (400 MHz, MeOD) δ_{H} 7.11 (2H, d, $^3J_{2',3'} = 9.3$ Hz, H-2', H-6'), 7.21 (2H, d, $^3J_{3',2'} = 9.3$ Hz, H-3', H-5'), 7.81 (2H, d, $^3J_{2,3} = 9.0$ Hz, H-2, H-6), 7.81 (1H, s, CH=NNH), 8.21 (2H, d, $^3J_{3,2} = 9.3$ Hz, H-3, H-5); ^{13}C NMR (100 MHz, MeOD) δ_{C} 115.0 (C-3', C-5'), 125.0 (C-2', C-6'), 125.7 (C-4'), 127.2 (C-3, C-5), 130.1 (C-2, C-6), 135.3 (C-1', CH=NNH), 144.0 (C-1), 148.2 (C-4); **m/z** (FI⁺) calc. for [M]⁺ = 275.0462, found = 275.0456.

7.2.4 (*E*)-1-(4-Methoxybenzylidene)-2-(4'-nitrophenyl)hydrazone²¹⁴ (**4**)



To a stirred solution of *p*-methoxybenzaldehyde (0.16 mL, 1.3 mmol, 1 eq.) in EtOH (10 mL, 0.13 M) at 78 °C was added *p*-nitrobenzyl hydrazine (200 mg, 1.3 mmol, 1 eq.). After heating at reflux the solution for 2.5 h, NEt₃ (0.17 mL, 1.3 mmol, 1 eq.) was added. The reaction mixture was refluxed for 1 h until t.l.c. analysis indicated complete consumption of the starting material. It was cooled to room temperature, and the solvent was removed under reduced pressure. The crude product was purified flash column chromatography on silica gel (petrol. ether/CH₂Cl₂ 4:1 with 2% of 7 N NH₃ in MeOH) to give purified hydrazone **1** as a purple solid (80.8 mg, 28%). ^1H NMR (500 MHz, CDCl₃) δ_{H} 3.85 (s, 3H, OCH₃), 6.94 (d, $^3J_{3,2} = 8.7$ Hz, 2H, H-3, H-5), 7.10 (d, $^3J_{2',3'} = 9.1$ Hz, 2H, H-2', H-6'), 7.63 (d, $^3J_{2,3} = 8.7$ Hz, 2H, H-2, H-6), 7.77 (s, 1H, CH=NNH), 7.99 (s, 1H, CH=NNH), 8.17 (d, $^3J_{3',2'} = 9.2$ Hz, 2H, H-3', H-5'); ^{13}C NMR (500 MHz, CDCl₃) δ_{C} 55.5 (OCH₃), 111.7 (C-2', C-6'), 114.5 (C-3, C-5), 126.4 (C-3', C-5'), 127.0 (C-1), 128.4 (C-2, C-6), 140.2 (C-1'), 141.5 (CH=NNH), 149.8 (C-4'), 161.1 (C-4). Data were consistent with the literature values.³⁵³

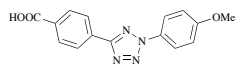
7.2.5 (*E*)-1-(4-Methoxybenzylidene)-2-(4'-methoxyphenyl)hydrazone²¹⁵ (**5**)



Chopped NaOH pellets (46.0 mg, 1.15 mmol, 1 eq.), *p*-methoxyphenyl hydrazine hydrochloride (200 mg, 1.15 mmol, 1 eq.), silica (115 mg, 10 mmol/g), and NEt₃ (0.15 mL, 1.15 mmol, 1 eq.) were mixed together in an agate mortar. *p*-Methoxybenzaldehyde (0.14 mL, 1.15 mmol, 1 eq.) was added, and the mixture was subjected to grinding for 40 min until the powder was dry. The solid was dissolved in CH₂Cl₂ (30 mL) and HCl (12 mL of a 1 M aqueous solution) and extracted with CH₂Cl₂ (2 x 10 mL). The combined organic fractions were washed with NaHCO₃ (15 mL of a saturated aqueous solution) and brine (15 mL), and dried over MgSO₄. The solvent was removed under reduced pressure, and the crude product was purified by recrystallisation from EtOH to give purified hydrazone **5** as light brown crystals (102.4 mg, 35%). **R_f** 0.50 (petrol. ether/EtOAc 3:1); **m_p** 125-127 °C (from EtOH); **UV/vis** λ_{max}(CDCl₃) [nm] = 355, 312, 260 and 230; **IR** (film) ν_{max} [cm⁻¹] 3310 (amine, C=C-H), 3007, 2957, 2929 (C_{aryl}H), 2836 (-OMe), 1608 (C=C_{arom}), 1569, 1521, 1508, 1477 (amine), 1441, 1417, 1353, 1314, 1295, 1249, 1182, 1167, 1137, 1100, 1032, 945, 905, 827 (C=C-H), 773, 644; **¹H NMR** (400 MHz, CDCl₃) δ_H 3.78 (3H, s, OCH₃'), 3.83 (3H, s, OCH₃), 6.86 (2H, d, ³J_{3',2'} = 9.0 Hz, H-3', H-5'), 6.90 (2H, d, ³J_{3,2} = 8.8 Hz, H-3, H-5), 7.05 (2H, d, ³J_{2',3'} = 9.0 Hz, H-2', H-6'), 7.36 (1H, s, CH=NNH), 7.58 (2H, d, ³J_{2,3} = 8.8 Hz, H-2, H-6), 7.63 (1H, s, CH=NNH); **¹³C NMR** (100 MHz, CDCl₃) δ_C 55.5 (OCH₃), 55.8 (OCH₃'), 114.1 (C-3', C-5'), 114.2 (C-3, C-5), 114.9 (C-2', C-6'), 127.6 (C-2, C-6), 128.4 (C-1), 137.3 (CH=NNH), 139.3 (C-1'), 153.8 (C-4'), 160.0 (C-4); **m/z** (ESI⁺) calc. for [M+Na]⁺ = 279.1104, found = 279.1103.

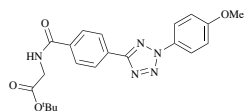
7.3 Tetrazole Syntheses

7.3.1 4-(2'-(4''-Methoxyphenyl)-2*H*-tetrazol-5'-yl)benzoic acid (**12**)



Tetrazole **12** was kindly donated by Dr. Sander van Berkel.³⁵⁴

7.3.2 (*tert*)-Butyl 2-(4'-(2''-(4'''-methoxyphenyl)-2*H*-tetrazol-5''-yl)benzamido) acetate (**13**)

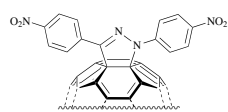


To a stirred solution of tetrazole **12** (50.0 mg, 0.17 mmol, 1 eq.) in anhydrous DMF (2.8 mL, 60 mM) at room temperature was added HATU (128.3 mg, 0.34 mmol, 2 eq.) and DIPEA (58.8 μL, 0.34 mmol, 2 eq.) under an atmosphere of argon. The solution was stirred for 20 min until t.l.c. analysis indicated complete consumption of the starting material. To the yellow mixture was added H-Gly-O^tBu (42.4 mg, 0.25 mmol, 1.5 eq.). The reaction mixture was stirred at room temperature for 40 min. The solvent was removed *in vacuo*, and the crude product was dissolved in CHCl₃ (15 mL) and extracted with H₂O (4 x 15 mL). The combined organic fractions were washed with brine (25 mL) and dried over MgSO₄. The solvent was removed under reduced pressure,

and the crude product was purified by flash column chromatography on silica gel (CHCl_3) to give tetrazole **13** as a white solid (56.4 mg, 67%). R_f 0.20 (CHCl_3); **UV/vis** $\lambda_{max}(\text{CDCl}_3)$ [nm] = 260 (ϵ [$\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$] 42000); **IR** (film) ν_{max} [cm^{-1}] 3335 (amine, C=C-H), 2979, 2934 (C_{aryl}H), 1746 (C=O), 1643 (CONH), 1552 (heterocycle), 1512, 1467, 1367, 1305 (N-N=N), 1255, 1224, 1153, 1018 & 998 (heterocycle), 918, 835 (C=C-H), 741, 624; $^1\text{H NMR}$ (400 MHz, CDCl_3) δ_H 1.51 (9H, s, $\text{C}(\text{CH}_3)_3$), 3.88 (3H, s, OCH_3), 4.16 (2H, d, $^3J_{\text{Gly,NH}} = 4.9$ Hz, H_{Gly}), 6.83 (1H, t, $^3J_{\text{NH,Gly}} = 4.7$ Hz, NH), 7.05 (2H, d, $^3J_{3'',2'''} = 9.1$ Hz, H-3'', H-5''), 7.95 (2H, d, $^3J_{3',2'} = 8.4$ Hz, H-3', H-5'), 8.09 (2H, d, $^3J_{2'',3'''} = 9.1$ Hz, H-2'', H-6''), 8.29 (2H, d, $^3J_{2',3'} = 8.4$ Hz, H-2', H-6'); $^{13}\text{C NMR}$ (100 MHz, CDCl_3) δ_C 28.2 ($\text{C}(\text{CH}_3)_3$), 42.7 (Gly), 55.8 (OCH_3), 82.8 ($\text{C}(\text{CH}_3)_3$), 114.8 (C-3'', C-5''), 121.5 (C-2'', C-6''), 127.2 (C-2', C-6'), 127.8 (C-3', C-5'), 130.4 (C-1''), 135.5 (C-4'), 160.7 (C-4''), 164.2 (C-1'), 166.7 (CONH), 169.3 (COO^tBu); m/z (ESI^+) calc. for $[\text{M}+\text{H}]^+ = 410.1828$, found = 410.1816.

7.4 Fulleropyrazoline Syntheses

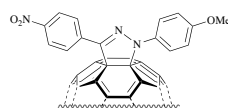
7.4.1 1-(4'-Nitrophenyl)-3-(4-nitrobenzylidene)-4,5-fullerene-pyrazoline (6)



To a stirred solution of hydrazone **1** (19.9 mg, 69.4 μmol , 1 eq.) in anhydrous CHCl_3 (10 mL, 7 mM) at room temperature was added recrystallised NBS (18.5 mg, 104.1 μmol , 1.5 eq.) under an atmosphere of argon. The solution was stirred for 35 min until t.l.c. analysis indicated complete consumption

of the starting material and the solvent removed quickly under reduced pressure. The brown solid was solved in anhydrous toluene (12 mL, 5.2 mM). After adding C_{60} (50 mg, 69.4 μmol , 1 eq.) and NEt_3 (13 μL , 104.1 μmol , 1.5 eq.) the reaction mixture was stirred under microwave irradiation ($T = 160^\circ\text{C}$, 88 W) for 1.5 h. The solvent was removed *in vacuo* and the crude product was purified twice by flash column chromatography on silica gel ($\text{CS}_2 \rightarrow \text{toluene}$) to give unconsumed C_{60} (31.5 mg, 63%) and the monocycloadduct **6** (10.0 mg, 15%, 25% based on recovered C_{60}). R_f 0.85 (toluene/ EtOAc 24:1); $^1\text{H NMR}$ (500 MHz, CDCl_3) δ_H 8.28 (d, $^3J_{2',3'} = 9.3$ Hz, 2H, H-2', H-6'), 8.37 (d, $^3J_{3',2'} = 9.3$ Hz, 2H, H-3', H-5'), 8.40 (d, $^3J_{2,3} = 8.9$ Hz, 2H, H-2, H-6), 8.52 (d, $^3J_{3,2} = 8.9$ Hz, 2H, H-3, H-5); $^{13}\text{C NMR}$ (500 MHz, CDCl_3) δ_C 81.8 (C_{60-1}), 91.5 (C_{60-2}), 120.3 (C-2', C-6'), 124.4 (C-2, C-6), 125.5 (C-1), 125.6 (C-3', C-5'), 128.4 ($\text{CH}=\text{NNH}$), 129.2 (C-1'), 129.8 (C-3, C-5), 136.7, 137.2, 138.0 (1/2C), 138.1 (1/2C), 139.8, 140.8, 142.2, 142.2, 142.4, 142.6, 142.7, 143.2, 143.3, 143.3 (1/2C), 143.5, 144.4, 144.5 (1/2C), 144.5, 144.6, 145.3, 145.4, 145.4 (2C), 145.7, 145.8, 146.2, 146.4 (2C), 146.6, 146.7, 147.5 (1/2C), 147.9 (1/2C), 148.5 (1/2C), 149.2 (C-4'). Data were consistent with the literature values.²¹⁴

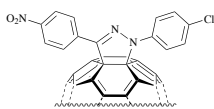
7.4.2 1-(4'-Methoxyphenyl)-3-(4-nitrobenzylidene)-4,5-fullerene-pyrazoline (7)



To a stirred solution of hydrazone **2** (42.7 mg, 69.4 μmol , 1 eq.) in anhydrous CHCl_3 (10 mL, 7 mM) at room temperature was added recrystallised NBS (18.5 mg, 104.1 μmol , 1.5 eq.) under an atmosphere of argon. The solution

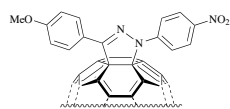
was stirred for 30 min until t.l.c. analysis indicated complete consumption of the starting material and the solvent removed quickly under reduced pressure. The brown solid was solved in anhydrous toluene (12 mL, 5.2 mM). After adding C₆₀ (50 mg, 69.4 μ mol, 1 eq.) and NEt₃ (26 μ L, 208.1 μ mol, 3 eq.) the reaction mixture was stirred under microwave irradiation (T = 110 °C, 60 W) for 3 h. The solvent was removed *in vacuo* and the crude product was purified by flash column chromatography on silica gel (CS₂ $\rightarrow$ toluene $\rightarrow$ toluene/EtOAc 50:1) to give unconsumed C₆₀ (12.4 mg, 25%) and the monocycloadduct **7** (27.6 mg, 40%, 65% based on recovered C₆₀). ¹H NMR (500 MHz, CDCl₃) δ_H 3.86 (s, 3H, OCH₃), 7.03 (d, ³J_{2',3'} = 9.0 Hz, 2H, H-2', H-6'), 7.82 (d, ³J_{3',2'} = 9.0 Hz, 2H, H-3', H-5'), 8.35 (d, ³J_{2,3} = 9.0 Hz, 2H, H-2, H-6), 8.57 (d, ³J_{3,2} = 9.0 Hz, 2H, H-3, H-5); ¹³C NMR (500 MHz, CDCl₃) δ_C 55.7 (OCH₃), 80.4 (C₆₀-1), 93.4 (C₆₀-2), 114.8 (C-3', C-5'), 124.2 (C-2', C-6'), 127.1 (C-3, C-5), 128.4 (CH=NNH), 128.9 (C-2, C-6), 129.2 (C-1'), 136.3, 136.9, 138.0, 139.3 (1/2C), 140.2, 140.3 (1/2C), 140.5, 142.0, 142.3 (2C), 142.4, 142.5, 142.6, 143.0, 143.1, 144.3 (1/2C), 144.4, 144.5, 145.0, 145.1, 145.4, 145.5, 145.7, 145.7, 145.9, 146.0, 146.2, 146.2, 146.4, 146.5, 147.4 (1/2C), 147.6 (1/2C), 147.8 (1/2C), 158.4 (C-4'); m/z (MALDI) calc. for [M] = 989.08, found = 989.73 (C₆₀ standard m/z = 720.44). Data were consistent with the literature values.¹⁰⁷

7.4.3 1-(4'-Chlorophenyl)-3-(4-nitrobenzylidene)-4,5-fullerene-pyrazoline (**8**)



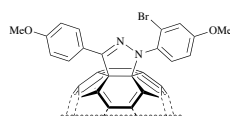
To a stirred solution of hydrazone **3** (10.7 mg, 34.7 μ mol, 1 eq.) in anhydrous CHCl₃ (5 mL, 7 mM) at room temperature was added recrystallised NBS (9.3 mg, 52.0 μ mol, 1.5 eq.) under an atmosphere of argon. The solution was stirred for 30 min until t.l.c. analysis indicated complete consumption of the starting material. The solvent was removed under reduced pressure and the brown solid was solved in anhydrous toluene (12 mL, 2.6 mM). After adding C₆₀ (25 mg, 34.7 μ mol, 1 eq.) and NEt₃ (13.4 μ L, 104.0 μ mol, 3 eq.) the reaction mixture was stirred at room temperature for 2 h and heated at T = 85 °C for a further 2 h. The solvent was removed *in vacuo* and the crude product was purified by flash column chromatography on silica gel (CS₂ $\rightarrow$ CS₂/toluene 1:1) to give unconsumed C₆₀ (11.6 mg, 46%) and the monocycloadduct **8** (17.8 mg, 51%, 97% based on recovered C₆₀). UV/vis λ_{max} (toluene) [nm] = 254 (ϵ [dm³mol⁻¹cm⁻¹] 56600) and 314 (20400); IR (film) ν_{max} [cm⁻¹] 2962-2851 (CH₃, CH₂, CH), 1594 (C=C_{arom}), 1544, 1514, 1487, 1405 (CH₂), 1335, 1260, 1219, 1090 (C_{aryl}-Cl), 1017, 850, 797, 773, 689, 667, 621; ¹H NMR (500 MHz, CDCl₃) δ_H 7.47 (2H, d, ³J_{2',3'} = 8.8 Hz, H-2', H-6'), 7.91 (2H, d, ³J_{3',2'} = 8.8 Hz, H-3', H-5'), 8.37 (2H, d, ³J_{2,3} = 8.9 Hz, H-2, H-6), 8.55 (2H, d, ³J_{3,2} = 8.9 Hz, H-3, H-5); ¹³C NMR (125 MHz, CDCl₃) δ_C 80.9 (C₆₀-1), 92.6 (C₆₀-2), 124.3 (C-3', C-5'), 125.3 (C-2', C-6'), 129.2 (C-3, C-5), 129.7 (C-2, C-6), 131.4 (CH=NN), 136.6, 136.8, 138.8 (1/2C), 140.1, 140.6, 142.0, 142.1, 142.3, 142.3, 142.3, 142.6, 142.6, 142.6 (1/2C), 143.1, 143.1, 144.4, 144.3, 144.4 (2C), 144.9, 145.3, 145.4, 145.5, 145.5, 145.7, 145.8, 146.1, 146.2, 146.2, 146.5, 146.6, 147.5 (1/2C), 147.8 (1/2C), 147.9 (1/2C); m/z (MALDI) calc. for [M] = 993.03, found = 993.61 (C₆₀ standard m/z = 720.44).

7.4.4 1-(4'-Nitrophenyl)-3-(4-methoxybenzylidene)-4,5-fullerene-pyrazoline (9)



To a stirred solution of hydrazone **4** (25.5 mg, 69.4 μ mol, 1 eq.) in anhydrous CHCl_3 (10 mL, 7 mM) at room temperature was added recrystallised NBS (18.5 mg, 104.1 μ mol, 1.5 eq.) under an atmosphere of argon. The solution was stirred for 35 min until t.l.c. analysis indicated complete consumption of the starting material and the solvent removed quickly under reduced pressure. The brown solid was solved in anhydrous toluene (12 mL, 5.2 mM). After adding C_{60} (50 mg, 69.4 μ mol, 1 eq.) and NEt_3 (13 μ L, 104.1 μ mol, 1.5 eq.) the reaction mixture was stirred under microwave irradiation ($T = 160^\circ\text{C}$, 88 W) for 3 h. The solvent was removed *in vacuo* and the crude product was purified twice by flash column chromatography on silica gel (toluene $\rightarrow$ toluene/EtOAc 24:1) to give unconsumed C_{60} (19.0 mg, 38%), the monocycloadduct **9** (16.0 mg, 24%, 39% based on recovered C_{60}), and the disubstituted cycloadduct (9.2 mg, 11%, 17% based on recovered C_{60}). R_f 0.80 (toluene/EtOAc 24:1); $^1\text{H NMR}$ (500 MHz, CDCl_3) δ_H 3.89 (s, 3H, OCH_3), 7.07 (d, $^3J_{3,2} = 8.9$ Hz, 2H, H-3, H-5), 8.19 (d, $^3J_{2,3} = 8.9$ Hz, 2H, H-2, H-6), 8.26 (d, $^3J_{2',3'} = 9.4$ Hz, 2H, H-2', H-6'), 8.33 (d, $^3J_{3',2'} = 9.4$ Hz, 2H, H-3', H-5'); $^{13}\text{C NMR}$ (500 MHz, CDCl_3) δ_C 55.6 (OCH_3), 82.8 (C_{60-1}), 90.5 (C_{60-2}), 114.6 (C-3, C-5), 119.3 (C-2', C-6'), 124.0, 125.4 (C-1), 125.6 (C-3', C-5'), 128.4 (CH=NNH), 129.2 (C-1'), 130.8 (C-2, C-6), 136.3, 137.2, 138.0 (1/2C), 139.5, 140.6, 142.1, 142.3 (2C), 142.6 (2C), 143.1, 143.2, 143.2 (2C), 143.4, 143.5, 144.3, 144.6, 144.8, 145.4, 145.5, 145.6, 145.8 (2C), 146.1, 146.3, 146.3, 146.5, 146.6, 146.6, 147.0 (1/2C), 147.4 (1/2C), 147.8 (1/2C), 150.0 (C-4'), 161.2 (C-4); m/z (MALDI) calc. for $[\text{M}] = 989.72$, found = 987.67 (C_{60} standard $m/z = 718.85$). Data were consistent with the literature values.²¹⁴

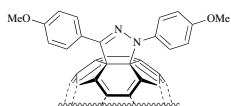
7.4.5 1-(2'-Bromo-4'-methoxyphenyl)-3-(4-methoxybenzylidene)-4,5-fullerene-pyrazoline (77)



To a stirred solution of hydrazone **5** (17.8 mg, 69.4 μ mol, 1 eq.) in anhydrous CHCl_3 (10 mL, 7 mM) at room temperature was added recrystallised NBS (18.5 mg, 104.1 μ mol, 1.5 eq.) under an atmosphere of argon. The solution was stirred for 30 min until t.l.c. analysis indicated complete consumption of the starting material and the solvent removed quickly under reduced pressure. The red solid was solved in a mixture of anhydrous toluene/ CHCl_3 11:1 (12 mL, 5.2 mM). After adding C_{60} (50 mg, 69.4 μ mol, 1 eq.) and NEt_3 (13 μ L, 104.1 μ mol, 1.5 eq.) the reaction mixture was stirred under microwave irradiation ($T = 160^\circ\text{C}$, 83 W) for 3 h. The solvent was removed *in vacuo* and the crude product was purified by flash column chromatography on silica gel ($\text{CS}_2 \rightarrow$ toluene) to give unconsumed C_{60} (40.3 mg, 80%) and an unseparable 5:2 mixture of the brominated monocycloadduct **77** and the chlorinated monocycloadduct (11.9 mg, 16%, 84% based on recovered C_{60}). R_f 0.70 (toluene/EtOAc 24:1); $^1\text{H NMR}$ (500 MHz, CDCl_3) δ_H 3.85 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 6.98 (1H, dd, $^4J_{5',3'} = 2.8$ Hz, $^3J_{5',6'} = 8.8$ Hz, H-5'), 7.03 (2H, d, $^3J_{3,2} = 8.9$ Hz, H-3, H-5), 7.28 (1H, d, $^4J_{3',5'} = 2.8$ Hz, H-3'), 7.81 (1H, d, $^3J_{6',5'} = 8.8$ Hz, H-6'), 8.25 (2H, d, $^3J_{2,3} = 8.9$ Hz, H-2, H-6); $^{13}\text{C NMR}$ (500 MHz, CDCl_3) δ_C 55.5 (OCH_3), 55.9 (OCH_3),

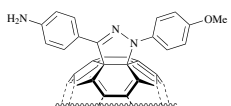
81.3 (C₆₀-1), 93.0 (C₆₀-2), 114.4 (C-5'), 114.4 (C-3, C-5), 119.5 (C-3'), 125.1 (CH=NNH), 130.3 (C-2, C-6), 132.9 (C-6'), 135.6 (C-1'), 140.0 (1/2C), 140.3, 141.9, 142.3, 142.4, 142.4, 142.5, 142.5, 142.9 (C-1), 142.9, 142.9, 143.2, 144.4, 144.4, 145.1 (1/2C), 145.2, 145.4, 145.5, 145.9, 145.9, 146.0, 146.1, 146.1, 146.3, 146.4, 146.5, 146.5 (1/2C), 147.3 (1/2C), 147.7 (1/2C), 159.7 (C-4'), 160.6 (C-4); **m/z** (MALDI) calc. for [M] = 1052.02, found = 1050.74 (C₆₀ standard **m/z** = 718.92).

7.4.6 1-(4'-Methoxyphenyl)-3-(4-methoxybenzylidene)-4,5-fullerene-pyrazoline²⁰⁴ (10)



To a stirred solution of hydrazone **5** (17.8 mg, 69.4 μ mol, 1 eq.) and C₆₀ (50 mg, 69.4 μ mol, 1 eq.) in anhydrous toluene (14 mL, 5 mM) was added (diacetoxyiodo)benzene (33.5 mg, 104.1 μ mol, 1.5 eq.) under an atmosphere of argon at 40 °C. The solution was stirred for 3.5 h and the solvent removed quickly under reduced pressure. The crude product was purified by flash column chromatography on silica gel (CS₂ $\rightarrow$ CS₂/toluene 1:1 $\rightarrow$ toluene) to give unconsumed C₆₀ (38.3 mg, 76%) and the monocycloadduct. The solid was washed with MeOH (2 x 1.5 mL) and Et₂O (0.5 mL) to give purified **10** as a brown solid (0.8 mg, 1%, 5% based on recovered C₆₀). **R_f** 0.80 (CHCl₃/EtOAc 1:19); **UV/vis** λ_{max} (toluene) [nm] = 255 (ϵ [dm³mol⁻¹cm⁻¹] 25300) and 312 (88000); **¹H NMR** (500 MHz, CDCl₃) δ_H 3.84 (3H, s, OCH₃'), 3.87 (3H, s, OCH₃), 7.00 (2H, d, $^3J_{3',2'} = 8.9$ Hz, H-3', H-5'), 7.03 (2H, d, $^3J_{3,2} = 8.8$ Hz, H-3, H-5), 7.80 (2H, d, $^3J_{2',3'} = 8.9$ Hz, H-2', H-6'), 8.23 (2H, d, $^3J_{2,3} = 8.8$ Hz, H-2, H-6); **¹³C NMR** product was not sufficiently stable to enable us to record the spectrum; **m/z** (MALDI) calc. for [M] = 974.11, found = 972.81 (C₆₀ standard **m/z** = 718.90).

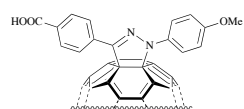
7.4.7 1-(4'-Aminophenyl)-3-(4-methoxybenzylidene)-4,5-fullerene-pyrazoline^{203,217} (11)



To a stirred solution of fulleropyrazoline **7** (32.8 mg, 33.1 μ mol, 1 eq.) and tin powder (2.36 g, 19.9 mmol, 600 eq.) in CHCl₃ (83 mL, 0.4 mM) was added HCl (33 mL of a 1 M aqueous solution, 1 mM) portionwise at room temperature. The solution was heated at reflux for 6 h, cooled to room temperature and neutralised with NaOH_{aq} to pH 11. The aqueous phase was extracted with CHCl₃ (3 x 50 mL), the organic fractions were combined, washed with brine (2 x 50 mL), dried over MgSO₄, and the solvent removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (CHCl₃) to give unconsumed **7** (7.3 mg, 22%) and the monocycloadduct **11** as a brown solid (1.9 mg, 6%, 8% based on recovered **7**). **R_f** 0.50 (CHCl₃); **UV/vis** λ_{max} (toluene) [nm] = 256 (ϵ [dm³mol⁻¹cm⁻¹] 38900) and 311 (15800); **¹H NMR** (500 MHz, CDCl₃) δ_H 3.84 (3H, s, OCH₃), 6.79 (2H, d, $^3J_{3',2'} = 8.5$ Hz, H-3', H-5'), 6.99 (2H, d, $^3J_{3,2} = 8.9$ Hz, H-3, H-5), 7.80 (2H, d, $^3J_{2',3'} = 8.9$ Hz, H-2', H-6'), 8.09 (2H, d, $^3J_{2,3} = 8.6$ Hz, H-2, H-6); **¹³C NMR** product was not sufficiently stable to enable us to

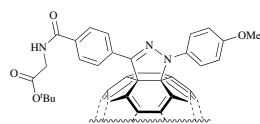
record the spectrum; m/z (MALDI) calc. for $[M] = 959.11$, found = 959.35 (C_{60} standard $m/z = 720.24$).

7.4.8 1-(4'-Carboxyphenyl)-3-(4-methoxybenzylidene)-4,5-fullerene-pyrazoline^{211,219} (**14**)



To a stirred solution of tetrazole **12** (3.3 mg, 11.1 μ mol, 1 eq.) in toluene (14 mL, 0.8 mM) was added C_{60} (40 mg, 55.5 μ mol, 5 eq.) at room temperature. The solution was stirred for 3.5 h under irradiation of a medium pressure mercury lamp. The solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (toluene/EtOAc 25:1 $\rightarrow$ CHCl₃/MeOH 1:20) to give unconsumed C_{60} (27.2 mg, 68%) and the monocycloadduct. The solid was washed with MeOH (3 x 25 mL) to give purified **14** as a brown solid (4.3 mg, 39%). R_f 0.20 (toluene/EtOAc 4:1); **UV/vis** λ_{max} (toluene) [nm] = 255 (ϵ [dm³mol⁻¹cm⁻¹] 54300) and 317 (18800); **IR** (film) ν_{max} [cm⁻¹] 2961-2851 (CH₃, CH₂, CH), 1507 (C=C_{arom}), 1462, 1409 (CH₂), 1259, 1222, 1086 (C_{aryl}-Cl), 1015, 863, 794, 700, 662; **¹H NMR** (500 MHz, CDCl₃) δ_H 3.86 (3H, s, OCH₃), 7.02 (2H, d, $^3J_{3,2} = 8.4$ Hz, H-3, H-5), 7.82 (2H, d, $^3J_{2',3'} = 8.4$ Hz, H-2', H-6'), 8.21 (2H, d, $^3J_{2,3} = 8.1$ Hz, H-2, H-6), 8.45 (2H, d, $^3J_{3',2'} = 8.2$ Hz, H-3', H-5'); **¹³C NMR** (125 MHz, CDCl₃) δ_C 55.7 (OCH₃), 80.9 (C_{60-1}), 93.2 (C_{60-2}), 114.8 (C-3, C-5), 126.9 (C-2', C-6'), 128.6 (C-3, C-5), 130.8 (C-2, C-6), 136.3 (C-1'), 136.8, 137.5, 137.9, 140.1, 140.4, 141.9, 142.0, 142.3, 142.4, 142.4, 142.5, 142.6, 143.0, 143.0, 143.3, 143.3, 144.4, 144.4, 145.2, 145.3, 145.4, 145.4, 145.6, 145.9, 145.9, 145.9, 146.0, 146.1, 146.1, 146.4, 146.5, 147.4, 147.8, 158.2 (C-4'), 190.1 (COOH); m/z (MALDI) calc. for $[M] = 988.08$, found = 720.80 (C_{60} standard $m/z = 720.24$).

7.4.9 *tert*-Butyl 2-(4'-(1''-(4'''-methoxyphenyl)-4'',5''-fulleropyrazoline)benzamido) acetate^{211,219} (**15**)



To a stirred solution of tetrazole **13** (5.7 mg, 14.0 μ mmol, 1 eq.) in toluene (14 mL, 1 mM) was added C_{60} (50.4 mg, 14.0 μ mol, 5 eq.) at room temperature. The solution was stirred for 5.5 h under irradiation of a medium pressure mercury lamp at $\lambda = 313nm$. The solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (toluene $\rightarrow$ toluene/EtOAc 9:1) to give unconsumed C_{60} (42.0 mg, 83%) and the monocycloadduct **15** as a white solid (3.8 mg, 25%). R_f 0.40 (toluene/EtOAc 9:1); **UV/vis** λ_{max} (toluene) [nm] = 257 (ϵ [dm³mol⁻¹cm⁻¹] 55200) and 322 (183800); **IR** (film) ν_{max} [cm⁻¹] 3238 (NH), 2955-2852 (CH₃, CH₂, CH), 1737 (C=O), 1669, 1603 (CONH), 1492 (C=C_{arom}), 1459, 1399 (CH₂), 1376, 1363, 1248, 1210, 1190, 1160, 1125, 1082 (C_{aryl}-Cl), 1026, 999, 968, 907, 881, 858, 824, 771; **¹H NMR** (500 MHz, CDCl₃) δ_H 1.51 (9H, s, C(CH₃)₃), 3.85 (3H, s, OCH₃), 6.69 (1H, t, $^3J_{NH,Gly} = 4.7$ Hz, NH), 7.02 (2H, d, $^3J_{3''',2'''} = 8.9$ Hz, H-3''', H-5'''), 7.82 (2H, d, $^3J_{2''',3'''} = 8.9$ Hz, H-2''', H-6'''), 7.94 (2H, d, $^3J_{3',2'} = 8.4$ Hz, H-3', H-5'), 8.40 (2H, d,

$^3J_{2',3'} = 8.2$ Hz, H-2', H-6'); ^{13}C NMR (150 MHz, CDCl_3) δ_{C} 28.2 ($\text{C}(\text{CH}_3)_3$), 42.7 (C_{Gly}), 55.7 (OCH_3), 81.1 (C_{60-1}), 82.9 ($\text{C}(\text{CH}_3)_3$), 93.0 (C_{60-2}), 114.7 (C-3'', C-5''), 126.9 (C-2'', C-6''), 127.7 (C-3', C-5'), 128.9 (C-2', C-6'), 134.2 (C-1''), 136.0 (C-4'), 136.3, 136.7, 137.7 (C-1'), 140.1, 140.4, 142.0, 142.3, 142.4, 142.4, 142.5, 142.6, 143.0, 143.0, 143.3, 144.4 (2C), 145.2, 145.3, 145.5, 145.6, 145.9, 146.0, 146.1, 146.1, 146.4, 146.5, 147.4 (1/2C), 147.8 (1/2C), 158.1 (C-4''), 166.8 (CONH), 169.3 (COO^tBu); **m/z** (MALDI) calc. for $[\text{M}] = 1102.07$, found = 1103.85 (C_{60} standard $\text{m/z} = 723.42$).

7.5 Light Induced Cleavage

7.5.1 C_{60} (16), C_{60}O (17) and Ethyl 4-nitrobenzoate (19)

To a stirred solution of pyrazoline **7** (2.3 mg, 2.2 μmol , 1 eq.) in freeze-pump-thaw degassed toluene (2.2 mL, 1 mM) at room temperature was added recrystallised maleic anhydride (6.5 mg, 66 μmol , 30 eq.) under an atmosphere of argon. The solution was irradiated with a medium pressure mercury lamp (125 W) for 1 h until t.l.c. analysis indicated complete consumption of the starting material. The crude product was purified by flash column chromatography on silica gel ($\text{CS}_2 \rightarrow \text{toluene} \rightarrow \text{toluene}/\text{EtOAc}$ 25:1) to give an inseparable mixture of C_{60} and C_{60}O (0.9 mg, 56%) and the nitrobenzylethyl ester (0.7 mg, 41%).

C_{60} (16)



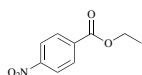
R_f 1.00 (toluene/EtOAc 25:1); **UV/vis** λ_{max} (toluene) [nm] = 256 (ϵ [$\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$] 16700) and 329 (5000); ^1H NMR (500 MHz, toluene- d_8) δ_{H} only solvent peaks; ^{13}C NMR (125 MHz, toluene- d_8) δ_{C} 143.2; **m/z** (MALDI) calc. for $[\text{M}] = 720.00$, found = 720.42. Data were consistent with the values of the commercial sample.

C_{60}O (17)



R_f 1.00 (toluene/EtOAc 25:1); **UV/vis** λ_{max} (toluene) [nm] = 251 (ϵ [$\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$] 32800) and 321 (9200); ^1H NMR (500 MHz, toluene- d_8) δ_{H} only solvent peaks; ^{13}C NMR (125 MHz, toluene- d_8) δ_{C} 80.9 (C-O), 140.9, 142.1, 142.4, 142.5, 142.7 (1/2C), 143.2, 143.2, 143.2 (C_{60}), 143.7 (1/2C), 144.1, 144.5, 144.5, 145.2 (1/2C), 145.3, 145.4, 145.4; **m/z** (MALDI) calc. for $[\text{M}+\text{O}] = 735.99$, found = 736.43 (C_{60} standard $\text{m/z} = 720.42$). Data were consistent with the literature values.³⁵⁵

Ethyl 4-nitrobenzoate (19)



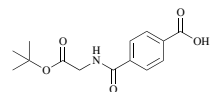
^1H NMR (700 MHz, CDCl_3) δ_{H} 1.43 (3H, t, $^3J = 7.2$ Hz, CH_2CH_3), 4.44 (2H, q, $^3J = 7.1$ Hz, CH_2CH_3), 8.22 (2H, d, $^3J_{2,3} = 8.7$ Hz, H-2, H-6), 8.29 (2H, d, $^3J_{3,2} = 8.7$ Hz, H-3, H-5); ^{13}C NMR (150 MHz, toluene- d_8) δ_{C} 14.4 (CH_2CH_3),

62.1 ($\underline{\text{CH}_2\text{CH}_3}$), 123.7 (C-3, C-5), 130.8 (C-2, C-6), 136.0 (C-1), 150.7 (C-4), 164.9 (COO). Data were consistent with the literature values.²⁴³

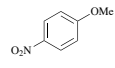
7.5.2 4-((2'-(*tert*-Butoxy)-2'-oxoethyl)carbamoyl)benzoic acid (**78**) and oxidised anisidines (**79**, **80**)

A stirred solution of pyrazoline **15** (1 mg, 0.9 μmol , 1 eq.) in $\text{CDCl}_3/\text{D}_2\text{O}$ 1:1 (1.8 mL, 1 mM) at room temperature was irradiated with a medium pressure mercury lamp (125 W) for 2 h at which point t.l.c. analysis indicated complete consumption of the starting material. The crude products were characterised.

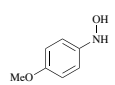
4-((2'-(*tert*-Butoxy)-2'-oxoethyl)carbamoyl)benzoic acid (**78**)

 **UV/vis** $\lambda_{\text{max}}(\text{CDCl}_3)$ [nm] = 258 (ϵ [$\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$] 35000) and 327 (16500); **IR** (film) ν_{max} [cm^{-1}] 2977-2849 ($\text{C}_{\text{aryl}}\text{H}$), 1702 (C=O), 1653&1601 (CONH), 1541, 1504, 1459, 1421, 1259, 1155, 1142, 1099, 1027, 841 (C=C-H), 802, 669; **^1H NMR** (400 MHz, CDCl_3) δ_{H} 1.51 (9H, s, $\text{C}(\text{CH}_3)_3$), 4.16 (2H, s, H_{Gly}), 6.83 (1H, t, $^3J_{\text{NH,Gly}}$ = 4.9 Hz, NH), 7.95 (2H, d, $^3J_{2,3}$ = 8.3 Hz, H-2, H-6), 8.29 (2H, d, $^3J_{3,2}$ = 8.2 Hz, H-3, H-5); **^{13}C NMR** (100 MHz, CDCl_3) δ_{C} 28.2 ($\text{C}(\text{CH}_3)_3$), 42.5 (Gly), 82.9 ($\text{C}(\text{CH}_3)_3$), 127.7 (C-2, C-6), 131.0 (C-3, C-5), 134.3 (C-4), 138.1 (C-1), 166.7 (COOH), 169.1 (COO^tBu), 180.8 (CONH); **m/z** (ES^+) calc. for $[\text{M-H}]^-$ = 278.1034, found = 278.1036.

1-Methoxy-4-nitrobenzene (**79**)

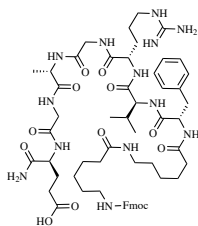
 **UV/vis** $\lambda_{\text{max}}(\text{CDCl}_3)$ [nm] = 258 (ϵ [$\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$] 35000) and 327 (16500); **IR** (film) ν_{max} [cm^{-1}] 2977-2849 ($\text{C}_{\text{aryl}}\text{H}$), 1702 (C=O), 1653&1601 (CONH), 1541, 1504, 1459, 1421, 1259, 1155, 1142, 1099, 1027, 841 (C=C-H), 802, 669; **^1H NMR** (400 MHz, CDCl_3) δ_{H} 3.95 (3H, s, OCH_3), 7.06 (2H, d, $^3J_{3,2}$ = 8.9 Hz, H-3, H-5), 8.04 (2H, d, $^3J_{2,3}$ = 8.9 Hz, H-2, H-6); **^{13}C NMR** (100 MHz, CDCl_3) δ_{C} 55.8 (OCH_3), 114.6 (C-3, C-5), 126.5 (C-2, C-6), 164.4 (C-4), 146.7 (C-1); **m/z** (ES^+) calc. for $[\text{M-MeOH}]^-$ = 138.02, found = 138.02.

N-(4-Methoxyphenyl)hydroxylamine (**80**)

 **UV/vis** $\lambda_{\text{max}}(\text{CDCl}_3)$ [nm] = 258 (ϵ [$\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$] 35000) and 327 (16500); **IR** (film) ν_{max} [cm^{-1}] 2977-2849 ($\text{C}_{\text{aryl}}\text{H}$), 1702 (C=O), 1653&1601 (CONH), 1541, 1504, 1459, 1421, 1259, 1155, 1142, 1099, 1027, 841 (C=C-H), 802, 669; **^1H NMR** (400 MHz, CDCl_3) δ_{H} 3.81 (3H, s, OCH_3), 6.91 (2H, d, $^3J_{3,2}$ = 8.5 Hz, H-3, H-5), 7.29 (2H, d, $^3J_{2,3}$ = 8.8 Hz, H-2, H-6); **^{13}C NMR** (100 MHz, CDCl_3) δ_{C} 55.8 (OCH_3), 114.0 (C-3, C-5), 129.5 (C-2, C-6), 158.6 (C-1); **m/z** (ES^+) calc. for $[\text{M-H}]^-$ = 138.06, found = 138.07.

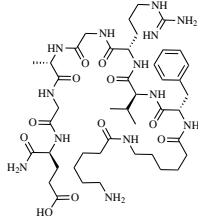
7.6 Peptide Linker Syntheses

7.6.1 NHFmoc-Ahx-Ahx-Phe-Val-Arg-Gly-Ala-Gly-Glu-CONH₂ (**27**)



The Fmoc-protected amino acids glutamic acid (1.79 g, 2 eq.), glycine (1.25 g, 2 eq.), alanine (0.68 g, 1 eq.), arginine (2.72 g, 2 eq.), valine (0.75 g, 1 eq.), phenylalanine (0.85 g, 1 eq.), and 6-aminohexanoic acid (Ahx) (1.48 g, 2 eq.) and the MBHA Rink resin (0.847 g) were put together on the peptide synthesiser from Biotage. The activator HBTU (11.379 g) was presolved in DMF, the activator base DIPEA (10.5 mL) in NMP (19.5 mL), the deprotector piperidine (60 mL), and the solvents DMF (2.6 L) and CHCl₃ (83 mL) were added to the machine. The peptide was completed after 7.5 h. The resin was washed with DMF (3 x), DCM (3 x), and Et₂O (3 x), and dried under reduced pressure. The peptide was cleaved from the resin with a mixture of TFA/H₂O/ Tis 95:2.5:2.5 (10 mL) for 1 h.³⁵⁶ The resin was filtered and washed with TFA, and the solvent was removed under a continuous blow of nitrogen. The crude peptide was purified by precipitation in Et₂O (45 mL), centrifugation (2 x 10 min, 7830 rpm), and decanting to give **27** as a white powder (880 mg). **HPLC** (Phenomenex Jupiter 4u Proteo 90A (250 · 4.6 mm) t = 15.9 min (H₂O/MeCN 95:5→5:95); ¹H NMR (500 MHz, MeOD) δ_H 0.99 (bd, *J* = 5.7 Hz, 10H), 1.45 (bd, *J* = 6.4 Hz, 18H), 1.66-2.29 (m, 25H), 2.44 (t, *J* = 6.8 Hz, 5H), 2.76-3.26 (m, 18 H), 3.85-4.04 (m, 8H), 4.18-4.45 (m, 11H), 4.59-4.72 (m, 3H), 7.30 (bs, 9H), 7.37 (m, 2H), 7.46 (t, *J* = 7.2 Hz, 2H), 7.69 (d, *J* = 7.0 Hz, 2H), 7.86 (d, *J* = 7.3 Hz, 2H); **m/z** (EI+) calc. for [M+2H]²⁺ = 613.8009, found = 613.8009; **MS/MS** R_t=32.83 min includes calc. for [M+H]⁺ = 1182.63, found = 1182.3, sequence confirmed. Data were consistent with the literature values.²⁶⁰

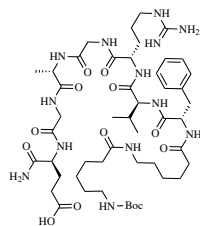
7.6.2 NH₂-Ahx-Ahx-Phe-Val-Arg-Gly-Ala-Gly-Glu-CONH₂ (**20**)



To a stirred solution of peptide **27** (100 mg, 0.08 mmol, 1 eq.) in DMF (42 mL, 2 mM) was added piperidine (2.1 mL, 5% v/v) at room temperature. After 30 min the solution was removed *in vacuo*. The crude product was purified by trituration with Et₂O, the solid was filtered with a 0.2 μm membrane nylon filter, and washed with *i*PrOH (200 mL) and Et₂O (100 mL) to give the purified unprotected peptide **20** as a white solid (72.8 mg, 91%). **HPLC** (Phenomenex Jupiter 4u Proteo 90A (250 · 4.6 mm) t = 9.8 min (H₂O/MeCN 95:5→5:95); **IR** (ATR-FTIR) ν_{max} 3300 (OH, NH), 3067, 2930, 2867 (CH₃, CH₂, CH), 1644 (C=O amide), 1543 (NH amide), 1450 (CH₂), 1416, 1367, 1251, 1168, 1087, 1048, 1025, 847, 769, 740, 698, 660, 620; ¹H NMR (700 MHz, DMSO-d₆) δ_H 0.84 (d, ³*J* = 6.7 Hz, 3H, H_{Val}-4a), 0.86 (d, ³*J* = 6.7 Hz, 3H, H_{Val}-4b), 1.08 (ddd, ³*J* = 5.0 Hz, ³*J* = 10.4 Hz, ³*J* = 17.6 Hz, 2H, H_{Ahx1}-4), 1.25 (d, ³*J* = 7.2 Hz, 5H, H_{Ahx2}-5, H_{Ala}-3), 1.30 (dt, ³*J* = 7.1 Hz, ³*J* = 14.5 Hz, 2H, H_{Ahx1}-5), 1.34 (dt, ³*J* = 7.3 Hz, ³*J* = 15.0 Hz, 2H, H_{Ahx1}-3), 1.48 (dt, ³*J* = 7.4 Hz, ³*J* = 14.7 Hz, 2H, H_{Ahx2}-3), 1.53 (m, 3H, H_{Ahx2}-4, H_{Arg}-4a), 1.77 (m, 3H, H_{Arg}-3, H_{Glu}-3a), 1.90 (m, 1H, H_{Glu}-3b), 2.02 (dt,

$^3J = 7.2$ Hz, $^3J = 29.5$ Hz, 4H, H_{Ahx1-2} , H_{Ahx2-2}), 2.11 (m, 3H, H_{Val-3} , H_{Glu-4}), 2.75 (m, 1H, H_{Arg-5a}), 2.82 (t, 1H, $H_{Ahx2-6a}$), 2.96 (dd, $^3J = 5.7$ Hz, $^3J = 11.6$ Hz, 3H, H_{Ahx1-6} , $H_{Ahx2-6b}$), 3.04 (dd, $^3J = 9.5$ Hz, $^3J = 12.6$ Hz, 2H, H_{Phe-3a} , H_{Arg-5b}), 3.12 (bs, 1H, H_{Phe-3b}), 3.52 (dd, $^3J = 5.2$ Hz, $^3J = 16.3$ Hz, 1H, $H_{Gly2-2a}$), 3.59 (d, $^3J = 12.9$ Hz, 1H, $H_{Gly1-2a}$), 3.69 (dd, $^3J = 6.4$ Hz, $^3J = 16.5$ Hz, 1H, $H_{Gly2-2b}$), 3.87 (dd, $^3J = 6.7$ Hz, $^3J = 16.5$ Hz, 1H, $H_{Gly1-2b}$), 3.99 (dd, $^3J = 8.4$ Hz, $^3J = 12.7$ Hz, 1H, H_{Glu-2}), 4.13 (m, 2H, H_{Val-2} , H_{Ala-2}), 4.27 (bs, 1H, H_{Arg-2}), 4.51 (m, 1H, H_{Phe-2}), 6.95 (s, 1H, CNHNH₂), 7.15 (t, $^3J = 7.2$ Hz, 1H, PhH_{Phe-4}), 7.22 (t, $^3J = 7.6$ Hz, 2H, PhH_{Phe-3} , PhH_{Phe-5}), 7.26 (t, $^3J = 7.0$ Hz, 2H, PhH_{Phe-2} , PhH_{Phe-6}), 7.40 (s, 1H, CONH₂), 7.78 (t, $^3J = 5.3$ Hz, 1H, CNHNH_{Arg}), 8.12 (bs, 2H, CONH_{Val}, CONH_{Glu}), 8.26 (d, $^3J = 7.6$ Hz, 1H, CONH_{Phe}), 8.31 (s, 1H, CONH_{Ala}), 8.64 (s, 1H, CONH_{Arg}), 8.69 (s, 1H, CONH_{Gly2}), 8.82 (s, 1H, CONH_{Gly1}), 8.97 (s, 1H, CONH_{Ahx2}); ^{13}C NMR (175 MHz, DMSO-*d*₆) δ_C 15.2, 17.3 (C_{Ala-3}), 18.4 (C_{Val-4}), 19.3, 22.0, 22.6 (C_{Arg-4}), 24.3, 24.8 (C_{Ahx2-4}), 24.9 (C_{Ahx2-3}), 25.4 (C_{Ahx1-3}), 25.5 (C_{Ahx2-5}), 25.8 (C_{Ahx1-4}), 26.8, 27.7 (C_{Gln-3}), 28.1 (C_{Arg-3}), 28.7 (C_{Ahx1-5}), 30.4 (C_{Val-3}), 30.7, 32.6 (C_{Gln-4}), 35.1 (C_{Ahx1-2}), 35.2 (C_{Ahx2-2}), 37.0 (C_{Arg-5}), 38.2 (C_{Ahx2-6}), 39.5 (C_{Phe-3}), 42.4 (C_{Gly1-2}), 42.9 (C_{Gly2-2}), 44.0 (C_{Ahx1-6}), 49.2 (C_{Ala-2}), 52.2 (C_{Arg-2}), 53.2 (C_{Gln-2}), 54.4 (C_{Phe-2}), 58.7 (C_{Val-2}), 62.0, 64.9, 79.2, 126.1 ($C_{Phe-4'}$), 127.9 ($C_{Phe-3'}$, $C_{Phe-5'}$), 129.2 ($C_{Phe-2'}$, $C_{Phe-6'}$), 138.3 ($C_{Phe-1'}$), 157.1 ($C_{Arg}NHNH_2$), 169.1, 169.3 (C_{Gln-2}), 171.2, 171.5, 171.8, 172.2, 172.3, 172.9, 173.9; **m/z** (LD⁺) calc. for $[M+H]^+ = 960.56$, found = 960.86.

7.6.3 NHBoc-Ahx-Ahx-Phe-Val-Arg-Gly-Ala-Gly-Glu-CONH₂ (**28**)

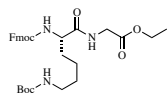


To a stirred solution of peptide **20** (60.0 mg, 63.5 μ mol, 1 eq.) in anhydrous DMF (63.5 mL, 1 mM) was added *tert*-butoxycarbonyl (Boc)₂O (14.6 μ L, 63.2 μ mol, 1 eq.) at room temperature. After stirring for 72 h, the precipitate was filtered with a 0.2 μ m membrane nylon filter and washed with CHCl₃ (2 x 150 mL), and dried under reduced pressure to give a mixture of cyclised **20** and Boc-protected peptide **28** 1:4 (40.9 mg, 62%).

HPLC (Phenomenex Jupiter 4u Proteo 90A (250 $\times$ 4.6 mm) $t = 14.1$ min ($H_2O/MeCN$ 95:5 $\rightarrow$ 5:95); **IR** (film) ν_{max} 3291 (OH, NH), 3070, 2935, 2866 (CH₃, CH₂, CH), 1643 (C=O amide), 1543 (NH amide), 1455 (CH₂), 1392, 1367, 1349, 1251, 1170, 1032, 700, 669; 1H NMR (700 MHz, DMSO-*d*₆) δ_H 0.84 (m, 6H, H_{Val-4}), 1.06 (dd, $^3J = 6.5$ Hz, $^3J = 13.4$ Hz, 2H, H_{Ahx1-4}), 1.18 (m, 2H, H_{Ahx2-5}), 1.24 (d, $^3J = 6.9$ Hz, 3H, H_{Ala-3}), 1.28 (dd, $^3J = 6.7$ Hz, $^3J = 14.1$ Hz, 2H, H_{Ahx1-3}), 1.34 (d, $^3J = 6.1$ Hz, 2H, H_{Ahx1-5}), 1.36 (s, 9H, *t*Bu), 1.45 (ddd, $^3J = 8.8$ Hz, $^3J = 16.0$ Hz, $^3J = 27.4$ Hz, 2H, H_{Ahx2-3}), 1.52 (m, 3H, H_{Ahx2-4} , H_{Arg-4a}), 1.66 (m, 1H, H_{Arg-3a}), 1.74 (m, 2H, H_{Arg-3b} , H_{Glu-3a}), 1.92 (m, 1H, H_{Glu-3b}), 2.02 (m, 5H, H_{Ahx1-2} , H_{Ahx2-2} , H_{Val-3}), 2.17 (m, 2H, H_{Glu-4}), 2.75 (t, $^3J = 11.8$ Hz, 1H, H_{Arg-5a}), 2.87 (t, $^3J = 6.8$ Hz, $^3J = 12.9$ Hz, 2H, H_{Ahx1-6}), 2.93 (dt, $^3J = 6.4$ Hz, $^3J = 12.6$ Hz, 2H, H_{Ahx2-6}), 3.04 (m, 2H, H_{Phe-3a} , H_{Arg-5b}), 3.12 (m, 1H, H_{Phe-3b}), 3.59 (m, 1H, $H_{Gly2-2a}$), 3.65 (d, $^3J = 12.8$ Hz, 1H, $H_{Gly2-2b}$), 3.71 (dd, $^3J = 5.7$ Hz, $^3J = 16.3$ Hz, 1H, $H_{Gly1-2a}$), 3.81 (dd, $^3J = 5.8$ Hz, $^3J = 16.3$ Hz, 1H, $H_{Gly1-2b}$), 4.10 (m,

1H, H_{Glu}-2), 4.17 (m, 2H, H_{Val}-2, H_{Ala}-2), 4.26 (m, 1H, H_{Arg}-2), 4.56 (m, 1H, H_{Phe}-2), 6.74 (s, 1H, CNH_{NH}2), 7.01 (d, $^3J = 9.8$ Hz, 1H, PhH_{Phe}-4), 7.17 (d, $^3J = 6.7$ Hz, 2H, PhH_{Phe}-3, PhH_{Phe}-5), 7.24 (t, $^3J = 9.0$ Hz, 2H, PhH_{Phe}-2, PhH_{Phe}-6), 7.40 (s, 1H, CONH₂), 7.67 (s, 1H), 7.89 (d, $^3J = 6.6$ Hz, 1H, CNH_{NH}Arg), 7.96 (s, 1H), 8.09 (d, $^3J = 8.2$ Hz, 1H, CONH_{Phe}), 8.13 (bs, 1H, CONH_{Val}), 8.18 (d, $^3J = 7.6$ Hz, 1H, CONH_{Glu}), 8.36 (s, 1H, CONH_{Ala}), 8.49 (s, 1H, CONH_{Arg}), 8.53 (s, 1H, CONH_{Gly}2); ^{13}C NMR (175 MHz, DMSO-d₆) δ_C 18.1 (C_{Ala}-3), 19.3 (C_{Val}-4), 24.5 (C_{Arg}-4), 25.1 (C_{Ahx2}-3), 25.4 (C_{Ahx1}-5), 25.9 (C_{Ahx1}-4), 26.0 (C_{Ahx2}-4), 27.3 (C_{Gln}-3), 28.3 (C(CH₃)₃), 28.8 (C_{Arg}-3), 28.9 (C_{Ahx2}-5), 29.3 (C_{Ahx1}-3), 30.6 (C_{Gln}-4), 30.7 (C_{Val}-3), 35.1 (C_{Ahx1}-2), 35.2 (C_{Ahx2}-2), 37.0 (C_{Arg}-5), 38.8 (C_{Ahx2}-6), 39.8 (C_{Ahx1}-6), 40.0 (C_{Phe}-3), 42.1 (C_{Gly1}-2), 42.5 (C_{Gly2}-2), 48.9 (C_{Ala}-2), 52.2 (C_{Arg}-2, C_{Gln}-2), 53.9 (C_{Phe}-2), 57.9 (C_{Val}-2), 77.3 (C(CH₃)₃), 126.1 (C_{Phe}-4'), 127.9 (C_{Phe}-2', C_{Phe}-6'), 129.2 (C_{Phe}-3', C_{Phe}-5'), 138.2, 155.6, 156.8, 168.9, 171.0, 171.4, 171.8, 171.9, 172.1, 172.8, 173.4; **m/z** (LD+) calc. for [M+H]⁺ = 1060.62, found = 1061.05.

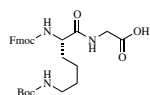
7.6.4 (S)-Ethyl 2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-((tert-butoxycarbonyl)amino)hexanamido)acetate – Fmoc-Lys(Boc)-Gly-OEt (32)



The solution of Fmoc-Lys(Boc)-OH **30** (1 g, 2.13 mmol, 1 eq.), HATU (1.22 g, 3.20 mmol, 1.5 eq.), and DIPEA (0.93 mL, 5.34 mmol, 2.5 eq.) in anhydrous DMF (34 mL, 63 mM) was stirred under an atmosphere of argon at 0 °C for 10 min. To the yellow mixture was added the HCl salt of Gly-OEt **31** (387.3 mg, 2.77 mmol, 1.3 eq.) and the solution stirred at 0 °C for 1 h, warmed to room temperature and stirred for 1.5 h. The solvent was removed under reduced pressure and the residue was solved in H₂O_{dest} (50 mL), and then the aqueous layer was extracted with DCM (3 x 40 mL) and EtOAc (5 x 60 mL). The organic layers were combined and washed with brine (120 mL), dried over MgSO₄, and filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (DCM/EtOAc 19:1→DCM/MeOH 97:3) and trituration with Et₂O (250 mL) to give the dipeptide **32** as a white solid (989.0 mg, 84%). **IR** (ATR-FTIR) ν_{max} 3306 (OH, NH), 3065, 2935, 2860 (CH₃, CH₂, CH), 1729, 1678, 1651 (C=O amide), 1530 (NH amide), 1447 (CH₂), 1391, 1366, 1341, 1301, 1269, 1247, 1209, 1167, 1130, 1102, 1088, 1033, 1020, 983, 935, 842, 778, 766, 737, 644, 621; ^1H NMR (400 MHz, CDCl₃) δ_H 1.26 (t, $^3J = 7.1$ Hz, 3H, OCH₂CH₃), 1.42 (s, 9H, C(CH₃)₃), 1.42 (m, 2H, H_{Lys}-5), 1.47 (m, 2H, H_{Lys}-4), 1.70 (m, 1H, H_{Lys}-3a), 1.87 (m, 1H, H_{Lys}-3b), 3.13 (m, 2H, H_{Lys}-6), 4.00 (m, 2H, H_{Gly}), 4.20 (m, 4H, H_{Lys}-1, COOCH₂CH₃, COOCH₂CH), 4.39 (d, $^3J = 6.9$ Hz, 1H, COOCH₂CH), 4.72 (bs, 1H, OCONH), 5.66 (d, $^3J = 6.3$ Hz, 1H, OC(O)NH), 6.80 (bs, 1H, CONH), 7.30 (dd, $^3J_{2,1} = ^3J_{2,3} = 7.4$ Hz, 2H, H_{Fmoc}-2, H_{Fmoc}-7), 7.39 (dd, $^3J_{3,2} = ^3J_{3,4} = 7.3$ Hz, 2H, H_{Fmoc}-3, H_{Fmoc}-6), 7.58 (d, $^3J_{1,2} = 7.3$ Hz, 2H, H_{Fmoc}-1, H_{Fmoc}-8), 7.75 (d, $^3J_{4,3} = 7.5$ Hz, 2H, H_{Fmoc}-4, H_{Fmoc}-5); ^{13}C NMR (125 MHz, CDCl₃) δ_C 14.2 (CH₂CH₃), 22.4 (C_{Lys}-4),

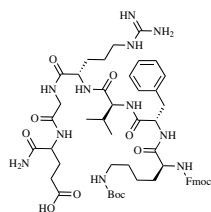
28.5 ($C(\underline{CH}_3)_3$), 29.7 (C_{Lys-5}), 32.1 (C_{Lys-3}), 39.9 (C_{Lys-6}), 41.4 (C_{Gly}), 47.2 (C_{Fmoc-9}), 54.8 (C_{Lys-2}), 61.7 ($\underline{CH}_2CH_3$), 67.2 ($OCH_2CH_2NHFmoc$), 79.3 ($\underline{C}(\underline{CH}_3)_3$), 120.1 (Fmoc C-1, C-8), 125.2 (Fmoc C-2, C-7), 127.2 (Fmoc C-4, C-5), 127.8 (Fmoc C-3, C-6), 141.4 (Fmoc C-4a, C-5a), 143.8 (Fmoc C-1a, C-8a), 156.4 ($NHCO_2$), 169.8 ($COOCH_2$), 172.2 ($NHCOCH_2$); **m/z** (ESI+) calc. for $[M+Na]^+ = 576.2680$, $[M+Na]^+$ found = 576.2675.

7.6.5 (*S*)-2-(2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-6-((*tert*-butoxy-carbonyl)amino)hexanamido)acetic acid – Fmoc-Lys(Boc)-Gly-OH³⁵⁷ (**29**)



To a stirred solution of dipeptide **32** (100 mg, 0.18 mmol, 1 eq.) in 1,4-dioxane (1.5 mL, 0.12 M) was added $LiOH_{aq}$ (9.5 mg, 2.2 eq., 0.12 M) at 0 °C. After 1 h the reaction mixture was quenched with 0.05M HCl_{aq} (20 mL) and the aqueous layer was extracted with dichloromethane (DCM) (30 mL) and EtOAc (3 x 40 mL). The organic layers were combined and washed with brine (30 mL), dried over $MgSO_4$, and filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (DCM/MeOH 4:1+1 drop AcOH) to give the deprotected peptide **29** (59.7 mg, 63%). **UV/vis** λ_{max} (DMSO/DMF 1:1) [nm] = 301 (ϵ [$dm^3 mol^{-1} cm^{-1}$] 68400) and 291 (68300); **IR** (ATR-FTIR) ν_{max} 3309 (OH, NH), 3067, 2938, 2831 (CH_3 , CH_2 , CH), 1682, 1657 (C=O amide), 1608, 1537 (NH amide), 1447 (CH_2), 1414, 1367, 1251, 1168, 1105, 1088, 1025, 849, 759, 740, 660, 619; **¹H NMR** (400 MHz, MeOD) δ_H 1.41 (s, 9H, $C(CH_3)_3$), 1.41 (m, 4H, H_{Lys-4} , H_{Lys-5}), 1.64 (m, 1H, H_{Lys-3a}), 1.82 (m, 1H, H_{Lys-3b}), 3.01 (m, 2H, H_{Lys-6}), 3.76 (q, $^3J = 17.4$ Hz, 2H, H_{Gly}), 4.09 (m, 1H, H_{Lys-1}), 4.21 (t, $^3J = 6.7$ Hz, 1H, $COOCH_2CH$), 4.34 (m, 1H, $COOCH_{2a}CH_3$), 4.41 (m, 1H, $COOCH_{2b}CH_3$), 7.30 (dd, $^3J_{2,1} = ^3J_{2,3} = 7.4$ Hz, 2H, H_{Fmoc-2} , H_{Fmoc-7}), 7.38 (dd, $^3J_{3,2} = ^3J_{3,4} = 7.4$ Hz, 2H, H_{Fmoc-3} , H_{Fmoc-6}), 7.66 (dd, $^4J_{1,3} = 2.6$ Hz, $^3J_{1,2} = 7.3$ Hz, 2H, H_{Fmoc-1} , H_{Fmoc-8}), 7.78 (d, $^3J_{4,3} = 7.5$ Hz, 2H, H_{Fmoc-4} , H_{Fmoc-5}); **¹³C NMR** (125 MHz, MeOD) δ_C 22.6 (C_{Lys-4}), 28.8 ($C(\underline{CH}_3)_3$), 24.2 (C_{Lys-5}), 30.6 (C_{Lys-3}), 32.7 (C_{Lys-6}), 41.1 (C_{Gly}), 44.3 (C_{Fmoc-9}), 56.6 (C_{Lys-2}), 68.1 ($OCH_2CH_2NHFmoc$), 79.9 ($\underline{C}(\underline{CH}_3)_3$), 120.9 (Fmoc C-1, C-8), 126.2 (Fmoc C-2, C-7), 128.2 (Fmoc C-4, C-5), 128.8 (Fmoc C-3, C-6), 142.6 (Fmoc C-4a, C-5a), 145.1 ($NHCOCH_2$), 145.3 (Fmoc C-1a, C-8a), 158.6 ($NHCO_2$), 179.1 (COOH); **m/z** (ESI+) calc. for $[M+Na]^+ = 548.2367$, $[M+Na]^+$ found = 548.2364.

7.6.6 NBocFmoc-Lys-Phe-Val-Arg-Gly-Glu-NH₂ (**33**)



Peptide synthesiser The Fmoc-protected amino acids glutamic acid(*O*^tBu) (1.79 g, 2 eq.), glycine (0.65 g, 2 eq.), arginine(pbf) (2.72 g, 2 eq.), valine (0.75 g, 1 eq.), phenylalanine (0.85 g, 1 eq.), and lysine(Boc) (1.03 g, 1 eq.) and the preswollen rink amide MBHA resin (0.847 g, 0.5 mol, 100-200 mesh) were put together on the peptide synthesiser Liberty from CEM. The activator HBTU (2.655 g, 0.2 M) and HOBt monohydrate (0.536 g, 0.1 M) were

predissolved in DMF (35 mL), the activator base DIPEA (7 mL, 2 M) in NMP (13 mL), the deprotector piperidine 20% v/v (50 mL) in DMF (250 mL), and the solvents DMF (1.9 L) and DCM (83 mL) were added to the machine. The peptide was completed after 5.5 h. The resin was washed with DMF (3 x 25 mL), DCM (3 x 25 mL), and Et₂O (3 x 25 mL).

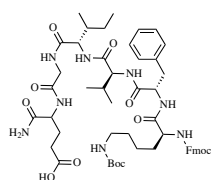
Acidic cleavage The peptide was cleaved from the resin with a mixture of TFA/Tis/H₂O 95:2.5:2.5 (10 mL/g) for 1 h, agitation every 15 min.³⁵⁶ The resin was filtered and washed with TFA (3 x 2 mL), and the solvent was removed under a continuous flow of nitrogen. The crude peptide was purified by precipitation. Trituration with Et₂O (3 x 40 mL), centrifugation (10 min, 7830 rpm), and decanting gave crude peptide **35** (410.0 mg, 114%).

Boc protection To the dissolved peptide (362.8 mg, 0.38 mmol) in DMF (50 mL, 7.6 mM) was added Boc₂O (1.16 mL, 5.0 mmol, 13 eq.) at room temperature. After stirring for 18 h, *N,N*-diisopropylethylamine (DIPEA) (175.8 μ L, 1 mmol, 2 eq.) was added. The reaction mixture was stirred for 5 h, then the solvent was removed under reduced pressure. Trituration with Et₂O (40 mL), centrifugation (10 min, 7830 rpm), and decanting gave the crude mixture (349.4 mg, 85%), which was purified by HPLC (semi-preparative column) to leave white solid **33** (164.2 mg, 40%).

HPLC (Phenomenex Synergi 4u Hydro-RP 80A (100 x 21.2 μ m, 4 micron), t_R = 4.7-7.0 min H₂O/MeCN 60:40→5:95, 15 min); **IR** (film) ν_{max} 3727-3596, 3286 (OH, NH), 3068-2826 (CH₃, CH₂, CH), 1684 (C=O acid), 1647 (C=O amide), 1625 (C=C), 1541 (NH amide), 1456 (CH₂), 1397, 1274, 1249, 737, 669, 654; **¹H NMR** (500 MHz, DMSO-d₆) δ_H 0.81 (m, 6H, H_{Val}-4), 1.12 (m, 1H, H_{Lys}-4a), 1.20 (m, 1H, H_{Lys}-4b), 1.29 (m, 2H, H_{Lys}-5), 1.36 (s, 9H, ^{*t*}Bu), 1.51 (m, 5H, H_{Lys}-3, H_{Arg}-3, H_{Arg}-4), 1.79 (m, 2H, H_{Glu}-3), 1.97 (m, 2H, H_{Val}-3, H_{Glu}-4a), 2.13 (m, 2H, H_{Glu}-4b), 2.84 (m, 3H, H_{Lys}-6, H_{Phe}-3a), 3.05 (m, 3H, H_{Phe}-3b, H_{Arg}-5a), 3.18 (m, 1H H_{Arg}-5b), 3.53 (m, 1H, H_{Gly}-2a), 3.70 (dd, ^{*3*}*J* = 4.6 Hz, ^{*3*}*J* = 16.5 Hz, 1H, H_{Gly}-2b), 3.92 (m, 1H, H_{Lys}-2), 3.96 (m, 11H, H_{Glu}-2), 4.20 (m, 3H, H_{Fmoc}-9', H_{Val}-2), 4.27 (m, 2H, H_{Fmoc}-1, H_{Arg}-2), 4.58 (dd, ^{*3*}*J* = 8.3 Hz, ^{*3*}*J* = 12.5 Hz 1H, H_{Phe}-2), 6.74 (s, 1H, NH_{Lys}Boc), 6.91 (s, 1H), 6.97 (s, 1H), 7.13 (m, 1H, PhH_{Phe}-4), 7.20 (m, 4H, PhH_{Phe}-2, PhH_{Phe}-3, PhH_{Phe}-5, PhH_{Phe}-6), 7.32 (t, ^{*3*}*J* = 7.3 Hz, 2H, H_{Fmoc}-2', H_{Fmoc}-7'), 7.41 (t, ^{*3*}*J* = 7.4 Hz, 2H, H_{Fmoc}-3', H_{Fmoc}-6'), 7.46 (d, ^{*3*}*J* = 7.3 Hz, 1H, CONH_{Lys}), 7.57 (dd, ^{*3*}*J* = 6.7 Hz, ^{*3*}*J* = 24.2 Hz, 2H, CONH_{Lys}), 7.70 (t, ^{*3*}*J* = 6.3 Hz, 2H, H_{Fmoc}-1', H_{Fmoc}-8'), 7.89 (d, ^{*3*}*J* = 7.4 Hz, 3H, H_{Fmoc}-4', H_{Fmoc}-5', CONH_{Val}), 8.01 (d, ^{*3*}*J* = 7.5 Hz, 1H, CONH_{Phe}), 8.15 (d, ^{*3*}*J* = 6.7 Hz, 1H, CONH_{Arg}), 8.55 (s, 1H, CONH_{Gly}), 8.93 (s, 1H, CONH_{Glu}), 9.45 (s, 1H, NH_{Arg}NHNH₂); **¹³C NMR** (125 MHz, DMSO-d₆) δ_C 18.0 (C_{Val}-4a), 19.2 (C_{Val}-4b), 22.8 (C_{Lys}-4), 24.7 (C_{Lys}-3), 26.9 (C_{Glu}-3), 28.3 (C(CH₃)₃), 28.5 (C_{Arg}-3), 29.3 (C_{Lys}-5), 31.7 (C_{Val}-3, C_{Arg}-4), 33.5 (C_{Glu}-4), 37.6 (Phe C-3), 39. (C_{Arg}-5), 39.8 (C_{Lys}-6), 43.2 (C_{Gly}-2), 46.7 (C_{Fmoc}-9'), 51.6 (C_{Arg}-2), 53.5 (Phe C-2), 54.1 (C_{Glu}-2), 54.8 (C_{Lys}-2), 57.5 (C_{Val}-2), 65.7 (C_{Fmoc}-1), 77.4 (C(CH₃)₃), 121.4 (C_{Fmoc}-4', C_{Fmoc}-5'), 125.3 (C_{Fmoc}-1', C_{Fmoc}-8'), 126.2 (PhC_{Phe}-4), 127.3 (C_{Fmoc}-2', C_{Fmoc}-7'), 128.0 (C_{Fmoc}-3', C_{Fmoc}-6', PhC_{Phe}-2, PhC_{Phe}-6), 129.3 (PhC_{Phe}-3, PhC_{Phe}-5), 137.5 (PhC_{Phe}-1),

139.4 (C_{Fmoc} -4a', C_{Fmoc} -4b'), 143.8 (C_{Fmoc} -8a'), 143.9 (C_{Fmoc} -9a'), 155.6 (COO_{Boc}), 155.9 (COO_{Fmoc}), 169.1 ($CONH_{Gly}$), 170.8 ($CONH_{Phe}$), 170.9 ($CONH_{Val}$, $NHCNHNH_2$), 171.9 ($CONH_{Lys}$), 172.9 ($CONH_{Arg}$), 174.0 ($CONH_2Glu$), 177.8 ($COOH_{Glu}$); m/z (ES^+) calc. for $[M+H]^+ = 1055.5440$, $[M+2H]^{2+} = 528.7793$, $[M+2H]^{2+}$ found = 528.8233; α_D not soluble enough.

7.6.7 NBocFmoc-Lys-Phe-Val-Ile-Gly-Glu-NH₂ (34)



Peptide synthesiser The Fmoc-protected amino acids glutamic acid(O^t Bu) (1.79 g, 2 eq.), glycine (0.65 g, 2 eq.), isoleucine (0.78 g, 1 eq.), valine (0.75 g, 1 eq.), phenylalanine (0.85 g, 1 eq.), and lysine(Boc) (1.03 g, 1 eq.) and the preswollen rink amide MBHA resin (0.847 g, 0.5 mol, 100-200 mesh) were put together on the peptide synthesiser Liberty from CEM. The activator HBTU

(2.655 g, 0.2 M) and HOBt monohydrate (0.536 g, 0.1 M) were predissolved in DMF (35 mL), the activator base DIPEA (7 mL, 2 M) in NMP (13 mL), the deprotector piperidine 20% v/v (50 mL) in DMF (250 mL), and the solvents DMF (1.9 L) and DCM (83 mL) were added to the machine. The peptide was completed after 4.8 h. The resin was washed with DMF (3 x 25 mL), DCM (3 x 25 mL) and Et₂O (3 x 25 mL).

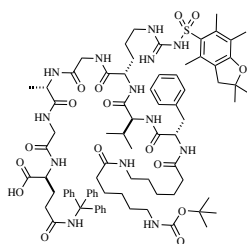
Acidic cleavage The peptide was cleaved from the resin with a mixture of TFA/Tis/H₂O 95:2.5:2.5 (10 mL/g) for 1 h, agitation every 15 min.³⁵⁶ The resin was filtered and washed with TFA (4 mL), the solvent was removed under a continuous flow of nitrogen. The crude peptide was purified by precipitation. Trituration with Et₂O (3 x 40 mL), centrifugation (10 min, 7830 rpm), and decanting gave crude peptide **36** (397.8 mg, 87%).

Boc protection To the dissolved peptide (360.5 mg, 0.39 mmol) in DMF (39 mL, 10 mM) was added Boc₂O (0.91 mL, 3.9 mmol, 10 eq.) at room temperature. After stirring for 61 h, DIPEA (137.5 μ L, 0.8 mmol, 2 eq.) was added. The reaction mixture was stirred for 2 h, then the solvent was removed under reduced pressure. Trituration with Et₂O (45 mL), centrifugation (10 min, 7830 rpm), and decanting gave the crude mixture (383.6 mg, 96%), which was purified by HPLC (semi-preparative column) to leave white solid **34**.

HPLC (Phenomenex Synergi 4u Hydro-RP 80A (100 x 21.2 μ m, 4 micron), $t_R = 14.7$ min H₂O/MeCN 95:5→5:95, 16 min); **¹H NMR** (500 MHz, DMSO-d₆) δ_H 0.82 (m, 12H, H_{Val-4} , $H_{Ile-3'}$, H_{Ile-5}), 1.11 (m, 1H, H_{Ile-4a}), 1.22 (m, 2H, H_{Lys-4}), 1.31 (m, 2H, H_{Lys-5}), 1.36 (s, 9H, t Bu), 1.46 (m, 3H, H_{Lys-3} , H_{Ile-4b}), 1.75 (m, 2H, H_{Ile-3} , H_{Glu-3a}), 1.95 (m, 2H, H_{Val-3} , H_{Glu-3b}), 2.17 (t, $^3J = 7.8$ Hz, 2H, H_{Glu-4}), 2.83 (m, 3H, H_{Lys-6} , H_{Phe-3a}), 3.00 (dd, $^3J = 4.0$ Hz, $^3J = 13.7$ Hz, 1H, H_{Phe-3b}), 3.73 (d, $^3J = 5.5$ Hz, 2H, H_{Gly-2}), 3.90 (dd, $^3J = 8.4$ Hz, $^3J = 13.4$ Hz, 1H, H_{Lys-2}), 4.12 (m, 2H, H_{Ile-2} , H_{Glu-2}), 4.21 (m, 4H, $H_{Fmoc-9'}$, H_{Fmoc-1} , H_{Val-2}), 4.57 (dd, $^3J = 8.2$ Hz, $^3J = 12.5$ Hz 1H, H_{Phe-2}), 6.75 (d, $^3J = 4.7$ Hz, 1H, $NH_{Lys}Boc$), 7.03 (s, 1H), 7.12 (m, 1H, PhH_{Phe-4}), 7.19 (m, 4H, PhH_{Phe-2} , PhH_{Phe-3} ,

PhH_{Phe}-5, PhH_{Phe}-6), 7.32 (t, $^3J = 7.2$ Hz, 2H, H_{Fmoc}-2', H_{Fmoc}-7'), 7.41 (t, $^3J = 7.4$ Hz, 2H, H_{Fmoc}-3', H_{Fmoc}-6'), 7.45 (d, $^3J = 4.7$ Hz, 1H), 7.57 (dd, $^3J = 6.7$ Hz, $^3J = 24.2$ Hz, 2H, CONH_{Lys}), 7.70 (t, $^3J = 6.4$ Hz, 2H, H_{Fmoc}-1', H_{Fmoc}-8'), 7.89 (d, $^3J = 7.5$ Hz, 2H, H_{Fmoc}-4', H_{Fmoc}-5'), 8.03 (t, $^3J = 8.3$ Hz, 2H), 8.09 (m, 1H, CONH_{Phe}), 8.17 (t, $^3J = 5.4$ Hz, 1H); ¹³C NMR (125 MHz, DMSO-d₆) δ_C 11.1 (C_{Ile}-5), 15.3 (C_{Ile}-3'), 18.2 (C_{Val}-4a), 19.2 (C_{Val}-4b), 22.3 (C_{Lys}-4), 24.5 (C_{Lys}-3, C_{Ile}-4), 27.4 (C_{Glu}-3), 28.3 (C(CH₃)₃), 29.3 (C_{Lys}-5), 30.4 (C_{Val}-3), 30.5, 31.3 (C_{Glu}-4), 36.4 (C_{Ile}-3), 37.7 (Phe C-3), 40.8 (C_{Lys}-6), 42.0 (C_{Gly}-2), 46.7 (C_{Fmoc}-9'), 52.2 (C_{Ile}-2), 53.1 & 53.6 (Phe C-2), 55.2 (C_{Lys}-2), 57.4 (C_{Glu}-2), 57.6 (C_{Val}-2), 65.6 (C_{Fmoc}-1), 77.4 (C(CH₃)₃), 121.4 (C_{Fmoc}-4', C_{Fmoc}-5'), 125.3 (C_{Fmoc}-1', C_{Fmoc}-8'), 126.2 (PhC_{Phe}-4), 127.1, 127.3 (C_{Fmoc}-2', C_{Fmoc}-7'), 127.9 (PhC_{Phe}-2, PhC_{Phe}-6), 128.9 (C_{Fmoc}-3', C_{Fmoc}-6'), 129.3 (PhC_{Phe}-3, PhC_{Phe}-5), 137.6 (PhC_{Phe}-1), 140.7 (C_{Fmoc}-4a', C_{Fmoc}-4b'), 143.8 (C_{Fmoc}-8a'), 143.9 (C_{Fmoc}-9a'), 155.6 (COO_{Boc}), 155.9 (COO_{Fmoc}), 168.6 (CONH_{Gly}), 170.8 (CONH_{Phe}), 170.9 (CONH_{Val}), 171.2 (CONH_{Ile}), 171.8 (CONH_{Lys}), 173.1 (CONH_{2Glu}), 174.8 (COOH_{Glu}); **m/z** (LD+) calc. for [M+Na]⁺ = 1035.5162, found = 1035.8560, (ES-) calc. for [M-H]⁻ = 1011.5196, found = 1011.6742; α_D not soluble enough.

7.6.8 NHBoc-Ahx-Ahx-Phe-Val-Arg-Gly-Ala-Gly-Gln-OH²⁶³ (37)



Preloading To the in DMF (5 mL) swollen HMBA-AM resin was added a filtered solution of Fmoc-Gln(trt)-OH (1.02 g, 1.67 mmol, 3 eq.), *N,N'*-dicyclohexylcarbodiimide (460.5 mg, 2.23 mmol, 4 eq.) and 4-(dimethylamino) pyridine (6.8 mg, 56 μ mol, 0.1 eq.) in DMF (5 mL). After shaking for 2 h, the resin was washed with DMF (2 x 12 mL) and the coupling repeated. After washing the resin with DMF (5 x 12 mL) and swelling in DMF (5 mL), acetic anhydride (0.32 mL, 3.35 mmol, 6 eq.) and DMAP (6.8 mg, 56 μ mol, 0.1 eq.) were added. After shaking for 1 h at room temperature, the resin was washed thoroughly with DMF, DCM (3 x 12 mL), and Et₂O (2 X 12 mL) and dried under *vacuo*. The loading was measured to be 0.784 mmol/g.

Peptide synthesiser The Fmoc-protected amino acids glycine (0.65 g, 2 eq.), alanine (0.37 g, 1 eq.), arginine(pbf) (1.43 g, 2 eq.), valine (0.41 g, 1 eq.), phenylalanine (0.46 g, 1 eq.), and Ahx (0.78 g, 2 eq.) and the preloaded HMBA-AM resin (0.321 g) were put together on the peptide synthesiser Liberty from CEM. The activator HBTU (5.69 g, 0.5 M) was presolved in DMF (30 mL), the activator base DIPEA (7 mL) in NMP (13 mL), the deprotector piperidine 20% v/v (46 mL), monohydrate HOBt (0.1 M, 3.22 g) in DMF (184 mL), and the solvents DMF (1.7 L) and DCM (83 mL) were added to the machine. The peptide was completed after 6.7 h. The resin was washed with DMF (3 x 12 mL) and DCM (3 x 12 mL).

Boc protection To the swollen resin in DMF (5 mL) was added Boc₂O (0.06 mL, 10 eq.) at room temperature. After shaking for 16 h, DIPEA (0.87 mL, 5.0 mmol, 10 eq.) was added. The

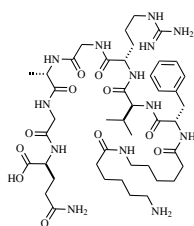
reaction mixture was agitated for another hour, then the resin was washed with DMF (3 x 12 mL), DCM (3 x 12 mL), and Et₂O (3 x 12 mL), and dried under reduced pressure.

Basic cleavage The peptide was cleaved from the resin with NaOH_{aq.} (10 M, 3 mL) in 1,4-dioxane (9 mL) for 15 min. The peptide was washed with H₂O_{dest.}, H₂O/MeCN, and MeCN. The aqueous solution was neutralised to pH 6 with 1 M HCl_{aq.}, and the solvent was removed under reduced pressure. The crude mixture was purified by HPLC (semi-preparative column) to give a white solid.

HPLC (Phenomenex Synergi 4u Hydro-RP 80A (100 x 21.2 μm, 4 micron), t_R = 10.0 min H₂O/MeCN 55:45→5:95, 12 min)¹**H NMR** (500 MHz, MeOD) δ_H 0.93 (dd, *J* = 4.9 Hz, *J* = 6.4 Hz, 6H, H_{Val-4}), 1.17 (m, 2H, H_{Ahx2-4}), 1.31 (dt, ³*J* = 7.5 Hz, ³*J* = 15.0 Hz, 2H, H_{Ahx1-4}), 1.37 (m, 4H, H_{Ala-3}, H_{Gln-3}), 1.42 (s, 17H, ^tBu, H_{pbf-Me}, H_{Ahx2-5}), 1.49 (m, 6H, H_{Ahx1-5}, H_{Ahx2-3}, H_{Arg-4a}), 1.61 (dq, ³*J* = 7.4 Hz, ³*J* = 15.1 Hz, 3H, H_{Arg-3a}, H_{Ahx1-5}), 1.75 (td, ³*J* = 7.5 Hz, ³*J* = 13.7 Hz, 1H, H_{Arg-3b}), 2.05 (s, 4H, H_{Arg-4b}, H_{Val-3}, H_{pbf-CH₂}), 2.15 (dd, 5H, H_{Ahx1-2}, H_{Ahx2-2}, H_{Arg-5a}), 2.41 (m, 2H, H_{Gln-4}), 2.50 (s, 3H, H_{pbf-1Me}), 2.57 (s, 3H, H_{pbf-6Me}), 2.86 (dd, ³*J* = 10.0 Hz, ³*J* = 14.0 Hz, 1H, H_{Phe-3a}), 2.96 (s, 2H, H_{pbf-5Me}), 3.01 (t, ³*J* = 7.0 Hz, 2H, H_{Ahx1-6}), 3.08 (t, ³*J* = 6.5 Hz, 3H, H_{Ahx2-6}, H_{Arg-5b}), 3.14 (dd, ³*J* = 5.1 Hz, ³*J* = 14.0 Hz, 1H, H_{Phe-3b}), 3.82 (m, 4H, H_{Gly1-2}, H_{Gly2-2}), 4.07 (m, 0H, H_{Ala-2}), 4.11 (m, 1H, H_{Val-2}), 4.20 (q, ³*J* = 7.5 Hz, 2H, H_{Arg-2}, H_{Gln-2}), 4.67 (dd, ³*J* = 5.1 Hz, ³*J* = 9.8 Hz, 1H, H_{Phe-2}), 7.18 (m, 4H, PhH_{Phe-4}, PhH_{trt-4}), 7.24 (m, 16H, PhH_{Phe-2}, PhH_{Phe-3}, PhH_{trt-2}, PhH_{trt-3}); ¹**H NMR** (500 MHz, DMSO-d₆) δ_H 0.80 (m, 6H, H_{Val-4}), 1.03 (bs, 2H, H_{Ahx2-4}), 1.18 (m, 5H, H_{Ahx1-4}, H_{Ala-3}), 1.25 (m, 2H, H_{Ahx2-5}), 1.31 (s, 2H, H_{Ahx2-3}), 1.34 (s, 12H, ^tBu, H_{Ahx1-5}), 1.37 (s, 7H, H_{pbf-Me}), 1.43 (m, 2H, H_{Ahx1-3}), 1.45 (m, 2H, H_{Arg-3a}, H_{Arg-4a}), 1.66 (bs, 2H, H_{Arg-3b}, H_{Gln-3a}), 1.84 (bs, 1H, H_{Gln-3b}), 1.97 (bm, 8H, H_{Ahx1-2}, H_{Ahx2-2}, H_{Val-3}, H_{pbf-CH₂}), 2.26 (bs, 2H, H_{Gln-4}), 2.40 (s, 3H, H_{pbf-6Me}), 2.45 (s, 3H, H_{pbf-2Me}), 2.70 (t, ³*J* = 11.9 Hz, 1H, H_{Phe-3a}), 2.85 (d, ³*J* = 5.3 Hz, 2H, H_{Ahx1-6}), 2.92 (bs, 4H, H_{Ahx2-6}), 2.97 (s, 1H, H_{Phe-3a}), 3.00 (m, 2H, H_{Arg-5}), 3.15 (s, 2H, H_{pbf-5Me}), 3.67 (bm, 4H, H_{Gly1-2}, H_{Gly2-2}), 4.09 (bs, 1H, H_{Ala-2}), 4.12-4.20 (bs, 3H, H_{Val-2}, H_{Arg-2}, H_{Gln-2}), 4.56 (bs, 1H, H_{Phe-2}), 6.72 (s, 1H, CONH_{Ahx2}), 7.14 (d, ³*J* = 7.0 Hz, 10H, PhH_{Phe-4}, PhH_{trt-3}, PhH_{trt-4}, PhH_{trt-5}), 7.22 (d, ³*J* = 7.9 Hz, 10H, PhH_{Phe-2}, PhH_{Phe-3}, PhH_{Phe-5}, PhH_{Phe-6}, PhH_{trt-2}, PhH_{trt-4}), 7.65 (s, 1H, CONH_{Ahx1}), 7.86 (d, ³*J* = 7.2 Hz, 1H, CONH_{Ala}), 8.01 (bm, 1H, CONH_{Arg}), 8.02 (bm, 1H, CONH_{Phe}), 8.06 (bm, 1H, CONH_{Val}), 8.20 (m, 2H, CONH_{Gly1}, CONH_{Gly2}), 8.61 (s, 1H, CNHNH_{Arg}); ¹³**C NMR** (125 MHz, DMSO-d₆) δ_C 17.6 (C_{pbf-2Me}), 17.8 (C_{Ala-3}), 18.1 (C_{Val-4a}), 19.0 (C_{pbf-6Me}), 19.2 (C_{Val-4b}), 25.0 (C_{Ahx2-3}), 25.1 (C_{Ahx1-3}), 25.9 (C_{Ahx2-4}, C_{Arg-4}), 26.0 (C_{Ahx1-4}), 28.3 (2C_{pbf-Me}, C_{Gln-3}), 28.3 (C(C_{CH₃})₃), 28.9 (C_{Ahx2-5}), 29.3 (C_{Ahx1-5}, C_{Arg-3}), 30.6 (C_{Val-3}), 32.8 (C_{Gln-4}), 35.1 (CH₂_{pbf}), 35.4 (C_{Ahx1-2}, C_{Ahx2-2}), 37.2 (Phe C-3), 38.3 (C_{Ahx2-6}), 40.4 (C_{Ahx1-6}, C_{Arg-5}), 42.5 (C_{Gly1-2}, C_{Gly2-2}), 48.6 (C_{pbf-5Me}, C_{Ala-2}), 52.4 (C_{Arg-2}, C_{Gln-2}), 53.6 (Phe C-2), 57.5 (C_{Val-2}), 69.2, 77.3 (C(C_{CH₃})₃), 86.3, 116.3 (PhC_{pbf-2}), 124.3 (PhC_{pbf-5}), 126.1 (PhC_{Phe-4}), 126.3 (PhC_{trt-4}), 127.5 (PhC_{trt-2}, PhC_{trt-6}), 127.9 (PhC_{Phe-2}, PhC_{Phe-6}), 128.5 (PhC_{trt-3}, PhC_{trt-5}), 129.3 (PhC_{Phe-3}, PhC_{Phe-5}), 131.4 (PhC_{pbf-6}), 134.3 (PhC_{pbf-1}), 137.3

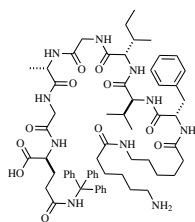
(PhC_{pbf}-3), 138.1, 144.9 (PhC_{Phe}-1, PhC_{trt}-1), 155.6 (COOBoc), 156.3, 157.4 (PhC_{pbf}-4), 168.7, 170.8, 171.4, 171.6, 171.7 (CONH_{Ahx2}), 171.8, 172.1 (C_{quart.pbf}), 172.5 (COOH); **m/z** (EI⁺) calc. for [M+H]⁺ = 1554.8065, found = 1554.8110.

7.6.9 NH₂-Ahx-Ahx-Phe-Val-Arg-Gly-Ala-Gly-Gln-OH (76)



To compound **37** (9.8 mg, 6.4 μ mol, 1 eq.) was added a mixture of TFA/thioanisole 10:1 (3.5 mL, 1.8 mM). The reaction mixture was stirred for 30 min at room temperature until complete deprotection was observed by thin layer chromatography (TLC). The solvent was removed under a continuous nitrogen flow for 10 min. The crude product was triturated with Et₂O (11 mL), centrifuged (10 min, 7830 rpm), and decanted to give the product **76**

(6.9 mg, quantitative). **R_f** 0.0 (CHCl₃/MeOH 19:1+1 drop FA); **HPLC** Phenomenex Synergi 4u Hydro-RP 80A (100 x 21.2 μ m, 4 micron), *t_R* = 11.6 min H₂O/MeCN 95:5→5:95, 24 min, 1 mL/min, 210 nm; **IR** (film) ν_{max} 3289 (OH, NH), 3066, 2937-2872 (CH₃, CH₂, CH), 1652 (C=O amide), 1543 (NH amide), 1455 (CH₂), 1419, 1202, 1179, 1134, 1051, 1027, 1007, 833, 801, 721, 669, 607; **¹H NMR** (500 MHz, DMSO-d₆) δ_H 0.83 (dd, ³*J* = 6.7 Hz, ³*J* = 15.1 Hz, 6H, H_{Val}-4), 1.04 (m, 2H, H_{Ahx2}-4), 1.22 (d, ³*J* = 7.1 Hz, 3H, H_{Ala}-3), 1.27 (m, 4H, H_{Ahx1}-4, H_{Ahx2}-5), 1.34 (m, 2H, H_{Ahx2}-3), 1.48 (m, 6H, H_{Ahx1}-3, H_{Ahx1}-5, H_{Arg}-4), 1.54 (m, 1H, H_{Gln}-3a), 1.58 (m, 1H, H_{Arg}-3a), 1.70 (m, 1H, H_{Arg}-3b), 1.77 (m, 1H, H_{Gln}-3b), 1.94 (m, 2H, H_{Val}-3), 2.00 (t, ³*J* = 7.4 Hz, 2H, H_{Ahx2}-2), 2.04 (t, ³*J* = 7.4 Hz, 2H, H_{Ahx1}-2), 2.10 (m, 2H, H_{Gln}-4), 2.74 (m, 3H, H_{Ahx1}-6, H_{Phe}-3a), 2.94 (dd, ³*J* = 6.7 Hz, ³*J* = 13.1 Hz, 2H, H_{Ahx2}-6), 3.01 (dd, ²*J* = 3.9 Hz, ³*J* = 13.8 Hz, 1H, H_{Phe}-3b), 3.09 (d, ³*J* = 5.9 Hz, 2H, H_{Arg}-5), 3.70 (d, 2H, H_{Gly}-2), 3.74 (d, ³*J* = 5.2 Hz, 1H, H_{Gly}-2), 4.11 (dd, ³*J* = 7.4 Hz, ³*J* = 12.8 Hz, 1H, H_{Gln}-2), 4.20 (m, 1H, H_{Val}-2), 4.26 (m, 2H, H_{Arg}-2, H_{Ala}-2), 4.56 (td, ³*J* = 4.1 Hz, ³*J* = 10.3 Hz, 1H, H_{Phe}-2), 6.74 (s, 1H), 7.18 (m, 2H, PhH_{Phe}-4), 7.24 (d, ³*J* = 4.3 Hz, 14H, PhH_{Phe}-2, PhH_{Phe}-3, PhH_{Phe}-5, PhH_{Phe}-6), 7.29 (m, 2H), 7.71 (m, 3H, CONH_{Ahx2}), 7.80 (d, ³*J* = 8.7 Hz, 1H, CONH_{Val}), 7.85 (bs, 1H, CONH_{Gln}), 8.07 (t, ³*J* = 8.2 Hz, 2H, CONH_{Phe}, CONH_{Ala}), 8.19 (d, ³*J* = 5.6 Hz, 2H, CONH_{Arg}, CONH_{Gly2}), 8.25 (bs, 1H, CONH_{Gly1}); **¹³C NMR** (125 MHz, DMSO-d₆) δ_C 17.9 (C_{Ala}-3), 18.0 (C_{Val}-4a), 19.2 (C_{Val}-4b), 24.8 (C_{Ahx1}-3), 24.9 (C_{Ahx1}-5), 25.0 (C_{Ahx2}-3), 25.5 (C_{Ahx2}-5), 25.9 (C_{Ahx2}-4), 26.8 (C_{Arg}-4), 27.4 (C_{Gln}-3), 28.9 (C_{Ahx1}-4, C_{Arg}-3), 30.8 (C_{Val}-3), 31.4 (C_{Gln}-4), 35.1 (C_{Ahx1}-2), 35.1 (C_{Ahx2}-2), 37.1 (Phe C-3), 38.3 (C_{Ahx2}-6), 38.7 (C_{Ahx1}-6), 40.3 (C_{Arg}-5), 42.0 (C_{Gly1}-2, C_{Gly2}-2), 48.6 (C_{Ala}-2), 52.0 (C_{Gln}-2), 52.3 (C_{Arg}-2), 53.7 (Phe C-2), 57.4 (C_{Val}-2), 126.2 (PhC_{Phe}-4), 128.0 (PhC_{Phe}-3, PhC_{Phe}-5), 129.2 (PhC_{Phe}-2, PhC_{Phe}-6), 138.1 (PhC_{Phe}-1), 156.8, 157.9, 158.1 (CNHNH_{Arg}), 168.5 (CONH_{Gly}), 168.6 (CONH_{Gly}), 170.8 (CONH_{Val}), 171.4 (CONH_{Ahx1}), 171.6 (CONH_{Phe}), 171.7 (CONH_{Ahx2}), 172.2 (CONH_{Ala}), 172.5 (CONH_{Arg}), 173.7 (CONH_{Gln}); **m/z** (ES⁺) calc. for [M+H]⁺ = 960.5625, found = 960.5544.

7.6.10 NH₂-Ahx-Ahx-Phe-Val-Ile-Gly-Ala-Gly-Gln(trt)-OH (**40**)

Peptide synthesiser The Fmoc-protected amino acids glycine (0.65 g, 2 eq.), alanine (0.37 g, 1 eq.), isoleucine (0.42 g, 1 eq.), valine (0.41 g, 1 eq.), phenylalanine (0.46 g, 1 eq.), and Ahx (0.78 g, 2 eq.) and the glutamine(trt) preloaded 2-chlorotrityl resin (0.368 g, 0.68 mmol/g) were put together on the peptide synthesiser Liberty from CEM. The activator HBTU (1.897 g, 0.2 M)

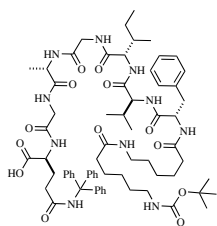
was presolved in DMF (25 mL), the activator base DIPEA (5.2 mL) in NMP (9.8 mL), the deprotector piperidine 20% v/v (40 mL) in DMF (160 mL), and the solvents DMF (1.6 L) and DCM (83 mL) were added to the machine. The peptide was completed after 5.5 h. The resin was washed with DMF (3 x 10 mL), DCM (3 x 10 mL), and Et₂O (2 X 10 mL), and dried under *vacuo*.

Acidic cleavage The peptide was cleaved from the resin with a mixture of HFIP/DCM 1:4 (10 mL, 27 mL/g) with agitation for 10 min.²⁶⁴ The resin was filtered and washed with DCM (2 x 5 mL), MeCN (2 x 8 mL), MeCN/H₂O 1:1 (10 mL), MeOH (10 mL), DCM (2 x 8 mL), and Et₂O (10 mL), and the solvent was removed under reduced pressure. The crude peptide was purified by trituration with cold Et₂O/hexane 1:1 (40 mL), centrifugation (15 min, 7830 rpm), and decanting to give crude peptide **40** (113.0 mg, 26%). Then, 30 mg of the crude mixture was purified by HPLC (semi-preparative column) to give a white solid (19.0 mg, 4%).

HPLC Phenomenex Synergi 4u Hydro-RP 80A (100 x 21.2 μm, 4 micron), t_R = 15.2 min H₂O/MeCN 95:5→5:95, 24 min, 1 mL/min, 210 nm; **IR** (film) ν_{max} 3726-3629, 3289 (OH, NH), 2938 (CH₃, CH₂, CH), 1626 (C=O amide), 1541 (NH amide), 1457 (CH₂), 1054, 1033, 1012, 667, 650, 625; **¹H NMR** (500 MHz, DMSO-d₆) δ_H 0.82 (m, 12H, H_{Ile}-3', H_{Ile}-5, H_{Val}-4), 1.08 (m, 3H, H_{Ahx2}-4, H_{Ile}-4a), 1.22 (d, 3J = 6.6 Hz, 5H, H_{Ahx1}-4, H_{Ala}-3), 1.28 (m, 3H, H_{Ahx2}-5), 1.34 (m, 2H, H_{Ahx1}-3), 1.47 (m, 5H, H_{Ahx1}-5, H_{Ahx2}-3, H_{Ile}-4b), 1.72 (bs, 2H, H_{Ile}-3, H_{Gln}-3a), 1.84 (bs, 1H, H_{Gln}-3b), 2.01 (m, 5H, H_{Ahx1}-2, H_{Ahx2}-2, H_{Val}-3), 2.25 (bs, 2H, H_{Gln}-4), 2.75 (m, 3H, H_{Ahx1}-6, H_{Phe}-3a), 2.94 (bs, 2H, H_{Ahx2}-6), 3.01 (dd, 2J = 3.2 Hz, 3J = 13.5 Hz, 1H, H_{Phe}-3b), 3.71 (m, 4H, H_{Gly1}-2, H_{Gly2}-2), 4.00 (bs, 1H, H_{Gln}-2), 4.10 (t, 3J = 7.7 Hz, 1H, H_{Ile}-2), 4.23 (dd, 3J = 7.2 Hz, 3J = 14.7 Hz, 2H, H_{Val}-2, H_{Ala}-2), 4.53 (td, 3J = 4.0 Hz, 3J = 10.2 Hz, 1H, H_{Phe}-2), 7.18 (m, 11H, PhH_{Phe}-4, PhH_{trt}-2, PhH_{trt}-4, PhH_{trt}-6), 7.24 (m, 10H, PhH_{Phe}-2, PhH_{Phe}-3, PhH_{Phe}-5, PhH_{Phe}-6, PhH_{trt}-3, PhH_{trt}-5), 7.49 (d, 3J = 4.5 Hz, 1H, CONH_{Gln}), 7.75 (bs, 1H, CONH_{Ahx2}), 8.01 (d, 3J = 6.4 Hz, 1H, CONH_{Ala}), 8.11 (d, 3J = 6.7 Hz, 1H, CONH_{Val}), 8.20 (d, 3J = 8.2 Hz, 1H, CONH_{Phe}), 8.26 (m, 2H, CONH_{Ile}, CONH_{Gly2}), 8.44 (m, 1H, CONH_{Gly1}), 8.76 (s, 1H, CONH_{Gln}-trt); **¹³C NMR** (125 MHz, DMSO-d₆) δ_C 11.0 (C_{Ile}-5), 15.3 (C_{Ile}-3'), 17.9 (C_{Ala}-3), 18.2 (C_{Val}-4a), 19.3 (C_{Val}-4b), 24.6 (C_{Ile}-4), 24.8 (C_{Ahx2}-3), 25.0 (C_{Ahx1}-3), 25.6 (C_{Ahx1}-4), 25.9 (C_{Ahx2}-4), 26.8 (C_{Arg}-4), 27.0 (C_{Ahx1}-5), 28.9 (C_{Ahx2}-5), 29.2 (C_{Gln}-3), 30.7 (C_{Val}-3), 33.3 (C_{Gln}-4), 35.1 (C_{Ahx1}-2, C_{Ahx2}-2, C_{Ile}-3), 36.2 (C_{Phe}-3), 37.2 (C_{Ahx2}-6), 38.6 (C_{Ahx1}-6), 40.4, 42.0 (C_{Gly}-2), 42.4 (C_{Gly}-2), 48.4 (C_{Ala}-2), 53.4 (C_{Gln}-2), 54.0 (C_{Phe}-2), 57.6 (C_{Ile}-2), 57.8 (C_{Val}-2), 69.2 (C_{Gln}(trt)₃), 126.1

(PhC_{Phe}-4), 126.3 (PhC_{trt}-4), 127.5 (PhC_{trt}-3, PhC_{trt}-5), 128.0 (PhC_{Phe}-3, PhC_{Phe}-5), 128.6 (PhC_{trt}-2, PhC_{trt}-6), 129.2 (PhC_{Phe}-2, PhC_{Phe}-6), 138.2 (PhC_{Phe}-1), 145.0 (PhC_{trt}-1), 167.9 (CONH_{Gly}), 168.6 (CONH_{Gly}), 171.0 (CONH_{Val}), 171.4 (CONH_{Ile}), 171.4 (CONH_{Phe}), 171.8 (CONH_{Ahx1}), 172.0 (CONH_{Gln}-trt), 172.2 (CONH_{Ahx2}), 172.5 (CONH_{Ala}), 174.0 (COOH); **m/z** (ESI+) calc. for [M+H]⁺ = 1159.6538, found = 1159.6550.

7.6.11 NHBoc-Ahx-Ahx-Phe-Val-Ile-Gly-Ala-Gly-Gln(trt)-OH (**39**)

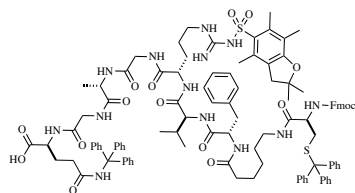


To a stirred dispersion of **40** (83.1 mg, 71.7 μ mol, 1 eq.) in anhydrous DMF (9 mL, 8 mM) was added Boc₂O (0.21 mL, 0.91 mmol, 13 eq.) at room temperature. After stirring for 14.5 h, DIPEA (78.9 μ L, 0.45 mmol, 6 eq.) was added. The reaction mixture was stirred for another hour, then the solvent was removed under reduced pressure. The crude peptide was purified by trituration with cold Et₂O/hexane 1:1 (2 x 30 mL), centrifugation (10 min, 7830 rpm), and decanting to give crude peptide **39** (87.7 mg, 97%). Then the

crude mixture was purified by HPLC (semi-preparative column) to give a white solid (40.7 mg, 45%). **R_f** 0.70 (CHCl₃/MeOH/FA 40:10:1); **HPLC** Phenomenex Synergi 4u Hydro-RP 80A (100 x 21.2 μ m, 4 micron), *t_R* = 18.2 min H₂O/MeCN 95:5→5:95, 24 min, 1 mL/min, 210 nm; *t_R* = 19.2 min is an isomeric form (2.8 mg, 3%); **IR** (film) ν_{max} 3725-3629, 3283 (OH, NH), 2966-2867 (CH₃, CH₂, CH), 1627 (C=O amide), 1523 (NH amide), 1456 (CH₂), 1396, 1366, 1252, 1170, 1055, 1033, 1013, 669, 649, 618; **¹H NMR** (500 MHz, DMSO-d₆) δ_H 0.82 (m, 12H, H_{Ile}-3', H_{Ile}-5, H_{Val}-4), 1.07 (m, 3H, H_{Ahx2}-4, H_{Ile}-4a), 1.19 (m, 6H, H_{Ahx1}-4, H_{Ala}-3), 1.27 (m, 2H, H_{Ahx2}-5), 1.33 (m, 4H, H_{Ahx1}-5), 1.36 (s, 9H, C(CH₃)₃), 1.44 (m, 3H, H_{Ahx1}-3, H_{Ahx2}-3, H_{Ile}-4b), 1.72 (m, 2H, H_{Ile}-3, H_{Gln}-3a), 1.89 (m, 1H, H_{Gln}-3b), 1.98 (m, 5H, H_{Ahx1}-2, H_{Ahx2}-2, H_{Val}-3), 2.30 (m, 2H, H_{Gln}-4), 2.73 (dd, ³*J* = 10.4 Hz, ³*J* = 13.8 Hz, 1H, H_{Phe}-3a), 2.87 (dd, ³*J* = 6.8 Hz, ³*J* = 12.9 Hz, 2H, H_{Ahx1}-6), 2.93 (dd, ³*J* = 6.8 Hz, ³*J* = 12.9 Hz, 2H, H_{Ahx2}-6), 2.99 (dd, ²*J* = 3.9 Hz, ³*J* = 13.8 Hz, 1H, H_{Phe}-3b), 3.70 (dd, ³*J* = 5.3 Hz, ³*J* = 11.8 Hz, 4H, H_{Gly1}-2, H_{Gly2}-2), 4.11 (m, 1H, H_{Ile}-2), 4.13 (m, 1H, H_{Gln}-2), 4.21 (m, 1H, H_{Val}-2), 4.25 (m, 1H, H_{Ala}-2), 4.58 (td, ³*J* = 4.1 Hz, ³*J* = 10.1 Hz, 1H, H_{Phe}-2), 6.75 (t, ³*J* = 5.4 Hz, 1H, NH_{Ahx1}), 7.18 (m, 11H, PhH_{Phe}-4, PhH_{trt}-2, PhH_{trt}-4, PhH_{trt}-6), 7.25 (m, 10H, PhH_{Phe}-2, PhH_{Phe}-3, PhH_{Phe}-5, PhH_{Phe}-6, PhH_{trt}-3, PhH_{trt}-5), 7.68 (t, ³*J* = 5.4 Hz, 1H, CONH_{Ahx2}), 7.88 (d, ³*J* = 7.2 Hz, 1H, CONH_{Gln}), 7.95 (m, 3H, CONH_{Val}, CONH_{Ile}, CONH_{Ala}), 8.05 (d, ³*J* = 8.4 Hz, 1H, CONH_{Phe}), 8.21 (m, 2H, CONH_{Gly1}, CONH_{Gly2}), 8.64 (s, 1H, CONH_{Gln}-trt); **¹³C NMR** (125 MHz, DMSO-d₆) δ_C 11.0 (C_{Ile}-5), 15.3 (C_{Ile}-3'), 18.0 (C_{Ala}-3), 18.1 (C_{Val}-4a), 19.2 (C_{Val}-4b), 24.5 (C_{Ile}-4), 25.0 (C_{Ahx2}-3), 25.1 (C_{Ahx1}-3), 25.9 (C_{Ahx1}-4), 26.0 (C_{Ahx2}-4), 27.6 (C_{Gln}-3), 28.3 (C(CH₃)₃), 28.9 (C_{Ahx2}-5), 29.3 (C_{Ahx1}-5), 30.6 (C_{Val}-3), 32.7 (C_{Gln}-4), 35.1 (C_{Ahx1}-2), 35.4 (C_{Ahx2}-2), 36.4 (C_{Ile}-3), 37.3 (C_{Phe}-3), 38.3 (C_{Ahx2}-6), 39.6 (C_{Ahx1}-6), 40.4, 41.9 (C_{Gly}-2), 41.9 (C_{Gly}-2), 48.4 (C_{Ala}-2), 51.9 (C_{Gln}-2), 53.7 (C_{Phe}-2), 57.2 (C_{Ile}-2), 57.6 (C_{Val}-2), 69.2 (C_{Gln}(trt)₃), 77.3 (C(CH₃)₃), 126.1 (PhC_{Phe}-4), 126.3 (PhC_{trt}-4), 127.5 (PhC_{trt}-3, PhC_{trt}-5), 127.9 (PhC_{Phe}-3, PhC_{Phe}-5), 128.5 (PhC_{trt}-2, PhC_{trt}-6), 129.2 (PhC_{Phe}-2,

PhC_{Phe}-6), 138.1 (PhC_{Phe}-1), 144.9 (PhC_{trt}-1), 155.6 (COOBoc), 168.5 (CONH_{Gly}), 168.6 (CONH_{Gly}), 170.9 (CONH_{Val}), 171.2 (CONH_{Phe}), 171.4 (CONH_{Ahx1}), 171.4 (CONH_{Gln}-trt), 171.8 (CONH_{Ala}), 172.1 (CONH_{Ahx2}), 172.5 (CONH_{Ile}), 173.4 (COOH); **m/z** (ESI+) calc. for [M+H]⁺ = 1259.7059, found = 1259.7075.

7.6.12 NHFmoc-Cys(trt)-Ahx-Phe-Val-Arg(pbf)-Gly-Ala-Gly-Gln(trt)-OH (**38**)



Peptide synthesiser The Fmoc-protected amino acids glycine (0.95 g, 3 eq.), alanine (0.37 g, 1 eq.), arginine(pbf) (1.43 g, 2 eq.), valine (0.41 g, 1 eq.), phenylalanine (0.46 g, 1 eq.), Ahx (0.42 g, 1 eq.), and cysteine(trt) (0.70 g, 1 eq.) and the glutamine(trt) preloaded 2-chlorotrityl resin (0.368 g, 0.68 mmol/g) were put together on the peptide synthesiser Liberty from CEM. The activator HBTU (2.276 g, 0.2 M) was presolved in DMF (30 mL), the activator base DIPEA (7 mL) in NMP (13 mL), the deprotector piperidine 20% v/v (40 mL) in DMF (160 mL), and the solvents DMF (1.7 L) and DCM (83 mL) were added to the machine. The peptide was completed after 7 h. The resin was washed with DMF (3 x 12 mL), DCM (3 x 12 mL), and Et₂O (2 X 12 mL), and dried under *vacuo*.

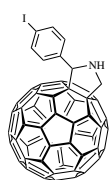
Acidic cleavage The peptide was cleaved from the resin with a mixture of HFIP/DCM 1:4 (27 mL/g) with agitation for 10 min.²⁶⁴ The resin was filtered and washed with DCM (2 x 5 mL), MeCN (2 x 8 mL), MeCN/H₂O 1:1 (2 x 8 mL), MeOH (8 mL), DCM (8 mL), and Et₂O (8 mL), and the solvent was removed under reduced pressure. The crude peptide was purified by trituration with Et₂O (40 mL), centrifugation (10 min, 7830 rpm), and decanting to give crude peptide **38** (171.8 mg, 36%). Then the crude mixture was purified by HPLC (semi-preparative column) to give a white solid (89.1 mg, 19%).

R_f 0.75 (CHCl₃/MeOH 19:1+drop FA); **HPLC** Phenomenex Synergi 4u Hydro-RP 80A (100 x 21.2 μm, 4 micron), *t_R* = 15.8-17.1 min H₂O/MeCN 50:50(2 min)→(4 min) 25:75(7 min)→(3 min)5:95(2 min), 1 mL/min, 210 nm; **IR** (film) *ν*_{max} 3709-3629, 3318 (OH, NH, SH), 2981-2826 (CH₃, CH₂, CH), 2075, 1653 (C=O amide), 1541 (NH amide), 1455 (CH₂), 1372, 1346, 1321, 1250, 1170, 1055, 1033, 1013, 742, 700, 669, 623; **¹H NMR** (500 MHz, DMSO-d₆) *δ*_H 0.83 (dd, ³*J* = 6.7 Hz, ³*J* = 12.5 Hz, 6H, H_{Val}-4), 1.01 (d, ³*J* = 7.1 Hz, 2H, H_{Ahx}-4), 1.22 (m, 3H, H_{Ala}-3), 1.29 (m, 5H, H_{Ahx}-3, H_{Ahx}-5), 1.39 (s, 6H, H_{pbf}-Me), 1.43 (m, 2H, H_{Arg}-4), 1.49 (m, 1H, H_{Arg}-3a), 1.63 (m, 1H, H_{Arg}-3b), 1.71 (m, 1H, H_{Gln}-3a), 1.88 (m, 1H, H_{Gln}-3b), 1.94 (m, 3H, H_{Ahx}-2, H_{Val}-3), 1.99 (s, 3H, H_{pbf}-5_{Me}), 2.32 (m, 4H, H_{Cys}-3, H_{Gln}-4), 2.42 (s, 3H, H_{pbf}-2_{Me}), 2.47 (s, 3H, H_{pbf}-6_{Me}), 2.72 (dd, ³*J* = 10.4 Hz, ³*J* = 13.7 Hz, 1H, H_{Phe}-3a), 2.87 (m, 1H, H_{Ahx}-6a), 2.94 (s, 2H, H_{pbf}-CH₂), 3.00 (m, 4H, H_{Ahx}-6b, H_{Phe}-3b, H_{Arg}-5), 3.71 (m, 4H, H_{Gly1}-2, H_{Gly2}-2), 4.01 (dd, ³*J* = 8.4 Hz, ³*J* = 14.6 Hz, H_{Cys}-2), 4.19 (m, 5H, H_{Fmoc}-9', H_{Fmoc}-1a, H_{Val}-2, H_{Arg}-2, H_{Gln}-2), 4.28 (m, 2H, H_{Fmoc}-1b, H_{Ala}-2), 4.59 (td, ²*J* = 4.2 Hz, ³*J* = 10.1 Hz, 1H, H_{Phe}-2), 6.43 (bs, 1H), 7.16 (m, 12H, PhH_{Phe}-4,

PhH_{trt}), 7.24 (m, 13H, PhH_{Phe-2}, PhH_{Phe-3}, PhH_{Phe-5}, PhH_{Phe-6}, PhH_{trt}), 7.30 (m, 13H, H_{Fmoc-2'}, H_{Fmoc-7'}, PhH_{trt}), 7.40 (td, $^4J = 2.2$ Hz, $^3J = 7.5$ Hz, 2H, H_{Fmoc-3'}, H_{Fmoc-6'}), 7.63 (d, $^3J = 4.7$ Hz, 1H, NH_{Cys}), 7.73 (dd, $^4J = 2.0$ Hz, $^3J = 7.3$ Hz, 2H, H_{Fmoc-1'}, H_{Fmoc-8'}), 7.81 (d, $^3J = 5.5$ Hz, 1H, CONH_{Ahx}), 7.85 (d, $^3J = 8.7$ Hz, 1H, CONH_{Val}), 7.88 (d, $^3J = 7.6$ Hz, 2H, H_{Fmoc-4'}, H_{Fmoc-5'}), 7.96 (d, $^3J = 6.1$ Hz, 1H, CONH_{Gln}), 8.00 (m, 2H, CONH_{Phe}, CONH_{Ala}), 8.06 (d, $^3J = 6.9$ Hz, 1H, CONH_{Arg}), 8.17 (m, 2H, CONH_{Gly1}, CONH_{Gly2}), 8.61 (s, 1H, CONH_{Gln-trt}); ^{13}C NMR (125 MHz, DMSO-d₆) δ_{C} 12.3 (C_{pbf-5Me}), 17.6 (C_{pbf-6Me}), 18.0 (C_{Ala-3}), 18.1 (C_{Val-4a}), 19.0 (C_{pbf-2Me}), 19.2 (C_{Val-4b}), 24.9 (C_{Ahx-3}), 25.3 (C_{Arg-4}), 25.7 (C_{Ahx-4}), 27.4 (C_{Gln-3}), 28.3 (2C_{pbf-Me}), 28.6 (C_{Ahx-5}), 29.2 (C_{Arg-3}), 30.7 (C_{Val-3}), 32.5 (C_{Gln-4}), 34.2 (C_{Cys-3}), 35.1 (C_{Ahx-2}), 37.2 (C_{Phe-3}), 38.5 (C_{Ahx-6}), 39.8 (C_{Arg-5}), 41.7 (C_{Gly-2}), 41.9 (C_{Gly-2}), 42.5 (CH_{2pbf}), 46.6 (C_{Fmoc-9'}), 48.4 (C_{Ala-2}), 51.6 (C_{Gln-2}), 52.4 (C_{Arg-2}), 53.5 (C_{Phe-2}), 53.8 (C_{Cys-2}), 57.5 (C_{Val-2}), 65.8 (C_{Fmoc-1}), 65.9 (C_{Cys(trt)}₃), 69.2 (C_{Gln(trt)}₃), 86.3 (C_{quart.pbpf}(CH₃)₂), 116.3 (PhC_{pbf-6}), 120.1 (C_{Fmoc-4'}, C_{Fmoc-5'}), 124.3 (PhC_{pbf-3}), 125.4 (C_{Fmoc-1'}), 125.4 (C_{Fmoc-8'}), 126.1, 126.3 (PhC_{Phe-4}), 126.8 (PhC_{Phe-3}, PhC_{Phe-5}), 127.0 (C_{Fmoc-2'}), 127.1 (C_{Fmoc-7'}), 127.5 (PhC_{trt}, PhC_{trt}), 127.6, 127.9 (C_{Fmoc-3'}, C_{Fmoc-6'}), 128.1 (PhC_{trt}), 128.5 (PhC_{trt}), 129.1 (PhC_{Phe-2}, PhC_{Phe-6}, PhC_{trt}), 129.2, 131.4 (PhC_{pbf-6}), 134.2 (PhC_{pbf-1}), 137.3 (PhC_{pbf-5}), 138.1 (PhC_{Phe-1}), 140.7 (C_{Fmoc-4a'}, C_{Fmoc-4b'}), 143.7, 143.8 (C_{Fmoc-8a'}, C_{Fmoc-9a'}), 144.3 (PhC_{trt-1}), 144.9, 155.6, 156.1 (COOFmoc), 157.5 (PhC_{pbf-4}), 163.1, 168.6 (CONH_{Gly}), 168.6 (CONH_{Gly}), 169.5 (CONH_{Cys}), 170.9 (CONH_{Val}), 171.3 (CONH_{Gln-trt}), 171.4 (CONH_{Ahx}), 171.6 (CONH_{Phe}), 172.1 (CONH_{Arg}), 172.4 (CONH_{Ala}), 173.3 (COOH); **m/z** (ESI⁺) calc. for [M]⁺ = 1908.8577, found = 1908.8568.

7.7 Modifying Fullerenes

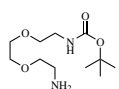
7.7.1 1-(4'-Iodophenyl)-3,4-fulleropyrrolidine⁴ (**21**)



To a stirred solution of C₆₀ fullerene (C₆₀) (403.5 mg, 56.0 mmol, 1 eq.) and *para*-iodobenzaldehyde **23** (42.0 mg, 56.0 mmol, 1 eq.) in anhydrous toluene/DMF 9:1 (200 mL, 3 mM) was added glycine (**24**, 129.9 mg, 56.0 mmol, 1 eq.) under an atmosphere of argon. The solution was refluxed for 3.5 h, and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (CS₂→CS₂/toluene 1:1→toluene) to give unconsumed C₆₀ (142.7 mg, 35%) and the monocycloadduct **21** (184.4 mg, 34%, 53% based on recovered C₆₀). **R_f** 0.65 (toluene); **IR** (ATR-FTIR) ν_{max} 321 (NH), 3093, 2962, 2928, 2857 (CH₃, CH₂, CH), 1643, 1547 (C=C_{arom}), 1452 (CH₂), 1273, 1251, 1090, 1027, 877, 735, 701, 615; ^1H NMR (300 MHz, CDCl₃) δ_{H} 4.69 (bs, 1H, NH), 4.86 (d, $^3J_{5a,5b} = 10.4$ Hz, 1H, H-5a), 5.09 (d, $^3J_{5b,5a} = 10.4$ Hz, 1H, H-5b), 5.74 (s, 1H, H-2), 7.59 (d, $^3J_{2',3'} = 7.3$ Hz, 2H, H-2', H-6'), 7.77 (d, $^3J_{3',2'} = 8.4$ Hz, 2H, H-3', H-5'); ^{13}C NMR (75 MHz, CDCl₃) δ_{C} 61.6 (C-5), 72.5 (C-2), 80.9 (C₆₀₋₁), 92.6 (C₆₀₋₂), 129.1, 130.0 (C-2', C-6'), 136.1, 136.9, 137.4, 137.9 (C-3', C-5'), 139.8, 140.0, 140.1, 140.3, 141.7,

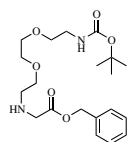
141.8, 142.0, 142.0 (2C), 142.1, 142.1, 142.2, 142.2, 142.3, 142.3, 142.4, 142.5, 142.7, 142.7, 142.7, 142.8, 143.1, 143.2, 144.4 (2C), 144.6, 144.7, 145.2, 145.3, 145.3, 145.3, 145.4 (2C), 145.4, 145.5, 145.5, 145.6, 145.7, 146.0 (2C), 146.1, 146.1, 146.2, 146.3, 146.3, 146.3, 146.4, 146.4, 146.6, 147.2 (2C), 152.4 (C-1'), 153.2 (2C), 153.7 (2C), 156.0 (C-4'); m/z (MALDI) calc. for $[M] = 964.97$, found = 964.80 (C_{60} standard $m/z = 718.99$).

7.7.2 *tert*-Butyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate⁴ (**64**)



To a stirred solution of diamine **25** (5.0 mL, 34.2 mmol, 6 eq.) in dioxane (28.5 mL) was slowly added a solution of di-*tert*-butyl dicarbonate (1.25 g, 5.7 mmol, 1 eq.) in 1,4-dioxane (5.7 mL) over 3 h at room temperature. After stirring for 67 h, the solvent was removed from the white precipitate *in vacuo*. The remaining mixture was solved in H_2O (50 mL), the aqueous phase was extracted with DCM (6 x 80 mL). The combined organic layers were washed with brine (90 mL), dried over $MgSO_4$, and the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography on silica gel (1% NH_3 (7 N in MeOH) in DCM/MeOH 9:1→4:1→7:3) to give the mono-Boc-protected diamine **64** as white liquid (1.318 g, 93%). R_f 0.10 (19:1 DCM/MeOH); 1H NMR (400 MHz, $CDCl_3$) δ_H 1.42 (s, 9H, tBu), 2.89 (t, $J = 5.2$ Hz, 2H, $OCH_2CH_2NH_2$), 3.30 (dt, $J = 4.7$ Hz, $J = 4.7$ Hz, 2H, OCH_2CH_2NHBoc), 3.52 (t, $J = 5.0$ Hz, 2H, $OCH_2CH_2NH_2$), 3.53 (t, $J = 4.8$ Hz, 2H, OCH_2CH_2NHBoc), 3.61 (s, 4H, OCH_2CH_2O), 5.18 (bs, 1H, NH_2); ^{13}C NMR (400 MHz, $CDCl_3$) δ_C 28.6 (CH_3-tBu), 40.5 ($OCH_2CH_2NH_2$), 41.7 (OCH_2CH_2NHBoc), 70.4, 70.4, 70.4 ($OCH_2CH_2OCH_2CH_2NH_2$), 73.1 (OCH_2CH_2NHBoc), 79.4 (C_q-tBu), 156.3 ($NHCO_2tBu$); m/z (EI+) calc. for $[M+H]^+ = 249.18$, found = 249.2. Data were consistent with the literature values.^{4,358}

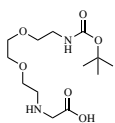
7.7.3 Benzyl 2,2-dimethyl-4-oxo-3,8,11-trioxa-5,14-diazaheptadecan-16-oate⁴ (**81**)



To a stirred solution of amine **64** (216.8 mg, 0.9 mmol, 3 eq.) in 1,4-dioxane (0.4 mL, 2 M) at 0 °C was added a solution of benzyl 2-bromo acetate (46.1 μL , 0.3 mmol, 1 eq) in 1,4-dioxane (0.7 mL, 0.4 M) over 20 min. The solution was warmed to room temperature and stirred for 19 h. The reaction mixture was quenched with H_2O_{dest} (15 mL) and extracted with DCM (6 x 15 mL) and EtOAc (3 x 25 mL). The combined organic layers were washed with brine (100 mL), dried over $MgSO_4$, filtered, and the solvent was removed *in vacuo*. The crude product was purified by flash chromatography on silica gel (EtOAc→EtOAc/ NH_3 99:1) to give purified double protected diamine **81** as a colourless oil (119.7 mg, 99%). 1H NMR (500 MHz, $CDCl_3$) δ_H 1.43 (s, 9H, $C(CH_3)_3$), 2.82 (t, $^3J = 4.8$ Hz, 2H, $OCH_2CH_2NHCH_2$), 3.30 (d, $^3J = 4.7$ Hz, 2H, OCH_2CH_2NHBoc), 3.50 (m, 4H, OCH_2CH_2O), 3.60 (m, 6H, $OCH_2CH_2NHCH_2$, OCH_2CH_2NHBoc), 5.16 (s, 2H, OCH_2Ph), 5.20 (bs, 1H, NH), 7.34 (m, 5H, Ph); ^{13}C NMR (125 MHz, $CDCl_3$) δ_C 28.5 (CH_3-tBu), 40.5 (OCH_2CH_2NHBoc), 48.8 ($OCH_2CH_2NHCH_2$), 51.0 ($NHCH_2COOBn$), 66.7 (OCH_2Ph), 70.3 ($OCH_2CH_2NHCH_2$),

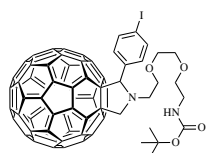
70.4 ($\text{OCH}_2\text{CH}_2\text{O}$), 70.7 ($\text{OCH}_2\text{CH}_2\text{NHBoc}$), 79.2 ($\text{C}_q\text{-}^t\text{Bu}$), 128.5 (Ph C-2, C-6), 128.7 (Ph C-3, C-5), 135.7 (Ph C-1), 156.2 (NHCO_2^tBu), 172.3 ($\text{CH}_2\text{CO}_2\text{Bn}$). Data were consistent with the literature values.⁴

7.7.4 2,2-Dimethyl-4-oxo-3,8,11-trioxa-5,14-diazahehexadecan-16-oic acid⁴ (**26**)



To a stirred solution of benzyl ester **81** (550.0 mg, 1.4 mmol, 1 eq.) in oxygen free, anhydrous MeOH (2.8 mL, 0.5 M) at room temperature was added Pd/C (13.9 mg, 0.1 mmol/mg) under an atmosphere of H_2 . The solution was stirred for 15.5 h and filtered through celite, the solvent was removed *in vacuo*. The crude product was triturated with Et_2O (100 mL), centrifuged (10 min, 7830 rpm), and decanted and then washed with Et_2O (50 mL) to give purified acid **26** as a colourless oil (295.7 mg, 59%). **¹H NMR** (500 MHz, CDCl_3) δ_H 1.43 (s, 9H, $\text{C}(\text{CH}_3)_3$), 3.24 (t, $^3J = 4.9$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{NHCH}_2$), 3.29 (d, $^3J = 4.0$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{NHBoc}$), 3.52 (t, $^3J = 5.0$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{NHCH}_2$), 3.59 (s, 2H, OCH_2Ph), 3.60 (m, 2H, $\text{OCH}_2\text{CH}_2\text{O}$), 3.65 (dd, $^2J = 2.9$ Hz, $^3J = 5.8$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{O}$), 3.82 (t, $^3J = 5.0$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{NHBoc}$), 5.53 (bs, 1H, NH); **¹³C NMR** (125 MHz, CDCl_3) δ_C 28.6 ($\text{CH}_3\text{-}^t\text{Bu}$), 40.5 ($\text{OCH}_2\text{CH}_2\text{NHBoc}$), 47.0 ($\text{OCH}_2\text{CH}_2\text{NHCH}_2$), 49.9 (NHCH_2COOH), 66.5 ($\text{OCH}_2\text{CH}_2\text{NHCH}_2$), 70.4 ($\text{OCH}_2\text{CH}_2\text{NHBoc}$), 70.5 ($\text{OCH}_2\text{CH}_2\text{O}$), 79.3 ($\text{C}_q\text{-}^t\text{Bu}$), 156.3 (NHCO_2^tBu), 170.5 ($\text{CH}_2\text{CO}_2\text{H}$); **m/z** (EI+) calc. for $[\text{M}+\text{H}]^+ = 307.19$, found = 307.0. Data were consistent with the literature values.⁴

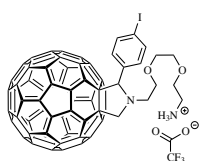
7.7.5 *tert*-Butyl (2-(2-(2-(2-(4'-iodophenyl)-3,4-fulleropyrrolidin-1-yl)-ethoxy)ethoxy)ethyl)carbamate⁴ (**82**)



To a stirred solution of C_{60} (37.7 mg, 50 μmol , 1 eq.) and α -amino acid **26** (15.9 mg, 50 μmol , 1 eq.) in anhydrous toluene/DMF 9:1 (20 mL, 3 mM) was added glycine (**24**, 12.0 mg, 50 μmol , 1 eq.) under an atmosphere of argon. The solution was refluxed for 18.5 h, and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (toluene $\rightarrow$ toluene/ EtOAc 9:1) to give unconsumed C_{60} (16.0 mg, 43%) and the monocycloadduct **82** (15.5 mg, 26%, 44% based on recovered C_{60}). **R_f** 0.40 (toluene/ EtOAc 9:1); **IR** (film) ν_{max} 3443-3351 (OH, NH), 2923+2853 (CH_3 , CH_2 , CH), 1714, 1677 ($\text{C}=\text{O}$ amide), 1588, 1503 (NH amide), 1482, 1463 (CH_2), 1430, 1406, 1389, 1364, 1265, 1172, 1121, 1099, 1060, 1045, 1006, 861, 825, 781, 756, 730 (C-I), 695, 66, 626; **¹H NMR** (500 MHz, CDCl_3) δ_H 1.45 (s, 9H, ^tBu), 2.86-2.91 (m, 1H, $\text{OCH}_2\text{CH}_a\text{H}_b\text{N}_{\text{pyrrolidine}}$), 3.27-3.37 (bm, 2H, $\text{OCH}_2\text{CH}_2\text{NHBoc}$), 3.37-3.45 (m, 1H, $\text{OCH}_2\text{CH}_a\text{H}_b\text{N}_{\text{pyrrolidine}}$), 3.61 (t, $J = 5.1$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{N}_{\text{pyrrolidine}}$), 3.76-3.76 (m, 4H, $\text{OCH}_2\text{CH}_2\text{O}$), 3.94-4.08 (m, 2H, $\text{OCH}_2\text{CH}_2\text{NHBoc}$), 4.30 (d, $^3J_{5a,5b} = 9.6$ Hz, 1H, H-5a), 4.69 (bs, 1H, NH), 5.12 (s, 1H, H-2), 5.20 (d, $^3J_{5b,5a} = 9.6$ Hz, 1H, H-5b), 7.58 (bs, 2H, H-2', H-6'), 7.75 (d, $^3J_{3',2'} = 8.4$ Hz, 2H, H-3', H-5'); **¹³C NMR** (125 MHz, CDCl_3) δ_C 28.6 ($\text{CH}_3\text{-}^t\text{Bu}$), 40.6 ($\text{OCH}_2\text{CH}_2\text{N}_{\text{pyrrolidine}}$), 52.2 ($\text{OCH}_2\text{CH}_2\text{NHBoc}$), 67.8, 69.3, 70.5 ($\text{OCH}_2\text{CH}_2\text{OCH}_2\text{CH}_2\text{N}_{\text{pyrrolidine}}$), 70.6 (C-5), 70.7 ($\text{C}_q\text{-}^t\text{Bu}$), 76.1 ($\text{OCH}_2\text{CH}_2\text{NHBoc}$), 82.0

(C₆₀-1), 94.5 (C₆₀-2), 125.4 (C-2), 128.4 (C-2', C-6'), 129.2 (C-3', C-5'), 131.5, 135.7, 136.0, 136.6, 137.1, 137.2, 138.0, 138.0, 139.7, 140.1, 140.4, 141.7, 141.8, 142.0, 142.0, 142.0, 142.1, 142.2 (2C), 142.3, 142.3, 142.3, 142.4, 142.4, 142.7, 142.7, 142.8, 142.9, 143.2, 143.2, 144.3, 144.5, 144.6, 144.7, 144.9, 145.3, 145.4, 145.5, 145.5 (2C), 145.6, 145.7, 145.7 (2C), 145.9, 146.1, 146.1, 146.3, 146.3, 146.4, 146.5, 146.5, 146.5, 146.7, 147.5, 147.5 (NHCO₂^tBu), 153.0 (C-1'), 153.2, 154.2, 156.4 (C-4'); **m/z** (MALDI) calc. for [M+H]⁺ = 1197.12, found = 1197.03 (C₆₀ standard **m/z** = 720.65).

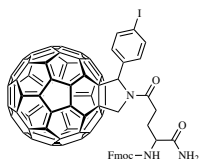
7.7.6 2,2,2-Trifluoro-*N*-(2-(2-(2-(2-(4'-iodophenyl)-3,4-fulleropyrrolidin-1-yl)ethoxy)ethoxy)ethyl)acetamide⁴ (**22**)



To a stirred solution of Boc-protected amine **82** (15.0 mg, 12.5 μmol, 1 eq.) in CHCl₃ (0.8 mL, 15 mM) was added trifluoroacetic acid (0.4 mL, 7.5 mM) at room temperature. The solution was stirred for 1.5 h, and the mixture was diluted with DCM (5 mL) and H₂O_{dest} (10 mL) and extracted with CHCl₃ (3 x 8 mL). The combined organic layers were washed with NaHCO₃ *aq* (25 mL) and brine (25 mL), dried over MgSO₄, filtered, and the solvent was removed *in vacuo* to give the free amine **22** (15.1 mg, 99%). **R_f** 0.20 (CHCl₃/MeOH 8:2); **UV/vis** λ_{max}(CHCl₃) [nm] = 432 (ε [dm³mol⁻¹cm⁻¹] 48400), 325 (34200 sh), 305 (36100), 258 (91500); **¹H NMR** (300 MHz, CDCl₃) δ_H 2.83-2.92 (m, 1H, OCH₂CH_aH_bN_{pyrrolidine}), 3.36-3.44 (m, 1H, OCH₂CH_aH_bN_{pyrrolidine}), 3.53-3.70 (m, 4H, OCH₂CH₂NH₃), 3.71-3.84 (m, 4H, OCH₂CH₂O), 3.93-4.08 (m, 4H, OCH₂CH₂N_{pyrrolidine}), 4.28 (d, ³J_{4a,4b} = 9.7 Hz, 1H, H-4a), 4.69 (s, 1H, NH), 5.12 (s, 1H, H-1), 5.21 (d, ³J_{4b,4a} = 9.7 Hz, 1H, H-4b), 7.57 (m, 2H, H-2', H-6'), 7.75 (d, ³J_{3',2'} = 8.4 Hz, 2H, H-3', H-5'); **¹³C NMR** (75 MHz, CDCl₃) δ_C 37.5 (OCH₂CH₂N_{pyrrolidine}), 55.3 (OCH₂CH₂NH₃), 69.3 (OCH₂CH₂OCH₂CH₂N_{pyrrolidine}), 70.8 (C-4), 76.2 (OCH₂CH₂NH₃), 82.0 (C₆₀-1), 94.5 (C₆₀-2), 127.6, 127.6 (C-1), 128.9 (C-2', C-6'), 131.5, 135.8, 136.1, 137.1, 137.2 (2C), 138.0, 139.7, 140.1 (C-3', C-5'), 140.3, 140.4, 141.7, 141.9, 142.0, 142.0, 142.1, 142.2 (2C), 142.3, 142.3, 142.3, 142.4, 142.4, 142.7, 142.7, 142.8, 142.9, 143.3, 143.3, 144.5, 144.6, 144.7, 144.9, 145.3, 145.4, 145.4, 145.5, 145.5, 145.6, 145.7 (2C), 145.7, 145.9, 146.1, 146.1 (2C), 146.3, 146.3, 146.4, 146.5, 146.5, 146.5, 146.7, 146.7, 147.5 (2C), 153.0 (C-1'), 153.2, 154.2, 156.5 (C-4'); **¹⁹F NMR** (400 MHz, CDCl₃) δ_F 75.68; **m/z** (MALDI) calc. for [M+H]⁺ = 1097.07, found = 1097.49 (C₆₀ standard **m/z** = 720.35).

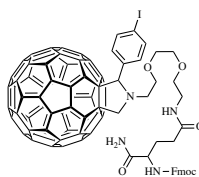
7.8 Peptide Coupling on fC₆₀

7.8.1 (9*H*-Fluoren-9-yl)methyl (1-amino-5-(2-(4'-iodophenyl)-3,4-fulleropyrrolidin-1-yl)-1,5-dioxopentane-2-yl)carbamate (**42**)



To a stirred solution of **21** (16.9 mg, 17.5 μ mol, 1 eq.) and glutamic acid **41** (6.6 mg, 18.0 μ mol, 1.03 eq.) in anhydrous CHCl₃ (5.8 mL, 3 mM) was added HATU (7.6 mg, 20.0 μ mol, 1.15 eq.) and DIPEA (3 μ L, 0.02 μ mol, 10⁻³ eq.) at room temperature under an atmosphere of argon. The solution was stirred for 24 h, and the solvent was removed under reduced pressure. The brown solid was washed with MeOH/H₂O 1:1 (3 x 3 mL) and filtrated. The crude product was purified by flash column chromatography on silica gel (CHCl₃/EtOAc 8:2→6:4) to give unconsumed **21** (8.5 mg, 50%) and the coupled amide **42** (4.4 mg, 19%, 39% based on recovered **21**). *R_f* 0.40 (CHCl₃/EtOAc 1:1); ¹H NMR (500 MHz, CDCl₃) δ_H 2.13 (m, 1H, H_{Glu}-4a), 2.45 (bs, 1H, H_{Glu}-3), 2.86 (bs, 1H, H_{Glu}-4b), 4.23 (m, 2H, H_{Glu}-2, H_{Fmoc}-9'), 4.44 (m, 2H, H_{Fmoc}-1), 5.48 (bs, 1H, H-5a), 5.54 (m, 1H, H-5b), 6.10 (m, 1H, H-2), 6.66 (bs, 1H, NH_{Fmoc}), 6.85 (s, 1H, CONH₂), 7.33 (m, 2H, H_{Fmoc}-2', H_{Fmoc}-7'), 7.39 (t, ³*J* = 6.6 Hz, 2H, H_{Fmoc}-3', H_{Fmoc}-6'), 7.54 (d, ³*J* = 8.2 Hz, 1H, H-2', H-6'), 7.62 (dd, ³*J* = 4.9 Hz, ³*J* = 6.5 Hz, 2H, H_{Fmoc}-1', H_{Fmoc}-8'), 7.73 (d, ³*J* = 8.1 Hz, 2H, H-3', H-5'), 7.82 (d, ³*J* = 6.1 Hz, 1H, H_{Fmoc}-4', H_{Fmoc}-5'); ¹³C NMR (125 MHz, CDCl₃) δ_C 18.3 (C_{Glu}-3), 29.5, 31.4, 38.8 (C_{Glu}-2), 47.2 (C_{Fmoc}-9'), 67.3 (C-5), 67.4 (C_{Fmoc}-1), 120.3 (C_{Fmoc}-4', C_{Fmoc}-5'), 125.2 (C_{Fmoc}-1', C_{Fmoc}-8'), 127.3 (C_{Fmoc}-2', C_{Fmoc}-7'), 128.0 (C_{Fmoc}-3', C_{Fmoc}-6'), 129.9 (C-3', C-5'), 136.9, 138.6, 140.0, 140.4, 140.4, 141.5, 141.5, 141.9, 142.0, 142.1, 142.1, 142.2 (2C), 142.3, 142.4, 142.8 (2C), 143.3, 144.4 (1/2C), 144.6 (1/2C), 144.7, 145.4, 145.5, 145.6, 145.6, 145.7, 145.8, 146.3 (2C), 146.3, 146.4 (1/2C), 146.5, 146.5, 147.7, 155.3, 156.7, 158.3, 173.7; *m/z* (MALDI) calc. for [M] = 1316.08, found = 1316.85 (C₆₀ standard *m/z* = 720.56).

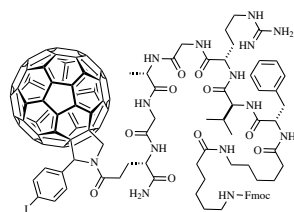
7.8.2 (9*H*-Fluoren-9-yl)methyl (1-amino-5-((2-(2-(2-(2-(4'-iodophenyl)-3,4-fulleropyrrolidin-1-yl)ethoxy)ethoxy)ethyl)amino)-1,5-dioxopentane-2-yl)carbamate (**43**)



To a stirred solution of **22** (15.4 mg, 12.9 μ mol, 1 eq.) and glutamic acid **41** (4.9 mg, 13.3 μ mol, 1.03 eq.) in anhydrous CHCl₃ (4.3 mL, 3 mM) was added HATU (5.6 mg, 14.8 μ mol, 1.15 eq.) and DIPEA (2.2 μ L, 0.01 μ mol, 10⁻³ eq.) at room temperature under an atmosphere of argon. The solution was stirred for 24 h, and the solvent was removed under reduced pressure. The brown solid was washed with MeOH/H₂O 1:1 (3 x 40 mL) and filtrated. The crude product was purified by flash column chromatography on silica gel (CHCl₃/MeOH 50:1→19:1) to give the coupled amide **43** (3.7 mg, 20%). *R_f* 0.20 (CHCl₃/MeOH 19:1); **IR** (ATR-FTIR) ν_{max} 3287 (OH, NH), 3066, 2956, 2918, 2850 (CH₃, CH₂, CH), 1649 (C=O amide), 1536 (NH amide), 1463 (CH₂), 1377, 1260, 1178, 1085, 1049, 1007, 800, 739, 664, 621; ¹H NMR (300 MHz, CDCl₃) δ_H

2.01 (m, 2H, H_{Glu}-3), 2.14 (m, 1H, H_{Glu}-4a), 2.36 (m, 1H, H_{Glu}-4b), 2.41 (m, 1H), 2.84 (m, 1H, OCH₂CH_aH_bN_{pyrrolidine}), 3.39 (m, 1H, OCH₂CH_aH_bN_{pyrrolidine}), 3.64 (s, 2H, OCH₂CH₂NHCO), 3.75 (m, 3H, OCH₂CH₂O), 3.98 (m, 2H, OCH₂CH₂N_{pyrrolidine}), 4.25 (m, 3H, OCH₂CH₂NHCO, H_{Glu}-2, H_{Fmoc}-9'), 4.39 (d, ³J = 5.8 Hz, 2H, H_{Fmoc}-1), 4.69 (s, 1H, NH), 5.08 (s, 1H), 5.17 (d, ³J = 9.1 Hz, 1H), 5.42 (bs, 1H), 6.19 (bs, 1H), 6.42 (bs, 1H NH_{Fmoc}), 6.94 (bs, 1H, NH₂), 7.32 (t, ³J = 7.4 Hz, 2H, H_{Fmoc}-2', H_{Fmoc}-7'), 7.40 (t, ³J = 7.4 Hz, 2H, H_{Fmoc}-3', H_{Fmoc}-6'), 7.55 (bs, 1H, H-2', H-6'), 7.61 (d, ³J = 7.0 Hz, 2H, H_{Fmoc}-1', H_{Fmoc}-8'), 7.74 (d, ³J = 8.4 Hz, 2H, H_{Fmoc}-4', H_{Fmoc}-5'), 7.76 (d, ³J = 7.5 Hz, 2H, H-3', H-5'); ¹³C NMR (125 MHz, CDCl₃) δ_C 23.3, 26.9, 29.5, 30.3, 32.1, 33.8, 37.5 (OCH₂CH₂N_{pyrrolidine}), 39.5, 47.3, 52.3, 53.8 (OCH₂CH₂NHCO), 67.2, 67.8, 69.2 (OCH₂CH₂OCH₂CH₂N_{pyrrolidine}), 70.0, 70.4, 70.7, 76.1 (OCH₂CH₂NHCO), 82.1, 94.6, 120.2 (C_{Fmoc}-4', C_{Fmoc}-5'), 125.3 (C_{Fmoc}-1', C_{Fmoc}-8'), 127.3 (C_{Fmoc}-2', C_{Fmoc}-7'), 127.9 (C_{Fmoc}-3', C_{Fmoc}-6'), 131.5, 135.7, 136.0, 136.6, 137.1, 138.0 (2C), 139.8, 140.1, 140.3, 140.7 (1/2C), 141.5 (2C), 141.5, 141.7, 141.8, 142.0, 142.0, 142.2, 142.3, 142.3 (2C), 142.3, 142.4, 142.7, 142.7, 142.8, 142.8, 143.2, 143.3, 143.9, 144.0, 144.5, 144.6, 144.7, 144.8 (1/2C), 145.3, 145.4, 145.5, 145.5 (2C), 145.5, 145.6, 145.7, 145.7, 145.8, 146.1 (1/2C), 146.1, 146.3, 146.3, 146.4, 146.4, 146.4, 146.5, 146.5, 146.6 (1/2C), 147.5 (2C), 153.1, 154.0, 173.1, 173.6; m/z (MALDI) calc. for [M+H]⁺ = 1447.27, found = 1448.27 (C₆₀ standard m/z = 720.72).

7.8.3 NFmoc-Ahx-Ahx-Phe-Val-Arg-Gly-Ala-Gly-Glu-CONH₂-1-(4'-iodo-phenyl)-3,4-fulleropyrrolidine (**44**)



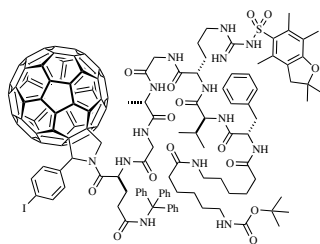
EDC coupling To a stirred solution of **21** (50 mg, 51.8 μmol, 1 eq.) and peptide **27** (73.5 mg, 62.1 μmol, 1.2 eq.) in anhydrous DMF (17.2 mL, 3 mM) was added EDC (14.8 mg, 77.7 μmol, 1.5 eq.), DMAP (6.3 mg, 51.8 μmol, 1 eq.), and DIPEA (22.5 μL, 129.4 μmol, 2.5 eq.) at 0 °C under an atmosphere of argon. The solution was stirred for 22 h, and the solvent was removed under reduced pressure. The brown

solid was washed with MeOH/H₂O 1:1 (6 x 45 mL) and filtrated to give the peptide-coupled product **44** (17.6 mg, 16%, 51% based on recovered **21**).

HATU coupling To a stirred solution of **21** (25.5 mg, 26.4 μmol, 1 eq.) in anhydrous CHCl₃ (8.1 mL, 3 mM) was added a solution of peptide **27** (37.5 mg, 31.7 μmol, 1.2 eq.), HATU (15.1 mg, 39.6 μmol, 1.5 eq.), and DIPEA (11.5 μL, 66.0 μmol, 2.5 eq.) in anhydrous DMF (0.7 mL, 30 mM), which was preactivated for 20 min at room temperature under an atmosphere of argon. The solution was stirred for 47 h, and then the preactivated acid (1.2 eq.) was added after 2.5 h and 23 h. The solvent was removed under reduced pressure. The brown solid was washed with MeOH/H₂O 1:1 (13 x 45 mL), MeOH (45 mL), CHCl₃ (3 x 45 mL, 2 x 14 mL), MeOH (14 mL), centrifuged (15 min, 7830 rpm), and decanted after every wash to give the fullerene coupled peptide **44** (13.4 mg, 24%).

R_f 0.10 (CHCl₃/MeOH 19:1); **UV/vis** λ_{max} (DMSO) [nm] = 430 (ϵ [dm³mol⁻¹cm⁻¹]) and 304; **IR** (film) ν_{max} 3361 (OH, NH), 2935, 2860 (CH₃, CH₂, CH), 1661 (C=O amide), 1541 (NH amide), 1447 (CH₂), 1296, 1221, 1153, 1044, 1016, 995, 814, 772, 668, 630, 617; **¹H NMR** (500 MHz, CDCl₃) δ_H 0.82 (dd, ³*J* = 5.8 Hz, ³*J* = 14.2 Hz, 6H, H_{Val}-4), 1.05 (m, 2H, H_{Ahx2}-4), 1.22 (m, 8H, H_{Ahx1}-4, H_{Ahx2}-3, H_{Ahx2}-5, H_{Ala}-3), 1.36 (m, 4H, H_{Ahx1}-5, H_{Arg}-4a), 1.49 (m, 4H, H_{Ahx1}-3, H_{Arg}-3a, H_{Arg}-4b), 1.68 (bs, 1H, H_{Arg}-3b), 1.98 (m, 5H, H_{Ahx2}-2, H_{Ahx1}-2, H_{Val}-3, H_{Gln}-3), 2.73 (m, 1H, H_{Phe}-3a), 2.96 (m, 5H, H_{Ahx1}-6, H_{Ahx2}-6, H_{Arg}-5a), 3.08 (d, 2H, ³*J* = 5.3 Hz, H_{Phe}-3a, H_{Arg}-5b), 3.75 (m, 4H, H_{Gly1}-2, H_{Gly2}-2), 4.20 (m, 6H, H_{Val}-2, H_{Arg}-2, H_{Ala}-2), 4.56 (bs, 1H, H_{Phe}-2), 4.86 (d, ²*J* = 10.3 Hz, 1H, H-5a), 5.13 (bs, 1H, H_{Gln}-2), 5.26 (d, ²*J* = 10.3 Hz, 1H, H-5b), 5.63 (s, 1H, H-2), 7.17 (s, 2H, PhH_{Phe}-4), 7.23 (m, 5H, PhH_{Phe}-2, PhH_{Phe}-3, PhH_{Phe}-5, PhH_{Phe}-6), 7.32 (t, ³*J* = 7.4 Hz, 2H, H_{Fmoc}-2', H_{Fmoc}-7'), 7.41 (t, ³*J* = 7.1 Hz, 2H, H_{Fmoc}-3', H_{Fmoc}-6'), 7.58 (d, ³*J* = 7.5 Hz, 1H, H-2', H-6'), 7.67 ((d, ³*J* = 7.5 Hz, 3H, H_{Fmoc}-1', H_{Fmoc}-8', CONH_{Ahx1}), 7.75 (d, ³*J* = 6.8 Hz, 2H, H-3', H-5'), 7.88 (d, ³*J* = 7.5 Hz, 3H, H_{Fmoc}-4', H_{Fmoc}-5'), 8.05 (d, ³*J* = 7.4 Hz, 1H), 8.13-8.24 (m, 2H); **¹³C NMR** (125 MHz, DMSO-d₆) δ_C 18.0 (C_{Ala}-3), 19.2 (C_{Val}-4), 25.0 (C_{Ahx1}-4, C_{Arg}-4), 25.1 (C_{Ahx1}-3, C_{Ahx2}-4), 25.9 (C_{Ahx1}-5), 28.9 (C_{Ahx2}-3, C_{Arg}-3), 29.2 (C_{Ahx2}-5), 30.8 (C_{Val}-3), 35.2 (C_{Ahx2}-2), 35.4 (C_{Ahx1}-2), 36.9 (C_{Arg}-5, C_{Gln}-4), 38.3 (Phe C-3), 40.1 (C_{Ahx2}-6), 40.3 (C_{Ahx1}-6), 41.9 (C_{Gly1}-2, C_{Gly2}-2), 46.8 (C_{Ala}-2), 48.6 (C_{Arg}-2), 52.0 (C_{Fmoc}-9'), 53.7 (Phe C-2), 57.4 (C_{Val}-2), 64.4 (C-5), 65.1 (C_{Fmoc}-1), 67.3, 76.5, 77.2 (C-2), 120.1 (C_{Fmoc}-4', C_{Fmoc}-5'), 125.1 (C_{Fmoc}-1', C_{Fmoc}-8'), 126.2 (PhC_{Phe}-4), 127.0 (C_{Fmoc}-2', C_{Fmoc}-7'), 127.6 (C_{Fmoc}-3', C_{Fmoc}-6'), 128.0 (PhC_{Phe}-3, PhC_{Phe}-5), 129.1 (PhC_{Phe}-2, PhC_{Phe}-6), 129.9 (C-3', C-5'), 132.2 (C-2', C-6'), 135.8, 136.2, 138.0 (PhC_{Phe}-1), 139.5, 140.8, 141.2, 141.5, 141.6, 141.7, 141.9, 142.0, 142.1, 142.5, 142.6, 143.9, 144.1, 144.6, 144.8, 145.4, 145.5, 145.8, 153.4, 156.1, 156.6, 168.6 (CONH₂_{Gln}), 170.9 (CONH_{Gln}), 171.4, 171.5, 171.8, 172.2, 173.4; **m/z** (MALDI) calc. for [M]⁺ = 2131.07, found = 2130.10 (C₆₀ standard m/z = 720.52).

7.8.4 NBoc-Ahx-Ahx-Phe-Val-Arg(pbf)-Gly-Ala-Gly-Glu(trt)-2-(4'-iodo-phenyl)-3,4-fulleropyrrolidine (45)

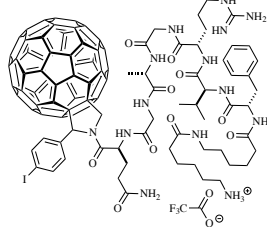


To a stirred solution of **21** (13.5 mg, 14.0 μ mol, 1 eq.) in anhydrous CHCl₃ (4.3 mL, 3 mM) was added a yellow solution of preactivated peptide. This was prepared by adding DIPEA (3.7 μ L, 21.0 μ mol, 1.5 eq.) to a stirred solution of peptide **37** (21.8 mg, 14.0 μ mol, 1 eq.) and HATU (8.0 mg, 21.0 μ mol, 1.5 eq.) in anhydrous DMF (0.45 mL, 30 mM) at room temperature after 20 min. The reaction mixture was stirred at room temperature for 1 h and 2.5 h at

T=60 °C. The solvent was evaporated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (CHCl₃→CHCl₃/MeOH 98:2→5:1) to give the coupled product **45** (14.9 mg, 43%, 81% based on recovered **21**). **R_f** 0.20 (CHCl₃/MeOH 19:1); **IR** (film) ν_{max} 3290 (OH, NH), 2963-2857 (CH₃, CH₂, CH), 1696 (C=O amide), 1626 (C=O

amide), 1519 (NH amide), 1447 (CH₂), 1367, 1248, 1167, 1107, 1091, 1006, 853, 752, 699 (C-I), 667; ¹H NMR (500 MHz, DMSO-d₆) δ_H 0.82 (m, 6H, H_{Val}-4), 1.04 (m, 2H, H_{Ahx2}-4), 1.13 (m, 5H, H_{Ahx1}-4), 1.22 (m, 3H, H_{Ala}-3), 1.23 (m, 2H, H_{Ahx2}-5), 1.28 (m, 2H, H_{Ahx2}-3), 1.29 (m, 2H, H_{Ahx1}-5), 1.36 (s, 9H, ^tBu), 1.38 (s, 7H, H_{pbf}-Me, H_{Arg}-4a), 1.40 (m, 2H, H_{Ahx1}-3), 1.46 (m, 2H, H_{Arg}-3a, H_{Arg}-4b), 1.64 (bs, 2H, H_{Arg}-3b), 1.95-1.98 (m, 7H, H_{Ahx1}-2, H_{Ahx2}-2, H_{Val}-3, H_{pbf}-CH₂, H_{Gln}-3), 2.38 (s, 3H, H_{pbf}-1_{Me}), 2.45 (s, 3H, H_{pbf}-6_{Me}), 2.69 (m, 1H, H_{Phe}-3a), 2.86 (m, 2H, H_{Ahx1}-6), 2.92 (bs, 3H, H_{Ahx2}-6, H_{pbf}-5_{Me}), 3.02 (m, 2H, H_{Phe}-3b, H_{Arg}-5), 3.71 (d, ³J = 8.9 Hz, 2H, H_{Gly1}-2), 3.84 (m, 2H, H_{Gly2}-2), 4.22 (m, 2H, H_{Val}-2, H_{Arg}-2, H_{Ala}-2), 4.57 (bs, 1H, H_{Phe}-2), 5.05 (bs, 3H, H_{Gln}-2), 5.70 (dd, ³J = 12.1 Hz, ³J = 28.7 Hz, 1H, H-5a), 6.06 (bm, 1H, H-5b), 6.38 (bs, 1H, H-2), 6.74 (t, ³J = 5.3 Hz, 1H, CONH_{Ahx2}), 7.15 (d, ³J = 7.6 Hz, 6H, PhH_{Phe}-2, PhH_{Phe}-6, PhH_{trt}-2, PhH_{trt}-6), 7.22 (m, 7H, PhH_{Phe}-3, PhH_{Phe}-5, PhH_{trt}-3, PhH_{trt}-5), 7.26 (m, 4H, PhH_{Phe}-4, PhH_{trt}-4), 7.67 (s, 1H, CONH_{Ahx1}), 7.71 (d, ³J = 12.0 Hz, 2H, H-2', H-6'), 7.81 (m, 1H, CONH_{Ala}), 7.86 (d, ³J = 8.0 Hz, 2H, H-3', H-5'), 8.03 (bm, 2H, CONH_{Phe}, CONH_{Val}, CONH_{Arg}), 8.18 (m, 1H, CONH_{Gly1}, CONH_{Gly2}), 8.39 (m, 1H, CONH_{Gln}), 8.72 (s, 1H, CNHNH_{Arg}); ¹³C NMR (125 MHz, DMSO-d₆) δ_C 17.7 (C_{Ala}-3), 17.8 (C_{pbf}-2_{Me}), 18.3 (C_{Val}-4), 18.9 (C_{pbf}-6_{Me}), 25.0 (C_{Ahx1}-3), 25.6 (C_{Ahx1}-4, C_{Ahx2}-4), 25.8 (C_{Arg}-4), 27.5 (C_{Gln}-3), 28.0 (C(CH₃)₃, 2C_{pbf}-Me), 28.9 (C_{Ahx2}-3, C_{Arg}-3), 29.0 (C_{Ahx2}-5), 29.5 (C_{Ahx1}-5), 30.2 (C_{Val}-3), 35.2 (C_{Ahx1}-2, C_{Ahx2}-2), 35.3 (CH₂_{pbf}), 36.8 (Phe C-3), 37.1 (C_{Arg}-5), 38.0 & 42.2 (C_{Ahx2}-6), 39.6 (C_{Ahx1}-6), 40.2 (C_{pbf}-5_{Me}), 41.7 (C_{Gly1}-2, C_{Gly2}-2), 48.4 (C_{Ala}-2), 50.6 (C_{Gln}-2), 52.4 (C_{Arg}-2), 53.4 (Phe C-2), 57.5 (C_{Val}-2), 58.4 (C_{pyrr}-5), 69.4 (C_{pyrr}-4'), 69.8 77.4 (C(CH₃)₃), 79.2, 86.3, 116.3 (PhC_{pbf}-2), 124.4 (PhC_{pbf}-5), 126.2, 126.4, 127.5 127.6 (PhC_{Phe}-4), 128.0 (PhC_{Phe}-2, PhC_{Phe}-6), 128.3 (PhC_{Phe}-3, PhC_{Phe}-5), 128.6, 129.0, 129.9, 130.0 (C_{pyrr}-2', C_{pyrr}-6'), 131.5 (PhC_{pbf}-6), 134.5 (PhC_{pbf}-1), 137.4 (PhC_{pbf}-3), 137.7 (C_{pyrr}-3', C_{pyrr}-5'), 138.1, 139.0, 139.4, 141.2, 141.5, 141.6, 142.1, 142.5, 143.8, 144.0, 144.1, 144.2, 144.7, 144.8, 144.8, 144.9, 145.0, 145.0, 145.3 (PhC_{Phe}-1, PhC_{trt}-1), 145.5, 145.6, 145.8, 145.8, 146.8, 154.5, 155.6 (COOBoc), 156.1, 157.5 (PhC_{pbf}-4), 162.4, 170.9, 171.4, 171.7, 171.9 (CONH_{Ahx2}), 171.9 (C_{pyrr}-1'), 172.2 (C_{quart.pbif}); m/z (MALDI) calc. for [M+Na]⁺ = 2523.75, found = 2523.95 (C₆₀ standard m/z = 718.90).

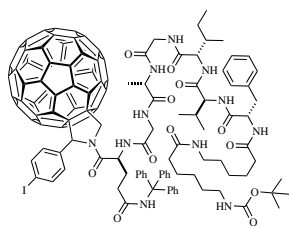
7.8.5 NH₂-Ahx-Ahx-Phe-Val-Arg-Gly-Ala-Gly-Glu-2-(4'-iodophenyl)-3,4-fulleropyrrolidine (46)



To a stirred solution of fullerene coupled peptide **45** (4.0 mg, 1 eq.) in TFA (10 mM) was added thioanisole (1 mM) at room temperature. After stirring for 40 min, the solvent was removed under N₂ flow. The solid was washed with Et₂O (4 x 2 mL), centrifuged (10 min, 2 kG), and decanted after every wash. The brown solid was dried *in vacuo* to give the free amine-functionalised fullerene **46** (2.5 mg, 81%). IR (film) ν_{max} 3307 (OH, NH), 3080, 2922, 2852 (CH₃, CH₂, CH), 1648 (C=O amide), 1540 (NH amide), 1430 (CH₂), 1261, 1202, 1183, 1135, 1007, 837, 801, 722, 700

(C-I); ^1H NMR (500 MHz, DMSO- d_6) δ_H 0.83 (m, 6H, $\text{H}_{\text{Val-4}}$), 1.05 (m, 3H, $\text{H}_{\text{Ahx2-4}}$), 1.25 (m, 8H, $\text{H}_{\text{Ahx1-4}}$, $\text{H}_{\text{Ahx2-5}}$, $\text{H}_{\text{Ala-3}}$), 1.34 (m, 2H, $\text{H}_{\text{Ahx2-3}}$), 1.49 (m, 6H, $\text{H}_{\text{Ahx1-3}}$, $\text{H}_{\text{Ahx1-5}}$, $\text{H}_{\text{Arg-4}}$), 1.54 (m, 1H, $\text{H}_{\text{Arg-3a}}$), 1.69 (bs, 2H, $\text{H}_{\text{Arg-3b}}$), 2.00 (m, 6H, $\text{H}_{\text{Ahx1-2}}$, $\text{H}_{\text{Ahx2-2}}$, $\text{H}_{\text{Val-3}}$), 2.76 (m, 3H, $\text{H}_{\text{Ahx1-6}}$, $\text{H}_{\text{Phe-3a}}$), 2.93 (bs, 2H, $\text{H}_{\text{Ahx2-6}}$), 2.99 (m, 1H, $\text{H}_{\text{Phe-3b}}$), 3.09 (m, 2H, $\text{H}_{\text{Arg-5}}$), 3.79 (m, 4H, $\text{H}_{\text{Gly1-2}}$, $\text{H}_{\text{Gly2-2}}$), 4.21 (bs, 1H, $\text{H}_{\text{Val-2}}$), 4.26 (bs, 1H, $\text{H}_{\text{Ala-2}}$), 4.56 (bs, 1H, $\text{H}_{\text{Phe-2}}$), 5.18 (dd, $^3J = 12.1$ Hz, $^3J = 28.7$ Hz, 1H, $\text{H}_{\text{Gln-2}}$), 5.89 (bm, 1H, H-5a), 6.29 (bs, 1H, H-5b), 7.17 (bs, 1H, $\text{PhH}_{\text{Phe-4}}$), 7.24 (m, 4H, $\text{PhH}_{\text{Phe-2}}$, $\text{PhH}_{\text{Phe-3}}$, $\text{PhH}_{\text{Phe-5}}$, $\text{PhH}_{\text{Phe-6}}$), 7.41 (m, 1H, $\text{CNHNH}_{\text{Arg}}$), 7.62 (m, 2H, $\text{NH}_2\text{Ahx1}$), 7.71 (m, 2H, $\text{CONH}_{\text{Ahx2}}$, H-2', H-6'), 7.87 (m, 2H, CONH_{Val} , H-3', H-5'), 8.03-8.45 (bm, 5H, CONH_{Phe} , $\text{CONH}_{\text{Gly1}}$, CONH_{Ala} , $\text{CONH}_{\text{Gly2}}$); ^{13}C NMR (125 MHz, DMSO- d_6) δ_C 18.0 ($\text{C}_{\text{Val-4a}}$, $\text{C}_{\text{Ala-3}}$), 19.2 ($\text{C}_{\text{Val-4b}}$), 24.8 ($\text{C}_{\text{Ahx1-3}}$), 25.0 ($\text{C}_{\text{Ahx2-3}}$), 25.5 ($\text{C}_{\text{Ahx1-4}}$), 25.9 ($\text{C}_{\text{Ahx2-3}}$), 26.9 ($\text{C}_{\text{Ahx1-5}}$, $\text{C}_{\text{Arg-4}}$), 29.0 ($\text{C}_{\text{Arg-3}}$), 29.1 ($\text{C}_{\text{Ahx2-5}}$), 30.8 ($\text{C}_{\text{Val-3}}$), 35.1 ($\text{C}_{\text{Ahx1-2}}$), 35.2 ($\text{C}_{\text{Ahx2-2}}$), 37.1 (Phe C-3), 38.3 ($\text{C}_{\text{Ahx2-6}}$), 38.8 ($\text{C}_{\text{Ahx1-6}}$), 40.5 ($\text{C}_{\text{Arg-5}}$), 41.9 ($\text{C}_{\text{Gly1-2}}$, $\text{C}_{\text{Gly2-2}}$), 44.5, 48.2, 52.3 ($\text{C}_{\text{Ala-2}}$), 53.6 (Phe C-2), 55.2, 57.4 ($\text{C}_{\text{Val-2}}$), 57.8, 61.4, 62.5, 64.8, 66.1, 67.9, 69.8, 74.8, 77.9 (C-5), 89.4, 102.1 (C-4'), 125.9, 126.2 ($\text{PhC}_{\text{Phe-4}}$), 128.0 ($\text{PhC}_{\text{Phe-2}}$, $\text{PhC}_{\text{Phe-6}}$), 128.9, 129.2 ($\text{PhC}_{\text{Phe-3}}$, $\text{PhC}_{\text{Phe-5}}$), 129.7 (C-2', C-6'), 132.1, 131.2, 137.6 (C-3', C-5'), 137.7, 138.1 ($\text{PhC}_{\text{Phe-1}}$), 142.2, 144.7, 144.8 (C-1'), 144.8, 145.5, 145.8, 147.8, 148.3, 154.3, 156.6, 157.7, 170.8, 170.9, 171.4, 171.4, 171.6 (CONH_{Phe}), 172.2 ($\text{CONH}_{\text{Ahx2}}$); ^{19}F NMR (376 MHz, DMSO- d_6) δ_F -73.4; **m/z** (MALDI) calc. for $[\text{M}+\text{H}]^+ = 1907.08$, found = 1909.17 (C_{60} standard **m/z** = 718.95).

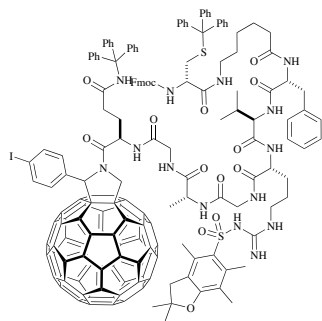
7.8.6 NBoc-Ahx-Ahx-Phe-Val-Ile-Gly-Ala-Gly-Glu(trt)-2-(4'-iodophenyl)-3,4-fulleropyrrolidine (47)



To a stirred solution of fulleropyrrolidine **21** (14.0 mg, 14.5 μmol , 1 eq.) in anhydrous CHCl_3 (4.2 mL, 3 mM) was added a yellow solution of preactivated peptide. This was prepared by adding DIPEA (3.79 μL , 21.8 μmol , 1.5 eq.) to a stirred solution of peptide **39** (18.3 mg, 14.5 μmol , 1 eq.) and HATU (16.6 mg, 43.6 μmol , 3 eq.) in anhydrous DMF (0.4 mL, 30 mM) at room temperature after 30 min. The reaction mixture was stirred for 21 h at $T=60^\circ\text{C}$. The reaction mixture was diluted with CHCl_3 (20 mL), washed with H_2O (20 mL), separated, and the aqueous layer was extracted with CHCl_3 (3 x 10 mL). The organic layers were combined, dried over MgSO_4 , filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel ($\text{CHCl}_3 \rightarrow \text{CHCl}_3/\text{MeOH}$ 49:1 $\rightarrow$ 5:1) to give the coupled product **47** (7.9 mg, 25%, 33% based on recovered **21**). **R_f** 0.50 ($\text{CHCl}_3/\text{MeOH}$ 9:1); **IR** (ATR-FTIR) ν_{max} 3300 (OH, NH), 3063, 2930, 2862 (CH_3 , CH_2 , CH), 1679, 1655 (C=O amide), 1623, 1519 (NH amide), 1448 (CH_2), 1392, 1367, 1248, 1209, 1168, 1090, 1034, 1005, 845, 760, 730, 697 (C-I), 621; ^1H NMR (500 MHz, DMSO- d_6) δ_H 0.82 (m, 12H, $\text{H}_{\text{Ile-3'}}$, $\text{H}_{\text{Ile-5}}$, $\text{H}_{\text{Val-4}}$), 1.06 (m, 3H, $\text{H}_{\text{Ahx2-4}}$, $\text{H}_{\text{Ile-4a}}$), 1.21 (m, 11H, $\text{H}_{\text{Ahx1-4}}$, $\text{H}_{\text{Ahx2-5}}$, $\text{H}_{\text{Ala-3}}$, $\text{H}_{\text{Gln-3}}$), 1.32 (m, 5H, $\text{H}_{\text{Ahx1-3}}$, $\text{H}_{\text{Ahx1-5}}$), 1.36 (s, 6H, $\text{C}(\text{CH}_3)_3$), 1.45 (m, 3H, $\text{H}_{\text{Ahx2-3}}$, $\text{H}_{\text{Ile-4b}}$), 1.68 (m, 1H,

H_{Ahx2-6} , H_{Phe-3b}), 3.74 (m, 4H, H_{Gly1-2} , H_{Gly2-2}), 4.15 (m, 1H, H_{Ile-2}), 4.24 (m, 2H, H_{Val-2} , H_{Ala-2} , H_{Gln-2}), 4.57 (m, 1H, H_{Phe-2}), 5.19 (m, 1H, H-2), 5.87 (dd, $^3J = 12.8$ Hz, $^3J = 59.2$ Hz, 1H, H-5a), 6.32 (dd, $^3J = 10.1$ Hz, $^3J = 83.5$ Hz, 1H, H-5b), 6.87 (s, 1H, NH_{Ahx1}), 7.16 (m, 1H, PhH_{Phe-4}), 7.21 (m, 4H, PhH_{Phe-2} , PhH_{Phe-3} , PhH_{Phe-5} , PhH_{Phe-6}), 7.32 (m, 1H), 7.64 (bs, 2H, NH_{2Ahx1}), 7.70 (bs, 1H, $CONH_{Ahx2}$), 7.76 (m, 2H, H-2', H-6'), 7.87 (m, 3H, $CONH_{Val}$, $CONH_{Ile}$, $CONH_{Ala}$, $CONH_{Gln}$, H-3', H-5'), 8.03 (m, 2H, $CONH_{Phe}$), 8.23 (m, 1H, $CONH_{Gly1}$, $CONH_{Gly2}$); ^{13}C NMR (125 MHz, DMSO- d_6) δ_C 11.1 (C_{Ile-5}), 15.3 ($C_{Ile-3'}$), 18.0 (C_{Ala-3}), 18.1 (C_{Val-4a}), 19.2 (C_{Val-4b}), 24.4 (C_{Ile-4}), 24.8 (C_{Ahx2-3}), 25.0 (C_{Ahx1-3}), 25.5 (C_{Ahx2-4}), 25.9 (C_{Ahx1-4}), 26.9 (C_{Ahx1-5}), 29.0 (C_{Ahx2-5} , C_{Gln-3}), 30.4 (C_{Val-3}), 30.6, 35.1 (C_{Ahx1-2} , C_{Ahx2-2}), 36.5 (C_{Ile-3}), 37.3 (C_{Phe-3}), 38.3 (C_{Ahx2-6}), 38.8 (C_{Ahx1-6}), 41.9 (C_{Gly-2} , C_{Gly-2}), 48.6 (C_{Ala-2}), 50.4 (C_{pyrr-2}), 53.6 (C_{Phe-2}), 57.0 (C_{Ile-2} , C_{Gln-2}), 57.5 (C_{Val-2} , C_{pyrr-5}), 69.9 (C-4'), 124.9, 126.1 (PhC_{Phe-4}), 127.9 (PhC_{Phe-3} , PhC_{Phe-5}), 129.2 (PhC_{Phe-2} , PhC_{Phe-6}), 130.0 (C-2', C-6'), 135.6, 136.3, 137.7 (C-3', C-5'), 138.1 (PhC_{Phe-1}), 139.1, 139.4, 139.6, 141.2, 141.5, 141.6, 141.6, 141.6, 141.7, 142.0, 142.1, 142.5, 142.6, 143.8, 144.0, 144.1, 144.2, 144.7, 144.8, 144.9, 145.0, 145.0, 145.3, 145.5, 145.6, 145.8, 145.8, 146.8, 146.9, 157.8, 168.6, 169.1, 170.5, 170.8, 171.2, 171.4, 171.6, 172.0, 172.5, 173.9; m/z (MALDI) calc. for $[M+H]^+ = 1864.51$, found = 1865.15 (C_{60} standard $m/z = 718.60$).

7.8.8 NFmoc-Cys(trt)-Ahx-Phe-Val-Arg(pbf)-Gly-Ala-Gly-Glu(trt)-2-(4'-iodophenyl)-3,4-fulleropyrrolidine (**50**)

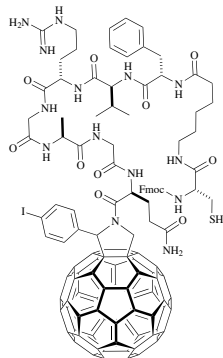


To a stirred solution of fulleropyrrolidine **21** (17.3 mg, 17.9 μ mol, 1 eq.) in anhydrous $CHCl_3$ (5.4 mL, 3 mM) was added a yellow solution of preactivated peptide. This was prepared by adding DIPEA (6.2 μ L, 35.8 μ mol, 2 eq.) to a stirred solution of peptide **38** (41.0 mg, 21.5 μ mol, 1.2 eq.) and HATU (13.6 mg, 35.8 μ mol, 2 eq.) in anhydrous DMF (0.6 mL, 30 mM) at room temperature after 30 min. The reaction mixture was stirred for 5.5 h at $T=60$ °C. The reaction mixture was diluted with $CHCl_3$ (20 mL), washed with H_2O (20 mL), separated, and the aqueous layer was extracted with $CHCl_3$

(3 x 10 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel ($CHCl_3 \rightarrow CHCl_3/MeOH$ 48:2 $\rightarrow$ 5:1) to give the coupled product **50** (13.7 mg, 27%, 39% based on recovered **21**, 48% based on recovered **38**). IR (ATR-FTIR) ν_{max} 3303 (OH, NH), 3059, 2926, 2868 (CH_3 , CH_2 , CH), 1649 (C=O amide), 1531 (NH amide), 1447 (CH_2), 1408, 1370, 1320, 1247, 1158, 1104, 1089, 1034, 1003, 902, 850, 804, 758, 740, 699 (C-I), 663, 639, 620; 1H NMR (500 MHz, DMSO- d_6) δ_H 0.82 (m, 6H, H_{Val-4}), 1.00 (m, 2H, H_{Ahx-4}), 1.25 (m, 8H, H_{Ahx-3} , H_{Ahx-5} , H_{Ala-3}), 1.37 (s, 6H, H_{pbf-Me}), 1.42 (m, 2H, H_{Arg-4}), 1.50 (m, 1H, H_{Arg-3a}), 1.63 (m, 1H, H_{Arg-3b}), 1.91 (m, 3H, H_{Ahx-2} , H_{Val-3} , H_{Gln-3a}), 1.97 (s, 3H, $H_{pbf-5Me}$), 2.17 (bs, 1H, H_{Gln-3b}), 2.32 (m, 2H, H_{Cys-3}), 2.38/40 (s, 3H, $H_{pbf-2Me}$),

2.43/46 (s, 3H, H_{pbf}-6_{Me}, H_{Gln}-4a), 2.57 (m, 1H, H_{Gln}-4b), 2.72 (m, 1H, H_{Phe}-3a), 2.87 (m, 1H, H_{Ahx}-6a), 2.91 (s, 2H, H_{pbf}-CH₂), 2.98 (s, 4H, H_{Ahx}-6b, H_{Phe}-3b, H_{Arg}-5), 3.78 (m, 4H, H_{Gly1}-2, H_{Gly2}-2), 4.00 (dd, ³*J* = 8.2 Hz, ³*J* = 14.5 Hz, H_{Cys}-2), 4.20 (m, 4H, H_{Fmoc}-9', H_{Fmoc}-1a, H_{Val}-2, H_{Arg}-2), 4.28 (dd, ³*J* = 10.7 Hz, ³*J* = 13.2 Hz, 2H, H_{Fmoc}-1b, H_{Ala}-2, H_{Gln}-2), 4.58 (bs, 1H, H_{Phe}-2), 5.05 (bs, 1H, H-2), 5.69 (dd, ³*J* = 12.9 Hz, ³*J* = 31.2 Hz, 1H, H-5a), 6.06 (m, 1H, H-5b), 6.38 (bs, 1H), 7.14 (m, 7H, PhH_{Phe}-4, PhH_{Gln}(trt)), 7.21 (m, 13H, PhH_{Phe}-2, PhH_{Phe}-3, PhH_{Phe}-5, PhH_{Phe}-6, PhH_{Cys}(trt)-4), 7.29 (m, 15H, H_{Fmoc}-2', H_{Fmoc}-7', PhH_{trt}), 7.39 (td, ⁴*J* = 1.8 Hz, ³*J* = 7.4 Hz, 2H, H_{Fmoc}-3', H_{Fmoc}-6'), 7.61 (d, ³*J* = 8.5 Hz, 1H, NH_{Cys}), 7.69 (d, ³*J* = 8.5 Hz, 1H, H-2', H-6'), 7.72 (d, ³*J* = 6.6 Hz, 2H, H_{Fmoc}-1', H_{Fmoc}-8'), 7.79 (m, 2H, CONH_{Ahx}), 7.84 (d, ³*J* = 6.1 Hz, 1H, CONH_{Val}, H-3', H-5'), 7.87 (d, ³*J* = 7.5 Hz, 2H, H_{Fmoc}-4', H_{Fmoc}-5'), 8.01 (bs, 1H, CONH_{Phe}, CONH_{Ala}), 8.06 (m, 1H, CONH_{Arg}), 8.18 (m, CONH_{Gly1}, CONH_{Gly2}), 8.39 (m, 1H), 8.70 (m, 1H, CONH_{Gln}-trt); ¹³C NMR (125 MHz, DMSO-d₆) δ_C 12.4 (C_{pbf}-5_{Me}), 17.7 (C_{pbf}-6_{Me}), 18.1 (C_{Ala}-3), 18.6 (C_{Val}-4_a), 19.0 (C_{pbf}-2_{Me}), 19.2 (C_{Val}-4_b), 25.0 (C_{Ahx}-3), 25.4 (C_{Arg}-4), 25.8 (C_{Ahx}-4), 27.3 (C_{Gln}-3), 28.3 (2C_{pbf}-Me), 28.6 (C_{Ahx}-5), 29.2 (C_{Arg}-3), 30.7 (C_{Val}-3), 32.0 (C_{Gln}-4), 34.2 (C_{Cys}-3), 35.1 (C_{Ahx}-2), 37.2 (C_{Phe}-3), 38.5 (C_{Ahx}-6), 40.1 (C_{Arg}-5), 41.9 (C_{Gly}-2), 42.0 (C_{Gly}-2), 42.5 (CH₂_{pbf}), 46.6 (C_{Fmoc}-9'), 48.4 (C_{Ala}-2), 50.3 (C_{pyrr}-2), 52.2 (C_{Gln}-2), 52.5 (C_{Arg}-2), 53.6 (C_{Phe}-2), 53.8 (C_{Cys}-2), 57.5 (C_{Val}-2), 58.7 (C_{pyrr}-5), 65.8 (C_{Fmoc}-1), 65.9 (C_{Cys}(trt)₃), 69.4/8 (C_{Gln}(trt)₃), 86.3 (C_{quart.pb}f(CH₃)₂), 116.3 (PhC_{pbf}-6), 120.1 (C_{Fmoc}-4', C_{Fmoc}-5'), 124.3 (PhC_{pbf}-3), 125.4 (C_{Fmoc}-1'), 125.4 (C_{Fmoc}-8'), 126.1, 126.3 (PhC_{Phe}-4), 126.8 (PhC_{Cys}(trt)-4), 127.1 (C_{Fmoc}-2', C_{Fmoc}-7'), 127.5 (PhC_{Gln}(trt)-4), 127.6 (C_{Fmoc}-3', C_{Fmoc}-6'), 127.7 (PhC_{Phe}-3, PhC_{Phe}-5), 128.1 (PhC_{Cys}(trt)), 128.6 (PhC_{Gln}(trt)), 129.1 (PhC_{Cys}(trt), PhC_{Phe}-2, PhC_{Phe}-6), 129.2 (PhC_{Gln}(trt)), 130.0 (C-2', C-6'), 131.5 (PhC_{pbf}-6), 134.2 (PhC_{pbf}-1), 137.4 (PhC_{pbf}-5), 137.7 (C-3', C-5'), 138.1 (PhC_{Phe}-1), 139.0, 139.4, 140.7 (C_{Fmoc}-4a', C_{Fmoc}-4b'), 141.1, 141.4, 141.5, 141.7, 142.0, 142.1, 142.1, 142.4, 142.5, 143.7 (C_{Fmoc}-8a', C_{Fmoc}-9a'), 143.8, 144.0, 144.0, 144.3, 144.6, 144.7, 144.8, 144.9, 145.2, 145.5, 145.5, 145.7, 146.8, 146.9, 155.6, 156.0 (COOFmoc), 157.5 (PhC_{pbf}-4), 168.5, 168.7, 169.0, 169.1, 169.6 (CONH_{Cys}), 170.9 (CONH_{Val}), 171.4, 171.5, 171.6, 172.1 (CONH_{Ahx}), 172.5 (CONH_{Ala}); **m/z** (MALDI) calc. for [M+Na]⁺ = 2877.80, found = 2876.92 (C₆₀ standard **m/z** = 718.56).

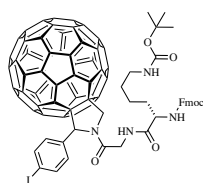
7.8.9 NFmoc-Cys-Ahx-Phe-Val-Arg-Gly-Ala-Gly-Glu-2-(4'-iodophenyl)-3,4-fulleropyrrolidine (49)



To the compound **50** (8.4 mg, 2.9 μmol , 1 eq.) was added a mixture of TFA/thioanisole 10:1 (1.6 mL, 1.8 mM). The reaction mixture was stirred for 50 min at room temperature until complete deprotection was observed by TLC. The solvent was removed under a continuous nitrogen flow. The crude product was purified by Et₂O (3 x 4 mL) washes, centrifugation (10 min, 7830 rpm), and decanting to give the deprotected product **49** (5.7 mg, 92%). **R_f** 0.0 (CHCl₃/MeOH 19:1); **IR** (film) ν_{max} 3710-3629, 3298 (OH, NH, SH), 2981-2826 (CH₃, CH₂, CH), 2074, 1670 (C=O amide), 1541 (NH amide), 1456 (CH₂), 1346, 1205, 1136, 1055, 1033, 1013, 802, 667, 648, 625; **¹H NMR** (500 MHz, DMSO-d₆) δ_{H} 0.83 (m, 6H, H_{Val}-4), 1.02 (m,

2H, H_{Ahx}-4), 1.29 (m, 8H, H_{Ahx}-3, H_{Ahx}-5, H_{Arg}-4a, H_{Ala}-3), 1.50 (m, 2H, H_{Arg}-3a, H_{Arg}-4b), 1.67 (m, 1H, H_{Arg}-3b), 1.98 (m, 4H, H_{Ahx}-2, H_{Val}-3, H_{Gln}-3a), 2.30 (m, 2H, H_{Cys}-3, H_{Gln}-3b), 2.47 (m, 1H, H_{Gln}-4b), 2.73 (m, 1H, H_{Phe}-3a), 2.88 (m, 1H, H_{Arg}-5a), 2.98 (m, 2H, H_{Ahx}-6, H_{Phe}-3b), 3.09 (m, 2H, H_{Arg}-5b), 3.77 (m, 4H, H_{Gly}1-2, H_{Gly}2-2), 4.18 (m, 3H, H_{Fmoc}-9', H_{Fmoc}-1a, H_{Val}-2), 4.27 (m, 3H, H_{Fmoc}-1b, H_{Cys}-2, H_{Arg}-2, H_{Ala}-2, H_{Gln}-2), 4.58 (bs, 1H, H_{Phe}-2), 5.18 (bs, 1H, H-2), 5.78 (m, 1H, H-5a), 6.26 (m, 1H, H-5b), 6.94 (bm, 2H), 7.14 (d, $^3J = 3.7$ Hz, 2H, PhH_{Phe}-4), 7.22 (bs, 4H, PhH_{Phe}-2, PhH_{Phe}-3, PhH_{Phe}-5, PhH_{Phe}-6, PhH_{Cys}(trt)-4), 7.29 (m, 3H, H_{Fmoc}-2', H_{Fmoc}-7'), 7.40 (td, $^4J = 10.6$ Hz, $^3J = 18.5$ Hz, 3H, H_{Fmoc}-3', H_{Fmoc}-6'), 7.72 (m, 6H, H_{Fmoc}-1', H_{Fmoc}-8', NH_{Cys}, CONH_{Ahx}, H-2', H-6'), 7.87 (m, 4H, H_{Fmoc}-4', H_{Fmoc}-5', CONH_{Val}, H-3', H-5'), 8.05 (bs, 2H, CONH_{Phe}), 8.14 (bs, 1H), 8.24 (m, 2H, CONH_{Gly}1, CONH_{Gly}2), 8.39 (bs, 1H); **¹³C NMR** (125 MHz, DMSO-d₆) δ_{C} 18.1 (C_{Val}-4a, C_{Ala}-3), 19.2 (C_{Val}-4b), 25.0 (C_{Ahx}-3, C_{Arg}-4), 25.8 (C_{Ahx}-4), 26.8 (C_{Gln}-3), 28.7 (C_{Ahx}-5), 29.0 (C_{Arg}-3), 30.7 (C_{Cys}-3, C_{Val}-3), 35.2 (C_{Ahx}-2), 37.1 (C_{Phe}-3), 38.7 (C_{Ahx}-6), 40.5 (C_{Arg}-5), 41.9 (C_{Gly}1-2, C_{Gly}2-2), 46.6 (C_{Fmoc}-9'), 48.4 (C_{Ala}-2), 50.5 (C_{pyrr}-2), 52.3 (C_{Gln}-2, C_{Arg}-2), 53.7 (C_{Phe}-2), 53.9 (C_{Cys}-2), 57.5 (C_{Val}-2), 58.8 (C_{pyrr}-5), 65.8 (C_{Fmoc}-1), 120.1 (C_{Fmoc}-4', C_{Fmoc}-5'), 122.1, 125.3 (C_{Fmoc}-1'), 125.4 (C_{Fmoc}-8'), 125.9, 126.2 (PhC_{Phe}-4), 127.1 (C_{Fmoc}-2', C_{Fmoc}-7'), 127.7 (C_{Fmoc}-3', C_{Fmoc}-6'), 128.0 (PhC_{Phe}-3, PhC_{Phe}-5), 128.9, 129.2 (PhC_{Phe}-2, PhC_{Phe}-6), 130.4 (C-2', C-6'), 132.4, 133.6, 136.2, 137.7 (C-3', C-5'), 138.1 (PhC_{Phe}-1), 139.4, 139.5, 140.7 (C_{Fmoc}-4a', C_{Fmoc}-4b'), 141.2, 141.5, 141.6, 141.7, 142.1, 142.5, 142.6, 143.7 (C_{Fmoc}-8a', C_{Fmoc}-9a'), 144.0, 144.1, 144.7, 144.8, 144.8, 144.9, 145.2, 145.5, 145.6, 145.8, 145.8, 146.9, 147.1, 155.9, 156.6 (COOFmoc), 169.7, 171.4 172.2; **m/z** (MALDI) calc. for [M+H]⁺ = 2122.5393, found = 2119.97 (C₆₀ standard m/z = 718.77).

7.8.10 (9*H*-Fluoren-9-yl)methyl *tert*-butyl (6-((2-(2'-(4'-iodophenyl)-3',4'-fulleropyrrolidin-1'-yl)-2-oxoethyl)amino)-6-oxohexane-1,5-diyl)di-carbamate (51**)**

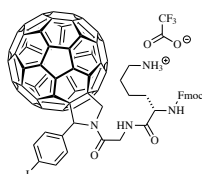


To a stirred solution of **21** (37.0 mg, 38.3 μ mol, 1 eq.) in anhydrous CHCl₃ (11.5 mL, 3 mM) was added a yellow solution of preactivated acid. This was prepared by adding DIPEA (13.3 μ L, 76.6 μ mol, 2 eq.) to a stirred solution of dipeptidic acid **29** (53.2 mg, 57.5 μ mol, 1.5 eq.) and HATU (29.1 mg, 76.6 μ mol, 2 eq.) in anhydrous DMF (1.3 mL, 30 mM) at room temperature.

The reaction mixture was stirred at room temperature for 1 h, and heated at 60 °C for 5 h until completed. The reaction was quenched by adding brine (25 mL), the organic layer was separated and the aqueous layer was extracted with CHCl₃ (2 x 20 mL). The organic layers were combined and washed with brine (20 mL), dried over MgSO₄, filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel (CHCl₃→CHCl₃/MeOH 99:1) to give the coupled product **51** (25.3 mg, 45%, 53% based on recovered **21**). *R_f* 0.60 (CHCl₃/MeOH 49:1); **IR** (film) ν_{max} 3311 (OH, NH), 2960-2852 (CH₃, CH₂, CH), 2246, 1650 (C=O amide), 1509 (NH amide), 1449 (CH₂), 1430, 1365, 1321, 1260, 1167, 1092, 1061, 1029, 1007, 907, 860, 803, 759, 730 (C-I), 668, 647, 621; **¹H NMR** (300 MHz, CDCl₃) δ_H 1.44 (s, 9H, C(CH₃)₃), 1.45 (m, 2H, H_{Lys}-5), 1.54 (m, 2H, H_{Lys}-4), 1.78 (m, 2H, H_{Lys}-3), 3.12 (m, 2H, H_{Lys}-6), 4.23 (t, ³*J* = 6.8 Hz, 1H, H_{Fmoc}-9'), 4.32 (m, 1H, H_{Lys}-2), 4.41 (d, ³*J* = 6.5 Hz, 2H, H_{Fmoc}-1), 4.67 (m, 2H, H_{Gly}-2), 5.64 (m, 2H, H-5), 6.94 (bs, 1H, H-2), 7.31 (m, 2H, H_{Fmoc}-2', H_{Fmoc}-7'), 7.39 (t, ³*J*_{3',2'} = ³*J*_{3',4'} = 7.3 Hz, 2H, H_{Fmoc}-3', H_{Fmoc}-6'), 7.55 (m, 2H, H-2, H-6), 7.61 (d, ³*J*_{1',2'} = 5.8 Hz, 2H, H_{Fmoc}-1', H_{Fmoc}-8'), 7.75 (d, ³*J*_{4',3'} = 7.4 Hz, 2H, H_{Fmoc}-4', H_{Fmoc}-5'), 7.83 (d, ³*J*_{3',2'} = 8.3 Hz, 2H, H-3, H-5); **¹H NMR** (500 MHz, DMSO-d₆) δ_H 1.37 (s, 9H, C(CH₃)₃), 1.40 (m, 2H, H_{Lys}-5), 1.61 (m, 1H, H_{Lys}-3a), 1.71 (m, 1H, H_{Lys}-3b), 2.92 (bs, 2H, H_{Lys}-6), 4.08 (bs, 1H, H_{Fmoc}-9'), 4.25 (m, 4H, H_{Gly}-2, H_{Fmoc}-1), 4.56 (m, 1H, H_{Lys}-2), 5.32 (m, 1H), 5.80 (m, 2H, H-5a), 6.94 (d, ²*J*_{5a,5b} = 12.3 Hz, 1H, H-5b), 6.79 (bs, 1H, NHBoc), 7.31 (m, 2H, H_{Fmoc}-2', H_{Fmoc}-7'), 7.40 (dd, ³*J* = 5.6 Hz, ³*J* = 8.7 Hz, 2H, H_{Fmoc}-3', H_{Fmoc}-6'), 7.61 (bs, 1H), 7.75 (dt, ³*J* = 7.7 Hz, ³*J* = 13.9 Hz, 3H, H-2', H-6', H_{Fmoc}-1', H_{Fmoc}-8'), 7.88 (t, ³*J* = 7.0 Hz, 4H, H-3', H-5', H_{Fmoc}-4', H_{Fmoc}-5'); **¹³C NMR** (75 MHz, CDCl₃) δ_C 22.7 (C_{Lys}-4), 28.6 (C(CH₃)₃), 29.8 (C_{Lys}-5), 32.4 (C_{Lys}-3), 40.0 (C_{Lys}-6), 42.8 (C_{Gly}-2), 47.3 (C_{Fmoc}-9'), 55.1 (C_{Lys}-2), 59.2 (C-5), 67.3 (C_{Fmoc}-1), 71.3 (C-2), 79.4 (C(CH₃)₃), 94.7 (C-4'), 120.2 (C_{Fmoc}-1', C_{Fmoc}-8'), 125.3 (C_{Fmoc}-2', C_{Fmoc}-7'), 127.3 (C_{Fmoc}-4', C_{Fmoc}-5'), 127.9 (C_{Fmoc}-3', C_{Fmoc}-6'), 129.4 (C-2', C-6'), 134.6, 135.4, 136.2, 137.0, 138.8 (C-3', C-5'), 140.0, 140.4, 140.5, 140.5, 141.4 (2C), 141.9, 141.9, 142.0, 142.0, 142.1, 142.2 (C_{Fmoc}-4a', C_{Fmoc}-5a'), 142.2, 142.3, 142.4, 142.9 (C-1'), 142.9, 143.3, 143.2, 142.3, 142.4, 142.8, 142.9, 143.3, 143.3, 143.9, 144.0, 144.2, 144.4, 144.6, 144.7, 144.7, 144.9, 145.4, 145.5, 145.5 (C_{Fmoc}-1a', C_{Fmoc}-8a'), 145.6, 145.7 (1/2C), 145.7, 145.8 (1/2C), 145.8 (1/2C), 146.3 (2C), 146.3, 146.5, 146.5, 146.5, 147.6, 147.7, 151.2, 153.6, 154.9,

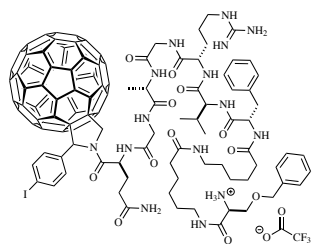
156.4 (NHCOO), 165.9 (CONR_2), 172.3 (COOH); m/z (MALDI) calc. for $[\text{M}+\text{K}]^+ = 1511.17$, found = 1511.27 (C_{60} standard $m/z = 720.13$).

7.8.11 (9*H*-Fluoren-9-yl)methyl (6-amino-1-((2-(2'-(4'-iodophenyl)-3',4'-fulleropyrrolidin-1'-yl)-2-oxoethyl)amino)-1-oxohexan-2-yl)carbamate (**52**)



To a stirred solution of fullerene coupled dipeptide **51** (10.0 mg, 6.8 μmol , 1 eq.) in CHCl_3 (0.7 mL, 10 mM) was added trifluoroacetic acid (TFA) (0.34 mL, 20 mM) and Et_3SiH (67.9 μL , 0.1 M) at room temperature. After stirring for 30 min, the solvent was removed under reduced pressure. The solid was washed with Et_2O (2 x 3 mL), centrifuged (10 min, 2 kG), and decanted after every wash. The brown solid was dried *in vacuo* to give the free amine-functionalised fullerene **52** (7.3 mg, 78%). R_f 0.35 ($\text{CHCl}_3/\text{MeOH}$ 4:1); **IR** (film) ν_{max} 3448 (OH, NH), 3016-2849 (CH_3 , CH_2 , CH), 1738, 1675, 1540 (NH amide), 1434 (CH_2), 1366, 1229, 1217, 1204, 1133, 1006, 906, 800, 759, 732 (C-I), 668, 618; **^1H NMR** (500 MHz, DMSO-d_6) δ_H 1.41 (m, 2H, $\text{H}_{\text{Lys-4}}$), 1.56 (m, 2H, $\text{H}_{\text{Lys-5}}$), 1.62 (m, 1H, $\text{H}_{\text{Lys-3a}}$), 1.76 (m, 1H, $\text{H}_{\text{Lys-3b}}$), 2.80 (m, 2H, $\text{H}_{\text{Lys-6}}$), 4.10 (bs, 1H, $\text{H}_{\text{Fmoc-9'}}$), 4.26 (dt, $^3J = 6.7$ Hz, $^3J = 15.0$ Hz, 4H, $\text{H}_{\text{Fmoc-1}}$, $\text{H}_{\text{Gly-2}}$), 4.57 (bs, 1H, $\text{H}_{\text{Lys-2}}$), 5.80 (bs, 1H, H-5a), 6.08 (t, $J = 17.4$ Hz, 1H, H-5b), 7.31 (m, 2H, $\text{H}_{\text{Fmoc-2'}}$, $\text{H}_{\text{Fmoc-7'}}$), 7.41 (t, $^3J_{3',2'} = ^3J_{3',4'} = 7.0$ Hz, 2H, $\text{H}_{\text{Fmoc-3'}}$, $\text{H}_{\text{Fmoc-6'}}$), 7.67 (bs, 4H, H-2), 7.74 (m, 4H, $\text{H}_{\text{Fmoc-1'}}$, $\text{H}_{\text{Fmoc-8'}}$, H-2', H-6'), 7.89 (t, $^3J_{4',3'} = 6.2$ Hz, 4H, $\text{H}_{\text{Fmoc-4'}}$, $\text{H}_{\text{Fmoc-5'}}$, H-3', H-5'); **^{13}C NMR** (125 MHz, DMSO-d_6) δ_C 22.5 ($\text{C}_{\text{Lys-4}}$), 26.6 ($\text{C}_{\text{Lys-5}}$), 31.2 ($\text{C}_{\text{Lys-3}}$), 39.8 ($\text{C}_{\text{Lys-6}}$), 42.1 ($\text{C}_{\text{Gly-2}}$), 46.7 ($\text{C}_{\text{Fmoc-9'}}$), 54.5 ($\text{C}_{\text{Lys-2}}$), 58.3 (C-5), 65.7 ($\text{C}_{\text{Fmoc-1}}$), 78.7 (C-2), 79.0 (C_{60-1}), 79.2 (C_{60-2}), 94.7 (C-4'), 120.2 ($\text{C}_{\text{Fmoc-1'}}$, $\text{C}_{\text{Fmoc-8'}}$), 125.3 ($\text{C}_{\text{Fmoc-2'}}$, $\text{C}_{\text{Fmoc-7'}}$), 127.1 ($\text{C}_{\text{Fmoc-4'}}$, $\text{C}_{\text{Fmoc-5'}}$), 127.7 ($\text{C}_{\text{Fmoc-3'}}$, $\text{C}_{\text{Fmoc-6'}}$), 129.9 (C-2', C-6'), 134.0, 134.7, 135.4, 135.4, 136.2, 137.9, 139.0, 139.4, 139.5 (C-3', C-5'), 139.8, 140.8 ($\text{C}_{\text{Fmoc-4a'}}$, $\text{C}_{\text{Fmoc-5a'}}$), 141.2, 141.4, 141.5, 141.6, 141.7, 141.8, 141.9, 142.1 (C-1'), 142.2 (1/2C), 142.6 (1/2C), 143.8 ($\text{C}_{\text{Fmoc-1a'}}$, $\text{C}_{\text{Fmoc-8a'}}$), 143.9, 144.0, 144.1, 144.2, 144.7, 144.9, 145.0, 145.3, 145.4 (1/2C), 145.5, 145.6, 145.8 (2C), 146.8, 146.9, 152.2, 153.8, 154.7, 156.0, 156.1, 156.1, 157.4, 157.6, 157.9, 158.1, 167.7 (NHCOGly , NHCOLys), 172.4 (NHCOO); m/z (MALDI) calc. for $[\text{M}+\text{H}]^+ = 1372.20$, found = 1373.15 (C_{60} standard $m/z = 720.13$).

7.8.12 NH_2 -Ser(Bn)-Ahx-Ahx-Phe-Val-Ile-Gly-Ala-Gly-Glu-2-(4'-iodophenyl)-3,4-fulleropyrrolidine (**56**)

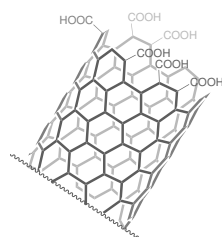


To a stirred solution of unprotected functionalised fulleropyrrolidine **46** (3.0 mg, 1.6 μmol , 1 eq.) in anhydrous DMF (0.5 mL, 0.8 mM) was added a yellow solution of preactivated peptide. This was prepared by adding DIPEA (0.4 μL , 2.4 μmol , 1.5 eq.) to a stirred solution of protected amino acid Boc-serine-OH (0.46 mg, 1.6 μmol , 1 eq.) and HATU (0.9 mg, 2.4 μmol , 1.5 eq.) in anhydrous DMF (0.5 mL, 0.8 mM) at room temperature after 35 min. The reaction

mixture was stirred for 14 h at room temperature until analysis by TLC showed completion. The solvent was removed under reduced pressure and the residue taken up in $\text{CHCl}_3/\text{MeOH}$ 20:1 (5 mL) and quenched with 1 M HCl_{aq} (5 mL). The aqueous layer was extracted with CHCl_3 (10 x 6 mL), the organic layers were combined, washed with brine (10 mL), dried over MgSO_4 , and filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel ($\text{CHCl}_3/\text{MeOH}$ 9:1→4:1) to give the coupled and Boc-protected product **56** (2.4 mg, 73%). R_f 0.55 ($\text{CHCl}_3/\text{MeOH}$ 4:1+drop FA); $^1\text{H NMR}$ (500 MHz, DMSO-d_6) δ_H 0.84 (m, 6H, H_{Val-4}), 1.04 (m, 2H, H_{Ahx2-4}), 1.23 (m, 11H, H_{Ahx1-4} , H_{Ahx2-5} , H_{Ala-3}), 1.36 (m, 8H, $t\text{Bu}$, H_{Ahx1-5} , H_{Ahx2-3}), 1.45 (m, 1H, H_{Ahx1-3} , H_{Arg-4}), 1.53 (m, 1H, H_{Arg-3a}), 1.68 (m, 1H, H_{Arg-3b}), 2.00 (m, 3H, H_{Ahx1-2} , H_{Ahx2-2} , H_{Val-3}), 2.72 (m, 1H, H_{Phe-3a}), 2.93 (dd, $^3J = 6.2$ Hz, $^3J = 13.4$ Hz, 2H, H_{Ahx2-6}), 3.00 (m, 1H, H_{Phe-3b}), 3.05 (m, 2H, H_{Ahx1-6}), 3.11 (m, 2H, H_{Arg-5}), 3.52 (m, 2H, H_{Ser-3}), 3.72 (m, 4H, H_{Gly1-2} , H_{Gly2-2}), 4.15 (m, 1H, H_{Arg-2}), 4.21 (m, 1H, H_{Ser-2} , H_{Val-2}), 4.27 (m, 1H, H_{Ala-2}), 4.47 (s, 2H, $\text{OCH}_2\text{Ph!}$), 4.57 (m, 1H, H_{Phe-2}), 5.18 (m, 1H, H_{Gln-2}), 5.89 (m, 1H, H-5a), 6.24 (d, $^3J = 12.4$ Hz, 1H, H-5b), 6.76 (d, $^3J = 8.0$ Hz, 1H, CONH_{Arg}), 7.17 (m, 1H, PhH_{Bn-4} , PhH_{Phe-4}), 7.23 (m, 2H, PhH_{Phe-2} , PhH_{Phe-3} , PhH_{Phe-5} , PhH_{Phe-6}), 7.31 (m, 4H, PhH_{Bn-2} , PhH_{Bn-3} , PhH_{Bn-5} , PhH_{Bn-6}), 7.49 (d, $^3J = 8.3$ Hz, 1H, CONH_{Ahx2}), 7.59 (m, 1H), 7.68 (bs, 2H, CONH_{Ahx1}), 7.74 (m, 1H, H-2', H-6'), 7.81 (m, 2H, CONH_{Val}), 7.89 (m, 4H, H-3', H-5'), 8.07 (m, 2H, CONH_{Phe}), 8.14 (m, 1H, CONH_{Ala}), 8.26 (m, 1H); $^{13}\text{C NMR}$ (125 MHz, DMSO-d_6) δ_C 17.7 (C_{Ala-3}), 17.9 (C_{Val-4a}), 19.3 (C_{Val-4b}), 22.0 (C_{Ahx1-4} , C_{Ahx2-5}), 24.4 (C_{Ahx1-3}), 24.6 (C_{Ahx1-5}), 24.7 (C_{Ahx2-3}), 25.6 (C_{Ahx2-4}), 27.7 (C_{Arg-3} , C_{Arg-4}), 28.2 ($\text{C}(\text{CH}_3)_3$), 30.6 (C_{Val-3}), 35.0 (C_{Ahx1-2} , C_{Ahx2-2}), 36.8 (C_{Phe-3}), 38.1 (C_{Ahx2-6}), 38.2 (C_{Ahx1-6}), 40.2 (C_{Arg-5}), 41.6 (C_{Gly-2} , C_{Gly-2}), 51.7 (C_{Gln-2}), 52.0 (C_{Ala-2}), 53.4 (C_{Phe-2}), 54.0 (C_{Arg-2}), 55.5 (C_{Ser-2}), 57.2 (C_{Val-2}), 62.6, 65.3, 69.8 (C_{Ser-3}), 70.1, 71.9 (OCH_2Ph), 78.2 ($\text{C}(\text{CH}_3)_3$), 126.2 (PhC_{Phe-4} , PhC_{Bn-4}), 127.4 (PhC_{Bn-2} , PhC_{Bn-6}), 127.9 (PhC_{Phe-3} , PhC_{Phe-5}), 128.2 (PhC_{Bn-3} , PhC_{Bn-5}), 128.5 (C-2', C-6'), 129.2 (PhC_{Phe-2} , PhC_{Phe-6}), 137.6 (C-3', C-5'), 138.1 (PhC_{Phe-1}), 138.2 (PhC_{Bn-1}), 142.2, too less material, not all carbon signals were found; m/z (MALDI) calc. for $[\text{M}+\text{Na}]^+ = 2108.01$, found = 2107.49 (C_{60} standard $m/z = 720.37$).

7.9 Functionalisation of Carbon Nanotubes

7.9.1 Oxidised CNTs^{284,288,289,313} (59)



Method A The suspension of carbon nanotubes (CNTs) (50.0 mg) in piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ 4:1, 50 mL) was stirred at room temperature for 1 h. The mixture was filtered (0.2 μm polycarbonate membrane) and washed thoroughly with $\text{H}_2\text{O}_{dest}$ (3 x 250 mL). The black solid was dried *in vacuo* at 60 °C to give oxidised CNTs **59** (51.4 mg).

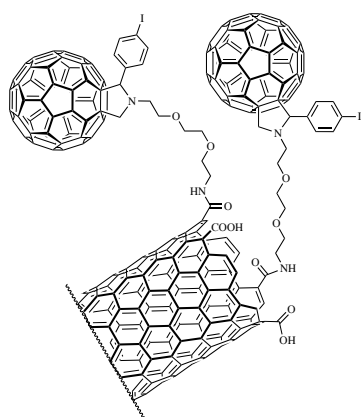
Method B The suspension of CNTs (51.4 mg) in HNO_3 (8 M, 25 mL) was sonicated for 30 min. The mixture was filtered (0.2 μm polycarbonate membrane) and washed thoroughly with $\text{H}_2\text{O}_{dest}$ (2 x 250 mL). The CNTs were taken up in NaOH_{aq} (4 M, 50 mL), sonicated for 15 min, and filtered and washed with $\text{H}_2\text{O}_{dest}$ (2 x 250 mL) until pH 5. The solid was taken up in HCl_{aq} (1 M, 100 mL), sonicated for 15 min, filtered and washed with $\text{H}_2\text{O}_{dest}$ (250 mL), taken up in $\text{H}_2\text{O}_{dest}$ (100 mL), sonicated, and filtered and washed with $\text{H}_2\text{O}_{dest}$ until pH 5. The black solid was dried *in vacuo* at 60 °C to give oxidised CNTs **59** (48.1 mg).

Method C The suspension of CNTs (56.1 mg) in HNO_3 (8 M, 25 mL) was sonicated for 1.5 h. The mixture was filtered (0.2 μm polycarbonate membrane) and washed thoroughly with $\text{H}_2\text{O}_{dest}$ (2 x 250 mL). The CNTs were taken up in NaOH_{aq} (4 M, 50 mL), sonicated for 15 min, and filtered and washed with $\text{H}_2\text{O}_{dest}$. Repeated NaOH-wash was done twice with sonication for 30 min and washing with $\text{H}_2\text{O}_{dest}$. The nanotubes were taken up in $\text{H}_2\text{O}_{dest}$ (70 mL), sonicated for 30 min, and filtered and washed with $\text{H}_2\text{O}_{dest}$ until pH 7. The solid was taken up in HCl_{aq} (1 M, 50 mL), sonicated for 30 min, filtered and washed with $\text{H}_2\text{O}_{dest}$ (250 mL), taken up in $\text{H}_2\text{O}_{dest}$ (80 mL), sonicated 30 min, and filtered and washed with $\text{H}_2\text{O}_{dest}$ until pH 5. The black solid was dried *in vacuo* at 60 °C to give oxidised CNTs **59** (47.7 mg).

7.9.2 Activate oxidised CNTs with SOCl_2 ^{52,284,304,315} (**70**)

To the oxidised CNTs **59** (17.1 mg) was added SOCl_2 (8.5 mL, 0.5 ml/mg) under an atmosphere of argon. The suspension was sonicated for 2 min and refluxed for 24 h at 70 °C. The solvent was removed under reduced pressure. The solid was washed with anhydrous THF (3 x 11 mL), centrifuged (20 min, 7197 G), decanted the yellow solution, and dried *in vacuo* to give the acyl chloride **70**. The crude black solid was used *in situ* for the next reaction steps.

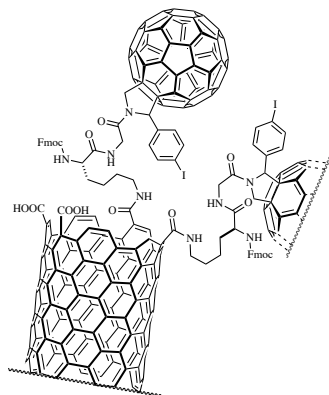
7.9.3 Synthesis of diethylene glycol-linked CNT- C_{60} ²⁹⁴ (**65**)



Condition A To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 6.7 mg, 1 eq.) in anhydrous CHCl_3 (7 mL, 1 ml/mg) was added the diethylene glycol-functionalised fulleropyrrolidine **22** (13.5 mg, 2 mass eq.) and DIPEA (4.3 μL , 2 eq. to amine) under an atmosphere of argon at room temperature. The mixture was sonicated for 5 min and stirred for 39.5 h. The solvent was removed by filtration (0.2 μm nylon membrane filter) with chloroform (CHCl_3) (200 mL). The crude product was washed with DMF (2 x 50 mL), sonicated for 5 min, and filtered. The procedure was repeated with H_2O (50 mL) and CHCl_3 (50 mL) until the eluent showed no residual fC_{60} by TLC ana-

lysis. The solid was dried *in vacuo* to give diethylene glycol-linked CNT-C₆₀ construct **65** as a black solid (7.3 mg).

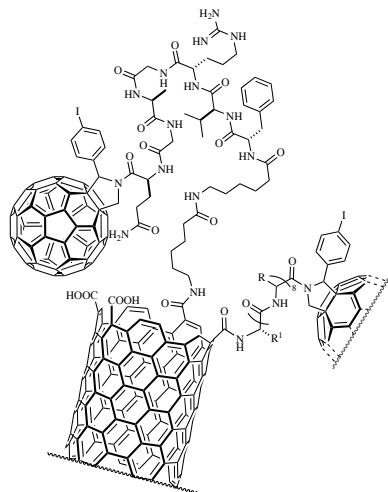
7.9.4 Synthesis of CNT-GK-C₆₀ **66**



Condition B To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 10.2 mg, 1 eq.) in anhydrous CHCl₃ (1 ml/mg) was added the dipeptide-functionalised fulleropyrrolidine **52** (13.9 mg, 1.4 mass eq.) and DIPEA (3.5 μ L, 2 eq. to amine) under an atmosphere of argon at room temperature. The mixture was sonicated for 5 min and stirred for 40.5 h. The solvent was removed by filtration (0.2 μ m nylon membrane filter) with CHCl₃ (60 mL). The crude product was washed with DMF (4 x 60 mL), sonicated for 30 min, and filtered. The procedure was repeated with H₂O (200 mL) and CHCl₃ (4 x 60 mL) until the eluent showed no residual fC₆₀ by

TLC analysis. The solid was dried *in vacuo* to give CNT-KG-C₆₀ construct **66** as a black solid (12.0 mg).

7.9.5 Synthesis of CNT-XXFVRGAGQ-C₆₀ **68**



Condition C To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 3.2 mg, 1 eq.) in anhydrous CHCl₃ (1 ml/mg) was added the peptide functionalised fulleropyrrolidine **46** (1.7 mg, 0.5 mass eq.) and DIPEA (0.3 μ L, 2 eq. to amine) under an atmosphere of argon at room temperature, and it was stirred for 87 h. The solvent was removed under reduced pressure. The crude mixture was washed with DMSO (3 x 3 mL), sonicated for 10 min, centrifuged (15 min, 2 kG), and decanted. The procedure was repeated with H₂O (2 mL), MeCN (2 x 2 mL), DCM (2 mL), and dried *in vacuo* to give CNT-XXFVRGAGQ-C₆₀ construct **68** as a black solid (3.9 mg).

Condition D To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 3.2 mg, 1 eq.) in anhydrous CHCl₃ (1 ml/mg) was added the peptide-functionalised fulleropyrrolidine **46** (1.7 mg, 0.5 mass eq.) and DIPEA (0.3 μ L, 2 eq. to amine) under an atmosphere of argon at 70 °C, and it was stirred for 87 h. The solvent was removed under reduced pressure. The crude mixture was washed with DMSO (3 x 3 mL), sonicated for 10 min, centrifuged (15 min, 2 kG), and then decanted. The procedure was repeated with H₂O (2 mL), MeCN (2 x 2 mL), DCM (2 mL), and dried *in vacuo* to give CNT-XXFVRGAGQ-C₆₀ construct **68** as a black solid (4.1 mg).

Condition E To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 3.2 mg, 1 eq.) in anhydrous CHCl₃/MeOH 6:1 (1 ml/mg) was added the peptide-functionalised fulleropyrrolidine **46** (1.7 mg, 0.5 mass eq.) and DIPEA (0.3 μ L, 2 eq. to amine) under an atmosphere of argon at room temperature, and it was stirred for 87 h. The solvent was removed under reduced pressure. The crude mixture was washed with DMSO (3 x 3 mL), sonicated for 10 min, centrifuged (15 min, 2 kG), and decanted. The procedure was repeated with H₂O (2 mL), MeCN (2 x 2 mL), DCM (2 mL), and dried *in vacuo* to give CNT-XXFVRGAGQ-C₆₀ construct **68** as a black solid (4.2 mg).

Condition F To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 4.2 mg, 1 eq.) in anhydrous DMF (0.5 ml/mg) was added the peptide-functionalised fulleropyrrolidine **46** (2.5 mg, 0.5 mass eq.) and DIPEA (0.5 μ L, 2 eq. to amine) under an atmosphere of argon at room temperature. It was sonicated for 10 min and stirred for 40 h. The crude mixture was washed with DMF (3 x 6 mL), sonicated for 10 min, centrifuged (30 min, 7197 G), and decanted. The procedure was repeated with H₂O (3 x 6 mL), DMF (2 x 6 mL), CHCl₃/MeOH 1:1 (3 x 6 mL), DCM (3 x 6 mL), and dried *in vacuo* to give CNT-XXFVRGAGQ-C₆₀ construct **68** as a black solid (6.3 mg).

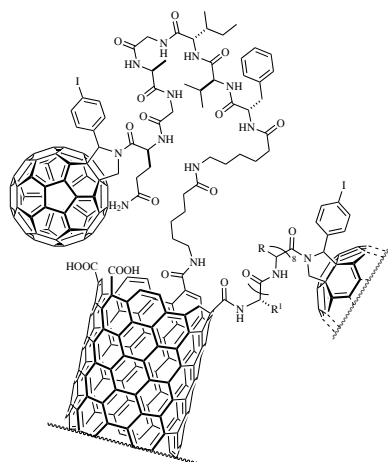
Condition G To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 4.2 mg, 1 eq.) in anhydrous DMF (0.5 ml/mg) was added the peptide-functionalised fulleropyrrolidine **46** (2.5 mg, 0.5 mass eq.) and DIPEA (0.5 μ L, 2 eq. to amine) under an atmosphere of argon at room temperature. It was sonicated for 10 min and stirred for 86 h. The crude mixture was washed with DMF (3 x 6 mL), sonicated for 10 min, centrifuged (30 min, 7197 G), and decanted. The procedure was repeated with H₂O (3 x 6 mL), DMF (2 x 6 mL), CHCl₃/MeOH 1:1 (3 x 6 mL), DCM (3 x 6 mL), and dried *in vacuo* to give CNT-XXFVRGAGQ-C₆₀ construct **68** as a black solid (8.3 mg).

Condition H To a suspension of oxidised CNT **59** (5.1 mg, 1 eq.) in anhydrous DMF (2.5 mL, 0.5 ml/mg) was added EDC (10 mg, 2 mass eq.), peptide-functionalised C₆₀ (**46**, 2.0 mg, 0.5 mass eq.) and DIPEA (0.4 μ L, 2 eq. to amine) under an atmosphere of argon. The mixture was sonicated for 1 h and stirred vigorously for 40 h at room temperature. The crude mixture was washed with DMF (3 x 6 mL), sonicated for 10 min, centrifuged (30 min, 7197 G), and then decanted. The procedure was repeated with H₂O (3 x 6 mL), DMF (2 x 6 mL), CHCl₃/MeOH 1:1 (3 x 6 mL), DCM (3 x 6 mL), and the product dried *in vacuo* to give CNT-XXFVRGAGQ-C₆₀ construct **68** as a black solid (6.8 mg).

Condition I Preactivated CNT-COCl (**70**, ~0.2 mg), peptide-functionalised fulleropyrrolidine **46** (~0.1 mg) and DIPEA (one drop) were grinded in an agatha mortar for 10 min under room temperature. The mixture was left over night, washed with H₂O (2 mL), sonicated for 10 min, centrifuged (15 min, 2 kG), decanted. The procedure was repeated with DMSO (2 x 2 mL),

H₂O (2 mL), MeCN (2 mL), DCM (2 mL), and dried *in vacuo* to give CNT-XXFVRGAGQ-C₆₀ construct **68** as a black solid (0.1 mg).

7.9.6 Synthesis of CNT-XXFVIGAGQ-C₆₀ **69**



Condition J To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 13.6 mg, 1 eq.) in anhydrous CHCl₃ (10 mL, 0.7 ml/mg) was added the peptide-functionalised fulleropyrrolidine **48** (6.9 mg, 0.5 mass eq.) and DIPEA (1.3 μ L, 2 eq. to amine) under an atmosphere of argon at room temperature. The mixture was sonicated for 10 min and stirred for 70 h. The solvent was removed under reduced pressure. The crude mixture was washed with DMSO (3 x 11 mL), sonicated for 10 min, centrifuged (30 min, 7197 G), and decanted. The procedure was repeated with PhCN (3 x 11 mL), toluene (3 x 11 mL), DCM (2 x 11 mL), and dried *in vacuo* to give CNT-XXFVIGAGQ-C₆₀ construct **69** as a black solid

(11.1 mg).

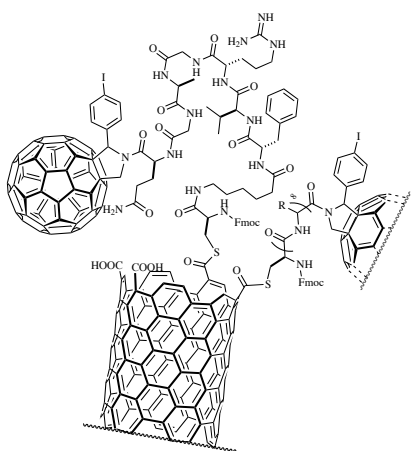
Condition K To a suspension of oxidised CNT **59** (5.0 mg, 1 eq.) in anhydrous DMF (2.5 mL, 0.5 ml/mg) was added EDC (10.0 mg, 2 mass eq.) and DIPEA (9.1 μ L, 2 eq. to amine) at room temperature under an atmosphere of argon. The mixture was sonicated for 1 h. Peptide-functionalised C₆₀ (**48**, 1.9 mg, 0.5 mass eq.) was added to the mixture, sonicated for 1 h, and stirred vigorously for 39 h at room temperature. The crude mixture was washed with H₂O (3 x 11 mL), sonicated for 30 min, centrifuged (20 min, 7197 G), and decanted. The washing procedure was repeated with DMF (3 x 11 mL), MeCN (3 x 11 mL), Et₂O (3 x 11 mL), and the product was dried *in vacuo* to give CNT-XXFVIGAGQ-C₆₀ construct **69** as a black solid (5.8 mg).

Condition L To a suspension of oxidised CNT **59** (5.0 mg, 1 eq.) in anhydrous DMF (2.5 mL, 0.5 ml/mg) was added EDC (10.0 mg, 2 mass eq.), NHS (6.0 mg, 1 eq. to EDC), and DIPEA (9.1 μ L, 2 eq. to amine) at room temperature under an atmosphere of argon. The mixture was sonicated for 1 h. Peptide-functionalised C₆₀ (**48**, 1.9 mg, 0.5 mass eq.) was added to the mixture, sonicated for 1 h and stirred vigorously for 39 h at room temperature. The crude mixture was washed with H₂O (3 x 11 mL), sonicated for 30 min, centrifuged (20 min, 7197 G), and then decanted. The washing procedure was repeated with DMF (3 x 11 mL), MeCN (3 x 11 mL), Et₂O (3 x 11 mL), and the product was dried *in vacuo* to give CNT-XXFVIGAGQ-C₆₀ construct **69** as a black solid (6.0 mg).

Condition M To a suspension of oxidised CNT **59** (5.0 mg, 1 eq.) in anhydrous DMF (2.5 mL, 0.5 ml/mg) was added HATU (10.0 mg, 2 mass eq.) and DIPEA (4.6 μ L, 2 eq. to coupling

reagent) at room temperature under an atmosphere of argon. The mixture was sonicated for 1 h. Peptide-functionalised C₆₀ (**48**, 1.9 mg, 0.5 mass eq.) was added to the mixture, sonicated for 1 h, and stirred vigorously for 39 h at room temperature. The crude mixture was washed with H₂O (3 x 11 mL), sonicated for 30 min, centrifuged (20 min, 7197 G), and decanted. The washing procedure was repeated with DMF (3 x 11 mL), MeCN (4 x 11 mL), Et₂O (3 x 11 mL), and the product was dried *in vacuo* to give CNT-XXFVIGAGQ-C₆₀ construct **69** as a black solid (5.8 mg).

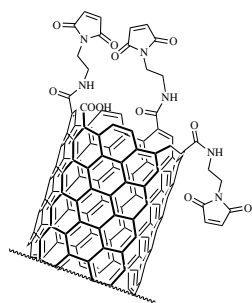
7.9.7 Synthesis of CNT-CXFVRGAGQ-C₆₀ **73**



To freshly prepared peptide-functionalised fulleropyrrolidine **49** (5.1 mg, 2.4 μ mol, 1 eq.) was added a suspension of preactivated CNT-COCl (**70**, 13.6 mg, 1 eq.) in anhydrous THF. The solvent was removed under reduced pressure. To the solid was added CHCl₃ (10 mL, 0.7 ml/mg) and DIPEA (0.8 μ L, 2 eq. to sulfide) under an atmosphere of argon at room temperature. The mixture was sonicated for 10 min and stirred vigorously for 70 h. The solvent was removed under reduced pressure. The crude mixture was washed with DMSO (2 x 11 mL), sonicated for 10 min, centrifuged (30 min, 7197 G), and then decanted. The procedure was repeated with PhCN (3 x 11 mL), toluene (3 x 11 mL), DCM

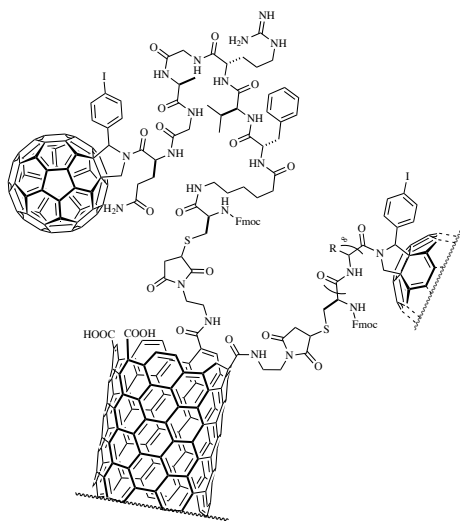
(2 x 11 mL), and dried *in vacuo* to give CNT-CXFVRGAGQ-C₆₀ construct **73** as a black solid (11.8 mg).

7.9.8 Synthesis of CNT-maleimide-CXFVRGAGQ-C₆₀ **74**



CNT-maleimide To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 6.5 mg, 1 eq.) in anhydrous CHCl₃ (6.5 mL, 1 ml/mg) was added the maleimide-containing amine (6.5 mg, 1 mass eq.) and DIPEA (4.5 μ L, 1 eq. to amine) under an atmosphere of argon at room temperature. The mixture was sonicated for 30 min and stirred for 65 h. The solvent was removed under reduced pressure. The crude mixture was washed with H₂O (3 x 11 mL), sonicated for 30 min, centrifuged (30 min,

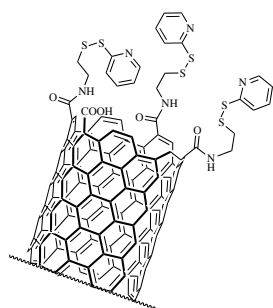
7197 G), and decanted. The procedure was repeated with MeCN (3 x 11 mL), Et₂O (3 x 11 mL), and then dried *in vacuo* to give CNT-maleimide **71** as a black solid (7.4 mg).



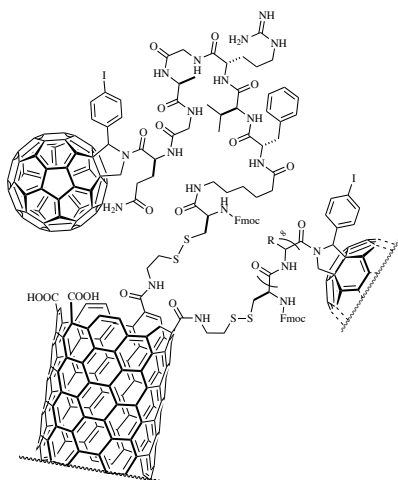
a black solid (1.8 mg).

Filled CNT-maleimide-CXFVRGAGQ-C₆₀ The suspension of maleimide-functionalised CNT **71** (1.5 mg, 1 eq.) in NaI_{aq} (300.0 mg in 1.5 mL, 1 ml/mg) was sonicated for 1 h and agitated for 18 h at room temperature. To the suspension was then added the peptide-functionalised fulleropyrrolidine **49** (0.84 mg, 0.5 mass eq.) in DMF (0.15 mL, 0.1 mL/mg) and DIPEA (0.1 μ L, 1 eq. to sulfide) at room temperature. The mixture was sonicated for 1 h and agitated for 32.5 h. The solvent was removed by centrifugation and decantation. The crude product was washed with DMF (2 mL), sonicated for 10 min, centrifuged (10 min, 2 kG), and then decanted. The procedure was repeated with H₂O (3 x 2 mL), DMF (3 x 2 mL), MeCN (2 x 2 mL), Et₂O (3 x 2 mL), and dried *in vacuo* to give NaI-filled CNT-maleimide-CXFVRGAGQ-C₆₀ construct **74** as a black solid (1.3 mg).

7.9.9 Synthesis of CNT-disulfide-CXFVRGAGQ-C₆₀ **75**



CNT-disulfide To a vigorously stirred suspension of preactivated CNT-COCl (**70**, 10.8 mg, 1 eq.) in anhydrous CHCl₃ (7.5 mL, 0.7 ml/mg) was added the disulfide-containing amine (8.7 mg, 0.8 mass eq.) and DIPEA (6.8 μ L, 1 eq. to amine) under an atmosphere of argon at room temperature. The mixture was sonicated for 30 min and stirred for 65 h. The solvent was removed under reduced pressure. The crude mixture was washed with H₂O (3 x 11 mL), sonicated for 30 min, centrifuged (30 min, 7197 G), and decanted. The procedure was repeated with MeCN (3 x 11 mL), Et₂O (3 x 11 mL), and dried *in vacuo* to give CNT-disulfide **72** as a black solid (10.8 mg).



CNT-disulfide-CXFVRGAGQ-C₆₀ To a suspension of protected disulfide-functionalised CNT **72** (1.5 mg, 1 eq.) in H₂O (1.5 mL, 1 ml/mg) was added the peptide-functionalised fulleropyrrolidine **49** (0.84 mg, 0.5 mass eq.) in DMF (0.15 mL, 0.1 mL/mg) and DIPEA (0.1 μ L, 1 eq. to sulfide) at room temperature. The mixture was sonicated for 30 min and agitated for 38.5 h. The crude mixture was washed with DMF (6 x 5 mL), sonicated for 20 min, centrifuged (20 min, 7197 G), and decanted. The procedure was repeated with MeCN (3 x 5 mL), Et₂O (2 x 5 mL), and dried *in vacuo* to give CNT-disulfide-CXFVRGAGQ-C₆₀ construct **75** as a black solid (2.0 mg).

Filled CNT-disulfide-CXFVRGAGQ-C₆₀ The suspension of protected disulfide-functionalised CNT **72** (1.5 mg, 1 eq.) in NaI_{aq} (300.0 mg in 1.5 mL, 1 ml/mg) was sonicated for 1 h and agitated for 18 h at room temperature. To the suspension was then added the peptide-functionalised fulleropyrrolidine **49** (0.84 mg, 0.5 mass eq.) in DMF (0.15 mL, 0.1 mL/mg) and DIPEA (0.1 μ L, 1 eq. to sulfide) at room temperature. The mixture was sonicated for 1 h and agitated for 32.5 h. The solvent was removed by centrifugation and decantation. The crude product was washed with DMF (2 mL), sonicated for 10 min, centrifuged (10 min, 2 kG), and then decanted. The procedure was repeated with H₂O (3 x 2 mL), DMF (3 x 2 mL), MeCN (2 x 2 mL), Et₂O (3 x 2 mL), and dried *in vacuo* to give NaI-filled CNT-disulfide-CXFVRGAGQ-C₆₀ construct **75** as a black solid (1.7 mg).

7.10 Enzymatic Cleavage

7.10.1 Enzymatic Cleavage of Peptide XXFVRGAGQ²⁶⁰

To 365 μ L citrate buffer (0.1 M, pH 5.5, containing 1 mM EDTA and 2 mM DTT) was added 125 μ L peptide **76** (0.2 mmol/mL DMSO) and 10 μ L activated cathepsin B solution (3.15 units/mL citrate buffer) at room temperature. The mixture was incubated at 37 °C under continuous shaking (600 rpm) for 24 h. The sample was heat-shocked at 100 °C for 2 min prior to analysis. **HPLC** (Phenomenex Synergi 4u Hydro-RP 80A (100 x 4.6 μ m, 4 micron), t_R = 6.3 min H₂O/MeCN 95:5→5:95, 24 min, λ = 210 nm); **LC/MS** H₂O/MeCN 95:5→5:95, 80 min gradient; t_R = 20.8 min, found $[M+2H]^{2+}$ = 480.72; t_R = 21.3 min, found $[M+2H]^{2+}$ = 480.80, $[M+H]^+$ = 534.35; t_R = 24.7 min, found $[M+2H]^{2+}$ = 580.34; t_R = 25.5 min, found $[M+H]^+$ = 392.32; t_R = 28.4 min, found $[M+2H]^{2+}$ = 480.80, $[M+2H]^{2+}$ = 508.84; t_R = 29.3 min, found $[M+Na]^+$ = 491.30.

7.10.2 Enzymatic Cleavage of XXFVRGAGQ-C₆₀

To 365 μ L citrate buffer (0.1 M, pH 5.5, containing 1 mM EDTA and 2 mM DTT) was added 125 μ L peptide-functionalised fC₆₀ **46** (0.2 mmol/mL DMSO) and 10 μ L activated cathepsin B solution (3.15 units/mL citrate buffer) at room temperature. The mixture was incubated at 37 °C under continuous shaking (600 rpm) for 24 h. The sample was heat-shocked at 100 °C for 2 min prior to analysis. **HPLC** (Phenomenex Synergi 4u Hydro-RP 80A (100 x 4.6 μ m, 4 micron), t_R = 6.4 min H₂O/MeCN 95:5→5:95, 24 min, λ = 210 nm); **LC/MS** H₂O/MeCN 95:5→5:95, 80 min gradient; t_R = 20.3 min, found $[M+2H]^{2+}$ = 416.29; t_R = 20.6 min, found $[M+2H]^{2+}$ = 387.76, t_R = 20.9 min, found $[M+2H]^{2+}$ = 480.79; t_R = 21.2 min, found $[M+2H]^{2+}$ = 471.79; t_R = 21.6 min, found $[M+H]^+$ = 534.39; t_R = 22.6 min, found $[M+2H]^{2+}$ = 388.26; t_R = 22.9 min, found $[M+2H]^{2+}$ = 401.78, $[M+2H]^{2+}$ = 462.82; t_R = 24.3 min, found $[M+2H]^{2+}$ = 400.78; t_R = 25.6 min, found $[M+H]^+$ = 392.28; t_R = 29.4 min, found $[M+Na]^+$ = 491.35; **m/z** (MALDI) calc. for $[M+Na]^+$ = 1930.85, found = 1930.66, 1209.93, 795.50 (C₆₀ standard m/z = 718.69).

7.10.3 Enzymatic Cleavage of CNT-XXFVRGAGQ-C₆₀

To 98 μ L citrate buffer (0.1 M, pH 5.5, containing 1 mM EDTA and 2 mM DTT) was added CNT-C₆₀ construct **68** (1.4 mg), and it was sonicated for 45 min. Then, 2 μ L activated cathepsin B solution (3.15 units/mL citrate buffer) was added at room temperature. The mixture was incubated at 37 °C under continuous shaking (1000 rpm) for 24 h. The sample was heat-shocked at 90 °C for 2 min. The crude mixture was centrifuged (10 min, 2 kG) and decanted. The solid was washed with citrate buffer (3 x 0.5 mL), sonicated for 10 min, centrifuged (10 min, 2 kG), and decanted. The procedure was repeated with H₂O (3 x 0.5 mL), DMF (6 x 1.8 mL), Et₂O (2 x 1.8 mL), and dried *in vacuo* to give cleaved CNT-XXFVR as a black solid. The DMF solution was dried *in vacuo* to give cleaved GAGQ-C₆₀ as a brown solid (<0.1 mg).

Appendix

A collection of supporting material comprising Raman and high resolution transmission electron microscopy (HRTEM) images is given below.

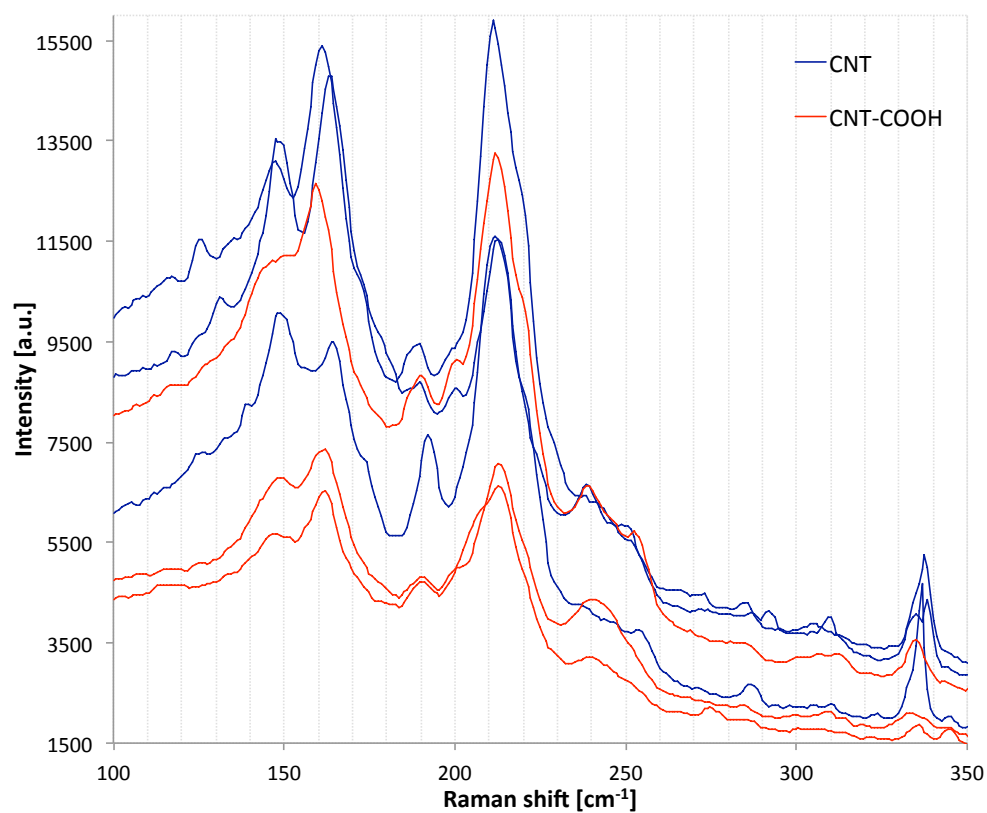


Figure 8.1: Pristine CNTs (blue spectra) have been sonicated in HNO_3 for 1.5 h (red spectra). A comparison of the RBM shows no significant up-shift as reported in literature.³⁵⁹

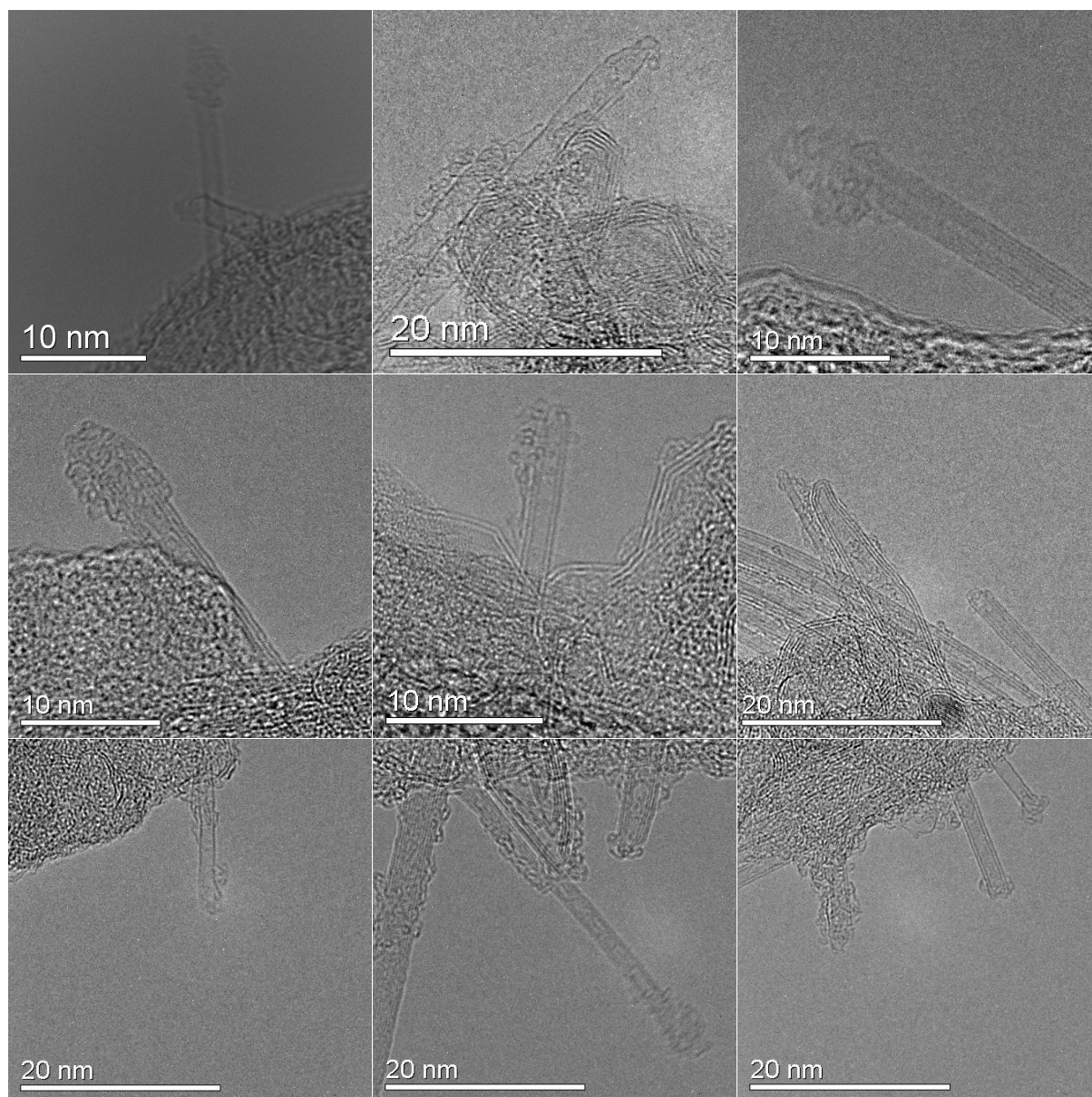
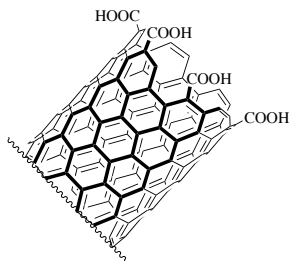


Figure 8.2: A selection of HRTEM images of fCNT **65** made with condition A.

An overview of the acquired, and when appropriate averaged data is given in the following tables. Conditions refer to Table 4.2, page 57.

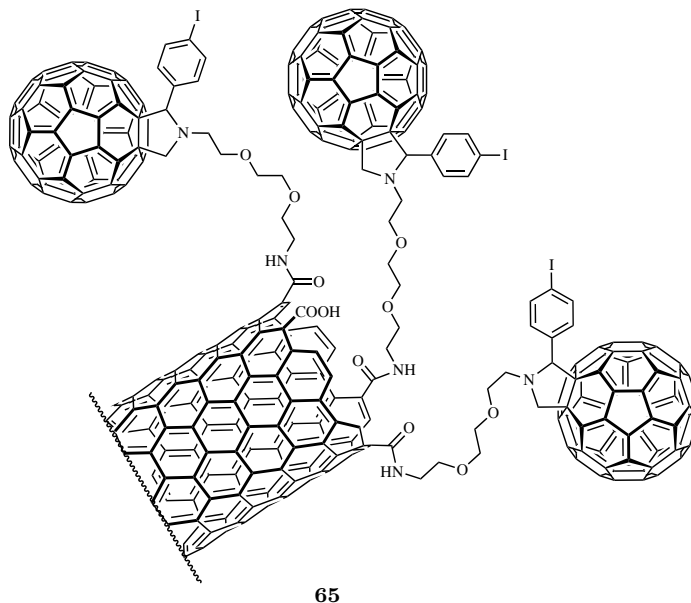
Table 8.1: Analytical results of the oxidised CNT **59**.



ENTRY	ANALYSIS	RESULT
1	ATR-FTIR	706, 1028, 1079, 1178, 1454, 1593, 1653(shoulder), 1755, 2411, 2868, 2922, 2953 cm^{-1}
2	Raman	$A_D/A_G = 0.1232$; 148, 161, 192, 214, 241, 285, 337, 345, 685, 898, 1055, 1320, 1576, 1709, 1906, 2606 cm^{-1}
3	HRTEM	fairly clean, some carbon debris
4	XRF	Cl, Ar, Ca, Cr, Fe, Ni in ascending energies
5	XPS	O and C present; 1 x O per 18 carbon
6	TGA	$T_{\text{combust.}} = 150.8, 603.8, 634\text{ }^{\circ}\text{C}(\text{sh}), 3.14\%$ residue
7	TGA ^a	$T_{\text{combust.}} = 355.4, 748.5\text{ }^{\circ}\text{C}, 3.6\%$ weight loss at 500 $^{\circ}\text{C}$

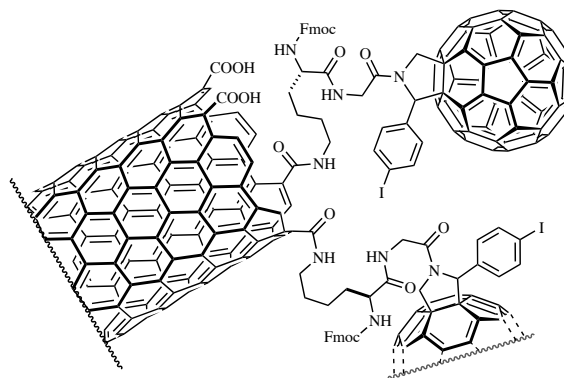
^a TGA was recorded in pure nitrogen gas.

Table 8.2: Analytical results of the diethylene glycol-linked CNT-C₆₀ construct **65** (condition A).

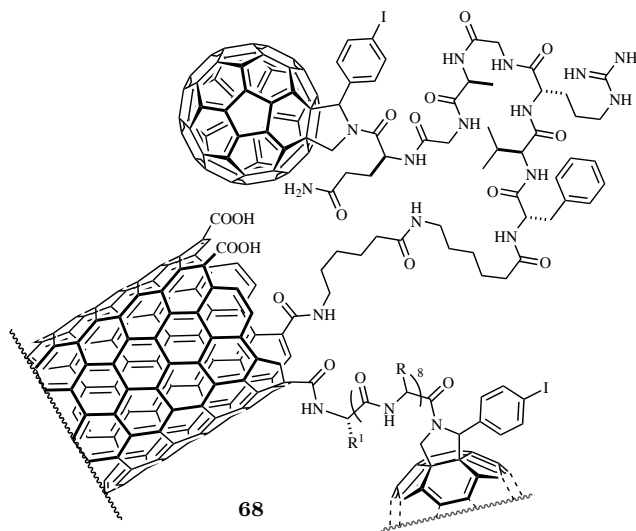


	ANALYSIS	RESULT	Y/N
1	ATR-FTIR	1010, 1223, 1265, 1377, 1468, 1491, 1597, 1686, 2595, 2853, 2920, 2951, 3671 cm^{-1}	~
2	Raman	$A_D/A_G = 0.1489$; 151, 164, 190, 214, 236-243, 285, 335, 344, 678, 887, 1053, 1320, 1577, 1708, 1903-1910, 2595-2609 cm^{-1}	–

	ANALYSIS	RESULT	Y/N
3	HRTEM	26 ends: 18 (69%) open thereof 15 (83%) functionalised tubes and 3 (17%) peapods	+
4	EDX	C, O, I, Si, Al, S, Cl, Na, Fe in decreasing quantity	+
5	XRF	Cl, Ar, Ca, I, Cr, Fe, Ni in ascending energies	+
6	XPS	I, O, N, C present; 1 x I per 117 carbon and 3 nitrogen; every 4th COOH functionalised	+

Table 8.3: Analytical results of KG functionalised fullerene coupled to CNT **66** (condition B).

ENTRY	ANALYSIS	RESULT	
1	ATR-FTIR	1125, 1267, 1468, 1597, 1686, 1751, 2344, 2855, 2901, 2928, 2953 cm^{-1}	+
2	Raman	$A_D/A_G = 0.1505$; 150, 163, 189, 214, 239, 284, 305, 335, 343, 680, 896, 1052, 1318, 1572-1584, 1707, 1904-1916, 2606 cm^{-1}	-
3	XPS	I, O, N, C present; 1 x I per 131 carbon and 3 nitrogen; every 10th COOH functionalised	+
4	Fmoc	183.9 nmol 52 /mg CNT equals functionalisation of ever 338th carbon	~

Table 8.4: Analytical results of the various coupling methods using CNT and XXFVRGAGQ functionalised C₆₀.

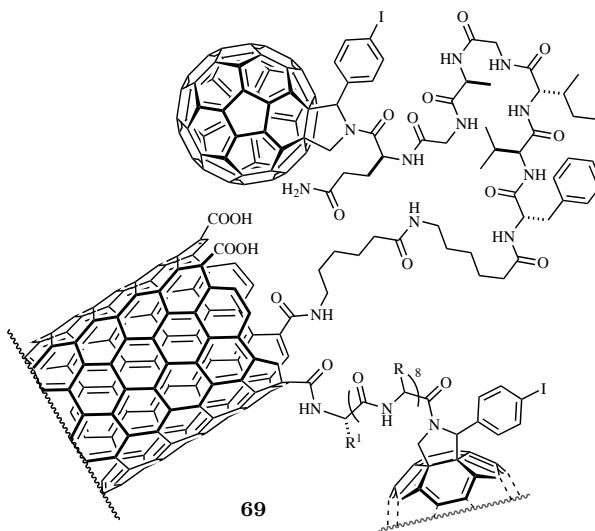
	SOLVENT	ANALYSIS	RESULT	Y/N
1	CHCl ₃ , 87 h, r.t., cond. C	ATR-FTIR	826, 1130, 1223, 1265, 1379, 1414, 1462, 1597, 1694, 2619, 2833, 2920, 2951 cm ⁻¹	+
2		Raman	$A_D/A_G = 0.1115$; 150, 161, 190, 214, 239, 285, 301, 309, 333, 344, 683, 703, 772, 897-907, 1052, 1318, 1573, 1709, 1905, 2602 cm ⁻¹	-
3		HRTEM	19 ends: 8 (50%) open thereof 6 (75%) functionalised and 2 (25%) filled tubes	+
4		EDX	C, Si, O, Cl, S, Al, I, Fe in decreasing quantity	+
5		XRF	Cl, Ar, Ca, I, Cr, Fe, Ni in ascending energies	+
6		XPS	I, O, N, C present; 1 x I per 412 carbon and 12 nitrogen; every 23rd COOH functionalised	+
7		TGA	$T_{combust.} = 270.6, 336.6(sh), 607.7$ °C, 22.46% residue	~
8	CHCl ₃ , 87 h,	ATR-FTIR	824, 1126, 1223, 1265, 1377, 1462, 1597, 1689, 2347, 2671, 2854, 2928, 2959 cm ⁻¹	+
9	70 °C, cond. D	Raman	$A_D/A_G = 0.1409$; 151, 163, 192, 214, 242, 270, 286, 301, 309, 335, 345, 678, 704, 775, 901, 1052, 1315, 1577, 1713, 1906, 2607 cm ⁻¹	~
10		HRTEM	11 ends: 10 (91%) open thereof 7 (70%) functionalised and 4 (40%) filled tubes	~
11		EDX	C, Si, O, Cl, S, I, Fe in decreasing quantity	+
12		XRF	S, Cl, Ar, Ca, I, Fe, Ni in ascending energies	+
13		XPS	I, O, N, C present; 1 x I per 321 carbon and 12 nitrogen; every 13th COOH functionalised	+
14		TGA	$T_{combust.} = 278.9, 335(sh), 623.1$ °C, 1.21% residue	~
15	CHCl ₃ / MeOH,	ATR-FTIR	824, 1138, 1225, 1265, 1379, 1464, 1601, 1697, 2363, 2675, 2853, 2920, 2953 cm ⁻¹	+

	SOLVENT	ANALYSIS	RESULT	Y/N
16	87 h, cond. E	Raman	$A_D/A_G = 0.1025$; 151, 163, 190, 214, 240, 285, 301, 309, 335, 345, 684, 705, 772, 899, 1049, 1316, 1575, 1709, 1904, 2605 cm^{-1}	–
17		HRTEM	31 ends: 3 closed, 13 (42%) open thereof 9 (69%) functionalised and 1 (8%) filled tube	~
18		EDX	C, Si, O, S, Cl, Al, I, Fe, Na in decreasing quantity	+
19		XRF	S, Cl, Ar, Ca, Cr, Fe, Ni in ascending energies	–
20		XPS	I, O, N, C present; 1 x I per 361 carbon and 11 nitrogen; every 16th COOH functionalised	+
21		TGA	$T_{\text{combust.}} = 274.8, 335.7, 518, 608.0$ °C, 4.14% residue	+
22	DMF, 40 h,	ATR-FTIR	1266, 1315, 1357, 1381, 1416, 1468, 1600, 1693, 2603, 2856, 2930, 2960 cm^{-1}	+
23	cond. F	Raman	$A_D/A_G = 0.1711$; 151, 163, 190, 215, 243, 270-284, 301-310, 335, 689, 770-781, 888-906, 1049-1062, 1320, 1576, 1720, 1906, 2602 cm^{-1}	–
24		HRTEM	21 ends: 17 (81%) open thereof 13 (76%) functionalised and 5 (29%) filled tubes	+
25		EDX	C, O, Si, Cl, Al, I, Fe, S in decreasing quantity	+
26		XPS	I, O, N, C present; 1 x I per 408 carbon and 13 nitrogen; every 16th COOH functionalised	+
27		TGA	$T_{\text{combust.}} = 338.4, 497.0, 593.9$ °C, 0.50% residue	+
28	DMF, 86 h,	ATR-FTIR	1128, 1239, 1266, 1379, 1468, 1595, 1688, 2567, 2855, 2928, 2958 cm^{-1}	~
29	cond. G	Raman	$A_D/A_G = 0.1483$; 149, 163, 191, 214, 241, 309, 335, 684, 771, 901, 1050, 1318, 1574, 1715, 1906, 2438, 2603 cm^{-1}	–
30		HRTEM	26 ends: 18 (69%) open thereof 16 (89%) functionalised tubes and 6 (33%) peapods	+
31		EDX	C, O, Cl, I, S, Si, Fe, Na in decreasing quantity	+
32		XPS	I, O, N, C present; 1 x I per 685 carbon and 20 nitrogen; every 10th COOH functionalised	+
33		TGA ^a	$T_{\text{combust.}} = 330(\text{sh}), 383.7, 687.5$ °C, 18.2% weight loss at 470 °C	+
34	DMF, ^b 40 h,	ATR-FTIR	871, 986, 1124, 1206, 1265, 1310, 1343, 1376, 1467, 1597, 1691, 1730(sh), 2363, 2855, 2927, 2954 cm^{-1}	+
35	cond. H	Raman	$A_D/A_G = 0.1528$; 150, 163, 191, 214, 241, 305, 336, 676, 772, 898, 1050, 1317, 1575, 1712, 1903, 2430, 2608 cm^{-1}	–
36		HRTEM	31 ends: 7 closed, 16 (52%) open thereof 11 (69%) functionalised tubes and 2 (13%) peapods	+
37		EDX	C, O, Si, Al, Cl, S, Fe, I in decreasing quantity	~
38		XPS	I, O, N, C present; 1 x I per 1320 carbon and 24 nitrogen; every 21st COOH functionalised	+
39		TGA	$T_{\text{combust.}} = 352.8, 572(\text{sh}), 628.4$ °C, 0.11% residue	+
40	neat, cond. I	Raman	$A_D/A_G = 0.3013$; 150, 162, 192, 217, 250, 337, 685, 709, 774, 896, 1062, 1319, 1582, 1720, 1908, 2612 cm^{-1}	–

SOLVENT	ANALYSIS	RESULT	Y/N
41	HRTEM	16 ends: 12 (75%) open thereof 5 (42%) functionalised and 3 (25%) filled tubes	–
42	EDX	C, O, Si, S, Cl, I, Fe in decreasing quantity	+

^a TGA was recorded in pure nitrogen gas; ^b activation of the carboxylic acid *via* EDC.

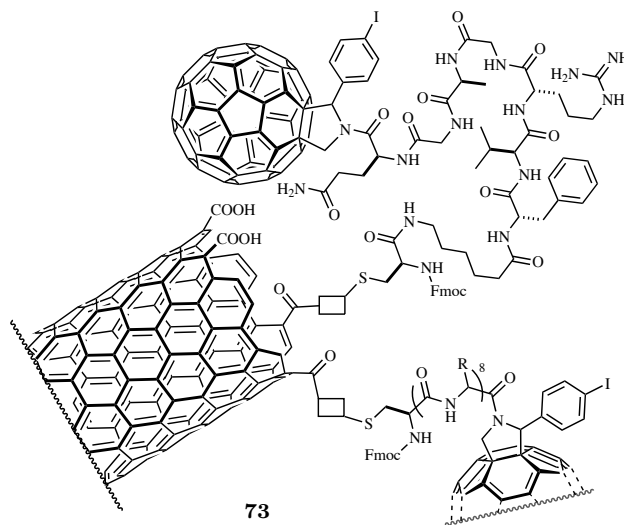
Table 8.5: Analytical results of the various coupling methods using CNT and XXFVIGAGQ functionalised C₆₀.



	ACTIV.	ANALYSIS	RESULT	Y/N
1	SOCl ₂ , cond. J	IR ^{a)}	1058-1200, 1385-1458, 1560, 1632, 1735, 2852, 2922, 3458 cm ⁻¹	+
		ATR-FTIR	1125, 1223, 1265, 1379, 1585, 1737, 2594, 2920 cm ⁻¹	–
2		Raman	A _D /A _G = 0.1141 (–11%); 151, 163, 215, 240, 286, 307, 334, 345, 670, 880, 1038, 1313-1320, 1574-1582, 1711, 1718, 1908, 2425, 2610 cm ⁻¹	–
3		HRTEM	15 ends: 8 (53%) open thereof 3 (20%) functionalised tubes	~
4		EDX	C, O, S, Cl, Na, Fe, I in decreasing quantity	~
5		XRF	S, Cl, Ar, Ca, I, Cr, Fe, Ni in ascending energies	+
6		XPS	I, O, N, C, S present; 1 x I per 504 carbon and 11 nitrogen; every 33rd COOH functionalised	+
7		TGA	T _{combust.} = 262.0, 367.9, 488, 600.1 °C, 11.6% residue	+
8	EDC, cond. K	ATR-FTIR	1123, 1267, 1354, 1505, 1607, 1694 (sh), 2590 cm ⁻¹ , weak spectrum	~
9		Raman	A _D /A _G = 0.1210 (+1%); 151, 162, 189, 214, 244, 284, 301, 309, 334, 345, 678, 773, 896, 1051, 1318, 1574, 1715, 1901, 2605 cm ⁻¹	~
10		HRTEM	35 ends: 12 closed, 13 (37%) open thereof 11 (85%) functionalised and 5 (38%) filled tubes	+
11		EDX	C, O, I, Fe, Ca, Si, S, Cl in decreasing quantity	~
12		XRF	Cl, Ar, Ca, I, Ba, Cr, Fe, Ni in ascending energies	+

ACTIV.	ANALYSIS	RESULT	Y/N	
13	XPS	I, O, N, C present; 1 x I per 613 carbon and 11 nitrogen; every 16th COOH functionalised	+	
14	TGA	T _{combust.} = 329.9, 596.9, 629(sh) °C, 1.64% residue	+	
15	EDC, NHS,	ATR-FTIR	1123, 1267, 1354, 1423, 1514, 1607, 1697, 2592 cm ⁻¹	+
16	cond. L	Raman	A _D /A _G = 0.1375 (+11%); 150, 163, 191, 214, 241, 306, 334, 676, 892, 1049, 1319, 1575, 1718, 1907, 2608 cm ⁻¹	-
17		HRTEM	19 ends: 7 closed, 9 (47%) open thereof 3 (33%) functionalised and 3 (33%) filled tubes	~
18		EDX	C, O, Si, Al, I, Fe, S, Cl, Na in decreasing quantity	~
19		XRF	Ar, Ca, I, Cr, Fe, Ni in ascending energies	+
20		XPS	I, O, N, C present; 1 x I per 691 carbon and 15 nitrogen; every 13th COOH functionalised	+
21		TGA	T _{combust.} = 333.5, 603.3 °C, 3.41% residue	~
22	HATU, cond. M	ATR-FTIR	1123, 1265, 1383, 1412, 1468, 1603, 1692, 1765, 2330, 2664, 2980 cm ⁻¹	+
23		Raman	A _D /A _G = 0.1347 (+9%); 150, 163, 191, 215, 240, 289, 301, 311, 333, 345, 685, 772, 904, 1056, 1318, 1576, 1714, 1906, 2605 cm ⁻¹	-
24		HRTEM	41 ends: 11 closed, 19 (46%) open thereof 17 (89%) functionalised and 3 (16%) filled tubes	+
25		EDX	C, O, I, Si, Br/Al, Fe, S, Cl in decreasing quantity	+
26		XRF	Cl, Ar, Ca, I, Ba, Cr, Fe, Ni in ascending energies	+
27		XPS	I, O, N, C present; 1 x I per 929 carbon and 12 nitrogen; every 22nd COOH functionalised	+
28		TGA	T _{combust.} = 345.1, 551(sh), 610.7 °C, 1.59% residue	+

a) IR spectrum measured through a KBr disk.

Table 8.6: Analytical results of the various coupling methods using CNT and CXFVRGAGQ functionalised C₆₀.

	ACTIV.	ANALYSIS	RESULT	Y/N
1	SOCl ₂	ATR-FTIR	779(b), 868(b), 1119, 1173(b), 1223, 1263, 1377, 1460, 1580, 1685(sh), 1734, 2444, 2523, 2853, 2891, 2961(b), 3156(b), 3640 cm ⁻¹	–
2		Raman	A _D /A _G = 0.1150; 151, 163, 190, 214, 333, 344, 670, 880, 1030, 1318, 1572, 1694, 1899, 2601 cm ⁻¹	–
3		HRTEM	17 ends: 13 (76%) open thereof 7 (54%) functionalised tubes, much carbon debris	~
4		EDX	C, O, S, Cl, Na, Fe, I in decreasing quantity	~
5		XRF	S, Cl, Ar, Ca, I, Ba, Cr, Fe, Ni in ascending energies	+
6		XPS	I, O, N, C, S present; 1 x I per 694 carbon, 10 nitrogen and 4 sulfur; every 46rd COOH functionalised	+
7		TGA	T _{combust.} = 279.2, 366.6, 515.1, 600.9 °C, 12.12% residue	+
8		Fmoc	no peak at 301 nm, high baseline	–
9	maleimide in DMF/H ₂ O	ATR-FTIR	1120, 1267, 1356, 1385, 1424, 1478, 1607, 1690, 1726 cm ⁻¹	+
10		Raman	A _D /A _G = 0.1475; 151, 160, 190, 215, 243, 285, 300, 309, 335, 683, 774, 902, 1052, 1320, 1576, 1712, 1907, 2605 cm ⁻¹	–
11		HRTEM	16 ends: 14 (88%) open thereof 12 (86%) functionalised tubes and 3 (21%) peapods	+
12		EDX	C, Si, O, Cl, Br, Fe, S, I, in decreasing quantity	~
13		XRF	Cl, Ar, Ca, I, Cr, Fe, Ni in ascending energies	~
14	maleimide in DMF/H ₂ O + NaI	ATR-FTIR	1126, 1223, 1265, 1352, 1379, 1414, 1468, 1599, 1688, 1722, 2384 cm ⁻¹	+
15		Raman	A _D /A _G = 0.1265; 163, 191, 215, 242, 286, 305, 335, 679, 707, 897, 1048, 1310-1319, 1577, 1705-1715, 1906, 2608 cm ⁻¹	–

ACTIV.	ANALYSIS	RESULT	Y/N
16	HRTEM	19 ends: 2 closed, 9 (47%) open thereof 7 (78%) functionalised and 2 (22%) filled tubes	+
17	EDX	C, Si, O, Cl, I, S, Fe in decreasing quantity	+
18	XRF	Cl, Ar, Ca, I, Cr, Fe, Ni in ascending energies	~
19	CNT-S-Spy in DMF/H ₂ O	ATR-FTIR 1049, 1125, 1265, 1354, 1385, 1468, 1605, 2434, 2820 cm ⁻¹	-
20	Raman	A _D /A _G = 0.1115; 151, 162, 215, 242, 307, 335, 679, 773, 892, 1047, 1319, 1573, 1713, 1905, 2605 cm ⁻¹	-
21	HRTEM	9 ends: 4 (44%) open thereof 2 (50%) functionalised and 3 (75%) filled tubes	~
22	EDX	C, O, S, Cl, Al, I, Fe, in decreasing quantity	+
23	XRF	Si, S, Cl, Ar, Ca, I, Cr, Fe, Ni in ascending energies	+
24	CNT-S-Spy in DMF/H ₂ O + NaI	ATR-FTIR 1055, 1125, 1265, 1352, 1379, 1514, 1599, 1692, 1742, 2646, 2851 cm ⁻¹	+
25	Raman	A _D /A _G = 0.1064; 164, 191, 214, 240, 300, 336, 680, 704, 896, 1051, 1320, 1574, 1712, 1906, 2601 cm ⁻¹	-
26	HRTEM	12 ends: 7 (58%) open thereof 5 (71%) functionalised tubes and 1 (24%) peapod	+
27	EDX	C, O, S, Cl, I, Si, Fe, in decreasing quantity	~
28	XRF	S, Cl, Ar, Ca, I, Cr, Fe, Ni in ascending energies	+

List of Abbreviations

ADF	annular dark field
AFM	atomic force microscopy
Ahx	6-aminohexanoic acid
ATR-FTIR	attenuated total reflection Fourier transformed infrared
Boc	<i>tert</i> -butoxycarbonyl
Bn	benzyl
C₆₀	C ₆₀ fullerene
C₆₀O	C ₆₀ O monooxidised fullerene
Cbz	carboxybenzyl
CHCA	α -cyano-4-hydroxycinnamic acid
CHCl₃	chloroform
CNT	carbon nanotube
COSY	correlation spectroscopy
CR	charge recombination
CS	charge separation
CVD	chemical vapour deposition
DABCO	1,4-diazabicyclo[2.2.2]octane
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
oDCB	<i>ortho</i> -dichlorobenzene
DCTB	<i>trans</i> -2-[3-4- <i>tert</i> -butylphenyl]-2-methyl-2-propenylidene]malononitrile
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide

DCM	dichloromethane
DDS	drug delivery system
DNA	deoxyribonucleic acid
DIPEA	<i>N,N</i> -diisopropylethylamine
DMAP	4-(dimethylamino) pyridine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DTT	dithiolthreitol
DWNT	double-walled carbon nanotube
EDC	<i>N</i> -(3-dimethylaminopropyl)- <i>N</i> '-ethylcarbodiimide hydrochloride
EDTA	ethylenediaminetetraacetic acid
EDX	energy dispersive X-ray spectroscopy
EM	electron microscope
Et₂O	diethyl ether
EtOAc	ethyl acetat
EtOH	ethanol
FA	formic acid
Fmoc	<i>N</i> -(9-fluorenylmethoxycarbonyl)
GlcNAc	<i>N</i> -acetyl-D-glucosamine
HAADF	high-angle annular dark field
HATU	1-[bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]pyridinium-3-oxid hexafluorophosphate
HBTU	<i>O</i> -(benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HFIP	hexafluoroisopropanol
HMBA-AM	4-hydroxymethylbenzoic acid aminomethyl polystyrene
HMBC	heteronuclear multiple bond correlation

HMM	hexamethylmelamine
HOBt	<i>N</i> -hydroxybenzotriazole
HOMO	highest occupied molecular orbital
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectroscopy
HRTEM	high resolution transmission electron microscopy
HSQC	heteronuclear single quantum correlation
ICMAB	Institut de Ciència de Materials de Barcelona
ICP-MS	inductively coupled plasma mass spectrometry
IR	infrared
ISC	intersystem crossing
LC/MS	liquid chromatography-mass spectrometry
LUMO	lowest occupied molecular orbital
MA	maleic anhydride
MALDI	matrix-assisted laser desorption/ionisation
MeCN	acetonitrile
MeOH	methanol
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mtr	4-methoxy-2,3,6-trimethylbenzenesulfonyl
μwave	microwave
MWNT	multi-walled carbon nanotube
NBS	<i>N</i> -bromosuccinimide
NCS	<i>N</i> -chlorosuccinimide
NEt₃	triethylamine
NHS	<i>N</i> -hydroxysuccinimide

NMP	<i>N</i> -methylpyrrolidone
NMR	nuclear magnetic resonance
NOESY	nuclear Overhauser effect spectroscopy
¹O₂	singlet oxygen
pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PET	positron emission tomography
PhCN	benzonitrile
ⁱPrOH	2-isopropanol
RBM	radial breathing mode
ROS	reactive oxygen species
SPPS	solid phase peptide synthesis
STEM	scanning transmission electron microscopy
SWNT	single-walled carbon nanotube
TBAF	tetra- <i>N</i> -butylammonium fluoride
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
TGA	thermogravimetric analysis
THF	tetrahydro furan
Tis	triisopropylsilane
TLC	thin layer chromatography
trt	triphenylmethyl
UV	ultra violet
UV/vis spectroscopy	ultraviolet/visible light spectroscopy
XPS	X-ray photoelectron spectroscopy
XRF	X-ray fluorescence spectroscopy

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Statement of Originality

By this letter I, **Reida Noëmi Rutte**, declare that I have written this DPhil thesis entitled **Selective Release from Carbon Nanocapsules**, supervised by **Prof. Dr. Benjamin G. Davis**, completely by myself and used no other sources or resources than the ones mentioned. Citations, as well as external sources and resources, have been stated in accordance with the rules and regulations of scientific practice. I am aware that I must not declare external texts or excerpts of texts to be written by myself. Furthermore, I am aware that, in the case of plagiarism, the examiners have the right to degrade the DPhil thesis or ask for required corrections, which have to be implemented for resubmission.

Moreover, I confirm that I have not handed in a DPhil thesis with the same or similar contents found elsewhere.

Mörtenbach, 26th October 2016



Publications

Reida N. Rutte, Thomas B. Parsons, and Benjamin G. Davis, *Modification of fulleropyrazolines modulates their cleavage by light. Chem. Comm.* **2014**, 50 (82), 12297-12299.

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Christopher J. Serpell, Reida N. Rutte, Kalotina Geraki, Elzbieta Pach, Markus Martincic, Magdalena Kierkowicz, Sonia De Munari, Kim Wals, Ritu Raj, Belén Ballesteros, Gerard Tobias, Daniel C. Anthony, and Benjamin G. Davis, *Carbon nanotubes allow capture of krypton, barium, and lead for multichannel biological X-ray fluorescence imaging. Nat. Commun.* **2016**, 7, 13118.

Modification of fulleropyrazolines modulates their cleavage by light†

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The extraordinary electrochemistry and the tunability of their energy levels allows the use of fulleropyrazolines in photovoltaics and charge-transfer systems. Here we show that substitution in position 1 tunes photolytic stability; electron-donating groups facilitate 1,3-dipolar cycloreversion to fullerene. This discovery has implications not only for photovoltaic stability but also highlights a potential strategy for photo-controlled fullerene release systems ('photo-caged'/'photo-activated' fullerene).

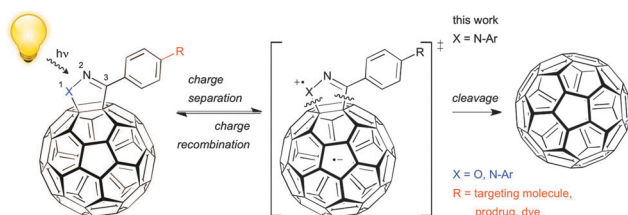
The unique electronic properties of fulleropyrazolines have enabled their use in photovoltaics.¹ Their photoactivity arises from the ability of fulleropyrazolines to undergo photonic excitement, charge separation and stabilisation, and then subsequent recombination (Scheme 1).² The effect of altering the C-3 pyrazoline substituent has been investigated both experimentally^{1a,2c} and computationally.^{2d} Modification of fulleropyrazolines at C-3 is essential for the energy transfer required in photovoltaic applications.³ Substituents on the *N*-phenyl group (*N*-1) increase electronic interaction with the fullerene moiety and have been found to be essential for the charge transfer process.⁴ However, to the best of our knowledge, the stability of fulleropyrazolines towards light has not been previously investigated.

The fundamental properties of photovoltaic substrates are the excitation energy and the lifetime of charge separated species.

The lifetime of these substrates, especially fulleropyrazolines, may be tuned by incorporation of groups that can participate in charge transfer and stabilise or destabilise the radical cation formed upon excitation and charge separation.^{2a,5} Intermediates of this type have the potential to disproportionate, as reported recently during the photocleavage of *para*-dimethylaminophenyl fulleroisoxazoline.⁶ Yet, it is notable that despite the wide range of cycloaddition-type reactions which have been utilised in functionalization of fullerenes, the only reports of photoinduced cycloreversion (de-functionalization) are limited to the [3+2]-isoxazoline systems and the inefficient [2+2] reaction.⁷ Limitations of these reactions are the uncontrollable equilibrium state of the photocycloaddition/-reversion as well as their lack of functionality.

Unlike isoxazolines, pyrazolines possess a second nitrogen atom (*N*-1) enabling further functionalization. This intriguing substrate class inspired us to combine dual functionalization and photocleavage in modified fulleropyrazolines (Scheme 1) for photoinduced cycloreversion as a technologically-useful delivery system for drugs,⁸ dyes,⁹ or targeting moieties.¹⁰ A representative range of 1,3-disubstituted fulleropyrazolines was prepared from the appropriate aryl hydrazones, which in turn were readily accessed by condensation of the corresponding 4-substituted-benzaldehydes & phenylhydrazines (Table 1).^{5b} Treatment of aryl hydrazones 1–4 with *N*-bromosuccinimide and triethylamine generated the nitrile imine *in situ*^{4a,11} which in the presence of C₆₀ fullerene gave the [3+2] fulleropyrazoline cycloadducts 6–9 in 15–51% yield. In all cases the pyrazoline was formed at the dipolarophilic 6,6-bond (ESI†).¹² Hydrazone 5 and fulleropyrazoline 10 were found to be very unstable in solution and could not be synthesised by this approach; instead basic milling¹³ was used to generate 5 which was then halogenated with (diacetoxyiodo)benzene^{4d} to give 10. The labile amino derivative 11 was accessed by reduction of 7 using tin.^{4c} To introduce a putative carboxylate handle for further modification the benzoic acid derivative 13 was also accessed by photolytic generation of the 1,3-dipole from tetrazole 12.¹⁴

We found that the modified fullerenes were soluble in a number of solvents. 1,3-Dipolar cycloreversion of fulleropyrazolines would, in principle, release pristine fullerene. Initial investigations into the



Scheme 1 Fulleroheterocycles can charge separate upon photoirradiation. Substituents have potential to tune reactivity & stability and to deliver cargo.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c4cc03859b

Table 1 Synthesis of hydrazones and fulleropyrazolines

Precursor	Fulleropyrazoline
 13% 1	 15% 6
 83% 2	 40% 7
 92% 3	 51% 8
 23% 4	 24% 9
 35% ^(b) 5	 1% ^(d) 10
 7	 6% 11
 12	 39% 13
 14	 25% 15

Reagents & conditions: (a) aldehyde, hydrazine, AcOH, EtOH, 78 °C;^{5b} (b) aldehyde, hydrazine, NaOH, silica, 40 min;¹³ (c) hydrazone, NBS, CHCl₃, r.t., 30 min; then C₆₀, NEt₃, toluene, microwave;^{5a,12} (d) hydrazone, C₆₀, (diacetoxyiodo)benzene, toluene, 40 °C, 3.5 h;^{4d} (e) Sn-HCl(aq), CHCl₃, reflux, 6 h, 6%;^{4c} (f) tetrazole, C₆₀, toluene, Hg-lamp.¹⁴

photocleavage reaction were conducted in degassed toluene with an excess of maleic anhydride. Based on previous reversible cleavage reactions^{6,15} we initially included this trapping alkene in the reaction mixture to prevent re-addition of the released 1,3-dipole with free C₆₀. Photocleavage reactions were monitored by t.l.c., HPLC, NMR, and mass spectrometry. Where possible, the

photocleavage products were isolated and fully characterised (see ESI†). Various light sources of different λ_{max} were investigated. Medium-pressure mercury, handheld U.V. ($\lambda = 365 \text{ nm}$),[‡] Rayonet ($\lambda = 350 \text{ nm}$), filtered medium-pressure mercury ($\lambda = 313 \text{ nm}$),¹⁶ and commercial halogen lamps all gave cycloreversion products with comparable efficiency (60–75% conversion after 1.5 hours). For ease of practicality all subsequent experiments used the Hg lamp.

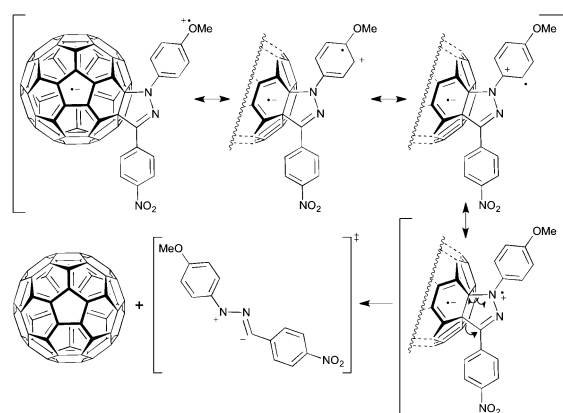
Fulleropyrazolines which possessed a *para*-methoxyphenyl substituents on *N*-1 (**7**, **10**, **11**, and **13**) underwent photo-induced cycloreversion to produce C₆₀ and C₆₀O whereas those carrying a *para*-nitro group (**6** and **9**) were unreactive under the same conditions (Table 2).§ We speculated that the strong positive mesomeric effect of the methoxy group¹⁷ stabilises the development of the ensuing radical cation during the transition state of the 1,3-dipolar cycloreversion (Scheme 2). This was confirmed by fulleropyrazoline **8** that bears a less +M *para*-chloro substituent, which was cleaved, but far more slowly than the methoxy analogue. The importance of the substituent at *N*-1 confirmed a key role of this atomic centre, perhaps through the lone pair of the sp³ nitrogen, in charge transfer processes.^{4d}

Notably, photocleavage of fulleropyrazolines gave not just pristine C₆₀ **16** but a mixture of monooxidised C₆₀O **17** with

Table 2 Photocleavage of fulleropyrazolines

R ¹	R ²	Fulleropyrazoline	Result
NO ₂	NO ₂	6	No reaction
NO ₂	OMe	7	Cleavage
NO ₂	Cl	8	Cleavage
OMe	NO ₂	9	No reaction
OMe	OMe	10	Cleavage
NH ₂	OMe	11	Cleavage
COOH	OMe	13	Cleavage
CONH ₂ -Gly-OBu ^f	OMe	15	Cleavage

Reagents & conditions: toluene or chloroform [1 mM], 20 eq. maleic anhydride, medium pressure mercury lamp, 4 min–12 h.



Scheme 2 Possible mechanism of fulleropyrazoline photocleavage.

traces of $C_{60}O_n$ in varying ratios. The proportions of C_{60} and $C_{60}O$ obtained did not show any simple correlation with reaction conditions (solvent, trapping alkene, degassed or anhydrous solvent, irradiation wavelength).[†] Irradiation of pristine C_{60} gave oxidation to $C_{60}O$ as previously reported.¹⁸ Oxidation of C_{60} occurred only at a 6,6-bond, an observation which is in accordance with previous reports.¹⁹ Surprisingly, the use of non-degassed solvent shortened the reaction time to around one quarter, but only marginally increased the ratio of $C_{60}O/C_{60}$. These results together suggested that $C_{60}O$ resulted from the *in situ* generation of singlet oxygen and subsequent reaction with the photo-released C_{60} and not as a direct result of photo-cleavage.

To test the necessity and role of dipolarophile traps, various other alkenes were investigated as additives in the photocycloreversion of fulleropyrazoline 7 (Table S2, ESI[†]). The rate of the photocleavage could be improved in the presence of 3,3-dimethylallylbromide or *para*-chlorostyrene. Pleasingly the cycloreversion also proceeded efficiently without alkene (Table S2, entry 8, ESI[†]), thus confirming the utility of this process even in the absence of a trapping partner.

The reaction rate for photocleavage showed clear solvent effects; it was enhanced in more polar solvents such as chloroform and benzonitrile and further enhanced in mixed aqueous-organic solvent systems. This supports suggested formation of a charge-separated diradical species (Scheme 2). Notably in the presence of base (pyridine as solvent, DABCO as additive) the photocleavage does not occur, presumably because the putative pyrazoline radical cation is quenched under these conditions.²⁰ In this mechanism, under photoirradiation fulleropyrazolines form a charge separated diradical species.^{2c,3,5b,6,21} The positive charge that is formed can be stabilised particularly effectively by the *N*-1 methoxy group, which promotes bond cleavage to release free C_{60} . The breakdown products are discussed further in the ESI.[†]

Finally to test utility in future applications, the reaction was tested in various mixed aqueous solvent systems (see ESI[†]); pleasingly, it proceeded efficiently in all and with rates enhanced by the presence of water. This could vitally enable the use of fulleropyrazolines under conditions suitable for biologically-relevant photo-controlled substrates when *e.g.* used in putative drug-delivery systems or for '1,3-dipole delivery' to olefin-containing biomolecules. The proof-of-principle for such a system was demonstrated by the construction of the amino-acid-carrying fulleropyrazoline 15 and subsequent successful 'photo-release' under aqueous conditions (see ESI[†]).

In conclusion we have shown that the reactivity of fulleropyrazolines may be tuned to enable highly efficient photocleavage. The reaction proceeds in various solvent systems and without the need for additional trapping agents to remove 1,3-dipolar species generated *in situ*. An electron donating substituent on *N*-1 of the pyrazoline is sufficient to enable the reaction to proceed; various substituents are tolerated at C-3. This synthetic flexibility and the aqueous compatibility could enable the use of C_{60} fullerenes as carriers for prodrugs or other photocontrolled elements.

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Notes and references

‡ Handheld lamp at $\lambda = 254$ nm gave 22% conversion after 3.5 hours.
§ This cleavage process was induced by exposure to light from a medium pressure mercury lamp and did not occur when 7, 10, 11, and 13 were subjected to heat. Cycloreversion of fulleropyrazolines was previously reported to occur only in the presence of $Cu(OTf)_2$.¹⁵

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Simultaneous atomic-resolution electron ptychography and Z-contrast imaging of light and heavy elements in complex nanostructures

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The aberration-corrected scanning transmission electron microscope (STEM) has emerged as a key tool for atomic resolution characterization of materials, allowing the use of imaging modes such as Z-contrast and spectroscopic mapping. The STEM has not been regarded as optimal for the phase-contrast imaging necessary for efficient imaging of light materials. Here, recent developments in fast electron detectors and data processing capability is shown to enable electron ptychography, to extend the capability of the STEM by allowing quantitative phase images to be formed simultaneously with incoherent signals. We demonstrate this capability as a practical tool for imaging complex structures containing light and heavy elements, and use it to solve the structure of a beam-sensitive carbon nanostructure. The contrast of the phase image contrast is maximized through the post-acquisition correction of lens aberrations. The compensation of defocus aberrations is also used for the measurement of three-dimensional sample information through post-acquisition optical sectioning.

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Forming an atomic-resolution image in the electron microscope that provides dose-efficient, high-contrast imaging of light elements, while also providing strong compositional contrast, has been a long-standing challenge. For light materials, such as graphene and biological structures, high-energy electrons are predominantly only scattered once in the sample. Such single scattering conditions can be described as a weak-phase object (WPO)¹, where only a small phase shift is introduced to the electron wave. Phase-contrast imaging using electrons has been shown to provide the highest dose efficiency for imaging WPOs, where radiation damage is a limiting factor², compared with inelastic electron scattering, as well as X-ray and neutron scattering. Electric and magnetic fields also lead to a phase shift of the electron beam. Phase-contrast imaging therefore plays an important role in the imaging of biological structures, light materials such as graphene and the detection of electric and magnetic fields. It is generally performed in the conventional transmission electron microscope (CTEM) using either deliberately injected lens aberrations or a phase plate³, to form image contrast of a WPO, or using off-axis holography^{4,5}, which requires a separate reference beam deflected using an electron bi-prism.

Providing a strong compositional sensitivity to element type, however, requires the detection of relatively high-angle scattering and this is performed using annular dark-field (ADF) imaging in the scanning TEM (STEM), as shown in Fig. 1, which forms an image with atomic-number contrast (Z-contrast). A beam of electrons is focused to form a probe of atomic dimensions that is raster scanned across the sample and the ADF detector collects the intensity of the high-angle scattering that is then plotted as a function of probe position to form an image.

The Z-contrast technique has developed into a key approach for atomic-resolution imaging, in particular as the development of hardware correctors for the inherent spherical aberration of the objective lens^{6,7}. It is an incoherent imaging technique, which means that the optimal conditions for imaging are the smallest, most intense illuminating probe possible⁸. This is achieved by tuning the aberration corrector for the smallest possible aberrations. Phase-contrast imaging, however, is

a coherent imaging mode and requires aberrations, such as a combination of spherical aberration and defocus, to form an effective phase plate. Such conditions are referred to as ‘Scherzer conditions’⁹. Alternative approaches that also make use of aberrations include negative spherical aberration conditions¹⁰ or the use of a focal series¹¹ in TEM. The optimal conditions for incoherent Z-contrast imaging are therefore different from those for coherent phase contrast, thus preventing their simultaneous optimal implementation. Furthermore, the principle of reciprocity¹² shows that the formation of a conventional phase-contrast image in STEM requires the use of a small axial bright-field (BF) detector, which collects only a small fraction of the transmitted electrons and is therefore dose inefficient.

Here we make use of electron ptychography to enable an efficient, quantitative phase imaging in STEM under optical conditions that are optimized for incoherent imaging so that the phase information is recorded simultaneously with the Z-contrast image, allowing a full analysis. Although ptychography has become well established for imaging with light¹³ and X-rays^{14–16}, its use with electrons has been more limited. The principle of ptychography is that a camera is used to record the intensity in the STEM detector plane as a function of probe position, resulting in a four-dimensional (4D) data set (two STEM probe coordinates and two coordinates within the detector plane), which can be used to reconstruct a phase image. Initial experimental demonstrations of electron ptychography were limited by the camera speed, microscope stability and the availability of sufficient computational processing power, and only very small images (typically 32×32 pixels) could be reconstructed¹⁷. More recent demonstrations have used highly defocused probes that illuminate a wider region of the sample to provide a larger field of view, while still limiting the number of probe positions and camera frames^{18–20}. Such defocused probes are incompatible with the conditions required for incoherent imaging and so cannot be used simultaneously with Z-contrast imaging. The subsequent formation of a Z-contrast image would require refocusing of the electron probe, which requires further electron dose on the sample and loses the ability to exactly register the phase and Z-contrast information.

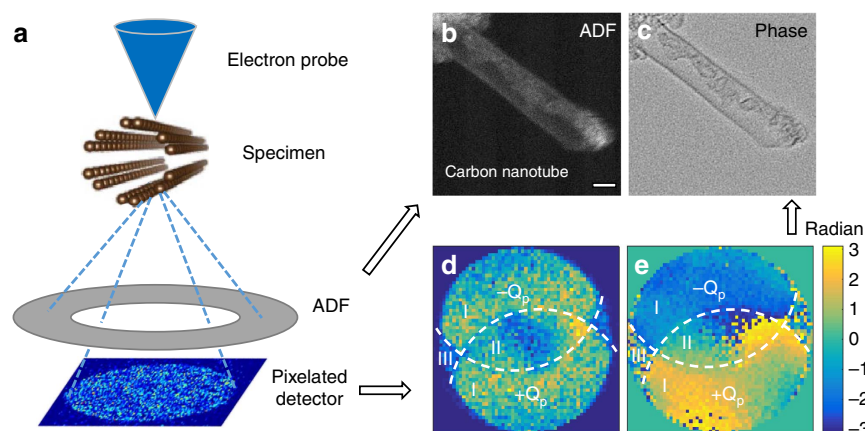


Figure 1 | Simultaneous atomic resolution incoherent and coherent imaging. (a) An ADF detector collects the dark-field signal to form (b) an incoherent Z-contrast image. (c) The simultaneous phase image is reconstructed using ptychography. Simultaneously, a fast pixelated detector records the coherent BF diffraction pattern at every probe position forming a 4D data set. Taking the Fourier transform of the 4D data set with respect to probe position results in a complex 4D matrix $G(\mathbf{K}_f, \mathbf{Q}_p)$, which carries the phase information of the interference between diffracted and undiffracted beams. (d,e) An example of the modulus and phase of the complex matrix $G(\mathbf{K}_f, \mathbf{Q}_p)$ at a single spatial frequency $\mathbf{Q}_p$ where two diffracted beams $+\mathbf{Q}_p$ and $-\mathbf{Q}_p$ (indicated by dashed lines) overlap with the undiffracted direct beam. (d) The areas labelled as area I are double-overlap regions where one diffracted beam interferes with the direct beam and area II is the triple-overlap region where both diffracted beams and the direct beam interfere. Area III has no interfering beams and is only noise and is therefore not used in the ptychography reconstruction. By analysing the phase information for all spatial frequencies in the image, (c) the phase image can be reconstructed. Scale bar, 1 nm.

A first proof-of-principle attempt to form simultaneous ADF and phase-contrast images²¹ was also limited in field of view by the camera speed. Differential phase contrast (DPC) images have been formed at atomic resolution in the STEM and used to detect ferroelectric polarization²², and the importance of adding phase-sensitive imaging as a STEM technique discussed²³, but the differential approach did not offer quantitative image phase measurement and does not offer the possibility of post-acquisition aberration correction demonstrated here. Ptychography has been shown to enable phase extraction using signals from segmented detectors including tri-sectors²⁴ and recently at atomic resolution using segments synthesized from pixelated data²⁵. Large detector pixels may lead to a non-isotropic transfer of phase information with reduced phase-contrast transfer efficiencies²⁶.

Here we show that new capabilities that have been enabled by recent developments in high-speed detector technology (described further in the Methods section) that provide frame speeds of 1 kHz or greater allowing image acquisition at scanning speeds much closer to those typically used in STEM. We show that the combination of Z-contrast and phase imaging reveals the structure of a complex nanomaterial that could not be determined using previous methods. Ptychography also allows for further post-acquisition detection and correction of residual lens aberrations, which optimizes the contrast of the phase image. We also show that ptychographic reconstruction can be used to access three-dimensional (3D) information through its intrinsic optical-sectioning effect.

Results

Data acquisition and processing. Figure 1a shows a schematic of the optical configuration for the experiment presented here, with single frames of the experimental diffraction patterns shown in Supplementary Fig. 1. Further experimental details are described in the Methods section. As a highly convergent beam is used in STEM, the diffracted beams observed in the far field broaden into discs. The phase information is found where these discs overlap. Using a fast pixelated STEM detector, diffraction patterns consisting of primarily the BF disc (direct beam) were recorded at every probe position during a raster scan, thereby forming a 4D

data set. The dark-field signal is simultaneously collected using an ADF detector, to form an incoherent Z-contrast image, as shown in Fig. 1b. In the detector plane, the recorded electron micro-diffraction patterns as a function of scattering vector $\mathbf{K}_f$ and probe position $\mathbf{R}_0$, can be described as the Fourier transform of product of the probe $P(\mathbf{R} - \mathbf{R}_0)$ and the specimen transmission function $\psi(\mathbf{R})$,

$$M(\mathbf{K}_f, \mathbf{R}_0) = \int P(\mathbf{R} - \mathbf{R}_0) \psi(\mathbf{R}) \exp(i2\pi \mathbf{K}_f \cdot \mathbf{R}) d\mathbf{R} \quad (1)$$

The intensity of the diffraction pattern $|M(\mathbf{K}_f, \mathbf{R}_0)|^2$ is recorded but the phase is lost. Various ptychography approaches in electron and X-ray microscopy have been developed. Here we used the Wigner distribution deconvolution (WDD) approach described by Rodenburg and Bates²⁷, and demonstrated experimentally using X-rays²⁸. WDD is non-iterative and therefore fast, especially under the conditions presented here where 256×256 image pixels results in 65,536 individual diffraction patterns to be processed. Iterative approaches^{14,18,29–31} and direct inversion methods¹⁹ have also been developed, but as yet they have not been demonstrated on the type of atomic-scale focused probe data used here, to enable the simultaneous incoherent imaging. The feasibility of using iterative methods on this type of data will be explored in further work.

WDD is performed by taking the forward Fourier transform of $|M(\mathbf{K}_f, \mathbf{R}_0)|^2$ in equation (1) with respect to the probe positions $\mathbf{R}_0$, leading to a complex 4D matrix denoted as $G(\mathbf{K}_f, \mathbf{Q}_p)$,²⁷

$$G(\mathbf{K}_f, \mathbf{Q}_p) = A(\mathbf{K}_f) A^*(\mathbf{K}_f + \mathbf{Q}_p) \otimes_{\mathbf{K}_f} \Psi(\mathbf{K}_f) \Psi^*(\mathbf{K}_f - \mathbf{Q}_p) \quad (2)$$

where $\otimes_{\mathbf{K}_f}$ denotes a convolution with respect to the scattering vector $\mathbf{K}_f$ and the two terms $A(\mathbf{K}_f) A^*(\mathbf{K}_f + \mathbf{Q}_p)$ and $\Psi(\mathbf{K}_f) \Psi^*(\mathbf{K}_f - \mathbf{Q}_p)$ are functions of the probe forming aperture, $A(\mathbf{K}_f)$, and the specimen transmission function, $\Psi(\mathbf{K}_f)$, respectively. They have been shown to follow the mathematical definition of the Fourier transform of a Wigner distribution function²⁷. $G(\mathbf{K}_f, \mathbf{Q}_p)$ contains information about the beam interferences at disc overlaps²⁶ (see Supplementary Methods), with an example shown in Fig. 1d,e. Such

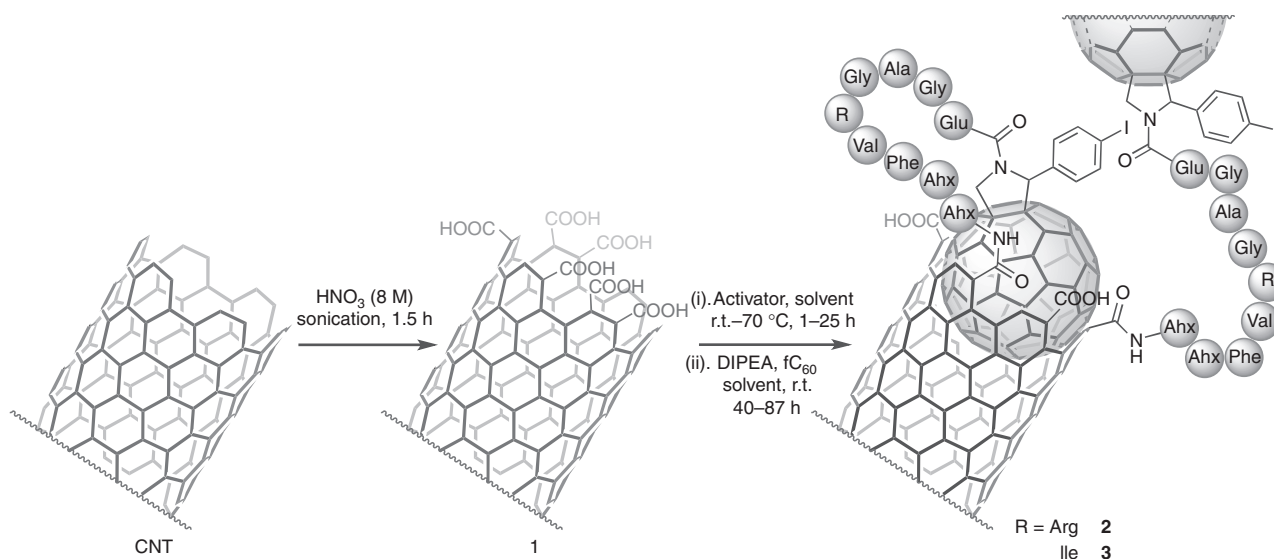


Figure 2 | Scheme of the intended hybrid synthesis. CNTs were oxidized to introduce carboxylic acid groups for functionalization. CNT-COOH **1** then underwent carboxyl preactivation and subsequent intended reaction with amine-bearing modified C_{60} peptido-fullerene **fC₆₀**, to give the target tethered hybrids **2** and **3**. Ahx denotes aminohexanoyl residue. For all other amino acid residues, standard 3-letter amino acid codes are used.

interference at disc overlaps contain both phase information of the specimen under illumination and the phase information due to residual lens aberrations, which will be discussed later (see Supplementary Fig. 2 and Supplementary Table 1). In the absence of lens aberrations, the two double-overlap regions (labelled as area I in Fig. 1d,e) should be uniform in phase, although small residual aberrations cause small variations in phase in these regions. Equation (2) shows that $G(\mathbf{K}_f, \mathbf{Q}_p)$ can be expressed as the result of a convolution between a function representing disc overlaps and a function derived from the specimen. The disc overlap function can be deconvolved from equation (2) and the object transmission function (Fig. 1c) can be reconstructed from $\Psi(\mathbf{K}_f)\Psi^*(\mathbf{K}_f - \mathbf{Q}_p)$ by setting $\mathbf{K}_f$ to zero. The WDD approach does not rely on the WPO approximation, but does require the transmission function to be a multiplicative object acting on the incident wave. As such, it is not strictly applicable to dynamical electron scattering, but none of the examples presented here would be regarded as leading to dynamical scattering conditions. The deconvolution step in the WDD enables residual lens aberrations, which affect the phase of the aperture function, $A(\mathbf{K}_f)$, to be corrected from the phase image post-processing. The aberration correction enabled by

WDD further allows 3D optical sectioning to be performed using one 4D data set at a fixed defocus without the need to run a focal-series experiment.

Simultaneous Z-contrast and phase of a complex nanostructure.

The combined ptychography and Z-contrast approach was tested on complex carbon nanotube (CNT) conjugates, which have exciting potential biological or medicinal applications, yet remain highly challenging to characterize. As a representative analytical substrate, candidate drug-delivery materials **2** and **3** (see Supplementary Methods) were chosen. As shown in Fig. 2, the intended design of this system aimed to create a peptidic covalently attached ‘tether’ between a single-walled CNT and a limited number of carbon fullerene (C_{60}) molecules. In this work, conventional analytical data (including infrared, Raman, X-ray photoemission spectroscopy and thermogravimetric analysis (TGA)) and the CTEM results suggested potentially consistent results with the intended synthesis (see Supplementary Figs 3–11); however, the absolute evidence for attachment and purity remained ambiguous.

Vitality, this ambiguity was resolved by the simultaneous Z-contrast and phase imaging demonstrated here. As shown in

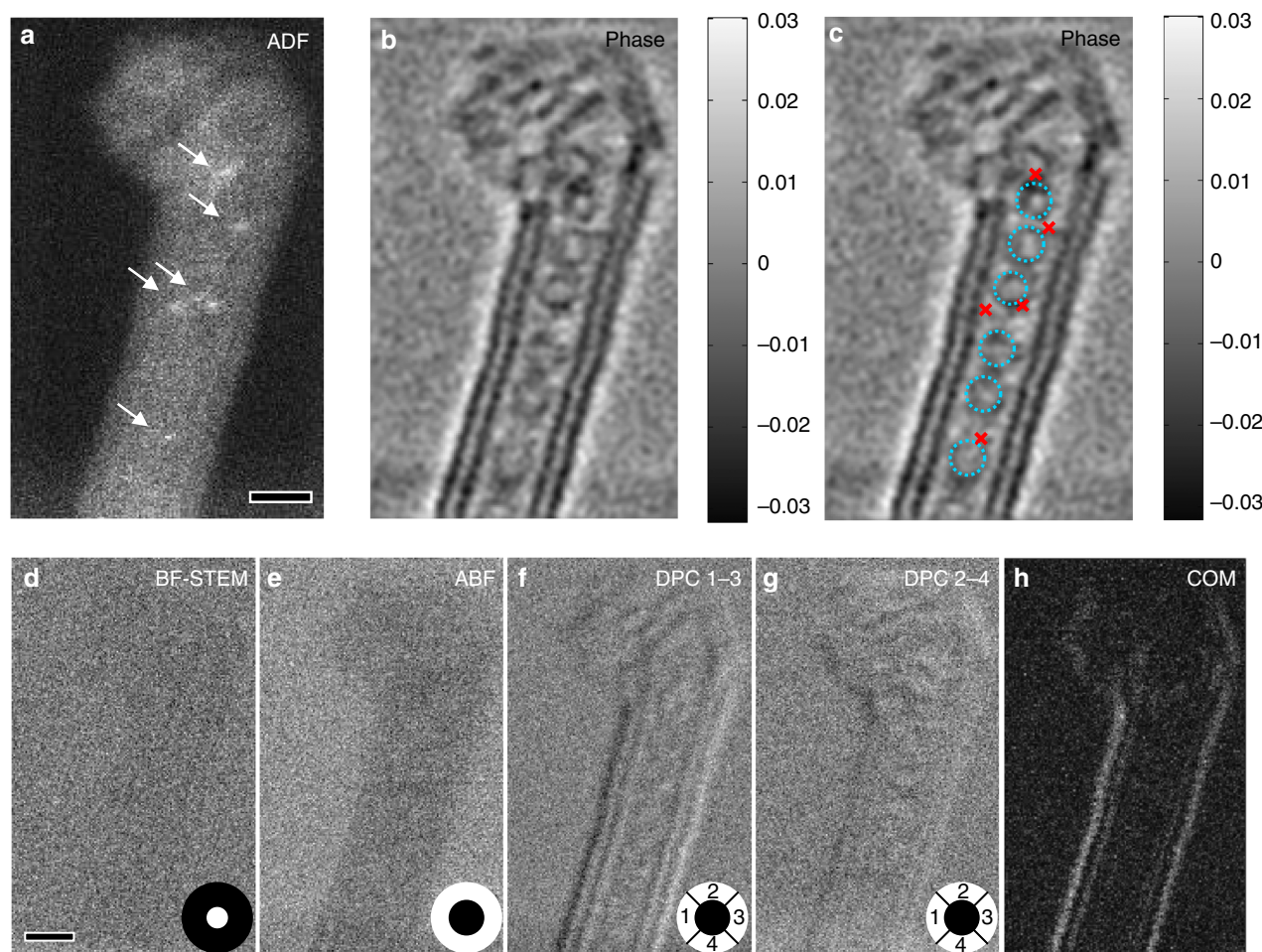


Figure 3 | Simultaneous Z-contrast and phase images of a double-wall CNT peapod. (a) Incoherent Z-contrast ADF image clearly shows the locations of the single iodine atoms indicated by the arrows. (b) The reconstructed phase image shows the presence of fullerenes inside the CNT. (c) Annotated phase image with the fullerenes labelled using dotted circles and iodine atoms labelled using cross marks based on their locations in the ADF image. It is clear that the iodine atoms are located close to but outside the fullerenes. For comparison, conventional phase-contrast images including BF, ABF, DPC and the DPC using the centre of mass (COM) approach were synthesized from the data and shown in **d-h**, respectively. The detector area of each imaging method is shown in white colour in **d-g**. The experiment was performed at an electron probe current of ~ 2.8 pA, pixel dwell time of 0.25 ms and a dose of $\sim 1.3 \times 10^4 \text{ e}^- \text{ \AA}^{-2}$. Scale bar, 1 nm; the grey scale of the phase in **b** is in unit of radians.

Fig. 3, the Z-contrast image reveals the position of the iodine atoms. These are not visible in the simultaneously acquired phase image because of its low atomic number sensitivity. Similarly, the carbon nanostructures are not clearly visible in the Z-contrast image. It is the ability created here to combine these imaging modes that allows the structure to be fully determined. The phase image clearly reveals a CNT containing deeply engulfed fullerene molecules that have entered the tubes to a depth that is inconsistent with the length of the shorter peptide tether. These observed ‘peapod’ structures thus uniquely revealed that not all of the intended attachment links between fullerene and CNT had been successful, and hence vitally informed redesign and function of the hybrid system.

Previous atomic resolution imaging of CNTs containing fullerenes has been achieved using phase contrast in the CTEM^{32,33} and heavy dopant atoms in these systems revealed using Z-contrast imaging in STEM^{34–36}, sometimes combined with BF imaging or electron energy loss spectroscopy. In the case of STEM, imaging of a WPO using BF imaging in STEM relies on deliberate defocus aberrations to form necessary contrast³⁷, which reduce the Z-contrast image resolution and contrast. To demonstrate this, other existing phase-sensitive imaging modes, including the conventional BF, annular BF^{38,39} (ABF), DPC²² and ‘first moment’ DPC imaging^{40,41} were also synthesized from the same 4D data set (in Fig. 3d–h); however, none of these modes are able to image the ‘peapod’ structure clearly at the electron dose used because of their worse signal-to-noise under conditions of near-zero aberrations. As expected, BF and ABF show the worst contrast of all imaging methods, because, similar to all symmetric detectors, they offer no weak-phase contrast under conditions of zero aberrations²⁶. Furthermore, the WDD method makes use of only the regions of the detector containing signal (for example, only regions I and II in Fig. 1d) and hence rejects noise more effectively than these other modes, which use the entire BF disc.

Measurement and correction of residual lens aberrations.

Equation (2) shows that WDD allows residual lens aberrations to be deconvolved from the 4D data set, leading towards aberration-free phase imaging. Here we demonstrate that the redundancy of information inside the 4D data also allows the illuminating probe function to be measured directly. This capability has been demonstrated with iterative methods for highly defocused data^{14,29} and here we implement a direct measurement for in-focus data of low- and high-order aberrations by solving a set of linear equations. Specifically, the phase of $G(\mathbf{K}_f, \mathbf{Q}_p)$ in the disc overlap regions can be mathematically described as a linear function of the lens aberration coefficients under the WPO approximation (see Supplementary Methods), and in this work we use this phase to measure the aberrations through a deterministic matrix inversion to solve such a set of linear equations (see Supplementary Fig. 2 and Supplementary Table 1). This aberration measurement method is a fast direct method that can be applied to both crystalline and non-crystalline specimens.

Figure 4 shows the result of applying the residual aberration correction to the phase image of another double-walled CNT structure recorded at $\sim 2.7 \times 10^4 \text{ e}^- \text{ \AA}^{-2}$ dose, which is twice that used for Fig. 3. Electron irradiation has destroyed the internal fullerene structures. Detection and subsequent deconvolution of a small residual aberration of 3.5 nm defocus and 2 nm twofold astigmatism allows the visibility of the lattice in the walls of the CNT to be significantly improved (Fig. 4d). Fringes from the lattice of the CNT are visible primarily along only one direction in the uncorrected phase (in Fig. 4c), whereas the corrected phase

(Fig. 4d) shows crossed fringes along with a visibly improved contrast. This aberration-corrected image demonstrates the quality of phase images that can now be achieved in a STEM using ptychography. The improved quality of phase images enabled by post-processing aberration correction can be quite beneficial for imaging beam-sensitive materials. Relaxing the requirement to manually adjust the probe defocus and astigmatism while imaging the sample, leads to a decrease in the electron dose and damage.

WDD optical sectioning from a 4D data set of single defocus.

A further advantage of the ptychographic 4D data set is that it contains 3D structure information of the object, as previously demonstrated for light microscopy^{42,43}. An object that is out of focus still has its information encoded in the 4D data set, but the disc overlap described by the $A(\mathbf{K}_f)A^*(\mathbf{K}_f + \mathbf{Q}_p)$ term in equation (2) will show a characteristic phase variation. We find that if the WDD process is performed using a defocus value that does not correspond to the depth of the object, the phase variations in the disc overlap region will not match the deconvolution kernel and will act to reduce the contribution of the object to the reconstructed phase image. Only when the correct defocus is used will the object fully appear and thus an ‘optical sectioning’ effect can be used to gain 3D structure information (see Supplementary Methods), even though the data has been recorded using only one experimental defocus with only one scan of the electron beam. Figure 5 shows an example of crossed CNTs located at different heights along the electron beam direction. A series of reconstructed phases were obtained from a single data set by deconvolving a series of defocus (C_1) values from the recorded 4D data set, whereupon the various CNTs located at different depths along the electron beam direction become most visible at different defocus (C_1) values used in WDD. For example, a small nanotube (indicated by arrows in Fig. 5) that shows no contrast in Fig. 5b,c becomes gradually visible in Fig. 5d,e, as the deconvolution kernel focuses at

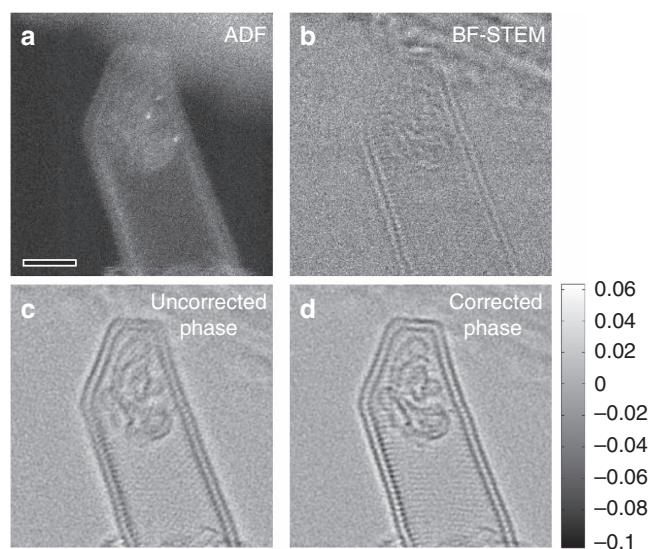


Figure 4 | Imaging a double-wall CNT at atomic resolution. (a) The ADF image shows the presence of single iodine dopants, whereas (b) the simultaneous BF image is rather noisy and shows little contrast of the CNT. (c,d) The phase image before and after correcting residual aberrations, respectively. This comparison shows the improved visibility of the CNT atomic fringes and the damaged carbonaceous structure inside the CNT due to aberration correction. Scale bar, 2 nm.

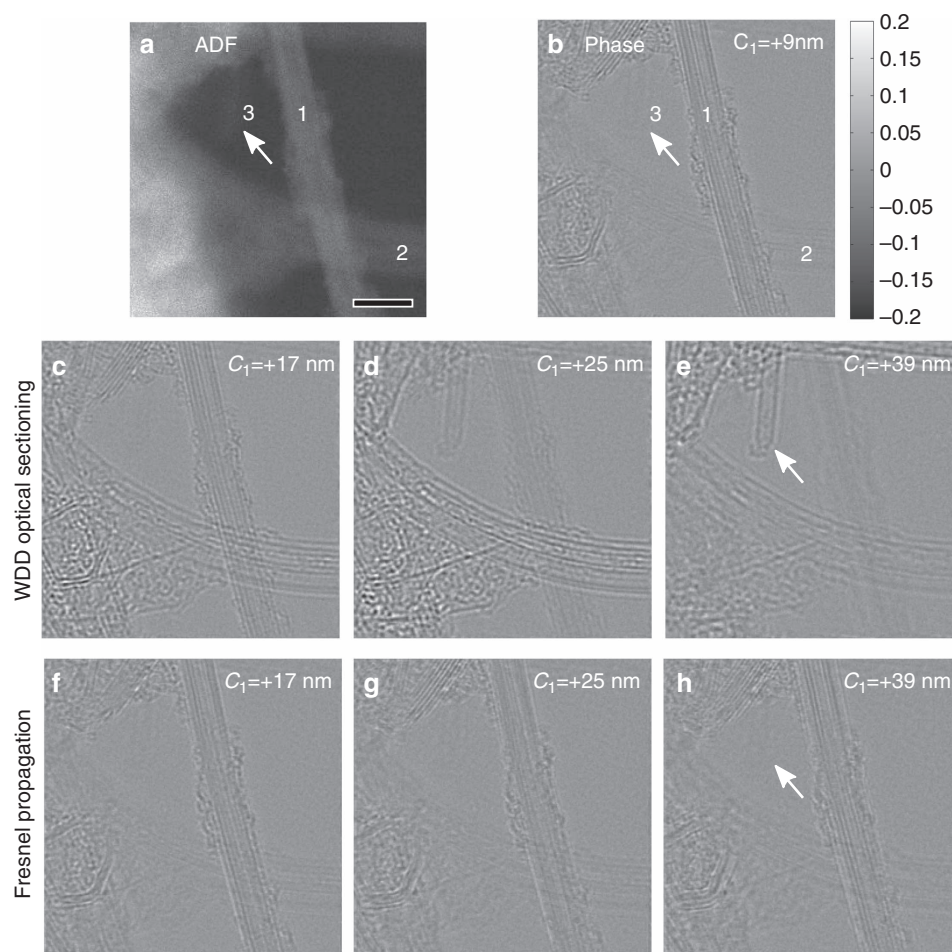


Figure 5 | WDD 3D optical sectioning of crossed CNTs of different heights. (a) The simultaneous ADF image shows that none of the tubes is exactly in focus in the experimental data. By deconvolving a series of defocus aberrations (C_1) from the 4D data set, a set of reconstructed phase images are obtained. The best phase image contrast of the two crossed nanotubes (labelled as 1 and 2 in **a**) are found to be at defocus (b) +9 nm and (d) +25 nm, respectively, with '+' being over-focus relative to the experimental focal plane, and the mid-plane between the two optimal focus is shown in **c** as a comparison. (e) At defocus +39 nm, a small CNT filled with fullerene (indicated by the arrow) becomes visible. WDD optical sectioning offers a depth sensitivity that is not equivalent to the simple Fresnel wave propagation of the reconstructed 'exit wave' and this is evidenced from **f,g,h** obtained by propagating the ptychographically reconstructed complex object function in **b** to the corresponding heights in **c-e**. The white arrow points to the small CNT, which becomes visible using WDD optical sectioning in **e**, but it remains invisible after applying Fresnel propagation in **h**, in comparison. Scale bar, 5 nm.

different depths (also see Supplementary Movie 1). The relative heights of the CNTs can be easily identified by analysing the contrast of the phase images.

It should be emphasized that this is a true optical sectioning effect enabled by WDD under the kinematical approximation and is not equivalent to a Fresnel propagation of the 'exit wave' as in high-resolution TEM (HRTEM)^{44,45}. As can be seen in Fig. 5f–h, Fresnel propagation of the complex transmission function retrieved using a ptychographic reconstruction using a defocus value giving the highest contrast for one CNT does not recover the phase information of the other CNTs located at different depths. Using WDD, by deconvolving the defocus aberration corresponding to a given depth, the deconvolution process acts to enhance the transfer of information at the depth selected by the deconvolution kernel and suppresses the transfer of out-of-focus phase information (see Supplementary Methods); therefore, each phase image resulted from WDD optical sectioning provides a unique identification of the 3D structure at its corresponding depth. We further demonstrate that WDD optical sectioning leads to a depth resolution and a bound to the 3D transfer function (see Supplementary Methods and

Supplementary Figs 12 and 13), which corresponds to that for ADF STEM optical sectioning⁴⁶. The fact that 3D optical sectioning can be achieved by scanning the sample only once at a fixed defocus without the need to run a focal series imaging significantly reduces the electron dose compared with the conventional STEM ADF optical sectioning methods^{47,48}. The ability to measure and correct high-order aberrations with post-processing enables further opening up of the probe forming aperture to reduce the depth of field of the microscope⁴⁹ for 3D phase imaging in future studies.

Discussion

We have shown that developments in camera and information handling technologies have allowed the use of ptychography to form phase images in the STEM under the zero-aberration conditions required for optimal simultaneous incoherent Z-contrast imaging. It is also shown that, for this type of focused-probe data set, the direct WDD approach provides high-quality reconstructions and allows correction of small residual aberrations. The ability to deconvolve aberrations leads

to the ability to optically section samples with a nanometre-scale depth resolution.

The ability to form simultaneous phase images and incoherent images has further potential, for example, simultaneous formation of energy-dispersive X-ray maps, or other spectroscopic and secondary electron imaging⁵⁰, with the phase image. Recording electron energy-loss spectra would, of course, require retraction of the fast camera, but phase and electron energy loss spectroscopy maps could be formed consecutively without any adjustment to the microscope electron optics required.

As the majority of the scattered electrons from the weak-scattering nanomaterials used here were detected, we would expect the dose requirements of the approach presented here to be similar to that for conventional HRTEM, but a detailed analysis is beyond the scope of this study. The ability to post-correct residual aberrations to improve contrast is shared with focal series exit-wave reconstruction in HRTEM⁵¹ but for low-dose imaging the re-registration of the focal series of images can be challenging, whereas no re-registration is required for ptychography.

Finally, we emphasise that 3D information is inherent in the data from a single scan and so optical sectioning can be performed post acquisition without the need for acquiring an experimental focal series of data.

Methods

pnCCD fast pixelated detector. The experiments were performed using the pnCCD (STEM) camera, a direct electron, radiation hard pixelated detector from PNDetector^{52,53}, mounted on the JEOL ARM200-CF aberration-corrected microscope with a 80 keV primary beam energy and 22 mrad convergence angle. This camera delivers full frame images of 264×264 pixels at a readout speed of 1,150 frames per second (fps) routinely and up to 20,000 fps through binning/windowing. The BF diffraction signal was set to cover almost the entire detector area by adjusting the camera length and the dark-field signal was collected by the ADF detector. This speed allows a typical 4D ptychographic data set, consisting of a 66×264 pixel image (fourfold binning) of the diffraction pattern acquired at each probe position in a 256×256 point grid, to be obtained in around 16 s. The data presented here were acquired in fourfold binning (4,000 fps) and twofold binning (2,000 fps) for the CNTs and the graphene, respectively. All data were taken in high charge handling mode⁵⁴.

Simultaneous synthetic images. Synthetic BF, ABF and DPC can also be obtained from the recorded diffraction patterns, with the full flexibility to adjust the collection angles and precise centering of the ABF detector, as well as the partition direction of DPC segments. Because of the focused probe being used, Z-contrast images can be recorded simultaneously using an ADF detector.

Wigner distribution deconvolution. The detailed procedure of WDD follows the previous literature by Rodenburg and Bates²⁷. The probe function is obtained by measuring the aberration measurement algorithm described in the Supplementary Methods. The only difference of this work compared with the previous work²⁷ is that a procedure called ‘stepping out’ was implemented into the WDD algorithm in the previous work, aiming for improving the resolution of the reconstructed phase, whereas in this work, ‘stepping out’ is not implemented, because the primary focus of WDD is for phase-sensitive imaging of weakly scattering objects instead of improving resolution. For the weakly scattering carbon nanostructures under study, ‘stepping out’, relying as it does on the so-called nonlinear interference, is not available.

Data availability. The numerical data shown in Figs 3–5 are available in TIFF format and the entire raw 4D data are all available. Links send on request to peter.nellist@materials.ox.ac.uk.

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Author contributions

H.Y. and P.D.N. designed the study and drafted the paper. H.Y., M.S. and M.H. performed the STEM experiment. H.Y. applied the WDD ptychography reconstruction method, which is developed from an earlier weak-phase method of T.J.P. H.Y. and P.D.N. developed the aberration-measurement method with contributions from R.S. R.N.R., M.L.H.G. and B.G.D. designed and synthesized the peapod specimens. L.J. contributed to the experimental design and data processing. M.S., H.R., M.H. and H.S. designed the fast pixelated detector and, along with Y.K., integrated it into the microscope hardware and control system.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing financial interests: M.S., H.R., M.H. and H.S. are employed by PNsens and PNdetect, which are commercial suppliers of detector hardware. R.S. and Y.K. are employed by JEOL Ltd, which supply electron microscopes. All remaining authors declare no competing financial interests.

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Carbon nanotubes allow capture of krypton, barium and lead for multichannel biological X-ray fluorescence imaging

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The desire to study biology *in situ* has been aided by many imaging techniques. Among these, X-ray fluorescence (XRF) mapping permits observation of elemental distributions in a multichannel manner. However, XRF imaging is underused, in part, because of the difficulty in interpreting maps without an underlying cellular ‘blueprint’; this could be supplied using contrast agents. Carbon nanotubes (CNTs) can be filled with a wide range of inorganic materials, and thus can be used as ‘contrast agents’ if biologically absent elements are encapsulated. Here we show that sealed single-walled CNTs filled with lead, barium and even krypton can be produced, and externally decorated with peptides to provide affinity for sub-cellular targets. The agents are able to highlight specific organelles in multiplexed XRF mapping, and are, in principle, a general and versatile tool for this, and other modes of biological imaging.

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Many imaging techniques that exploit electromagnetic radiation have been developed to probe and manipulate biological systems *in situ*. While certain regions of frequency have found widespread intensive application (Supplementary Fig. 1), such as hard X-rays (radiography, 3–30 EHz), radio waves (MRI, 3 KHz–300 MHz), UV–visible light (fluorescence microscopy, 430 THz–30 PHz) and gamma rays (SPECT/PET, >30 EHz), some potential uniquely powerful areas of the electromagnetic spectrum are notably underemployed. X-ray fluorescence (XRF) results from energy released when outer sphere electrons fall into holes in core atomic orbitals caused by ionization by the lower-energy end of hard X-ray radiation (500 PHz–5 EHz, equivalent to energies of 2–20 keV)¹. The resulting sharp, characteristic emission lines in unoccupied regions suggest quantitative, ultra-multi-channel imaging (>20) using a single, sharp excitation wavelength, well beyond the limited multi-modal strategies of current biological imaging. Advantageously, conventional XRF imaging is not sensitive to the oxidation state and bonding of the imaged chemical element. However, full use of this prospective unique precision and high informational content necessitates use of further elements that are either gaseous or toxic as ‘labels’. Here we show that the orthogonal filling and decoration of carbon nanotubes (CNTs) allows a versatile, modular system for the effective targeting of such intractable XRF contrast agent elements. These hermetically sealed CNT ‘nanobottles’ bearing organelle-specific peptides^{2,3} allowed not only systematic delineation of key sub-cellular structures (cell membrane, nucleus and endoplasmic reticulum), using toxic barium, lead or even gaseous krypton as ‘labels’, but also allowed vital insight into the versatility of CNTs as carriers that are trafficked inside cells. Given their robust control of contents, large filling capacity and ready modular functionalization, we envisage that such CNTs will prove to be general, targetable, encapsulating imaging agents for this and other novel-mode imaging techniques.

Results

Design of XRF nanobottle contrast agents. XRF elemental contrast agents were chosen that would not only allow distinct identification of emission peaks, but that would test the limits of both the filling capacity and proper hermetic sealing of any associated carrier system. Three different CNT filling ‘materials’/elements were selected—Ba, Pb and Kr—determined by critical factors relating to their XRF properties.

Vitality, elements with emission energies less than ~2 keV are typically not observed due to absorption by air between emitting sample and detector, whereas those requiring excitation energies >18 keV receive a reduced flux of photons (and hence emit less) due to the spectral properties of radiation from typical sources, including even synchrotron beamlines⁴, optimized for broad-range X-ray spectroscopy⁵. Furthermore, intervening emission regions of 2.0–3.7 and 5.9–8.6 keV are heavily populated by biologically abundant elemental XRF emissions that would therefore provide a high-potential background signal in any imaging applications (Supplementary Fig. 2). Barium ($L\alpha_1 = 4.5$ keV), lead ($L\alpha_1 = 10.5$ keV) and krypton ($K\alpha_1 = 12.6$ keV) occupy near ideal positions in the clear spectral ‘windows’ between these cluttered and/or low utility regions. Yet, their global toxicity (Pb), neurotoxicity (Ba) and gaseous state (Kr) would ordinarily prohibit their use. Their targeted use in isolation, or even in conventional conjugates as compounds, would therefore otherwise be extremely challenging to envisage in biological applications (either due to their toxic reactivity or essential lack of

reactivity) without due sequestration. While we have chosen three fillings here as illustrative examples, in principle any element or combination of elements may be contained with CNTs, providing the ability to produce highly multiplexed, ‘barcoded’ contrast agents.

We considered that proper capture and redirection of these elements might be uniquely achieved with CNTs for three reasons. First, CNTs have an exceptionally spacious cavity that can be filled with a wide variety of non-natural inorganic compounds (potential XRF tag elements) either through capillary force-induced uptake in the melt⁶ or, potentially, gaseous diffusion^{7,8}. Second, formation of impermeable hemispherical caps from open CNTs, for example, upon post-melt cooling⁹, creates totally isolated microphases within sealed CNTs through which nothing larger than a proton can traverse the graphene membrane^{10,11}. As a result, any toxicity or other biological activity of potential XRF tag elements is neutralized, since escape is prevented¹²; simultaneously any loss, leaching or degradation of the encapsulated, active label is prevented. Third, the outer covalent functionalization of these CNTs could, in principle, be achieved by a number of methods¹³, allowing ready and flexible attachment of putative targeting ligands and/or modulating groups to control both biological function and compatibility, as well as providing a platform for the testing of probe moieties. Here, peptidic ligands for cellular receptors and trafficking proteins provided a versatile class of targeting moieties^{2,3} to test the potential localization of these ‘nanobottles’ in an organelle-specific manner to sub-cellular structures (cell membrane, nucleus and endoplasmic reticulum). Together, these allowed the design and creation of a modular, addressable system (Fig. 1), through which we were able to systematically probe the effects of surface label upon localization of CNTs in cellular compartments.

Construction and characterization of nanobottles. Steam-opened and -shortened¹⁴ (ca. 400–450 nm) and purified¹⁵ single-walled CNTs (SWCNTs), which are widely regarded as biocompatible¹⁶, were first tested as containers for lead. Enclosed under vacuum (to prevent oxidation by air upon annealing) within sealed quartz ampoules, open-ended and empty SWCNTs, in the presence of lead oxide were heated to 100 °C above the melting temperature of the inorganic material (888 °C) to initiate molten-phase capillary filling¹⁷. After slow annealing to ambient temperature, the samples were thoroughly sonicated and washed with concentrated hydrochloric acid to remove unencapsulated, toxic material. Pleasingly, a range of methods (X-ray photoelectron spectroscopy (XPS; Supplementary Fig. 3), XRF, high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM) and thermogravimetric analysis (TGA)) confirmed the presence of successfully encapsulated lead; in particular, HAADF-STEM measurements showed well-packed, condensed phase lead content in all cases (Fig. 2a; Supplementary Fig. 4). Vitality, Raman spectroscopy (Supplementary Fig. 5) confirmed that the nanotubes were essentially intact, with only a slight increase in the magnitude of D-band resonances associated with defects¹⁸. TGA further confirmed this successful capture of PbO (~15.2%) (Supplementary Fig. 6). Most importantly, synchrotron XRF spectroscopy revealed a powerfully strong signal from the incarcerated materials (Fig. 2b) with the designated Pb emission lines, emerging well above even the strongest background signals in a clearly distinct spectral window. Capping was measured by further attempts to remove PbO by treatment with concentrated hydrochloric acid (Supplementary Methods). Inductively coupled plasma mass spectrometry (ICP-MS) revealed that 51% of the encapsulated lead could be

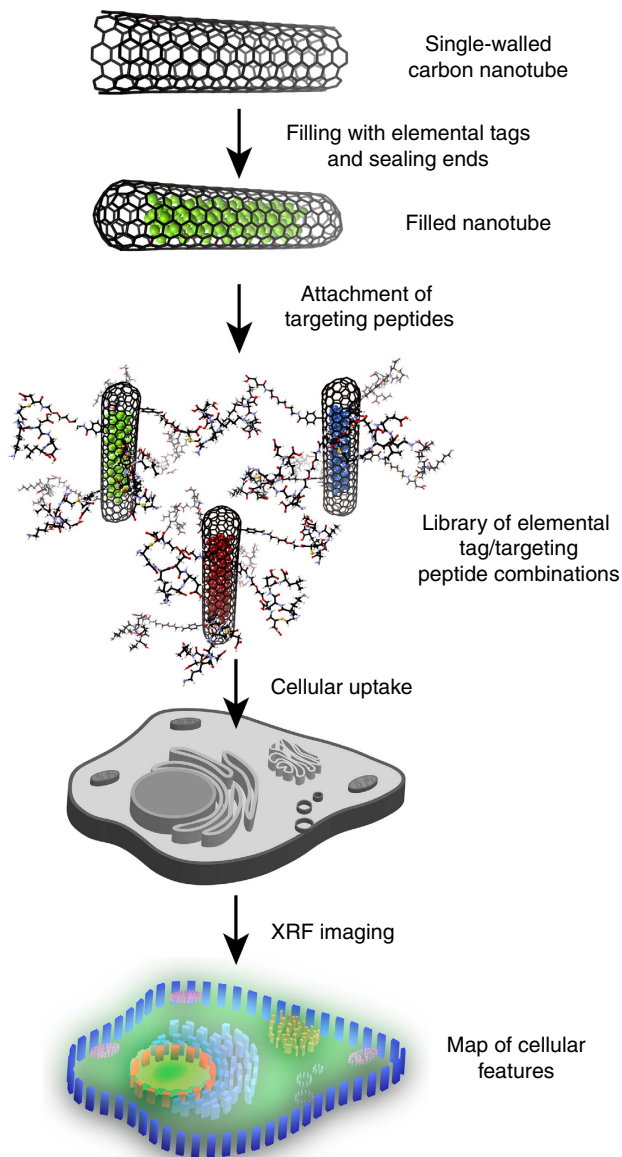


Figure 1 | Design of XRF-contrast carrier systems to probe cellular organelles through XRF mapping. By filling, sealing and modifying CNTs, ‘nanobottle’ systems with targeting ligands can be created based upon almost any appropriate elemental system in a modular and general manner. Colour coding in this figure indicates generic multiplexed imaging rather than specific materials.

extracted in a first wash, but subsequent washes were lead-free (Supplementary Table 1). This indicated that although closure of the tube ends was not 100% (attributed to the oxidizing nature of the filling material), a significant level of Pb was irreversibly contained within doubly-capped SWCNTs nonetheless.

Having demonstrated successful encapsulation of lead, we chose next to fill CNTs with both barium and iodine to create a potential, ‘dual-mode’ XRF-active probe system bearing two independent sets of distinct emission lines in useful windows (Ba $L\alpha_1 = 4.5$ keV, I $L\alpha_1 = 3.9$ keV) as a proof of principle. Barium iodide (Ba $L\alpha_1 = 4.5$ keV, I $L\alpha_1 = 3.9$ keV) is a highly soluble salt that would normally be readily dispersed in the body, with iodide being consequently naturally concentrated in the thyroid¹⁹, while barium ions are toxic, interfering with muscular and neuronal Ca^{2+} transport²⁰. Successful confinement of BaI_2 within a CNT^{21,22} would therefore provide

a powerful system for the alteration of this typical, toxic biodistribution. Essentially, identical methods for molten capillary filling (heating to 811 °C) followed by sonication and washing with water also proved successful for BaI_2 , thereby supporting the generality of the approach (corroborated by XPS (Supplementary Fig. 7); HAADF-STEM (Fig. 2a; Supplementary Fig. 8); Raman (Supplementary Fig. 5); TGA 8.9% wt (Supplementary Fig. 6)). Pleasingly, when tested as a putative XRF contrast agent (Fig. 2b), the resulting ‘Ba + I nanobottle’ provided dual signals in the lower spectral window, albeit with a lower relative intensity that is typical of L-lines used here for Ba and I, as opposed to K-lines observed for Pb, as well as the atomic number dependence of fluorescence yield²³. Further washes confirmed sealing of the CNT end caps by ICP-MS (Supplementary Table 1).

Finally, we tested krypton that, as an unreactive gas, was potentially our most challenging contrast element. Although Kr can potentially condense inside open CNTs at temperatures <200 K (refs 24,25), there are no reports of irreversible encapsulation. The SWCNTs were sealed into an silica ampoule under approximately atmospheric pressure of Kr, and then heated to 700 °C, before cooling slowly to permit closing of the open nanotube ends⁹. This was followed by work-up under high vacuum to remove any potentially physisorbed gas. Although high-resolution TEM confirmed closed ends (Fig. 2a; Supplementary Fig. 9), the presence of Kr gas was not evident from XPS, HAADF-STEM and TGA (Supplementary Fig. 6) analyses. However, vitally, XRF revealed a strong Kr signal (Fig. 2b) and provided additional confirmation of its unambiguous presence through the measurement of the absorption edge (measured: 14.325 keV; reported²⁶: 14.3256 keV; Supplementary Fig. 10). The difficulty in observing Kr using conventional methods for materials is unsurprising, given that gases are on the order of a thousand times less dense than condensed phases. Nonetheless, our first demonstrations of such Kr-encapsulation reveal that despite this lower level of filling, this element also provides a suitable signal for potential use in XRF contrast imaging in one of our identified spectral windows.

Functionalization of nanobottles for cellular targeting. Having successfully demonstrated both filling with our chosen contrast elements and the generation of useful XRF signal, we next converted these filled SWCNTs into putative cellular imaging agents through covalent surface attachment of peptides. Representative targeting peptides were selected that would allow us to map the trafficking of these agents at differing depths of penetration into cells: RGD²⁷ (Arg-Gly-Asp, cell surface adhesion), KDEL²⁸ (Lys-Asp-Glu-Leu, endoplasmic reticulum (ER) targeting) and two nuclear localization sequences (NLSs) taken from the SV40 virus^{29,30} and the human protein IL-1 α ³¹. A generalized bifunctional spacer was designed and synthesized (Fig. 2c, synthetic details in Supplementary Methods; Supplementary Figs 11–15) to ensure sufficient functional accessibility of peptides to corresponding cellular receptors. This was attached to the side-walls of the filled SWCNTs through the selective reaction of the aniline-moiety terminus of the spacer as its diazonium³², giving a usefully stable³³ C–C linkage, that leaves the opposite terminus of the linker free for further reaction.

Peptides were constructed through automated solid peptide synthesis using Fmoc (fluorenylmethyloxycarbonyl) chemistry (Supplementary Methods; Supplementary Figs 16–19) and terminated with *N*-terminal Fmoc-cysteine residues at either the *N*- or *C*-terminus, according to the location of the peptides within their native proteins of origin. This allowed a general strategy for chemoselective Michael-type conjugate addition for

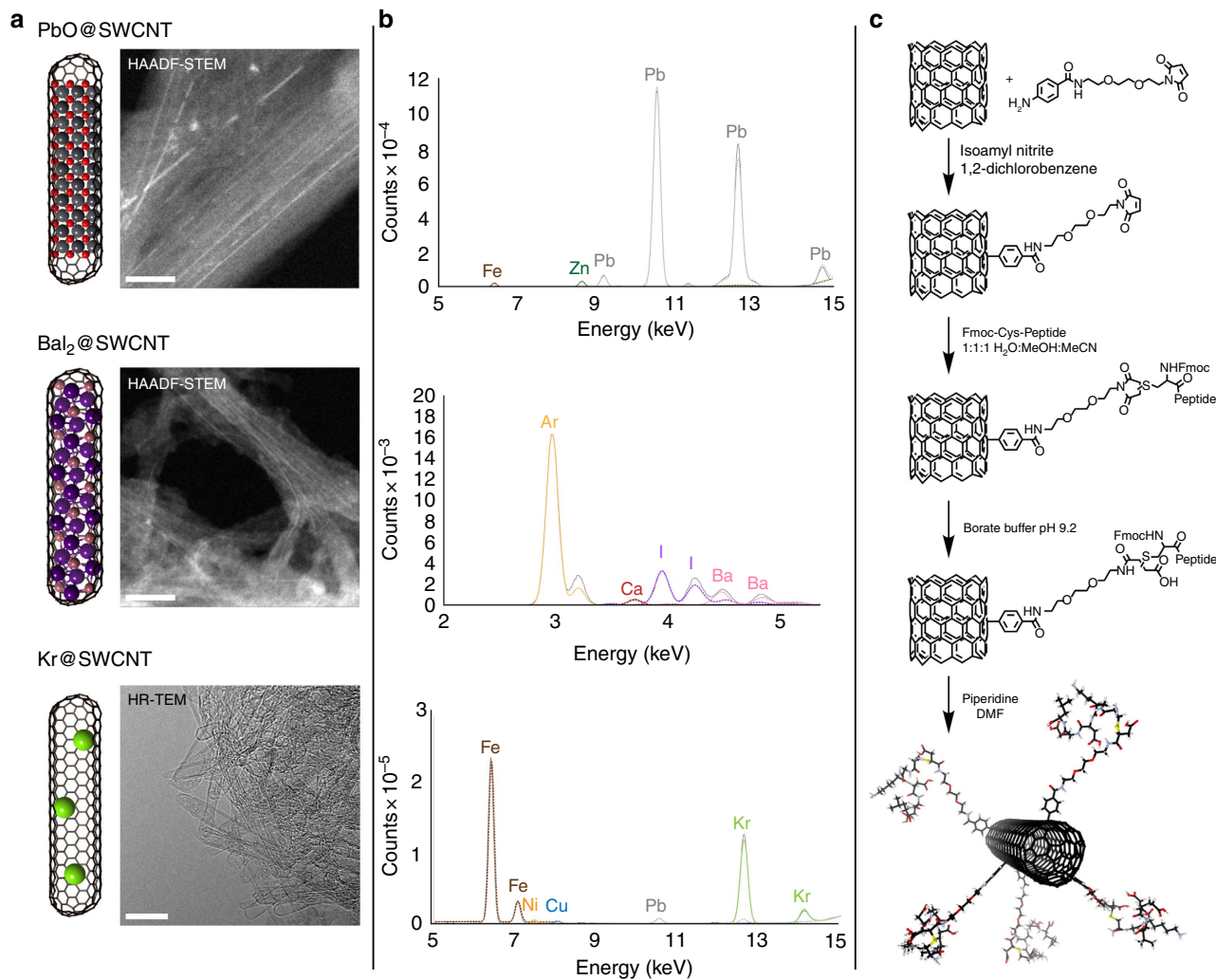


Figure 2 | Characterization of filled SWCNTs, and functionalization with targeting peptides. (a) HR-TEM characterization of filled SWCNTs (scale bars, 10 nm). HAADF-STEM images show bright lines indicating filling with high-Z species for PbO@SWCNT and Ba₂@SWCNT. Although the low density of Kr gas prevented observation of Kr in Kr@SWCNT in this way, the sealing of the ‘nanobottle’ ends was nonetheless confirmed in bright-field imaging. (b) XRF spectra of the filled tubes recorded using synchrotron radiation at excitation energies of 16, 6 and 15 keV for PbO, Ba₂ and Kr fillings, respectively, confirm the utility of not only Pb and Ba but also, strikingly, Kr. (c) Functionalization of the sealed SWCNTs with peptides was achieved using a novel, bifunctional linker. Further characterization can be found in Supplementary Figs 3–21.

attachment to the maleimide electrophile terminus found on the free end of the SWCNT-bound linkers. Subsequent ring-opening of the resulting succinimide-thioether in basic buffer converted this moiety to a more stable form and hence prevented possible exchange of the resulting conjugate with biological thiols^{34,35} during use as cellular probes. Finally, cleavage of the Fmoc group not only generated the final peptide moieties but also allowed ready, precise quantification of the coupling efficiency through UV measurement of the released Fmoc fulvene adduct³⁶ (which was also corroborated by TGA measurements, see Supplementary Figs 20 and 21); all conjugates showed good loading in the range of 0.2–1.0 $\mu\text{mol mg}^{-1}$ (Table 1; Supplementary Tables 2–4). Peptide attachment in this way noticeably improved dispersion in aqueous media in all cases compared with pristine or linker-functionalized SWCNTs, an important factor for biological use. Biocompatibilities of the peptide-functionalized and filled SWCNTs were tested using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell viability assay³⁷ in HeLa (human cervical cancer) cells, and revealed no measurable toxicity at concentrations even up to 50 $\mu\text{g ml}^{-1}$ (Supplementary Fig. 22; Supplementary Discussion), consistent with prior observations for

Table 1 | Yields for peptide decoration of filled SWCNTs according to correlated measurement by both Fmoc numbering and TGA.

Decoration	Target	Sequence	Conjugation yield (Fmoc) ($\mu\text{mol mg}^{-1}$)	Conjugation yield (TGA; $\mu\text{mol mg}^{-1}$)
Maleimide	NA	NA	NA	1.01
RGD	Cell adhesion	RGDC	0.81	0.37
KDEL	ER	CGKDEL	0.62	0.30
IL-1 α NLS	Nucleus	KVLKKRRC	0.27	0.21
SV40 NLS	Nucleus	PPKKRKVC	0.24	0.22

Italicized amino acids were added to the targeting sequence to permit conjugation.

other SWNT-based systems^{38,39}. Importantly, no correlation could be made between cell viability and SWCNT filling material, indicating that the XRF contrast component was isolated from cellular processes by the graphene membrane.

Cellular microscopy of XRF nanobottle contrast agents. With putative XRF contrast agents in hand, we first monitored their dynamic, cellular distribution using a range of complementary, CNT-associated imaging methods. First, dual modes of combined, simultaneous optical and Raman microscopy over a 24 h period (Fig. 3; Supplementary Figs 23–27) in HeLa cells allowed microscopic imaging that mutually correlated well: small aggregates of SWCNTs manifesting as optically opaque regions aligned with high G-band Raman intensity ($1,500\text{--}1,650\text{ cm}^{-1}$). No G-band intensity or dark aggregates were seen with untreated cells. Different targeting moieties dramatically controlled cellular distribution (Fig. 3). RGD-functionalization resulted in fine lines of SWCNT masses (dark and Raman G-band intense), which reliably followed the contours of the cells, consistent with cell adhesion. KDEL-SWCNTs were found in clumps consistent with intracellular localization on the ER (although it should be noted that it was not possible to observe the ER directly in this method). Co-localization of large, optically dark and Raman G-band intense features with the cell nuclei was conspicuous with both the SV40 and IL-1 α NLS peptides; uptake of SV40 NLS-SWCNTs was more voluminous, but the IL-1 α NLS-SWCNTs were more closely aligned with the nucleus.

Next, we examined the use of low-voltage HAADF-STEM to further test these observations on the intracellularly targeted SWCNTs at higher magnification. As a result of the low electron density (in the presence of contrast-reducing resin matrix) individual SWCNTs are not visible in cells with this technique. Instead, aggregates of targeted SWCNTs could be readily observed, manifesting as regions of moderately high contrast with a fibrous texture (Fig. 3c). This was unambiguously

confirmed through the use of EELS spectroscopy, which revealed characteristic graphitic carbon peaks at $\sim 292\text{ eV}$ (Supplementary Fig. 28). Strikingly, this aggregate-selective imaging revealed previously unobserved modes of dynamic, cellular trafficking of functionalized SWCNTs, for example, IL-1 α NLS-functionalized SWCNTs were observed during the process of endosomal uptake at various stages before nuclear localization (Fig. 3d; Supplementary Fig. 29). This excitingly uncovered ‘debundling’ of SWCNTs during internalization into complex endosomal compartments in the cytoplasm. Taken together with the dual optical-Raman microscopy, this suggests that, through such ‘debundling’, aggregates that are too large to traffic on to the nucleus are ‘teased-out’ as individual SWCNTs through a ‘Mikado-like’ mechanism before nuclear uptake. Similarly, SWCNTs targeted with the SV40 NLS peptide were also taken up to a significant degree, with aggregates found in numerous endosomal compartments, particularly close to the nucleus (Fig. 3c; Supplementary Fig. 30). Using the KDEL-functionalized SWCNTs, bundles were seen in compartments apparently within the ER consistent with the function of KDEL (Fig. 3b; Supplementary Fig. 31); however, in these cases it was harder to distinguish between SWCNTs and rough ER, and the result cannot be taken as fully conclusive.

With these key aspects of cellular trafficking both confirmed and revealed using the peptide-functionalized ‘nanobottles,’ we next tested their utility as designed XRF-contrast agents in the ‘mapping’ of a tissue-like cellular array as a proof of principle. An X-ray excitation energy of 10 keV was sufficient to excite the ‘Ba + I nanobottle’ contents, as well as the biologically present elements, but for the Kr and Pb nanobottles maps were compiled at 15 and 14 keV (respectively chosen for the elemental tags) with

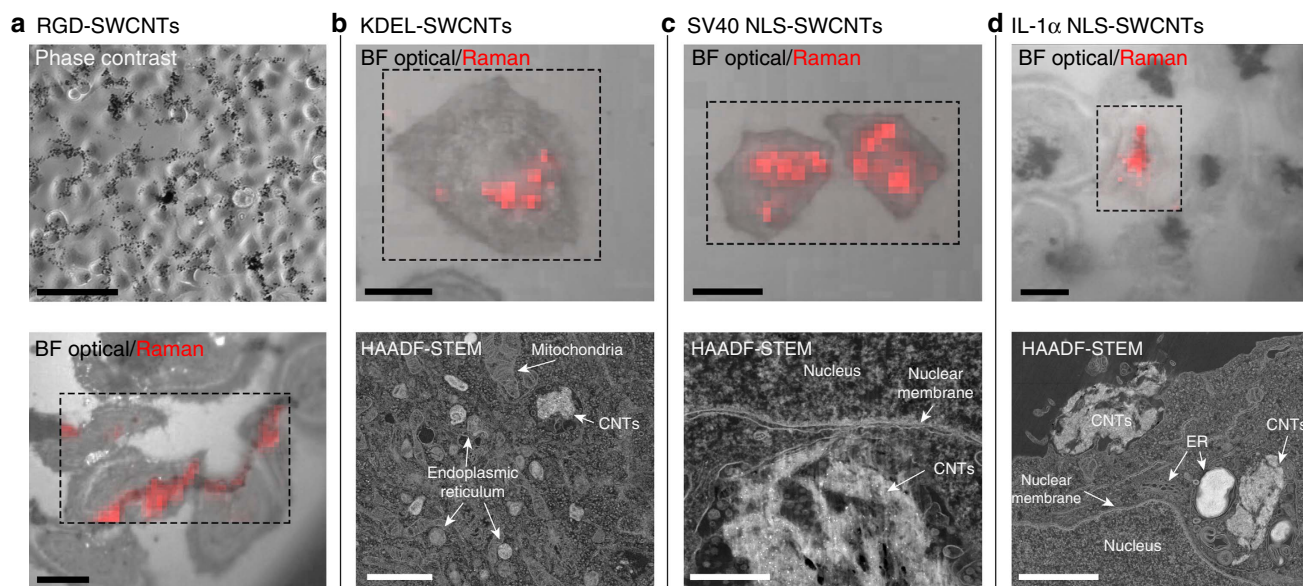


Figure 3 | Intracellular tracking of peptide-functionalized SWCNT nanobottles. Maps of the SWCNT Raman G-band ($1,500\text{--}1,650\text{ cm}^{-1}$) were recorded within the dashed boxes. In the HAADF-STEM images, SWCNTs can be identified by high-contrast regions speckled with even brighter spots (residual iron catalyst particles), a ‘fibrous’ texture, and a degree of smearing due to the tensile strength of SWCNTs resisting the diamond ultramicrotome knife. **(a)** RGD-functionalized SWCNTs interacting with HeLa cells imaged by phase contrast (scale bar, $100\text{ }\mu\text{m}$), and bright-field microscopy with Raman mapping (scale bar, $20\text{ }\mu\text{m}$). The SWCNTs are seen to follow the cell outline contours, consistent with the binding of RGD to cell-surface integrins. **(b)** KDEL-functionalized SWCNTs inside HeLa cells imaged by bright-field microscopy with Raman mapping (scale bar, $20\text{ }\mu\text{m}$) and HAADF-STEM (scale bar, $2\text{ }\mu\text{m}$). Raman mapping shows SWCNTs within the cells at locations consistent with the ER. HAADF-STEM found SWCNT bundles close to the ER, but could not unambiguously show association. **(c)** SV40 NLS-functionalized SWCNTs inside HeLa cells imaged by bright-field microscopy with Raman mapping (scale bar, $20\text{ }\mu\text{m}$) and HAADF-STEM (scale bar, 500 nm). Raman mapping showed SWCNTs within the cells, overlapping with the nuclei, and HAADF-STEM revealed interactions with the nuclear membrane, as expected for the association of the SV40 peptide with the nuclear pore. **(d)** IL-1 α NLS-functionalized SWCNTs inside HeLa cells imaged by bright-field microscopy with Raman mapping (scale bar, $20\text{ }\mu\text{m}$) and HAADF-STEM (scale bar, $2\text{ }\mu\text{m}$). Nuclear localization is clearly visible in the Raman maps. HAADF-STEM uncovered cellular uptake of the SWCNTs and their trafficking towards the nucleus. Further microscopy images can be found in the Supplementary Figs 23–31.

additional scans at 5 keV to provide adequate signal for the lower-Z biologically-abundant elements to provide sufficient contrast (Supplementary Figs 32–37). Maps of untreated cells (Fig. 4a; Supplementary Figs 38 and 39) showed the presence of biotic elements such as P, S, Cl and Zn ordered diffusely throughout the cells. Of these, the best contrast was obtained for S, and hence the S map was chosen as a marker of the cell outline, which can be used to assess the location of the filled SWCNTs relative to the cell.

In cells that had taken up ‘nanobottles’, the new contrast element signals could be used to map cellular architecture (Fig. 4b–d) by virtue, in part, of their designed-specific targeting to organelles (*vide supra*). Small levels of iron impurities are ubiquitous in SWCNTs, and advantageously could be used here as an additional co-localization marker of SWCNT positions⁴⁰ (for example, in green channel in Fig. 4b,d). Notably, certain biotic metals (Cu, Zn) were co-localized with the ‘nanobottles’ (Supplementary Figs 40–52), suggesting mutual influence of Cu- and/or Zn-dependent processes during SWCNT trafficking⁴¹.

Excitingly, the XRF maps of treated cells, enabled through the unique elemental contents of the ‘nanobottles’ (Fig. 4), revealed modes of uptake and trafficking consistent with those observed by

optical, Raman, and electron microscopy imaging detailed above. The location of RGD-functionalized SWCNTs at the cell surface, for example, could be clearly seen using any choice of ‘nanobottle’ filling material (Fig. 4b–d). Similarly, nuclear location of the SWCNTs was imaged using both the SV40 and IL-1 α peptides with all three of these ‘nanobottle’ contrast systems. The orthogonality of interior contrast agent and exterior peptide targeting is clearly shown. The distributions of barium and iodine in the binary, ‘dual-mode’ Ba + I ‘nanobottle’ system were conspicuously well correlated (Fig. 4c), as were all of the contrast elements with Fe, showing that escape was not occurring; this also reduced ambiguities in imaging for improved precision. The power of the ‘nanobottle’ method for encapsulation was perhaps most markedly evidenced in the fact that it was possible to use Kr as a precisely localized cellular imaging agent (Fig. 4d); this is the first time that this has been achieved to our knowledge.

Discussion

Scanning XRF microscopy has been used previously to reveal, for example, process-dependent alterations in bioelemental organization^{42–44}, including disease states⁴⁵, and to study the

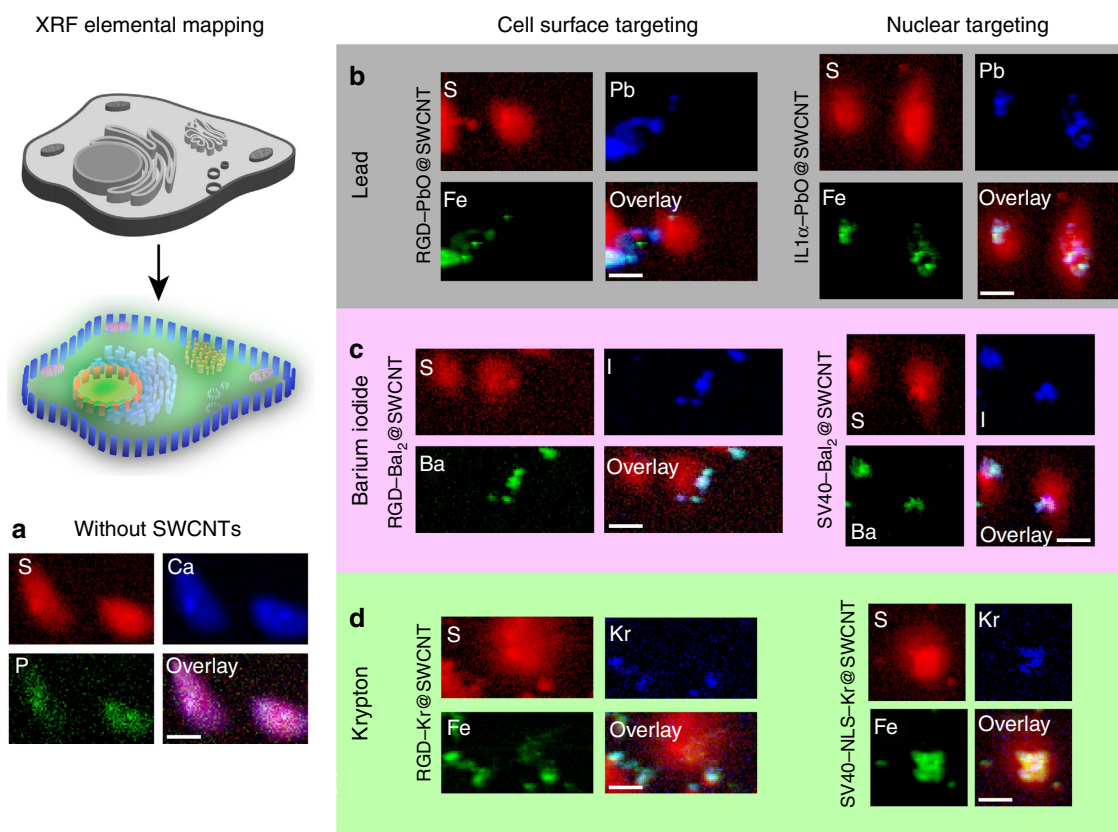


Figure 4 | Use of filled and decorated SWCNTs as biological XRF contrast agents. (a) Elemental distribution maps of untreated HeLa cells. Three biologically relevant channels are presented to show the cell features. Sulfur was chosen as the best marker of cell morphology. (b) XRF maps of cells treated with Pb@SWCNT-based contrast agents. Fe channel is included as a marker of SWCNT location, and correlates with Pb, showing the lack of escape of Pb from the SWCNTs. (c) XRF maps of cells treated with BaI₂@SWCNT-based contrast agents. Colocation of Ba and I confirm secure encapsulation of the elements. These elements also correlate with Fe (Supplementary Figs 44–47). (d) XRF maps of cells treated with Kr@SWCNT-based contrast agents. Fe channel is included as a marker of SWCNT location, and correlates with Kr, showing the lack of escape of Kr from the SWCNTs. Scale bars (20 μ m) in overlay apply to all channels. Maps are plotted on a logarithmic intensity scale; linearly-scaled maps of a wider range of elements (≥ 8), and details of deconvolution can be found in the Supplementary Figs 32–52. Synchrotron microprobe arrangements permitted a beam width of 2 μ m, with a rounded intensity profile. To maximize the effective resolution obtainable, the cells were seeded sparsely, which results in spread-out morphologies after chemical fixing, meaning that by scanning the beam at 1 μ m increments over the sample, the maximum spatial detail can be obtained (at the price of the cells looking ‘healthy’). Some peaks corresponding to other, unexpected, elements (for example, Ti, Cr, Ga and La) were also observed across all samples, but since their distribution was uniform (Supplementary Figs 38 and 39), they were included in the fitting calculations to prevent overestimation of the elements of interest, but can be regarded as background noise.

pathways of heavy metal poisons^{46,47}. The often diffuse biodistribution of naturally occurring elements in these prior studies means that features, such as organelles and membranes can be poorly defined and ambiguity arises/would arise from use of these elements as labels, thereby hindering interpretation of associated maps. While some elegant studies have made use of xenobiotic elements as contrast agents^{48–52}, there has been no general method until now that would permit wide-ranging variation of both targeting ligand and XRF tag element.

We believe that the generalizable encapsulation approach shown here will broaden XRF (and potentially other types of) imaging, which is otherwise underused in the biological arena, through the creation of modular and versatile ‘XRF contrast agents’. The incarceration of ‘difficult’ (toxic or gaseous) elements shown here has provided one of the more striking proofs of the impermeability of the atomically-thin graphene carbon membrane¹¹. That it is furthermore possible to image the noble gas Kr inside SWCNTs, inside cells, illustrates that such ultra-thin membranes can find useful applications in novel constructs, here as the ‘walls’ to such ‘nanobottles’ for imaging. The shielding provided to biological systems by this graphene membrane system from the toxic influence of lead and barium as ‘heavy’ metals, further illustrates its flexible utility. The elements used as high-contrast tags were chosen primarily according to their unique spectral and hence illustrative potential; given the range of CNT fillings already reported^{46,7,53}, there is no doubt that many alternative tags are available. This research anticipates the creation of a highly multiplexed library of XRF contrast agents that employ a wide range of elements; the use of binary and higher elemental combinations could even enable a complete cellular ‘barcode’ system.

It has been argued that the bane of carbon nanotechnology has been its unnecessary application to problems that do not require the unique strengths or properties of carbon nanomaterials⁵⁴. In contrast, here, (i) versatile, hermetic encapsulation via robust, ultra-thin membranes; (ii) diverse modes of imaging and (iii) ready surface-functionalization are essential exploited properties that arise through a unique combination found only in CNTs. The functionalization is achieved chemically and so can be readily extended to other ligands (for example, with an affinity for their organelles⁵⁵ or biological markers⁵⁶) in a near-unlimited manner.

Imaging using synchrotron sources spans a huge range of scales. Thus, with microprobe optics, it is now feasible to detect elements in the attogram sensitivity range with pixel sizes <100 nm;⁵⁷ while it is also possible to image live, adult humans at the tissue level⁵⁸. ‘Imaging’ is a word used to encompass all length scales, both microscopic and *in vivo*, but it is rare that a single platform or mode might provide direct access across the board. Correlation here of four distinct modes of ‘nanobottle’ detection (optical, XRF, Raman and electron microscope) with varying tissue penetration ability excitingly suggests new methods for correlating cellular microscopy with *in vivo* imaging. Additional modes of imaging can also be readily envisaged, for example, through the use of magnetic resonance imaging- or radiochemically active elemental fillings. Although today there are significant hurdles⁵³ to the use of filled CNT XRF contrast agents in imaging live humans for healthcare (X-ray safety, radiation penetration depths and governmental approval of internal use of CNTs being chief among them), this technology could be used in the near future for highly illustrative *ex vivo* imaging of animal models.

These unique probes have now revealed clear modes of cellular interaction that valuably illuminate the utility of CNTs. As well as the observed lack of toxicity, the effective trafficking of these nanobottles throughout cells (dependent on targeting) has highlighted striking flexibility. In particular, we have observed an

effective ‘debundling’ mechanism that also explains why, although CNT aggregation is fast on a microscopic, physico-chemical level⁵⁹, CNTs prove to be effective in their entry into cells, as shown here. These suggest that cellular systems can traffic such CNTs in a wholly effective and dynamic way⁶⁰, separating them from each other in a manner similar to intracellular lipid disassembly⁶¹. Together this suggests that far from being viewed by nature as ‘foreign’, the decoration of a hydrophobic carbon-based surface with appropriate surface moieties creates conjugates that are well tolerated and even processed, at least in part, naturally. The exploitation of such ‘well-accepted nanobottles’ could clearly have broad and wide-ranging applications in biology and medicine.

Methods

Commercially available materials were used without any pre-treatment unless otherwise stated. Dry solvents were obtained by purging with N₂ and then passing through an MBraun MPSP-800 column. H₂O was de-ionised and microfiltered using a Milli-Q Millipore machine. All other solvents and commercial grade reagents were used without further purification.

Routine nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVIIIHD 400 nanobay spectrometer with ¹H NMR operating at 400 MHz, proton-decoupled ¹³C at 100 MHz. Routine mass spectra were recorded on a Micromass LCT Premier XE spectrometer and accurate masses determined to four decimal places using Bruker microTOF and Micromass GCT spectrometers.

High-resolution TEM and scanning TEM images were acquired on either a JEOL JEM3000F, operating at 300 kV equipped with a JEOL HAADF detector for Z-contrast imaging or a FEI Tecnai G2 F20 microscope operating at 200 kV. Electron energy loss spectroscopy was performed using Gatan Imaging Filter Quantum SE 963 fitted with a 2 × 2 k charge-coupled device camera. Low-voltage HAADF-STEM images of cells were acquired at 20 kV on a FEI Magellan XHR 400L FE-SEM equipped with a dedicated STEM detector. Filled CNT samples were deposited from an ethanolic suspension onto lacey carbon copper grids (300 mesh).

Raman spectroscopy was performed using a Horiba Jobin Yvon Raman microscope, exciting using a 632.8 nm laser. Raman mapping was performed in the peripheral lab attached to beamline I18 at the Diamond Light Source synchrotron using a Renishaw InVia Raman microscope using a ×50 objective and exciting at 785 nm.

TGA was performed using a TGA Q5000 IR instrument. About 2 mg of sample were placed into a platinum boat and stabilized for 15 min at room temperature. The sample was then heated under flowing air (20 ml min^{−1}) at 10 °C min^{−1} up to 900 °C.

ICP-MS was conducted using a Thermo Finnigan Element 2. Calibrations were obtained using external standards (a series of standards of known trace element concentrations were prepared and analysed to gain a calibration linear, before the measurement of your samples). Dilutions from 10 p.p.m. Merck transition multi element standards traceable to National Institute of Standards and Technology’s Standard Reference Materials (NIST SRM’s) were used as calibration standards. For a quality check, an external standard was diluted and measured from a dilution of High-Purity Standards 10 p.p.m. ICP-MS-68A and B standard. Sample dilutions were made using a 2% HNO₃ solution, prepared using in-house distilled nitric acid and 18.2 MΩ deionized water.

UV/visible spectroscopy was achieved using a SPECTROstar Nano from BMG LABTECH, equipped with a charge-coupled device detector with a spectral range of 220–1,000 nm. The data were analysed with the MARS Data Analysis Software.

XRF and absorption spectroscopy, and XRF mapping were performed at the Diamond Light Source synchrotron, beamline I18. The X-ray beam is focused with a pair of Kirkpatrick Baez mirrors and the energy tuned by means of a Si double-crystal monochromator. XRF maps were analysed using PyMca⁶².

Purification and opening of CNTs. Chemical vapour deposition Elicarb SWCNTs from Thomas Swan & Co. Ltd. were supplied as dry powder. The as-received material was steam treated to remove carbonaceous impurities and open the ends of the CNTs, following a previously reported protocol¹⁵. In brief, 400 mg of SWCNTs were finely grounded with an agate mortar and pestle and spread on the centre part inside a silica tube of 5 cm in diameter. The silica tube, opened at both ends, was then placed into the alumina tube of the furnace and purged with argon to allow the complete removal of oxygen. Steam was introduced by flowing the incoming argon through a bubbler with hot water, resulting in a mixture of Ar/steam in contact with the sample. The temperature of the furnace was then increased at 20 °C min^{−1} up to 900 °C, and held at this temperature for 4 h. After this period of time the furnace was cooled down at a rate of 10 °C min^{−1} until room temperature in the presence of Ar/steam. Steam has been reported to remove graphitic particles that coat catalytic nanoparticles⁶³. Therefore, the sample was next treated with 6 M HCl to remove any exposed catalytic particles. Finally, the powder was collected by filtration through a 0.2 µm polycarbonate membrane and washed with distilled water until the pH of the filtrate was neutral and dried at

80 °C overnight. Raman A_D/A_G ratio = 4.85. TGA (air) mass loss peak 615 °C, residue remaining = 0.4 wt%.

PbO@SWCNT. PbO (10.0 mg) and SWCNTs (10.0 mg) were ground together, and placed in a quartz tube. The tube was evacuated and sealed. The sample was heated to 918 °C for 10 h, then 858 °C for 10 h. This cycle was repeated and then the sample was cooled to room temperature. The ampule was cut open and the solids sonicated with conc. HCl for 1 h before being collected on a polycarbonate membrane (0.2 µm pores), and washed with copious water. Some grey solids were observed, which were determined to be metallic Pb (likely reduced by graphitic impurities), which were separated by addition of ethanol to the vial. The floating carbonaceous material was pipetted from the surface, while the metallic species sank. After high vacuum drying, 9.3 mg of the product was obtained. The filling was also achieved by heating PbO (60 mg) and CNTs (20 mg) at 1,000 °C for 12 h, giving 17.5 mg of product after work-up. Raman A_D/A_G ratio = 7.43. TGA (air) mass loss peak 565 °C, residue remaining = 15.2 wt%.

BaI₂@SWCNT. SWCNTs (10 mg) and BaI₂ (30 mg) were ground together in a pestle and mortar, before being placed in a quartz tube. The tube was subjected to high vacuum for an hour before being flame sealed. The sample was heated at 5 °C min⁻¹ to 811 °C, and held at that temperature for 12 h. The mixture was then cooled at 1 °C min⁻¹ to 500 °C before allowing to equilibrate with room temperature. The ampoule was opened and water (15 ml) was added to the mixture, which was then sonicated. The CNTs were collected on a polycarbonate membrane (0.2 µm pores), washed with copious water and dried under vacuum, giving 7.5 mg of black powder as the product. Raman A_D/A_G ratio = 4.96. TGA (air) mass loss peaks at 596, 610 °C, residue remaining = 8.9 wt%.

Kr@SWCNT. SWCNTs (26 mg) were ground in a pestle and mortar and then transferred to a 15 cm long silica tube. The tube was carefully evacuated and filled with Kr from a balloon four times. The taps were closed, leaving the tube at around atmospheric pressure, and the bottom of the tube was inserted into liquid nitrogen to the maximum extent possible, leaving room for application of the blowtorch at the constriction point without risk of heating the Dewar. Due to the contraction of the gas, the reduced pressure in the tube enabled easy sealing. The sealed tube was then heated to 700 °C at 5 °C min⁻¹, held for 4 h, and then allowed to cool to ambient temperature. The tube was opened and the contents subjected to vacuum for 1 h, giving a total of 15.7 mg of product. Raman A_D/A_G ratio of starting material = 4.85, product = 6.88. TGA (air) mass loss peak 634 °C, residue remaining = 1.1 wt%.

General method for attachment of maleimide linker to SWCNTs.

Aniline–maleimide linker (0.180 g, 0.518 mmol) was sonicated with SWCNTs (31 mg, 2.59 mmol, 5 equivalents with respect to nanotube carbon atoms) in orthodichlorobenzene (25 ml) for 20 min. Isoamyl nitrite (84 µl, 73 mg, 0.622 mmol) was added and the mixture heated to 100 °C for 24 h. Ethanol (75 ml) was added, and the mixture centrifuged. The supernatant was decanted, and 50 ml EtOH was added, followed by sonication. Centrifugation/supernatant removal was performed two more times with EtOH and two times with water. The SWCNTs were collected on a polycarbonate membrane (0.2 µm pores) and dried under vacuum (51 mg). Raman A_D/A_G ratio of starting material = 4.85, product = 7.53. TGA (air) mass loss peaks 116, 361 °C (organics), 635 °C (CNTs), residue remaining = 0.2 wt%.

General prep for CNT peptide appendage and Fmoc cleavage.

Peptides (1.39 × 10⁻⁵ mol, 1.5 equiv. with respect to maleimide) were dissolved in 5 ml of water/methanol as necessary for solubility. Maleimide-SWCNTs were added (5 mg) and the mixture was sonicated for five minutes, before stirring overnight. The solid was collected by centrifugation and washed on a polycarbonate membrane (0.2 µm pores) with water. The liquid phases were evaporated to recover leftover peptide. The CNTs were then sonicated in 1 ml pH 9.2 borate buffer and placed in the thermomixer overnight at 37 °C. The CNTs were collected by filtration on a polycarbonate membrane, and washed with water. After vacuum drying, the CNTs were sonicated in 2 ml of 5% piperidine in DMF for 20 min. The mixture was centrifuged and the absorption of the supernatant measured at 301 nm to determine Fmoc concentration. Tenfold volume of water was added to the remaining CNTs, which were then collected by membrane filtration, washed with water and dried under high vacuum.

Cell studies. Cells were obtained from an existing HeLa line within the Department of Chemistry at the University of Oxford originally obtained from American Type Culture Collection. The specific line is available on request. When thawing cells from frozen, DMEM media containing 10% FBS and 1% pen/strep solution was used. After the first passage, the pen/strep was not used in subsequent growth steps. Antibiotics were used again when the CNTs were added, since this was performed in a fume hood under non-sterile conditions to be as close as possible to the sonicator. CNTs were administered at 1 mg ml⁻¹ after thorough

sonication in tissue culture-grade water. The suspension was added to cells in a ninefold volume of media to give a 100 µg ml⁻¹ final concentration. After incubation with CNTs, a PBS wash was performed before any analysis. Cells were tested for mycoplasma infection on a quarterly basis using Lonza MycoAlert Mycoplasma Detection Kit.

MTS assay. The effect of filled and decorated SWCNTs upon cell viability was assessed using CellTiter 96 Aqueous One Solution Cell Proliferation Assay from Promega, following the manufacturer's instructions. In brief, cells were seeded in a 96-well plate at a density of 10,000 per well, and permitted 2 h to adhere. The cells were washed with PBS and the media exchanged for 100 µl of media containing SWCNTs at 50 and 100 µg ml⁻¹ (three replicates per SWCNT sample). The cells were incubated for 24 h before washing and replacement of the media with that containing the MTS reagent. After 2 h incubation, absorption at 490 nm (which is approximately proportional to cell viability) was measured with a well plate reader. Control samples were cells untreated with CNTs, untreated cells killed with 10% DMSO, and 100 µg ml⁻¹ SWCNTs in media without any cells. The latter sample gave the same absorbance as the dead cells, indicating at SWCNT optical absorption has a negligible effect upon the assay.

Preparation of sapphire discs for XRF/Raman imaging of cells. Sapphire discs were used as a mount for both XRF and Raman imaging because they give neither XRF or Raman signals that overlap with areas of interest (pre-bought tissue culture treated coverslips have intense Raman signals close to the CNT G-band, as well as elemental impurities). After autoclaving, one side of the slides was made suitable for growth of adherent cells by coating in poly-D-lysine hydrobromide. Following the manufacturer's instructions, 50 ml of tissue culture-grade water was added to poly-D-lysine (5 mg) provided in a septum-capped bottle, followed by vortexing to ensure solution. The discs were laid out in Petri dish and enough of the solution was added to ensure complete coverage. After 5 min, the solution was removed by pipette and the discs were washed three times with tissue culture-grade water (theoretically removing the bromide), after which they were left to dry for 2 h in a sterile environment. The discs were then stored in an air-tight container until use. For cell culture on the discs, they were placed with the treated side up in a 24-well plate. Cells were plated at 30,000 per well in 0.6 ml of media. The cells were given 2–4 h to adhere, before the media was swapped for that containing CNTs well-sonicated (at least 30 min) in media at a concentration of 100 µg ml⁻¹. After incubation for 24 h (typically giving a confluency of 60%), the cells were washed with PBS twice, and fixed as follows. A 2.5% solution of glutaraldehyde in PBS (1 ml) was added to the cells and they were incubated for 1 h at room temperature. Next, the cells were washed with 2 × PBS and 2 × Milli-Q water, with 2 min permitted for diffusion in each wash. The cells were then dehydrated by incubation in 50% EtOH for 10 min, followed by 70, 90, and 95%, and then 100% EtOH for 20 min three times. After removal of the ethanol, hexamethyldisilazine was added and incubated for exactly 3 min. The hexamethyldisilazine was removed and the samples were placed in a vacuum desiccator overnight to ensure removal of all volatiles.

Raman imaging. Raman mapping was performed using a 50 × objective and exciting at 785 nm. Due to problems of drift and sample burnout (due to photothermal excitation of CNTs), the best maps were collected quickly—larger, multicell maps at higher resolutions with full spectra tended to become distorted and/or burnt, wasting many hours and destroying samples. In contrast, by mapping one cell at a time, using 100% laser power (785 nm) but moving quickly with half second collection times, 2 µm resolution and looking at only the region around the G-band (centred on 1,585 cm⁻¹) a single cell could be mapped with sufficient resolution to assess the distribution of CNTs. Cells were no more than ~70% confluent, giving them a more spread-out morphology. The sapphire discs were simply placed on a microscope slide, without further efforts to fix using glue or tape, each of which resulted in drift errors. For data analysis using WiRE 3.4, signal-to-baseline (1,500–1,650 cm⁻¹) quantification was used, since the baseline varied significantly from point to point.

Preparation of cell samples for STEM imaging. Samples for TEM were prepared on 13 mm circular glass Thermanox coverslips that had been pre-treated for tissue culture. After cell plating and dosing as above, the cells were fixed with freshly made pre-warmed 2.5% glutaraldehyde, 2% PFA in 0.1M PIPES buffer. Three to five washes of 15–30 min with 0.1 M PIPES containing 50 mM glycine was followed by incubation with 1% osmium tetroxide in PIPES at 4 °C for 1 h. After washing again with Milli-Q water, the samples were incubated with 0.5% uranyl acetate at 4 °C overnight in the dark, followed by further rinsing. The cells were dried using progressively higher ethanol concentrations as described above. Epoxy resin infiltration was achieved by incubation with 3:1 EtOH:Agar100 resin for 1 h, then a 1:1 mixture for 2–3 h and 1:3 for 1 h with rotation and finally with 100% Agar100 overnight and with two further changes in 24 h. The agar pieces were transferred to BEEM capsules filled with 100% Agar100 resin and were polymerized for 1–2 days in the 60 °C oven. Sections of 70 nm were then cut using an ultramicrotome. Four sections were taken from each sample, and 30–40 images were collected from a representative range of areas.

XRF imaging. XRF excitation energies were selected after initial testing to optimize visibility of the relevant elements. In particular, the lowest possible energy to give a good emission peak of the xenobiotic element (15 keV for Kr, 14 keV for Pb). A second scan of with an energy of 5 keV was required to observe the low-Z biological elements clearly if the first scan was > 10 keV. At 5 keV, the cells appeared to shrink during the course of the collection, so the lower-energy scan was performed after the higher energy. A beam size of $2.0\text{ (v)} \times 2.3\text{ (h)}\text{ }\mu\text{m}$ was scanned over the sample at 1 μm intervals, with an exposure time of 1 s per pixel. After the data collection, the total spectrum of the measured area was fitted to the relevant elements (a process that includes comprehensive deconvolution) using PyMca, and the particular settings saved in a configuration file. This was then checked against individual spots to ensure applicability. The data was then processed to obtain net XRF peak areas for all elements of interest at each map pixel.

Supplementary Methods detail the assessment of SWCNT end-sealing; synthesis of the maleimide linker (with corresponding NMR spectra presented in Supplementary Figs 11–15), peptide synthesis (with high-performance liquid chromatography traces and MS shown in Supplementary Figs 16–19), filling-specific details for maleimide decoration and in-depth XRF parameters; (Supplementary Table 5).

Data availability. The authors declare that all the data supporting the findings of this study (post processing) are available within the article and its Supplementary Information files. Unprocessed data relating to this manuscript are available from the corresponding author upon request and XRF, TEM, Raman, XPS, TGA, NMR and microscopy data have been deposited on the Oxford database ORA-data at doi: 10.5287/bodleian:9R2Xr7BAY.

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Author contributions

The project was conceived by B.G.D. and C.J.S. C.J.S. synthesized and characterized the constructs, C.J.S., R.R. and K.W. conducted the tissue culture; C.J.S. performed the associated experiments. CNT starting materials were provided by M.M. and G.T. K.G. provided support and consultation from the beamline. R.N.R. assisted C.J.S. with peptide synthesis. Electron microscopy studies were performed by R.N.R., E.P. and B.B. M.K. conducted TGA measurements. XRF and Raman mapping data were collected by C.J.S., R.N.R., S.D.M., K.W. and R.R. C.J.S., G.T., D.C.A. and B.G.D. analysed the data. All authors read, commented on and approved the manuscript.

Additional information

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