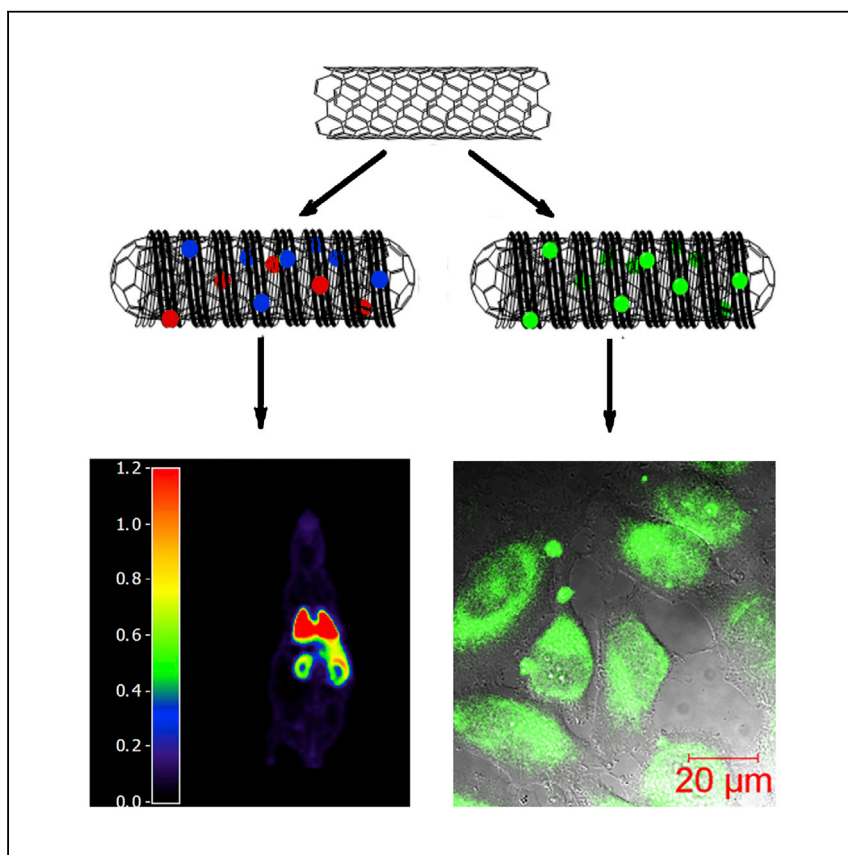


Article

Behavior of Supramolecular Assemblies of Radiometal-Filled and Fluorescent Carbon Nanocapsules *In Vitro* and *In Vivo*

Pascu and colleagues have designed a general, non-covalent directed self-assembly procedure to obtain radioactive and/or luminescent biocompatible carbon-based nanohybrids. They were able to fill carbon-nanocapsule-based tubular structures with the radionuclide of choice in water. Nanoproductions were investigated by UV-vis, fluorescence, Raman, NMR, and EDX spectroscopy; SEM, TEM, AFM, and confocal and two-photon fluorescence microscopy; and cytotoxicity assays. Furthermore, *in vivo* microPET imaging in Wistar rats showed localization in the lung and kidney.

Haobo Ge, Patrick J. Riss, Vincenzo Mirabello, ..., Jonathan R. Dilworth, Franklin I. Aigbirhio, Sofia I. Pascu

fia20@wbic.cam.ac.uk (F.I.A.)
s.pascu@bath.ac.uk (S.I.P.)

HIGHLIGHTS

Hierarchical functionalization of SWNTs by self-assembled processes in water

Rapid and high kinetic stability encapsulation of ^{64}Cu ions from water

Dynamic microPET imaging in Wistar rats shows almost exclusive lung biolocalization

Imaging and cell-viability tests confirm kinetic stability and biocompatibility

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Article

Behavior of Supramolecular Assemblies of Radiometal-Filled and Fluorescent Carbon Nanocapsules *In Vitro* and *In Vivo*

Haobo Ge,^{1,8} Patrick J. Riss,^{4,8} Vincenzo Mirabello,^{1,8} David G. Calatayud,^{1,3} Stephen E. Flower,¹ Rory L. Arrowsmith,¹ Tim D. Fryer,⁴ Young Hong,⁴ Steve Sawiak,⁴ Robert M.J. Jacobs,⁵ Stanley W. Botchway,⁷ Rex M. Tyrrell,^{1,2} Tony D. James,¹ John S. Fossey,⁶ Jonathan R. Dilworth,⁵ Franklin I. Aigbirhio,^{4,*} and Sofia I. Pascu^{1,9,*}

SUMMARY

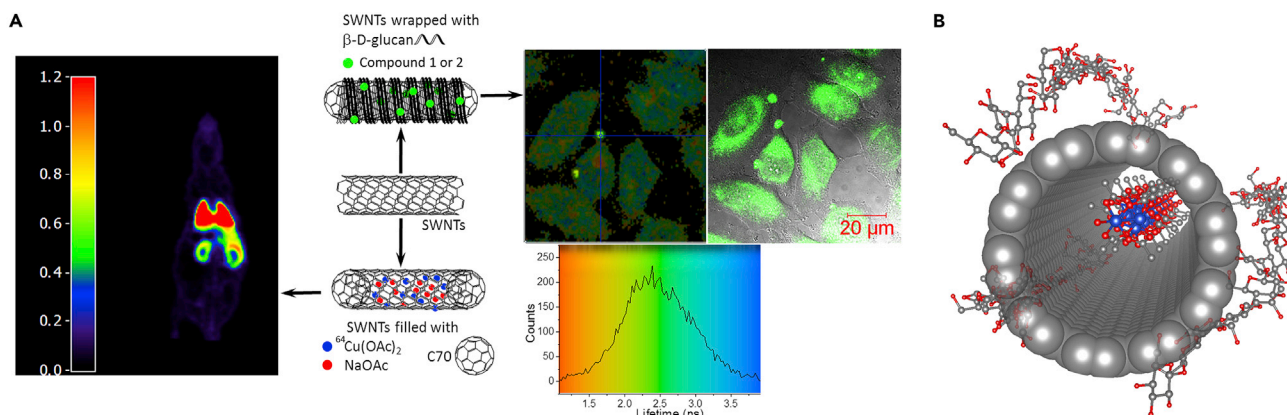
Hybrid materials based on supramolecularly assembled single-walled carbon nanotubes (SWNTs) are generated for positron emission tomography (PET), magnetic resonance imaging, and fluorescence imaging. The all-in-one imaging probe allows quantitative imaging from subcellular resolution to whole tissue regions. The SWNTs can be exposed to aqueous solutions of non-radioactive and radioactive metal salts in the presence of fullerenes and β -D-glucan. Encapsulating ^{64}Cu ions achieves a minimum of 69% incorporation of radiochemical. The results suggest that this method can be extended to other metal ions of medical relevance, such as zirconium(IV)-89 or rhenium(VII)-188, which are used for medical imaging or radiotherapy, respectively. The *in vivo* uptake of $^{64}\text{Cu}(\text{II})@\text{SWNT}@\beta\text{-D-glucan}$ in Wistar rats allows the investigation of organ bio-distribution by microPET. Radioactivity rapidly accumulates predominantly in the lungs and myocardium with peak uptakes of 4.8 ± 0.9 standardized uptake value. Furthermore, such materials are fully traceable in cells by multiphoton fluorescence lifetime imaging with near-infrared excitation (910 nm).

INTRODUCTION

The ^{64}Cu radioisotope is attracting considerable interest for use in both imaging and therapy,¹ because of its decay pathways involving both beta (0.579 MeV, 38%) and positron emissions (0.653 MeV, 18%) and 12.7-hr half-life. Copper is an essential biological element, and highly efficient copper sequestering agents abound in living systems.² The challenge in developing ^{64}Cu radiopharmaceuticals is to find a rapid and mild encapsulation method that will be kinetically stable *in vivo* as well as *in vitro* (Scheme 1). Any loss of copper will lead to the biodistribution being dominated by biological copper metabolic pathways.^{3,4} Ligands such as cross-bridged cyclams⁵ and cryptands⁶ are, to date, the most promising. Here, we report the potential of modified single-walled nanotubes (SWNTs) to both contain and deliver radiocopper in an aqueous environment as a superior alternative to chelators. Several allotropes of carbon such as fullerenes, carbon nanotubes (CNTs), graphene, and graphene-based functionalized derivatives (graphene oxides) have garnered interest in recent years.⁷ Our recent work in decorating carbon nanomaterials with biocompatible materials such as naphthylidiimides and porphyrin oligomers constituted a proof-of-principle study on how understanding and design at the molecular level could help develop new diagnostics or therapeutics.⁸ The possibilities of using carbon

The Bigger Picture

This work is an important step toward the biomedical implementation of functional materials of relevance to the radiopharmaceutical industry, nanomedicine, and early detection of cancers. We found that carbon nanotubes have the ability to redirect the trajectory of radiochemicals, which can lead to the *in vivo* imaging of the lung. This research supports the hypothesis that it is possible to use cutting-edge imaging techniques to develop biocompatible materials for sensing and imaging within the field of radiodiagnostics. Previous studies have shown that carbon nanotubes have high *in vivo* toxicity. The conceptual advance of our findings is that they can also act as scaffolds for the encapsulation of positron emitting radioisotopes in water, and their subsequent supramolecular wrapping leads to a diminished toxicity. The broader significance is that this work provides a simple and rapid approach to nanomedicine in water and addresses significant radiosynthetic and imaging challenges.



Scheme 1. Filling and Wrapping of SWNTs and Their Applications in microPET and Optical Imaging

(A) Schematic representation of the synergetic and/or fluorescent self-assembly synthetic approaches leading to the formation of the radiolabeled nanocarbon complexes in water for *in vivo* (microPET) as well as *in vitro* imaging by multi-photon fluorescent spectroscopies.

(B) Optimized structure of (10:10) SWNT with simple strands of glucan outside and $\text{Cu}(\text{OAc})_2$ nanocrystals encapsulated within the nanotube. The optimization was carried out with MM+ (Forcite) in Materials Studio and VESTA software (<http://jp-minerals.org/vesta/en/download.html>). The size of the $\text{Cu}(\text{OAc})_2$ was estimated from single-crystal X-ray diffraction data, taking into account its confinement inside the SWNT cavity.

nanotubes for biomedical applications are currently a highly active area of research.⁹ However, design protocols leading to smart nanotheranostics for future sensing and imaging application relying solely on the synthetic advantages offered by molecular recognition principles have not been previously reported. We describe our conceptual advances provided by recent directed- and self-assembly studies in water, supported by high-resolution transmission electron microscopy (HRTEM), surface analysis techniques such as atomic force microscopy (AFM), and molecular imaging on the nano- and microscale.

A variety of approaches have been adopted for the radiolabeling of carbon-based nanotubes, generally under conditions that are difficult to control under radiochemical conditions or involving significant loss of the radioactive tag in aqueous media. One approach involves the covalent attachment of a metal-specific chelating ligand system to the nanotube followed by standard radiolabeling with an appropriate radioisotope.¹⁰ This technique has been applied for decorating the exterior of SWNTs with single-photon emission computed tomography (SPECT), positron emission spectroscopy (PET), or therapeutic radionuclides, ^{57}Co ($t_{1/2} = 271.8$ days),¹¹ ^{64}Cu ($t_{1/2} = 12.70$ hr),¹² ^{86}Y ($t_{1/2} = 14.70$ hr),¹³ ^{89}Zr ($t_{1/2} = 78.42$ hr),¹⁴ $^{99\text{m}}\text{Tc}$ ($t_{1/2} = 6.00$ hr),¹⁵ ^{111}In ($t_{1/2} = 67.24$ hr),¹⁶ ^{235}Ac ($t_{1/2} = 10.0$ days).¹⁴ The first report of the filling of nanotubes by capillary absorption typically used molten salts at high temperatures, and this permitted the incorporation of salts of metals such as lanthanides,¹⁷ K or Ca,¹⁸ and Zr.¹⁹ However, this requires temperatures in excess of 300°C and extended reaction times; therefore, it is not generally appropriate for radiopharmaceutical applications, where aqueous media and mild conditions are often required. There is one report of the filling of SWNTs with “cold” metals (metals that do not generate radioactive decay), such as Cu, from aqueous solution. Fullerenes were used as stoppers to prevent leaching of the metal from the nanotube cavity. But the filling yield cannot be reliably estimated, and no kinetic stability evaluation or *in vivo* tracing has been shown thus far.²⁰ There have been no reports, to the best of our knowledge, on the filling of SWNTs with radioisotopes in water or on the applications of self- and directed-assembly in the construction of CNT-based hybrids tailored toward imaging and therapeutic radionanomedicines.

¹Department of Chemistry, University of Bath, Bath BA2 7AY, UK

²Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK

³Department of Electroceramics, Instituto de Ceramica y Vidrio, Kelsen 5, Campus de Cantoblanco, 28049 Madrid, Spain

⁴Wolfson Brain Imaging Centre, Addenbrooke's Hospital, Box 65, Cambridge CB2 0QQ, UK

⁵Chemistry Research Laboratory, Department of Chemistry, University of Oxford, 12 Mansfield Road, Oxford OX1 3TA, UK

⁶School of Chemistry, University of Birmingham, Edgbaston, Birmingham, West Midlands B15 2TT, UK

⁷Central Laser Facility, Rutherford Appleton Laboratory, Research Complex at Harwell, Science & Technology Facilities Council, Didcot OX11 0QX, UK

⁸These authors contributed equally

⁹Lead Contact

*Correspondence: fia20@wbic.cam.ac.uk (F.I.A.), s.pascu@bath.ac.uk (S.I.P.)

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The biocompatibility and cytotoxicity of carbon nanomaterials overall remain an issue of much debate.^{21–23} However, if engineered appropriately, pristine, opened, and shortened SWNTs offer an exceptional advantage as potential synthetic nanocarriers for radioactivity delivery, because it is possible to fill their interior space^{24–28} with a high dose of therapeutic or diagnostic radionuclides, and, in the case of PET or SPECT radiotracers, without attenuating their emitted gamma radiation. Here, we report the endohedral inclusion of ^{64}Cu in SWNTs from aqueous solutions and also the encapsulation of cold KReO_4 salt as a model for hot $^{188}\text{ReO}_4^-$ ions incorporation (^{188}Re half-life, $t_{1/2} = 17.0$ hr).

As-made, pristine SWNTs are not suitable for biomedical applications and require derivatization to increase biocompatibility and facilitate their dispersion in water. This has been achieved previously by covalent modification with multiple carboxyls, amines, or hydroxyl groups, but in the context of radiochemistry followed by *in vivo* imaging protocols, this is time consuming. The alternative is to wrap nanotubes in biocompatible polymers. A range of molecules have been used to help solubilize SWNTs, including glycosylated proteins,¹² carbohydrates,²⁹ polyethylene glycols,¹² and hyaluronates³⁰ but these approaches have not been evaluated in radionanomedicines.

Certain glucans have been reported to encapsulate SWNTs.^{31–33} Here, we report the use of readily accessible and sustainable β -D-glucan from barley for the recognition and simultaneous functionalization of SWNTs in water for use in biosensing and *in vivo*.

For functional imaging or therapeutic use, the attachment of a targeting vector is a prerequisite.³⁴ Although some bioactive molecules such as doxorubicin¹⁵ can be bound directly to the nanotube surface via strong hydrophobic interactions, there is only one current report on the attachment of fluorophore boronic acid to a nanotube-encapsulating glucan giving rise to a hierarchical material.³⁵

Furthermore, the intrinsic fluorescence of the carbon nanohybrids does not give sufficiently intense emission in the near-infrared (IR) region to trace the probes in cells. Therefore, two different dyes (1 and 2) incorporating fluorescein boronic acid were used to tag the exterior of the SWNT@ β -D-glucan assembly via boronic ester recognition of either 1,2- or 1,3-diols available on the surface of the glucan-wrapped supramolecular aggregate. Here, we used molecular recognition in solution as the assembly technique for the SWNT-based radionanoprobe designed, as well as cellular-based, fluorescence lifetime imaging microscopy (FLIM) techniques to assess the integrity of the resulting supramolecular composites in cells.^{36,37} This enabled tracing of the SWNT@ β -D-glucan@fluorophore system in a range of living cells, recording both confocal fluorescence images and two-photon induced fluorescence lifetime maps aiming to probe whether or not the nanohybrid system remains essentially intact in cells over the timescale of imaging methods *in vivo* and *in vitro*.^{38,39}

The behavior of the emergent supramolecular nanoprobes in living systems was imaged, and we also report here on the stability of the ^{64}Cu radiolabeled wrapped SWNTs systems in several different cell lines (human prostate cancer PC-3, Chinese hamster ovary [CHO], and healthy human primary skin [FEK4] cell lines). Our radioprobe nanohybrid of SWNTs was also injected into Wistar rats, and their potential for *in vivo* imaging was evaluated via microPET. The aim of most nanomedicine evaluations to date has been to derive and predict possible radiopharmaceutical

applications on the basis of the behavior in cells; however, microPET imaging is the ultimate reliable guide to *in vivo* behavior. We have therefore used PET imaging of $^{64}\text{Cu}@ \text{SWNT} @ \beta\text{-D-glucan}$ in a normal Wistar rat model coupled to metabolite studies in blood and compared the biodistribution with that of the simple $^{64}\text{Cu}(\text{OAc})_2$ salt. This approach also allows a detailed understanding of the surprisingly high kinetic stability of such supramolecular interactions in solution or the dispersed phase, as well as in the biological environment.

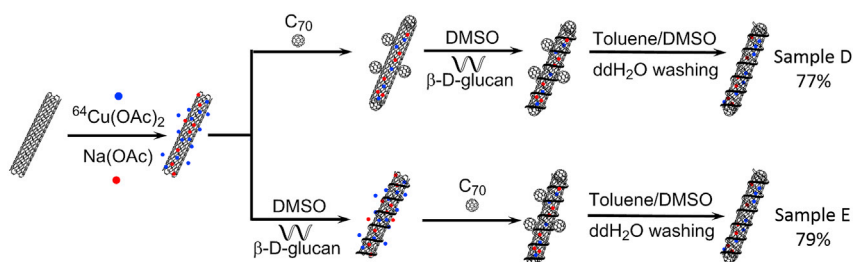
RESULTS AND DISCUSSION

There is an urgent and unmet clinical need for the development of rapid and efficient radiolabeling protocols, under mild conditions and in aqueous environments, suitable for manipulation within the regulatory and technical confines of most radiopharmaceutical laboratories. The main design criteria for any radioprobe rely on elements that include irreversibly incorporating the radionuclide while giving a kinetically stable product. We implemented a rapid, validated protocol commonly used for good manufacturing practice (GMP) radiosynthesis for constructing a nano-radiotracer assembled solely in aqueous media. Furthermore, the *in vivo* and *in vitro* functional behavior of our SWNT supramolecular nanoprobe as an intact object is explored.

SWNTs have attracted interest in the context of nuclear radiation delivery *in vivo* because of their attractive physical and chemical properties.^{40–45} The use of covalently functionalized SWNTs has already been explored,^{34,46,47} but the concept of using such materials as scaffolds in nanomedicine presents several recognized challenges.^{48–50} In this report, the scaffold of choice is a less than 500-nm long pristine CNT obtained by a top-down approach using a well-known steam purification method from SWNTs followed by the self-assembly of an all-in-one imaging probe (bottom-up approach) from molecular components, including radioactive metal species such as $^{64}\text{CuCl}_2$ (in the presence of NaOAc) and/or their complexes such as $^{64}\text{Cu}(\text{OAc})_2$. Its use eliminates the risk of release of the free radionuclide after administration.

A two-pronged approach was adopted to trace the supramolecular assemblies in cells and living organisms. Firstly, we show here that metal-filled SWNTs, which display high stability (with respect to metal loss), can be assembled rapidly in water, and it is this feature that makes them ideal for use in radioactive nanomedicine. We hypothesize that “empty” vehicles will behave in a largely similar manner to the corresponding cargo-laden vehicle, i.e., one can synthesize, characterize, and study radionuclide-free carbon tubes with minimum radiation exposure, followed by the rapid encapsulation of the radionuclide of choice, for therapeutic or diagnostic application. With appropriate formulation, such radioactive nanoprobe will give rise to future radiopharmaceuticals if the hypothesis holds true, and the payload of a filled nanotube does not affect the overall pharmacokinetics and biodistribution *in vivo*. Secondly, we hypothesize that it is possible to minimize or even eliminate the loss of encapsulated radionuclide to non-specific sites by using fullerenes of matching sizes, acting as stoppers for SWNTs of varying diameters. Such rapid and mild supramolecular derivatizations were carried out entirely in aqueous media.

Our synthetic protocol relies on the use of open, empty, and shortened SWNTs with lengths around 50–500 nm and diameters up to 2 nm, as determined by HRTEM and AFM. We found that, with the exception of the steam purification method used here, most other reported methods using fluorine, nitric acid, or hydrogen peroxide⁵¹



Scheme 2. Schematic Representation of the Different Synthetic Approaches Leading to the Formation of the Radiolabeled Nanocarbon Complexes

Conditions: 2 mg/mL glucan in DMSO, 0.5 mg/mL SWNT in H₂O, 50 μ L $^{64}\text{Cu}(\text{OAc})_2$, ca. 10^{-9} M copper; 10 mg NaOAc, 0.667 mg C₇₀ suspended in 1 mL H₂O for each sample. Representation of radiotracer preparation samples A–E emerging from corresponding methods denoted routes A–E are given in Scheme S3. Percentages refer to the decay-corrected ^{64}Cu ion incorporation.

caused extensive damage to the SWNT tubular structure, limiting the scope of radio metallation as a result of extensive leaking of the radioagent.^{52–56}

The steam purification method devised by Green et al.^{55,56} and scaled up by Thomas Swann Ltd. is acknowledged to give rise to shortened open nanotubes with high batch-to-batch purification reproducibility and sample consistency, as indicated by extensive HRTEM analysis and Raman spectroscopy studies. Imaging clearly showed that amorphous carbon and graphitic particles entangling the as-made SWNTs were successfully removed leaving behind pristine SWNT surfaces ready for functionalization. Such methods give rise to highly purified, pristine CNTs, containing only SWNT strands (when arc-made Carbolex-quality samples are used) but also SWNTs mixed with traces of the wider-diameter double-walled CNTs (when CVD-made Elicarb Thomas Swann Ltd. samples are used as the starting material). In both cases, pristine SWNTs with dimensions ranging between 50 and 500 nm are known to be obtained and have been used in this work.

Therefore, three distinct stages were involved in the formation of radiocopper-SWNT constructs, all relying on supramolecular chemistry in water: (1) filling and consequent endohedral radiolabeling of the tubes, (2) wrapping, and (3) external functionalization. These steps are summarized in Scheme 2, whereby (Elicarb) SWNTs with open ends from steam purification⁵⁵ were exposed to aqueous solutions of simple metal salts, such as CuX_2 ($\text{X} = \text{Br}^-$, Cl^- , or OAc^-) and their radioactive ionic species (i.e., aqueous $^{64}\text{Cu}^{2+}$ ions), with the capability of forming cation- π interactions with the aromatic network of the pristine SWNTs upon trapping, as well as being encapsulated within the cavity as a part of an entire complex nanocrystal, when formulated as the analogs $^{64}\text{CuCl}_2$ and/or $^{64}\text{Cu}(\text{OAc})_2$ (the latter species is likely to be generated *in situ* when excess NaOAc is used as the filling support; Table 1).

In addition to ^{64}Cu ($t_{1/2} = 12.7$ hr), this method is applicable to the encapsulation of other imaging and therapeutic metallodrugs, including radioisotopes such as ^{89}Zr (PET radiotracer, $t_{1/2} = 78.4$ hr). The filled tubes can be used as building blocks encased within inert carriers by rapid and simple chemistry based on non-covalent interactions, leading to the general construction of nanomedicines under GMP conditions. The use of NaOAc in carrier-added experiments is standard in PET tracer preparation and is deemed not to hinder the *in vivo* imaging methods, because the pH is normally adjusted to 8 in the final tracer formulation before *in vivo* work.

Table 1. Decay-Corrected ^{64}Cu Incorporation into SWNT@ β -D-Glucan Nanohybrid

Route ^a	Decay-Corrected ^{64}Cu Incorporation (%)
A	43.5
B	69.0
C	28.0
D	77.0
E	79.0

Decay-corrected ^{64}Cu incorporation into SWNT@ β -D-glucan complex via the encapsulation reaction routes A–E; $A = A_0 \times e^{-kt}$, $k = \ln 2/12.7$, $\text{hr} = 0.0546$ hr.

^aSee [Supplemental Information](#) for experimental details and schemes.

For cold (non-radioactive) Cu^{2+} @SWNT composites, obtained by filling with aqueous $\text{Cu}(\text{OAc})_2$, and for proof of concept also with CuBr_2 or CuCl_2 (in preliminary work), HRTEM analysis showed encapsulation in the form of short crystals seen inside the SWNTs ([Figure 1](#)). As a comparison, in the attempt to probe the possibility of using a different source of copper(II) as well as zinc(II), we also performed filling experiments of SWNTs with CuBr_2 and ZnI_2 via a molten salt route. Similar yields (estimated by HRTEM) were obtained only after ca. 8 hr heating at 400°C , which is well in accordance with literature reports.^{19,57,58}

A supramolecular assembly in water was therefore used for the encapsulation of ^{64}Cu ([Scheme 2](#) and [Table 1](#)). The supramolecular encapsulation of any radionuclide from aqueous media at low radiotracer concentration (10^{-9} M) proceeds under subtle kinetic control. The ^{64}Cu radiolabeling of SWNTs in water proceeded under the standard, no-carrier-added conditions whereby a saturated solution of NaOAc was used as an adjuvant in order to improve the yield of SWNT filling. NaOAc was deliberately chosen because it is also used in *in vivo* experiments to adjust the pH to 8 in the tracer formulation before *in vivo* imaging.^{59,60}

Yudasaka et al.⁶¹ observed that leakage from metal-filled but opened SWNTs could be attenuated by capping or co-filling with fullerene stoppers, which are irreversibly entrapped inside SWNTs in aqueous media by standard endohedral encapsulation. Here, we used simultaneous radioactivity trapping and encapsulation of C_{70} . By measuring ^{64}Cu leakage in the presence or absence of capping C_{70} , and comparing it with the use of C_{60} , we found that, because of its mean diameter of 7.4 ± 0.5 Å, C_{70} acted as a good match for the carbon vapor deposition (CVD) (pristine) SWNTs (1.02–1.7 nm) used to prevent leakage, whereas C_{60} was somewhat more suitable for arc-made (Rice Carbolex variety) tubes (1.6–1.7 nm). The Elicarb-CVD-made CNT samples contain a wider range of diameters, including some double-walled carbon nanotubes (DWNTs), which have an internal diameter of 2–4 nm, as observed by extensive HRTEM imaging of a number of batches used for the CVD-made steam-purified SWNTs used here. Subsequently, metal salt encapsulation was demonstrated by HRTEM in the form of short crystals visible inside the SWNTs ([Figure 1](#) and [Supplemental Information](#)). A disordered string of C_{70} molecules with interfullerene spacing within C_{70} @SWNT estimated to be ca. 11 Å was visible by HRTEM. These C_{70} molecules act as a plug for the open ends of the appropriate diameter SWNTs only, sealing the inner SWNT cavity in aqueous media. In our experiments, the excess C_{70} was removed by rapid extraction using cold toluene followed by phase separation and membrane microfiltration (pore size 2 μm) of the aqueous phase. The remaining solid suspension was then repeatedly washed with milliQ purified water followed by repeated filtration. Radio-thin-layer chromatography (TLC) analysis was then used to validate the absence of free ^{64}Cu species in the filtrate

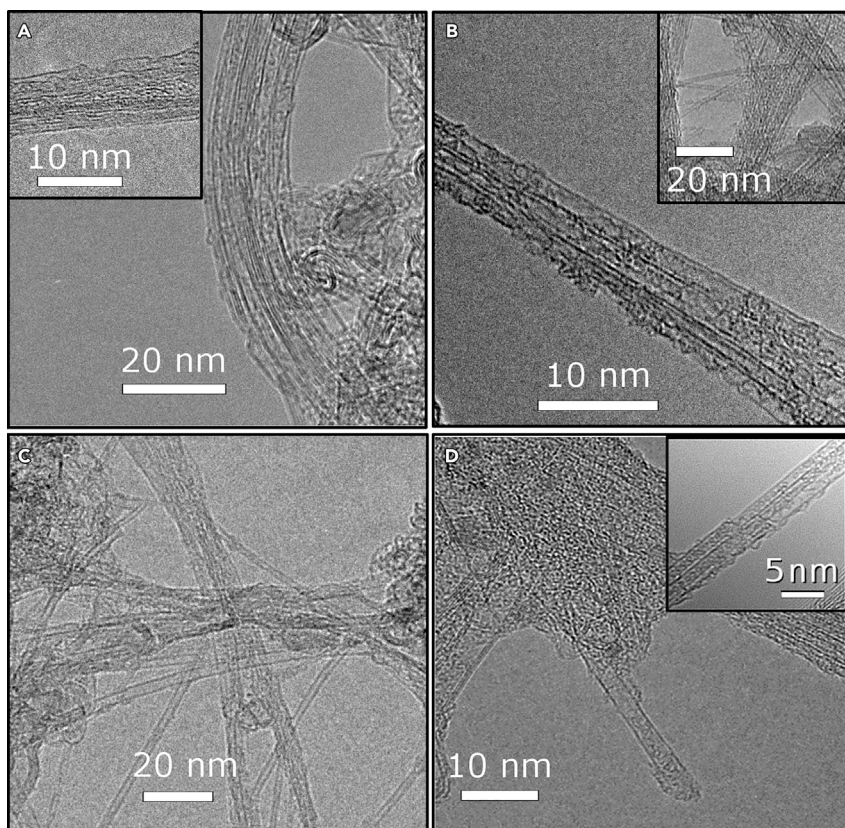


Figure 1. HRTEM Micrographs of the SWNT Nanohybrids

Typical HRTEM micrographs of (A) CuBr_2 @SWNT filled from the molten state method, followed by washing and filtration (inset: high-magnification micrograph showing an alternative image of CuBr_2 @SWNT); (B) CuBr_2 @SWNT filled from the molten-state method, followed by (1) mild aqueous-phase wash and (2) β -D-glucan wrapping protocols (inset shows an image of the pristine, steam-purified CVD SWNTs used); (C) $\text{Cu}(\text{OAc})_2$ @SWNT solution filling, followed by mild aqueous-phase wash; and (D) CuBr_2 @SWNT@ C_{70} from CuBr_2 and C_{70} solution filling experiments (inset: high-magnification micrograph showing two CuBr_2 @SWNT@ C_{70} strands where encapsulated C_{70} units can be observed). All filling experiments were repeated at least three times. Alternative HRTEM micrographs are given in [Supplemental Information](#).

(solution) phase. The optimized radiochemical incorporation for the radiometal was in the range of 69% ([Table 1](#)), and the filtrate showed by radio-high-performance liquid chromatography (HPLC) that the free, un-encapsulated $^{64}\text{Cu}(\text{OAc})_2$ could be recoverable.

After the redispersion of filled solids in aqueous media, no leaking of ^{64}Cu was observed over the course of 4 hr as monitored by radio-TLC and radio-HPLC. To investigate filling yields of pristine SWNTs, filling experiments were also performed with CuBr_2 , CuCl_2 , and ZnI_2 via the molten salt route. Samples were prepared by heating at 400°C for ca. 8 hr ([Supplemental Information](#)).^{19,57,58} These methods generated similar filling yields to those emerging from solution filling and C_{70} capping (estimated by HRTEM to be ca. 60%) for all salts investigated, so there was no apparent practical merit in using the molten salt route in radiochemical experiments.

The HRTEM analysis of MX_2 @SWNT composites emerging from molten salts procedures (for $\text{M} = \text{Cu}(\text{II})$, $\text{X} = \text{Cl}$, Br , and $\text{M} = \text{Zn}^{2+}$, $\text{X} = \text{I}$) as well as from solution filling

showed clean encapsulation simultaneously with the capping of SWNTs ends. The radionanohybrid denoted $^{64}\text{Cu}@\text{SWNT}@\beta\text{-D-glucan}$ was assembled via several alternative routes ([Supplemental Information](#)). To establish the most viable radiosynthesis protocol applicable to current practices in radiochemistry, the cold alternatives were prepared under identical conditions, and detailed microscopy investigations were undertaken (see [Supplemental Information](#) for details regarding the synthesis and characterization of the products).

Rapid supramolecular functionalization of radiolabeled SWNTs from water was achieved at the latest possible stage of the radiosynthesis protocol. This method generates well-dispersed radiotracer samples, which are biocompatible. In order to improve the dispersibility of the nanoradiotracer in aqueous media, wrapping of the outer tubular structure via non-covalent interactions with a little-studied $\beta\text{-D-glucan}$, from barley, which (unlike the yeast varieties) is fully water soluble, was used. This ensured that the intrinsic properties of the tubular aromatic system are largely preserved, and the complete and uniform coating of SWNTs with surfactants occurs rapidly, generally after less than 1 hr exposure time.^{62,63}

It is acknowledged generally that $\beta\text{-D-glucans}$ and their congeners have antitumor and antimicrobial activities *in vivo* through beneficial activation of endogenous mechanisms,^{64–66} so we do not anticipate any deleterious effects from their use as radiochemical adjuvants. The supramolecular wrapping of SWNTs by $\beta\text{-D-glucan}$ relies on a combination of hydrogen bonding (in DMSO or non-aqueous environments) or hydrophobic interactions (in aqueous media). Hydrogen bonding and hydrophobic interactions are well established as the main driving forces for glucans' self-organization into helical structures.^{67–69} Hydrophobic cavities of glucan are likely formed in solution in the presence of the hydrophobic surface of the SWNTs acting as a templating guest. This non-covalent interaction between the internal cavities of $\beta\text{-D-glucan}$ and the outer walls of SWNTs give rise to cylindrical shapes and flexible dimensions, perfectly adjustable to fit the circumference of SWNTs, without affecting the structure of the nanotubes. First, the $\text{SWNT}@\beta\text{-D-glucan}$ complex was prepared by mixing, in a 1:1 ratio, a solution of $\beta\text{-D-glucan}$ from barley (1 mg/mL), dissolved in DMSO, with an aqueous dispersion of the CNT (1 mg/mL) and incubating the mixture at 40°C, with either stirring or a mild periodic sonication (2 min sonication followed by a 5 min pause) over 1 hr. After isolation of the $\text{SWNT}@\beta\text{-D-glucan}$ (by filtration over a 2 μm membrane filter), the resulting composite showed excellent stability over 24 hr at 0.5 mg/mL concentrations, without precipitation in a variety of aqueous solutions (water, PBS, and Eagle's minimum essential medium [EMEM] or DMEM).

The radiotracer denoted $^{64}\text{Cu}@\text{SWNT}@\beta\text{-D-glucan}$ formed in the final stages of the radiosynthesis was collected by filtration over a membrane filter (Cyclopore, 2 μm) and re-suspended in either PBS or H_2O at ca. 1 mg/mL estimated concentration. The procedure was repeated independently at least four times. An average radiolabeling yield of ca. 70% was estimated by standard well-counter methods on centrifuged specimens, particularly those emerging from routes B, D, and E ([Scheme S3](#)). The average radiolabeling yield is assigned to the radioactivity incorporated into the SWNT, where typically a specific activity of 292 MBq/mg and a radiochemical purity of >98% were achieved within an average of 4-hr experiment time. The radiosynthesis protocols were repeated, and systematic variations in the operation sequences were introduced and evaluated in terms of radiolabeling yield and rapidity of operations to establish the most reliable radiolabeling protocol for open SWNTs in water. The SWNT filling experiments with $^{89}\text{Zr}(\text{OAc})_4$

in solution did not prove to be successful at a pH suitable for *in vivo* imaging applications. Only some low radiolabeling yield (ca. 10%) was achieved at acidic pH, despite the use of C₇₀ molecules at the ends of the SWNTs to block any possible significant leakage of the more oxophilic radiometal ⁸⁹Zr(IV) aqueous ions or aqueous clusters. The overwhelming majority of the radioactivity remained associated with the glucan when excess NaOAc was used as an adjuvant for ⁸⁹Zr(IV) oxalate ions. This is consistent with observations from HRTEM imaging, where less than 10% of cold Zr clusters were observed to fill the tubes, and these could only be visualized within the DWNT impurities under these conditions (Supplemental Information).

Batch-to-batch variability is an often encountered problem in nanomedicine that needs to be addressed. Thus, in order to establish reproducibility in the SWNT radiolabeling protocol with ⁶⁴Cu from water, a quality control test was performed via centrifugation of the formulated radiotracer, which separated the nanomaterials containing ⁶⁴Cu from any free ⁶⁴Cu ions in solution, and cross-validated the radioactivity associated with each fraction by radio-TLC. No leakage of the radionuclide content was observed over the duration of the radioprobe assembly and *in vivo* imaging (ca. 12 hr in total) and after being held over 24 hr in aqueous suspensions. Aqueous dispersions of ⁶⁴Cu@SWNT@β-D-glucan were subsequently stored for 4 weeks to allow for quantitative decay of the radioactive isotopes and subjected to the same microscopy and surface analysis investigations to independently confirm the size of the particulates and whether the filling and wrapping were maintained.

Tapping mode AFM (TM-AFM) images show that single strands of SWNTs are uniformly coated with a soft layer of organic material, presumably β-D-glucan. The SWNT strands are situated within the helix of β-D-glucan, and the resulting nanohybrid aggregates into small bundles ca. 10–20 nm in diameter (Figures 2 and S16). Raman spectra recorded in solution and in the solid phase showed that these aggregates are significantly more dispersible in water than their pristine SWNT precursors (Figures S25–S27). The structural integrity of the sp²-hybridized carbon atoms was validated by observing the G band (~1,590 cm⁻¹) and a disordered D band (~1,330 cm⁻¹, attributed to the disordered graphite structure of SWNTs). For dried samples, the CuCl₂@SWNT and CuCl₂@SWNT@β-D-glucan solid composites showed a slightly lower I_D/I_G band intensity ratio (ca. 9% in both cases) than did free SWNTs and SWNT@β-D-glucan (both ca. 12%), implying that functionalization had occurred without significant disruption of the aromatic network of the tubes. Single-particle detection and size analysis were carried out with a NanoSight HALO system, commonly used for visualization of particles (details are reported in the Supplemental Information and Figure S27).⁵

Model particle-size distribution was thus determined on the basis of the particle-by-particle analysis of the sample to show that for MX₂@SWNT@β-D-glucan (cold control samples, where MX₂ = CuCl₂, as well as the post-decay recovered nanohybrid ⁶⁴CuCl₂/NaOAc@SWNT@β-D-glucan, from samples B, D, or E), the average aggregated particle diameter in aqueous media is in the range of 240 nm (Supplemental Information). (The NanoSight HALO method estimates particle size on the basis of the Brownian motion of each particle, down to 10-nm resolution, leading to the estimation of the mean square displacement and diffusion coefficient, D_t. With particle tracking analysis, the real size of non-spherical particles [and the particles here have a very high aspect ratio] is difficult to assign precisely as the effective spherical size estimated here is very different to any of the real dimensions. Thus, the average size

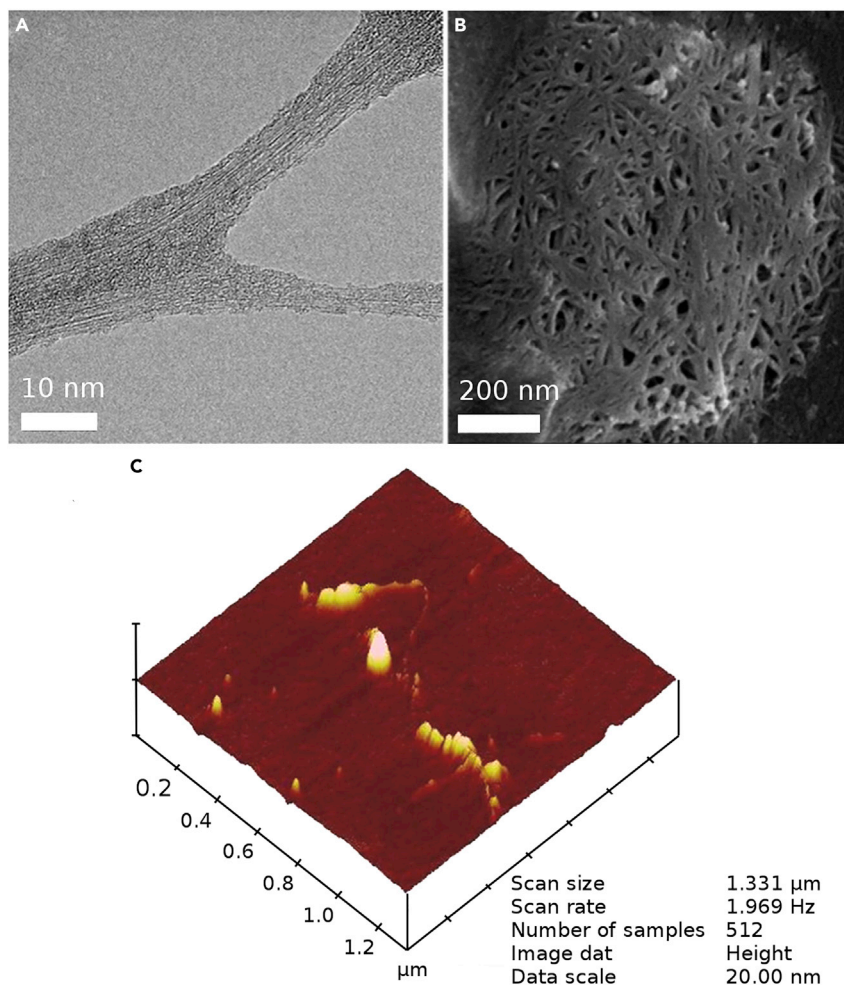


Figure 2. HRTEM, SEM, and AFM Images

(A) HRTEM of SWNT@ β -D-glucan.

(B) SEM of CuCl_2 @SWNT@ β -D-glucan.

(C) AFM of SWNT@ β -D-glucan.

estimated by the NanoSight HALO method for the aggregated particles is approximately three times larger than what is assumed for the individual composite particles.) AFM, HRTEM, and scanning electron microscopy (SEM) imaging experiments (Figure S18) revealed a population of small, fast-moving particles, and small numbers of larger, slow-moving particles of an average size of 80 nm were also observed in aqueous dispersions of CuCl_2 @SWNT@ β -D-glucan.

In order to verify the effective ability of β -D-glucan to increase the solubility of SWNT in the aqueous environment and to determinate the diffusion coefficient associated with the β -D-glucan nuclear magnetic resonance (NMR) spin systems, we employed diffusion ordered spectroscopy (DOSY). NMR diffusion experiments, like DOSY, are deemed to be a convenient spectroscopic tool to successfully identify inter- and intra-molecular self-assembled aggregates and aid the understanding of host-guest interactions in solution.⁷⁰ DOSY spectra of β -D-glucan and SWNT@ β -D-glucan were acquired at 298 K, and their spectra are shown in Figures S2 and S3. The D_{exp} values for β -D-glucan and SWNT@ β -D-glucan aggregates in D_2O were estimated to be

$8.03 \times 10^{-12} \pm 7.47 \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$ and $9.62 \times 10^{-12} \pm 8.11 \times 10^{-13} \text{ m}^2 \text{ s}^{-1}$ respectively. These observations, therefore, suggest that β -D-glucan succeeds in solubilizing the SWNTs in water and facilitates the formation of carbon hybrids with a size comparable with the glucan fibers when solubilized in D_2O .

***In Vitro* Kinetic Stability Radioassays and *In Vivo* Imaging**

To check the kinetic and radiochemical stability of the $^{64}\text{CuOAc/NaOAc@SWNT@glucan}$ nanocomposites (sample E), standard BSA challenge was performed; and details are given in [Figures S51–S52](#) and [Table S6](#). Stimulated by the *in vitro* testing, ^{64}Cu -labeled SWNT@ β -D-glucan (sample prepared via route E; [Scheme 1](#)) was used as proof-of-principle radiotracer PET imaging investigations in healthy male Wistar rats. A viable route for the radiosynthesis of ^{64}Cu -SWNTs via current standard radiopharmacy practices in aqueous phase ^{64}Cu chemistry is presented in [Scheme 2](#), and this was scaled up for animal experiments and tested by standard quality control. After optimization and scaleup, radiolabeled SWNTs formulated in glucan matrix ready for *in vivo* imaging were obtained with a yield of 79% and a radiochemical purity of >98% in the 1 mg/mL CNT sample used (as shown in [Figure 1](#)). Specifically, quality control (see [Supplemental Information](#)) was performed via centrifugation of the formulated radiotracer, which separates the nanosized tube assemblies containing $^{64}\text{Cu(II)}$ ions from any dissolved $^{64}\text{Cu(OAc)}_2$. The production of the total radiotracer sample ready for *in vivo* work in three Wistar rats followed pathway E ([Supplemental Information](#)) and could be performed in up to 2 hr.

After *in vivo* imaging, ^{64}Cu -SWNT blood distribution (discussed below) was performed on samples in triplicate, as described in the [Supplemental Information](#). [Table S7](#) shows the distribution of the radioactivity fraction in the blood ($n = 3$ rats), plasma, and blood cells. No metabolites of ^{64}Cu -SWNT were present in blood, instead $96.7\% \pm 3.9\%$ parent compound was present after 24 hr (see [Supplemental Information](#)).

Dynamic data acquisition was conducted on a CTI Focus 220 small-animal PET scanner, and the pharmacokinetics of the labeled SWNT assemblies after intravenous injection was observed for 60 min ([Figures 3 and 4](#)). The animals were then allowed to recover and kept overnight. Animals were killed 24 hr after injection, then underwent single-frame full-body PET imaging and T1-weighted 4.7 T MRI scanning to facilitate accurate anatomical localization of the regions of interest. Blood samples were drawn from all animals at the end of the study and the distribution of the radioactivity fraction in blood, plasma, and blood cells was investigated. Regional analysis of the scans revealed rapid uptake and strong retention of the $^{64}\text{Cu@SWNT@}\beta$ -D-glucan hybrid predominantly in the lungs and myocardium. Consistent with the well-known biodistribution of free radiocopper in rats,⁷¹ obtained by extensive PET imaging studies with either ^{61}Cu or ^{64}Cu , we also found in control experiments that [^{64}Cu] CuCl_2 , pre-formulated by normal saline (pH 6.5–7) in the presence of NaOAc and the β -D-glucan, shows almost exclusive excretion through the kidneys and some localization in the liver. It is known that the liver acts as a reservoir for metals *in vivo* especially copper.^{72,73} For the images of internalized $^{64}\text{Cu@SWNT@}\beta$ -D-glucan, peak uptakes of 4.8 ± 0.9 standardized uptake value (SUV) and 3.0 ± 0.8 SUV occurred in early frames (~ 5 min), followed by gradual washout within the next 55 min to 3.3 ± 0.9 SUV and 2.2 ± 0.5 SUV ([Figures 3 and 4](#)). On day 2, radioactivity in the lungs and kidneys accounted for 1.55 ± 1 SUV and 1.3 ± 0.3 SUV, respectively, suggesting a slightly stronger retention of this radiolabeled material in the myocardium. This is consistent with the distribution of fibroblast cells in these organs and could indicate binding of the β -D-glucan motif to cell-surface receptors.

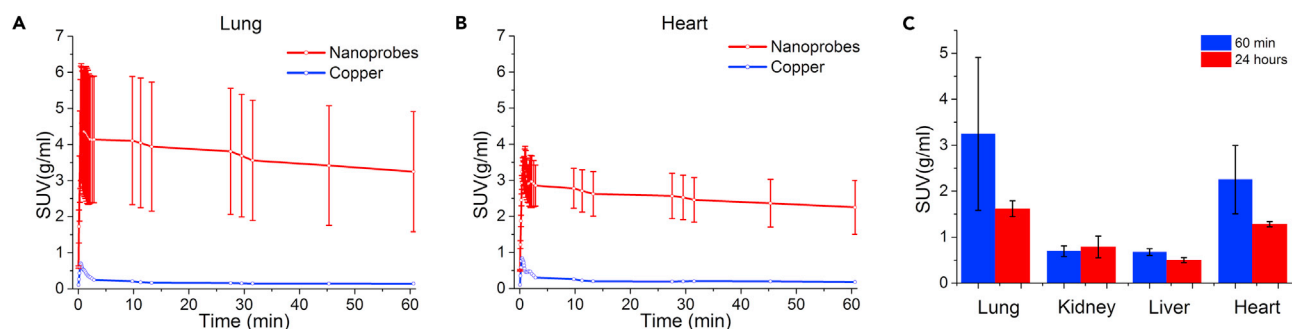


Figure 3. Standardized Uptake Values

Standardized uptake value (SUV) of nanoprobe and of free Cu^{2+} in (A) lungs and (B) heart over 60 min and SUV of nanoprobe in (C) lungs, heart, kidneys, and liver after 60 min (blue) and 24 hr (red) from full-body PET imaging of healthy male Wistar rats. Error bars represent one standard deviation (SD).

Hepato-biliary and renal excretion are balanced after 60 min, whereas the later scan shows some renal weighting, indicating slow but steady clearance of the radiotracer from the organism. It is also evident that SWNT encapsulation of ^{64}Cu bypasses the expected liver accumulation of free ^{64}Cu ions within the timescale of the experiment and leads to lung and heart accumulation and kidney excretion instead.

Cellular Behavior of the Fluorescent Supramolecular Carbon Nanocapsules

As stated above, rapid functionalization using fluorescein derivatives was used to secure the traceability of the constructs in cells. This protocol was chosen because proximal hydroxyl functions in the β -D-glucan can be functionalized by derivatization with boronic acids,^{74–76} which form boronic esters with 1,2- or 1,3-diols.^{77–81} In addition, boronic acids derivatized with biotin have been shown in our earlier works to retain their saccharide-binding potential upon streptavidin conjugation to microspheres⁷⁸ and gold surfaces.⁸²

Two different fluorescent boronic acids, i.e., a known derivative (giving rise to the composite SWNTs@ β -D-glucan@1) and a new derivative (giving rise to the new composite SWNTs@ β -D-glucan@2), each comprising a fluorescein unit linked to a boronic acid directly or via a hydrophobic linker, were used here and are shown as compounds 1 and 2, respectively, in Scheme 3.

Protected boronic acids, compounds 1 and 2, were thus exposed to dispersions of SWNT@ β -D-glucan; subsequent boronic ester formation gave nanohybrids denoted SWNT@glucan@1 and SWNT@ β -D-glucan@2, respectively. The incorporation of fluorescein boronic acids (1) or (2) into the glucan networks was used because it was expected to render these hybrids responsive to the 910-nm or 810-nm excitation wavelengths in multiphoton mode (Figure 6), as well as under 405-, 488-, and 532-nm single-photon lasers. Although such fluorescent probes offer high sensitivity, natural biological molecules found *in vitro* also absorb light effectively, and many of them are also emissive in the same spectral and temporal region. This gives rise to (1) absorption and scattering of UV and visible light by the sample, which limits the depth of sample that can be imaged, and (2) emission of light by the sample (autofluorescence), which limits the probe signal/background ratios. Two-photon fluorescence microscopy was used here because it allows for high-resolution cellular imaging providing deep penetration in various organs of living animals and minimizing cellular damage. In our earlier study on fluorophore-tagged nanomaterials, including carbon nanotubes and reduced graphene oxides, we discovered that multiphoton FLIM showed significant advantages for imaging of such

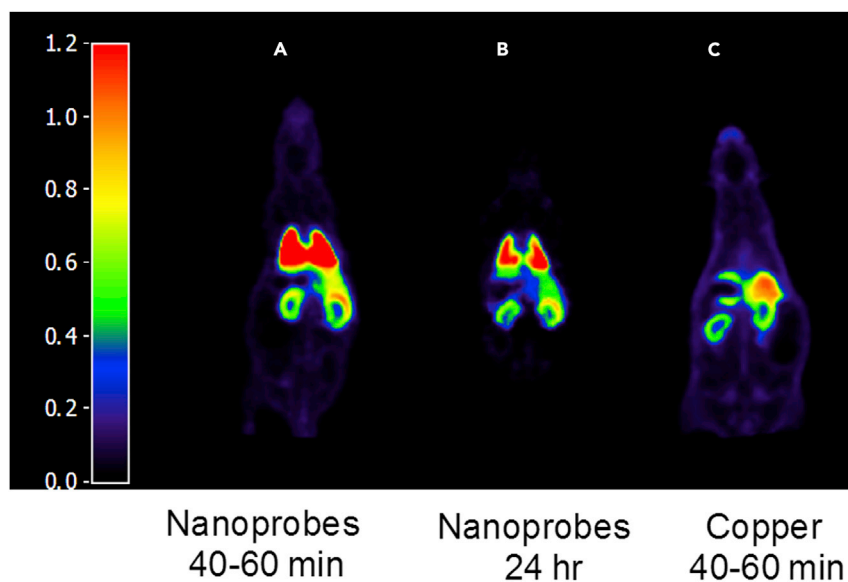
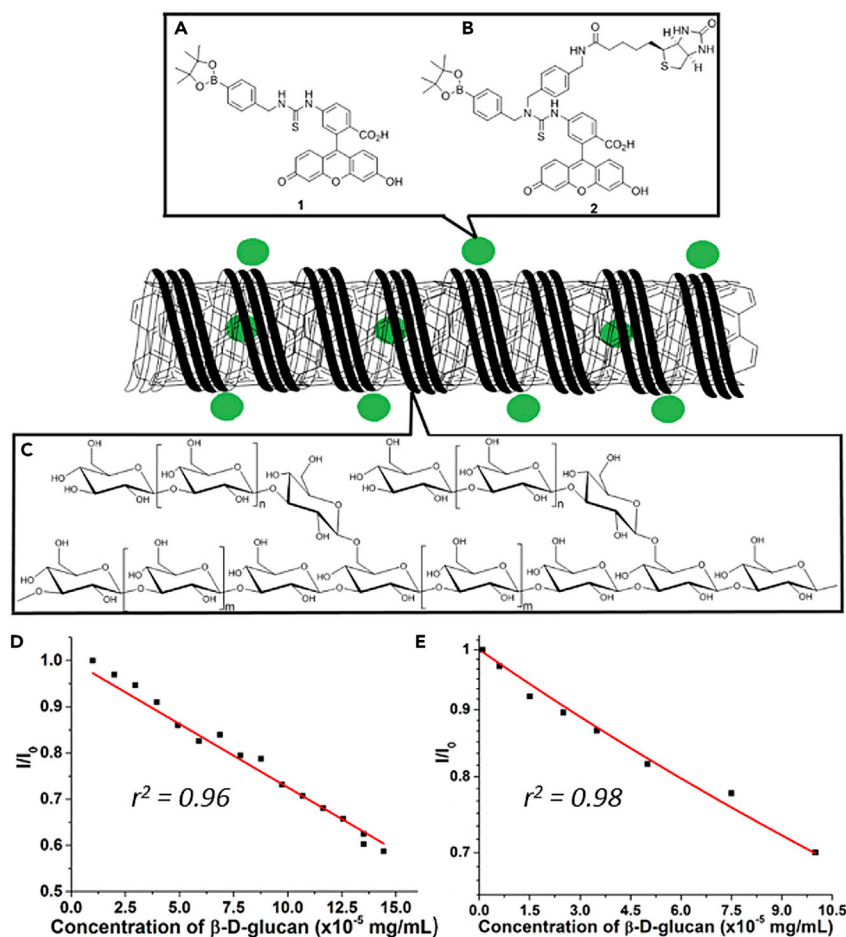


Figure 4. Whole-Body PET Imaging of Healthy Male Wistar Rats

PET imaging represents (A) the SUV of nanoprobe in the lung, heart, kidney, and liver from full-body PET imaging of healthy male Wistar rats over 40–60 min, (B) the SUV of nanoprobe in the lung, heart, kidney, and liver from full-body PET imaging of healthy male Wistar rats after 24 hr, (C) the SUV of free copper in the lung, heart, kidney, and liver from full-body PET imaging of healthy male Wistar rats over 40–60 min.

supramolecular complexes in cells, even when their emissive properties render them in the category of rather weak fluorophores. Carbon nanomaterial hybrids of organic materials minimize the autofluorescence, scattering, and photodamage when they were used as intracellular probes capable of cell-membrane penetration under 2P excitation. We also showed that emission lifetimes of uncoordinated (or free) fluorescent tags and their corresponding supramolecular complexes can differ by up to an order of magnitude, and that the cellular environment affects the fluorescence lifetime of the metalloprobe. Such an approach was also used here, for the first time, with the purpose of testing that the fluorophore-tagged supramolecular aggregates are fully traceable by multiphoton fluorescence lifetime imaging in cells with near-IR excitation (910 nm). This technique, which is extremely sensitive to the subtle changes in the fluorophore environment upon cellular uptake, acted as a strong predictor of kinetic stabilities with respect to dissociation in individual components for such supramolecular aggregates.

Confocal fluorescence microscopy imaging of standard healthy cells (CHO line), as well as HeLa cells, was performed (Figures 5, S37, S38, S41, and S42). Two-photon fluorescence lifetime measurements (Figure 6) were conducted in cells treated with SWNT@ β -D-glucan@2, and lifetime data were compared with data for the individual components: compound 2 and SWNT@ β -D-glucan composite.^{61,83,84} Time-correlated single-photon counting (TCSPC) of free compound 2, glucan@2, and SWNT@ β -D-glucan@2 allowed us to investigate the fluorescence lifetime decay of the photoexcited state of 2 and its nanohybrid in DMSO and/or in water (Figure 7). The TCSPC data of 2 free in solution and β -D-glucan@2 show a multicomponent exponential decay, and the fluorescence decay of SWNT@ β -D-glucan@2 can be fitted to a single-component system in these particular experimental conditions (Table 2). Lifetime data of this kind suggest that the SWNT@ β -D-glucan@2 has the potential to be more than the sum of the individual properties of the constituent



Scheme 3. Graphical Representation of the Supramolecular Assembly of Compounds 1 and 2, β -D-Glucan, and SWNT

(A–C) Structure of (A) compound 1, (B) compound 2, and (C) β -D-glucan from barley [β -(1,3) (1,4)-D-glucan].

(D) Titration of compound 1 with β -D-glucan; binding constant is $1.71 \times 10^3 \pm 0.06 \times 10^3 \text{ M}^{-1}$.

(E) Titration of compound 2 with β -D-glucan; binding constant is $3.68 \times 10^4 \pm 0.01 \times 10^4 \text{ M}^{-1}$.

Stock solutions for compounds 1 and 2: 25 μM in DMSO (pH 8.21). Aliquots of a 1 mg/mL suspension of β -D-glucan stock solution were gradually added (from 1×10^{-6} to 1.5×10^{-4} mg/mL).

molecules. Furthermore, a direct comparison of the values of fluorescence lifetime decay in solution (TCSPC) with those extrapolated in the cellular environments (FLIM) ensures that β -D-glucan@2 and SWNT@ β -D-glucan@2 are kinetically stable hybrids of the boronic acid (2) in DMSO/water suspension, as well as upon cellular internalization on the timescale of the imaging experiments performed here.

The cellular translocation behavior and fluorescent properties after cell uptake were investigated by confocal fluorescence imaging and fluorescence lifetime imaging. Both methods show that localization in subcellular (HeLa, PC-3, and CHO cells) regions and that the presence of this polysaccharide coating significantly enhances the cell-membrane translocation of SWNTs. Differential interference contrast (DIC) micrographs showed that cells remained viable throughout the experiment, consistent with the results of 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assays (see below). Confocal scanning microscopy both under single- and double-photon excitation modes suggest that the fluorescein-labeled nanocomposite is

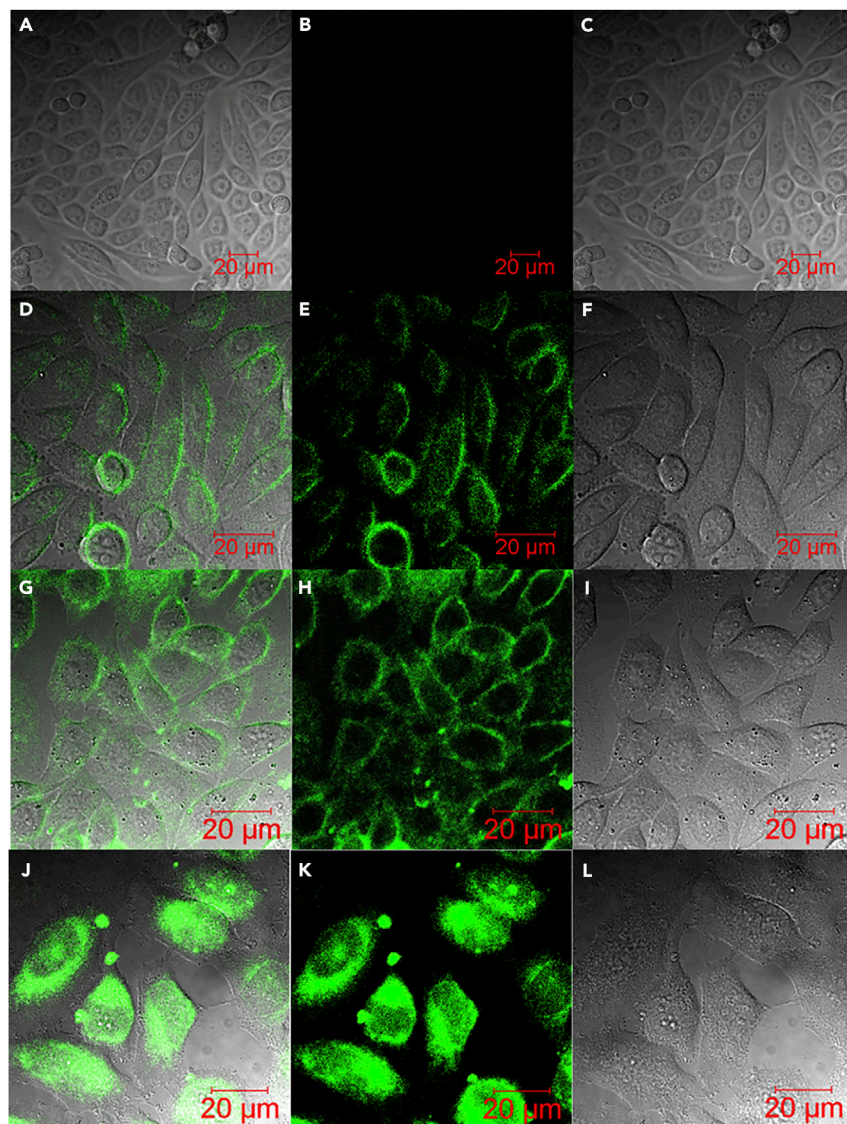


Figure 5. Confocal Microscopy

Confocal fluorescence images of CHO cells incubated at 37°C for 15 min with 10 μ L DMSO (1:99 DMSO/serum-free medium; control experiment) (A–C), compound **2** (100 μ M, 1:99 DMSO/serum-free medium) (D–F), β -D-glucan@**2** (10 μ g/mL, 1:99 DMSO/serum-free medium) (G–I), and SWNTs@ β -D-glucan@**2** (10 μ g/mL, 5:95 DMSO/serum-free medium) (J–L). (A, D, G, and J) Overlay of green channel (λ_{em} 516–530 nm) and differential interference contrast (DIC) channel; (B, E, H, and K) green channel; (C, F, I, and L) DIC channel. λ_{ex} = 488 nm.

taken up by cells. Compound **1** alone does not cross the nuclear membrane (Supplemental Information), remaining bound to the outer membrane, but in the presence of the corresponding SWNT-glucan nanoconstruct, cellular uptake can be observed. Similar observations were found for additional healthy cell lines such as FEK-4, as well as cancerous cells (PC-3). Interestingly, the cellular morphologies for cells treated by both **1** and **2** and the corresponding glucan and glucan-decorated SWNTs remain undamaged over the duration of the imaging experiment.

MTT assays confirm that pristine and purified SWNTs are toxic (Figure 8A). MTTs of β -D-glucan in FEK-4 show that this has a rather low toxicity, and it could improve the

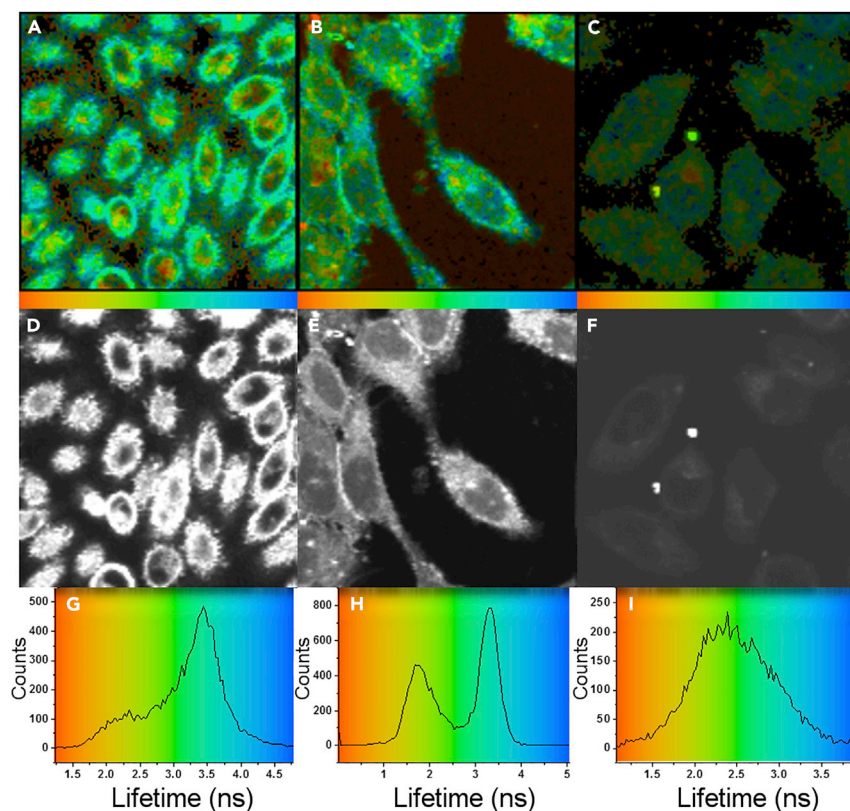


Figure 6. Two-Photon Laser Fluorescence Lifetime Maps

(A–C) Typical micrographs of CHO cells incubated for 15 min at 37°C with compound **2** (100 μ M, 1:99 DMSO/serum-free medium) show an apparently homogeneous distribution of compound **2** within the cell; lifetime mapping (A), intensity image (B), and corresponding lifetime distribution curve (C) are shown. λ_{ex} = 910 nm.

(D–F) Typical micrographs of CHO cells incubated for 15 min at 37°C with β -D-glucan@**2** complex (10 μ g/mL, 1:99 DMSO/serum-free medium); lifetime mapping (D), intensity image (E), and corresponding lifetime distribution curve (F) are shown. Bright spots in the cytoplasm region indicate that fluorescent β -D-glucan@**2** complex translocated into cells as intact objects.

(G–I) Typical micrographs of CHO cells incubated for 15 min at 37°C with SWNTs@ β -D-glucan@**2** composite (10 μ g/mL, 5:95 DMSO/serum-free medium). These contained 10 mM compound **2** anchored onto 1 mg/mL glucan-wrapped SWNT. Images show lifetime mapping (G), intensity image (H), and corresponding lifetime distribution curve (I). The intensity image clearly shows that compound SWNTs@ β -D-glucan@**2** mainly concentrates in the cytoplasm region without nuclear uptake.

Colors in (C), (F), and (I) provide a direct correlation between the lifetime maps and the lifetime histograms. Field of view in (A)–(F) is 100 μ m. λ_{ex} = 910 nm.

biocompatibility of SWNTs ($\sim 10^{-5}$ mg/mL).^{85–87} The half-maximal inhibitory concentrations (IC_{50}) of compounds **1** and **2** are $4.13 \times 10^{-6} \pm 1.43 \times 10^{-6}$ and $5.32 \times 10^{-4} \pm 1.03 \times 10^{-5}$ M, respectively, indicating rather non-toxic adjuvants, as expected (Figure 8B). These values appear to increase significantly when **1** and **2** are anchored onto the surface of SWNTs@ β -D-glucan, whereas both β -D-glucan and SWNTs@ β -D-glucan appear to be less toxic for the healthy cell line, FEK 4 (Figure 8C).

Conclusions

We have demonstrated a generic strategy that uses SWNTs as a multimodal nanoprobe platform for molecular imaging and diagnostics assembled entirely in water and is based on supramolecular recognition approaches in water. The nanoassembly

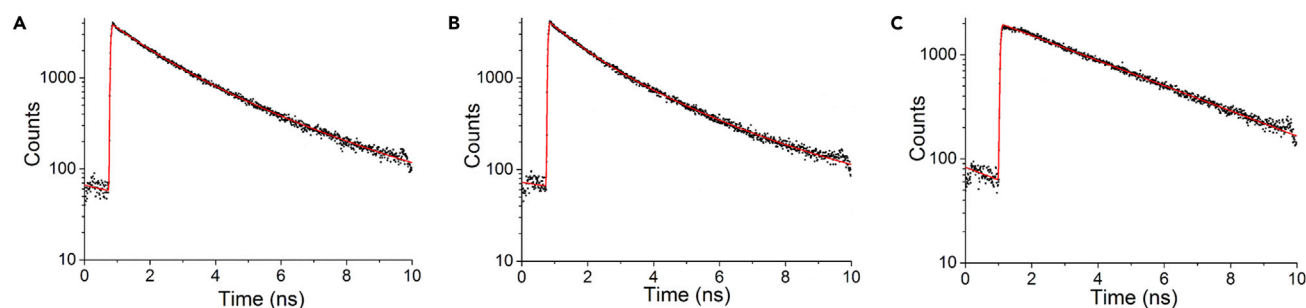


Figure 7. Time-Correlated Single-Photon Counting

Fluorescence lifetime decay traces ($\lambda_{exc} = 910$ nm) of (A) 10 mM compound **2** in DMSO, (B) 1 mg/mL β -D-glucan@**2** in DMSO, and (C) 1 mg/mL SWNTs@ β -D-glucan@**2** in water. Values of χ^2 describe the goodness of the fit, τ_1 and τ_2 (single- and multi-lifetime decay components of the fit), and associated percentages are reported in Table 2.

thus created has tunable functionality and remains intact in a biological environment. It is taken up *in vivo* with kinetic stability, as shown by PET imaging and metabolite analysis.

We report the successful, reproducible, and robust filling of SWNTs with a variety of Cu^{2+} ions (via radiochemical methods) through the use of hot ^{64}Cu ions anchored onto NaOAc and also a cold optimized procedure for $\text{Cu}(\text{OAc})_2$. The cold chemistry paralleled radiochemistry as the *modus operandi* for designing and testing radiotracers of importance for radiotherapy and/or PET, as well as a synthetic model for SPECT tracer encapsulation, such as aqueous species including simple $^{99\text{m}}\text{TcO}_4^-$ ions or for radiotherapy-relevant species labeled with ^{188}Re .

Our test-informed design, which prevents loss of the probe components en route to targeted organs (in this case, the lung) was verified. Proof of principle of all the steps required to construct an all-in-one biocompatible, targeted, molecular imaging probe for multimodal optical and PET imaging has been demonstrated. This paves the way for a general solution for the delivery of imaging agents, particularly, ^{64}Cu -based tracers, to cells, tissues, and organs, such as lungs, heart, liver, and kidneys. We are now focusing on further rationalizing the mechanism of nanoprobe-living organism interactions and the capability of carbon nanotubes to redirect the radioactive PET tracers *in vivo*, which will inform future synthetic programs and radiolabeling work involving shorter-lived therapeutic as well as diagnostic radioisotopes.

EXPERIMENTAL PROCEDURES

All purchased starting materials were used without further purification. All solvents were of reagent-grade quality and purchased commercially. The general methodology for UV-vis, fluorescence, and NMR spectroscopy; TCSPC; FLIM, TEM, SEM, and AFM; laser scanning confocal microscopy; purification of SWNTs; and synthesis of the intermediates of compound **2** is reported and discussed in detail in the [Supplemental Information](#).

All animal experiments were conducted in accordance with the UK Animal (Scientific Procedures) Act of 1986 (project license 80/2234). *In vivo* PET/MR imaging was performed on three Wistar rats (Charles River).

Synthesis of Compound **2**

Pinacol-protected boronic acid, Boc-protected *p*-xylylenediamine (200 mg, 0.44 mmol), was deprotected in trifluoroacetic acid (TFA) (1 mL) to remove the

Table 2. Time-Correlated Single Photon Counting

	χ^2	τ_1 (ns)	a_1 (%)	τ_2 (ns)	a_2 (%)
2	1.13	0.9	81.7	4.3	18.3
β -D-glucan@2	1.25	1.0	56.5	2.8	43.5
SWNTs@ β -D-glucan@2	1.30	3.6	100	–	–

Time-correlated single-photon counting ($\lambda_{exc} = 910$ nm) of 10 mM compound **2** in DMSO, 1 mg/mL β -D-glucan@2 in DMSO, and 1 mg/mL SWNTs@ β -D-glucan@2 in water.

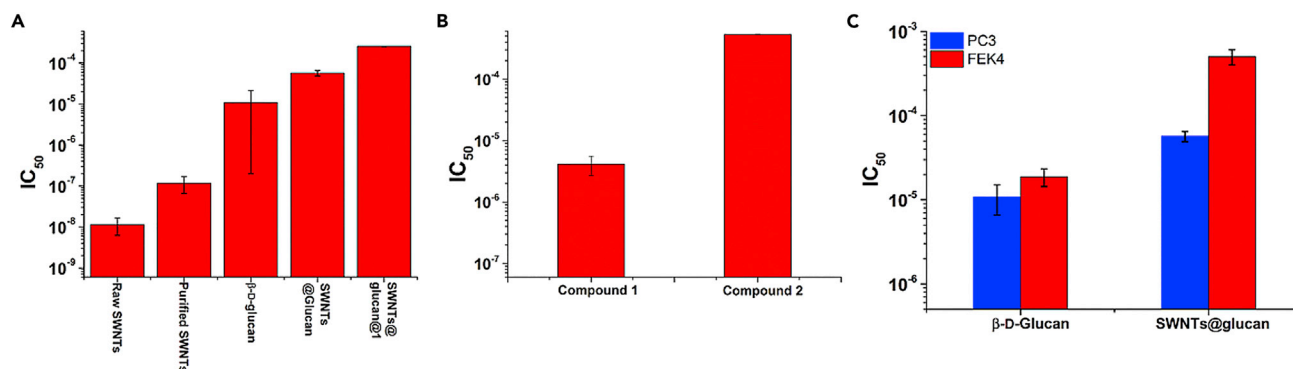
Boc group. Biotin-*N*-hydroxysuccinimide (150 mg, 0.44 mmol) was dissolved in dimethylformamide (DMF, 10 mL) and added to the deprotected boronic acid solution. Next, 4 equiv of triethylamine (TEA) was subsequently added. The mixture was stirred at room temperature overnight. The product was removed, yielding 220 mg white solid. Finally, DMF (10 mL) was added, followed by the addition of fluorescein isothiocyanate (148.1 mg, 0.38 mmol) and TEA (4 equiv, 1.76 mmol). The mixture was stirred overnight at room temperature covered with aluminum foil. The mixture was extracted with dichloromethane and water, products were isolated in the organic phase and dried over $MgSO_4$, and the solvent was evaporated under vacuum, affording compound **2** as a yellow solid. 1H NMR (300 MHz, CD_3OD , 298 K) $\delta = 7.65$ (d, 2H, $J = 7.8$ Hz), 7.28–7.02 (m, 5H), 7.00–6.53 (m, 6H), 4.95 (s, 2H), 4.40–4.28 (m, 1H), 4.21 (bs, 2H), 4.13–4.04 (m, 1H), 3.98 (bs, 2H), 3.04–2.93 (m, 1H), 2.82–2.68 (m, 1H), 2.59–2.47 (m, 1H), 2.18–2.05 (m, 2H), 1.65–0.70 (m, 12H) ppm. ^{13}C NMR (75 MHz, $DMSO-d_6$, 298 K) $\delta = 172.4$, 169.3, 169.0, 163.1, 162.6, 157.5, 156.1, 152.7, 146.4, 142.9, 141.6, 141.0, 139.0, 138.5, 134.9, 134.6, 129.5, 128.8, 128.4, 127.4, 127.2, 126.6, 126.4, 126.0, 125.9, 125.2, 83.6, 35.5, 33.7, 28.5, 28.4, 25.7, 25.3, 25.0, 24.8, 24.4 ppm. MALDI-TOF MS (m/z) calcd, $C_{52}H_{55}BN_5O_9S_2^+$, 968.35 [$M + H$] $^+$; found, 968.90.

SWNT Inner-Tube Labeling

To establish the metal-labeling protocol in water, we exposed a suspension of opened and pristine SWNTs to saturated aqueous solutions of metal salts, such as $CuBr_2$, $CuCl_2$, and $Cu(OAc)_2$, and then to solutions of their radioactive analog, i.e., ^{64}Cu or $CuCl_2$ supported onto a saturated solution of $NaOAc$ at room temperature. SWNTs were repeatedly re-suspended by mild sonication (15 s). This was carried out at 5-min intervals over the course of 1 hr to minimize damage to the tubular structure.⁸⁸ After fullerene encapsulation, the mixtures containing $M@C_{70}@SWNTs$ were filtered, and the solid residue was washed several times with distilled water to remove the free metal ions ($M = Cu(II)$, $Na(I)$, and oxo-species of $Zr(IV)$ and $Re(VII)$). A filling yield of ca. 60% (estimated by HRTEM and energy-dispersive spectroscopy) was obtained with pure $KReO_4$ in water from aqueous media (as a model for $^{188}ReO_4^-$ or $^{99m}TcO_4^-$ therapeutic or diagnostic radiotracers). The procedure for preparation of the five samples (A–E) for *in vivo* testing (based on the sequential addition of glucan, radioactive $^{64}Cu^{2+}$ aqueous solutions, and C_{70}) is shown in the [Supplemental Information](#).

In Vivo Experiments and microPET Imaging

All animal experiments were conducted in accordance with the UK Animal (Scientific Procedures) Act of 1986 (project license: 80/2234). Studies were performed on three Wistar rats (Charles River). Anesthesia was induced with 2% isoflurane administered in 1 mL/min oxygen and maintained throughout the experiment with less than 1% isoflurane. The left femoral vein was cannulated for intravenous administration of formulated nanoprobe, and the left femoral artery was cannulated to allow for the collection of blood samples. During all surgical procedures, body temperature



was maintained at 37°C with a heating blanket connected to a rectal thermistor probe. After PET scanning, the animals were allowed to recover and kept under standard conditions overnight. The next day animals were killed by intravenous injection of 1 mL (200 mg/mL) pentobarbital sodium (Euthanal), and the animals were rescanned.

PET data were acquired by a microPET Focus 220 scanner (Concorde Microsystems). The rats were placed prone on the scanner bed, and the head was fixed in a custom-made plastic frame using ear bars and a bite bar. Anesthesia and body temperature were maintained as described above. In addition, oxygen saturation, heart rate, and respiratory rate were measured and maintained within physiological limits throughout, with a non-invasive mouse OXTM (Starr Life Sciences) pulse oximeter sensor attached to the foot.

Before injection, single-mode transmission data were acquired for 8.5 min with a rotating ^{68}Ge point source (~ 20 MBq). An attenuation correction sinogram was produced from this scan and a blank scan of the same duration with the reconstruction and segmentation software on the Focus 220. In all experiments, ^{64}Cu -SWNT (8–16 MBq) was injected intravenously over 30 s, followed by a 15-s heparinized saline flush. The injected activity varied in order to keep the injected mass of SWNTs constant at 250 μg . List-mode data were histogrammed into sinograms for the following time frames: 12×5 s, 6×10 s, 3×20 s, 4×30 s, 5×1 min, 10×2 min, and 30×5 min (3 hr in total). Corrections were applied to random events, dead time, normalization, attenuation, scatter, and decay. Fourier rebinning (16) was used to compress the 4D sinograms to 3D before reconstruction with 2D filtered back projection with a Hann window cutoff at the Nyquist frequency. The image voxel size was $0.95 \times 0.95 \times 0.80$ mm, with an array size of $128 \times 128 \times 95$ mm. The reconstructed images were converted to kBq/mL with global and slice factors determined from imaging a uniform phantom filled with a ^{64}Cu -labeled cuprous acetate solution. This phantom acquisition was also used to cross-calibrate the scanner and the well counter used to measure blood radioactivity concentration.

In Vitro Fluorescence Lifetime Experiments

TCSPC and FLIM data were obtained with a two-photon microscope at the Central Laser Facility of the Rutherford Appleton Laboratory with some modifications. In brief, a two-photon microscope was constructed around a Nikon TE2000-U inverted microscope with custom-made X-Y galvanometers (GSI Lumonics) for the scanning system. Tunable laser light at the required wavelength of 910 nm was obtained from a mode-locked titanium sapphire laser (Mira, Coherent Lasers), producing 180-fs pulses at 75 MHz, pumped by a solid-state continuous-wave 532-nm laser (Verdi V18, Coherent Laser). The oscillator fundamental output of 910 ± 2 nm was also used. The laser beam was focused to a diffraction limited spot through a water immersion UV corrected objective (Nikon VC $\times 60$, numeric aperture 1.2) and specimens illuminated at the microscope stage of a modified Nikon TE2000-U with UV transmitting optics. The focused laser spot was raster scanned with an X-Y galvanometer (GSI Lumonics). Fluorescence emission was collected without de-scanning, bypassing the scanning system and then passed through a colored glass (BG39) filter. The scan was operated in normal mode, and line, frame, and pixel clock signals were generated and synchronized with an external fast microchannel plate photomultiplier tube used as the detector (R3809-U, Hamamatsu Photonics). These were linked via a time-correlated single-photon-counting PC module SPC830 (Becker and Hickl) to generate the multiphoton excited image with the associated characteristic decay at each pixel position. The decays were subsequently analyzed to generate the FLIM image. Before two-photon FLIM data collection, the probe-loaded cells were confirmed with a Nikon eC1 confocal microscope with excitation at 405, 488, and 543 nm where required. Data were analyzed with the SPCImage analysis software (Becker and Hickl). χ^2 values between 0.9 and 1.3 were taken to represent a good fitting curve, and where necessary, the decay profiles were fitted with two, rather than one exponential. The distribution of lifetime values within the region of interest were generated and displayed as a FLIM image.

Cell-Culture Preparation

PC-3 cells were cultured at 37°C in a 5% CO₂ atmosphere and diluted once a suitable confluency had been obtained. PC-3 cells were cultured in RPMI 1640 containing 10% heat-activated fetal calf serum (FCS), 0.5% penicillin and streptomycin (10,000 mg/mL), and 200 mM L-glutamine. The medium contained no fluorescent indicator dyes such as phenol red and was therefore suitable for use in fluorescence imaging studies. Excess supernatant containing dead cell constituents and excess proteins and metabolites was aspirated. The viable live adherent cells were washed with 2×10 mL aliquots of PBS to remove residual medium containing FCS. Cells were then re-suspended in 10 mL PBS with an additional 2–5 mL trypsin and incubated for a further 5 min at 37°C. After trypsinization, 5 mL medium containing 10% serum was added to inactivate the trypsin, and the suspension was centrifuged for 5 min (1,000 rpm) to remove residual dead cell constituents. The resulting supernatant was aspirated and 5 mL of medium was added. Cells were counted with a hemocytometer and seeded as appropriate.

Fresh DMEM (10% FCS) was added to the suspended cells to give a sufficient concentration of cells (ca. 300,000 cells/mL). The cells were plated in a glass-bottomed Petri dish (MatTek) and left for 24 hr to adhere before fluorescence imaging measurements were made.

MTT Assays Tests for IC₅₀ Estimation

PC-3 cells or FEK-4 cells (7×10^3 per well) were seeded in a sterile 96-well plate and incubated for 48 hr to adhere. Compounds 1 and 2, pristine SWNTs,

purified SWNTs, β -D-glucan, SWNTs@ β -D-glucan, SWNTs@ β -D-glucan@1, and SWNTs@ β -D-glucan@2 were subsequently loaded at different concentrations into the wells and cultured for 48 hr. The concentrations used ranged between 250 μ M (1% DMSO, 99% EMEM [10% FCS]), 100 μ M, 50 μ M, 10 μ M, 1 μ M, 0.5 μ M, 100 nM, and 1 nM for compounds 1 and 2 and between 10 μ g/mL, 1 μ g/mL, 0.5 μ g/mL, 0.1 μ g/mL, 50 ng/mL, 10 ng/mL, 1 ng/mL, and 0.01 ng/mL for pristine SWNTs, purified SWNTs, β -D-glucan, SWNTs@ β -D-glucan, SWNTs@ β -D-glucan@1, and SWNTs@ β -D-glucan@2. Each concentration was repeated six times in the experiment. Subsequently, cells were washed three times with PBS, 100 μ L of 3-MTT was added (0.5 mg/mL, 10% serum-free medium), and cells were incubated for 3 hr. After aspiration, 100 μ L DMSO was added and well plates were read by an ELISA plate reader (Versa Max BN02877, Molecular Devices). The absorption wavelength was 570 nm, and 630 nm wavelength was used as a reference.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, 53 figures, 7 tables, and 3 schemes and can be found with this article online at <http://dx.doi.org/10.1016/j.chempr.2017.06.013>.

AUTHOR CONTRIBUTIONS

S.I.P. conceived and directed the research, coordinated the experimental work, and interpreted all of the research results; she coordinated the team of authors, named collaborators, and technical service providers and was the primary supervisor and investigator. H.G. performed the synthesis of the boronic acid derivatives under the supervision of T.D.J. and J.S.F. with the contribution of V.M., D.G.C., and S.E.F. S.W.B. coordinated and performed confocal microscopy and FLIM experiments with the contribution of R.L.A., H.G., R.M.T., and V.M. for *in vitro* data processing, discussions, and analysis. H.G. carried out fluorescence titrations. H.G. also performed cell-culture and cytotoxicity experiments under the supervision of R.M.T. P.J.R., S.S., T.D.F., Y.H., and F.I.A. designed and carried out live animal experiments under the Cambridge University license given. R.M.J.J. performed the AFM work and helped to interpret the results. V.M. performed solution-state NMR experiments. D.G.C. also contributed to the solid-state characterization of the materials and compounds. S.I.P., J.S.F., J.R.D., D.G.C., and V.M. wrote the final version of the paper. All authors discussed the results, critically contributed, and commented on the various drafts of the manuscript.

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