
ON THE INTERACTION OF DNA NANOSTRUCTURES WITH LIPID BILAYERS

Céline Journot

A thesis submitted in fulfillment of
the requirements for the degree of
Doctor of Philosophy at the University of Oxford



Linacre College
Department of Physics
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ABSTRACT

Much of our knowledge of cellular biology arises from direct observation of active cellular functions. Tools and techniques have steadily developed over the past several hundreds of years to aid in our understanding and control of the nanoworld and are referred to as nanotechnologies. In the context of nanotechnology, DNA is not used as a carrier for genetic information (as it is in cell), but as a construction material. DNA offers unprecedented control over the construction of simplified biomimetic models for the study of biological processes. This thesis first introduces and defines the field of DNA nanotechnology, with particular emphasis on the interaction of synthetic DNA nanostructures with biological membranes. Inspired by the protein clathrin, three-fold symmetric DNA tile made of eight, short DNA strands and capable of polymerising is described and studied, with the aim to interact with and controllably bend a membrane bilayer. This structure presented challenges during construction so an enhanced three-armed DNA structure built with DNA origami was designed. The successful assembly of a rigid and functionalisable nanostructure is described. This origami structure was polymerised into large constructs in solution and on a supported lipid membrane. The shape of the structure was modulated to vary its curvature and apply a bending force to a lipid vesicle when anchored to it. Following the conclusion of this study, we present the construction of a small, unique DNA structure for enhanced electron microscopy imaging in cell lysate. This project is part of a developing technique to couple the interaction specificity of dyes in super-resolution microscopy and the high-resolution output of electron microscopy. Finally, the optimisation procedures and recommendations for TEM imaging of samples of DNA origami and lipid structures are discussed.

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TABLE OF CONTENT

1. INTRODUCTION

1.1	SCOPE OF THE THESIS	1
1.2	NANOTECHNOLOGY.....	2
1.3	DNA NANOTECHNOLOGY.....	3
1.3.1	DNA arrays.....	8
1.4	DNA ORIGAMI.....	10
1.4.1	Synthetic considerations for DNA origami.....	10
1.4.2	Functionalisation of DNA origami.....	14
1.5	CELLULAR MEMBRANE.....	15
1.5.1	Biological cellular membrane.....	15
1.5.2	Model cellular membrane.....	18
1.6	MEMBRANE-SHAPING PROTEINS.....	22
1.7	DNA NANOTECHNOLOGY AND LIPID MEMBRANES.....	26
1.8	DNA ORIGAMI CHARACTERISATION.....	27
1.8.1	Agarose Gel Electrophoresis (AGE).....	28
1.8.2	Atomic Force Microscopy (AFM).....	28
1.8.3	Transmission Electron Microscopy (TEM).....	29
1.8.4	Fluorescence microscopy.....	30
1.8.5	Confocal microscopy.....	31
1.9	AIMS AND OBJECTIVES.....	32
2.	CLATHRIN-INSPIRED DNA STRUCTURES	34
2.1	DESIGN OF CLATHRIN-INSPIRED DNA MODEL.....	34
2.1.1	General considerations from the clathrin protein.....	34
2.1.2	Design of a DNA 3-fold array.....	36
2.1.3	Interaction of DNA arrays with a lipid membrane.....	42
2.2	CONSTRUCTION OF DNA ARRAYS.....	46
2.2.1	Optimising the star polymerisation procedure.....	47

2.2.2	Formation of arrays and its surface interaction.	58
2.3	SUMMARY.	67
3.	CLATHRIN-INSPIRED DNA ORIGAMI.	68
3.1	DESIGN OF A CLATHRIN-INSPIRED DNA MODEL.	69
3.1.1	Design of a 3-fold DNA origami.	69
3.1.2	Design of a polymerisation method.	73
3.2	SYNTHESIS OF DNA ORIGAMI.	76
3.2.1	Optimising the triskelion folding procedure.	76
3.2.2	Optimising the triskelion polymerisation strength.	82
3.2.3	Optimising the triskelion polymerisation temperature.	87
3.2.4	A new polymerisation strategy.	90
3.2.5	Study of the polymerisation kinetics of triskelia.	97
3.2.6	Variation of the bridge length of a triskelion.	102
3.2.7	Improved triskelia polymerisation strategy observed by TEM	107
3.3	GOLD NANOPARTICLE FUNCTIONALISATION OF THE TRISKELION	109
3.3.1	Synthesis of AuNP-triskelia.	110
3.3.2	Variation of the bridge length of a AuNP-triskelion.	113
3.4	SUMMARY.	115
4.	CLATHRIN-INSPIRED DNA ORIGAMI IN INTERACTION WITH LIPID MEMBRANES	116
4.1	OPTIMISING COVERAGE OF LIPID VESICLES BY AUNP-TRISKELIA	116
4.2	LIPOSOMES DEFORMATION BY TRISKELIA OBSERVED BY TEM	122
4.3	TRISKELIA AND LIPID MEMBRANE ON EM GRID.	124
4.4	GUV DEFORMATION OBSERVED BY FLUORESCENCE MICROSCOPY ...	130
4.5	SUMMARY.	137
5.	DNA ORIGAMI AS CRYO-EM TAG.	139
5.1	CELLULAR IMAGING.	139
5.2	NUCLEIC ACID NANOSTRUCTURES IN CELLS.	141
5.3	DESIGN OF A CRYO-EM TAG.	143
5.4	DESIGN OF A DNA ORIGAMI.	145

5.5	SYNTHESIS OF THE DNA TETRAHEDRON.....	146
5.6	SUMMARY.....	151
6.	OPTIMISING PROCEDURE FOR TRANSMISSION ELECTRON MICROSCOPY.....	152
6.1	PREPARATION OF THE EM GRID.....	152
6.1.1	Formvar and carbon-coated grid.....	152
6.1.2	Glow-discharge.....	153
6.2	SAMPLE STAINING.....	156
6.2.1	Types of stain.....	158
6.2.2	Optimising staining methods.....	161
6.3	SAMPLE IMAGING.....	166
6.4	POSITIVE AND NEGATIVE STAINING.....	168
6.5	ADDITIONAL ARTEFACTS.....	169
6.6	SUMMARY.....	172
7	CONCLUSION.....	173
7.1	SUMMARY AND CONCLUSION OF THE THESIS.....	173
A.	ANNEX.....	177
A.1	BUFFERS.....	178
A.2	OLIGONUCLEOTIDES AND SCAFFOLDS.....	178
A.2.1	General.....	178
A.2.2	Chapter 2.....	179
A.2.3	Chapter 3 and 4.....	180
A.2.4	Chapter5.....	180
A.3	PURIFICATION OF DNA STRANDS (DENATURING PAGE).....	182
A.4	ANNEALING OF DNA STAR.....	183
A.5	ANALYSIS OF dsDNA HYBRIDISATION (NATIVE PAGE).....	184
A.6	NUPACK ANALYSIS.....	184
A.6.1	NUPACK analysis of S4 secondary structure (Chapter 2).....	184
A.6.2	NUPACK design and analysis of stiff bridges (Chapter 3).....	185
A.7	FOLDING AND ANALYSIS OF DNA ORIGAMI.....	189

A.8	PURIFICATION OF DNA ORIGAMI.	190
A.8.1	Sephacry stock.	190
A.8.2	Size-exclusion column	191
A.8.3	PEG precipitation	192
A.9	GOLD NANOPARTICLE ON DNA ORIGAMI.	192
A.9.1	Gold nanoparticle (AuNP) preparation.	192
A.9.2	Folding and purification of DNA origami with AuNP	193
A.10	LIPIDS	194
A.10.1	Formation of liposomes	194
A.10.2	Electroformation of GUV	195
A.11	FLUORESCENCE MICROSCOPY	196
A.11.1	Supported Lipid Bilayer (SLB) formation	196
A.11.2	Imaging GUV in fluorescence microscopy	197
A.12	ELECTRON MICROSCOPY (EM)	197
A.12.1	TEM grid preparation	198
A.12.2	Cryo-EM	198
A.13	ATOMIC FORCE MICROSCOPY (AFM)	199
A.14	MELTING CURVE.	199
A.15	ADDITIONAL TEM IMAGES FOR SECTION 3.2.6.	200
A.16	DNA SEQUENCES.	201
A.16.1	Chapter 2	201
A.16.2	Chapter 3 and 4	202
A.16.3	Chapter 5	209
A.17	CaDNA _{no} ROUTING FOR DNA ORIGAMI.	212
A.17.1	Triskelion	212
A.17.2	Tetrahedron	213
	BIBLIOGRAPHY	214

CHAPTER 1

INTRODUCTION

1.1 SCOPE OF THE THESIS

The aim of this work is the development and synthesis of new DNA structures for the study of cellular processes. The main objective focuses on membrane mechanisms, in particular bending, which required the design of biomimetic models (using DNA nanotechnology, section 1.1 to 1.4) capable of closely interact with a lipid bilayer (introduced in section 1.5) in order to exert a bending force on the membrane (section 1.6). In addition to the study and optimisation on these novel DNA nanostructures, this thesis discusses and analyses the effect of the anchorage of the DNA to model membranes made of a lipid bilayer (introduced in section 1.7).

The second objective of this thesis is the design of a DNA structure with a distinctive, angular shape, easily recognised by electron microscopy in a crowded cellular environment with the aim to develop a tag for cryo-electron tomography to study cellular processes. The success of these objectives will be assessed by standard characterisation methods for DNA nanotechnology (section 1.8.1 and 1.8.2), electron microscopy (Section 1.8.3) and fluorescence microscopy (section 1.8.4 and 1.8.5).

1.2 NANOTECHNOLOGY

The refinement of the microscopic lens in the 17th century by Antonie van Leeuwenhoek^[1] unveiled to scientists a hidden world of “animalcules” that would redefine generations of knowledge of the microscopic world. As these instruments peered further and further down into the tiny world, scientists became aware that the unaided eye was missing a plethora of detail and insight into natural phenomena. Intrigued by these discoveries, scientists were keen to interact with the new, microscopic world, but were hampered by inadequate instruments. As a lumberjack will not use a pair of scissors to fall a tree, a horticulturist will not use an axe to cut a flower, scientists realised that nanoscale instruments would be optimal to interact with and manipulate the nano-scope world. Tools and techniques have steadily developed over the past several hundreds of years, with nanoscale manipulation reached in the last 35 years^[2], to aid in our understanding and control of the nanoworld and are thus referred to as nanotechnologies.

Our observation and comprehension of biological processes at the nanoscale has transformed technologies that are applied in medicine^[3] (biodistribution, drug delivery), industry^[4] (cosmetics, preservatives) and the environment^[5] (food, decontamination). Biological systems and structures often provide inspiration in the development of new tools for nanotechnology^[6, 7]. Bionanotechnology can thus be defined as technology that involves biological material that can control processes at the molecular level^[8]. Recently, deoxyribonucleic acid (DNA) has emerged as a powerful molecule for bionanotechnology.

1.3 DNA NANOTECHNOLOGY

In the context of nanotechnology, DNA is not used as a carrier for genetic information (as it is in cell) but as a construction material^[9]. Several inherent characteristics of the structure of DNA underly its use as a building block for nano-instruments. The DNA backbone is a chain of nucleotides, each of which carry one of the four bases: adenine (A), cytosine (C), guanine (G) and thymine (T). The bases assemble pairwise with remarkable precision; T forms two hydrogen bonds with A, and C forms three hydrogen bonds with G (Figure 1.1A). The hybridisation of two DNA strands is energetically favourable (release of Gibbs free energy, ΔG) and the stability of the duplex is defined by the base-pairing efficiency. Therefore, the sequence of two, single strands of DNA will dictate how well they will interact with each other: if every base of the first strand matches the appropriate base on the second strand, the two strands will bind with each other and are called complementary (Figure 1.1B). The process of base pairing, including hydrogen bonding between complementary bases, is called hybridisation and involves two complementary anti-parallel strands of DNA. The directionality is based on the asymmetry of the sugar-phosphate backbone of the polymer. It is universally accepted that the sequence of DNA is written from the 5' carbon of the ribose ring (marked 5'-end) and finishes at the –OH functional group on the 3' carbon (marked 3'-end) of the ribose ring. A DNA strand can be represented by a linear arrow for simplicity, with the 3'-end as the arrow head (Figure 1.1B). Once hybridised, two DNA strands form a stable duplex which is coiled into a double helix approximately 2 nm in width and with each base pair spaced 0.34 nm apart. Each turn in the helix involves 10.5 base pairs for a helical pitch of 3.5 nm, which was first described by Watson, Crick and Franklin in 1953^[10, 11, 12] (Figure 1.1C). Two types of groove can be seen in the DNA double-helix molecule model first built by Watson and Crick (Figure 1.1C). The major and minor grooves are opposite each other, each runs continuously along the entire length of the DNA molecule, and arise from the antiparallel arrangement of the two

backbone strands^[13]. This distinction is important in biological processes, as the two grooves are involved in the recognition and attachment of proteins to specific sequences or regions of DNA^[14]. In addition to the base-pairing (hybridisation), the packing of two DNA strands localises the hydrophobic bases towards the inside of the helix, and the hydrophilic sugar-phosphate backbone towards the aqueous environment. This configuration minimises the interaction of the hydrophobic bases with the surrounding aqueous solution and further stabilises the interaction between two consecutive bases referred to as the base-stacking interaction.

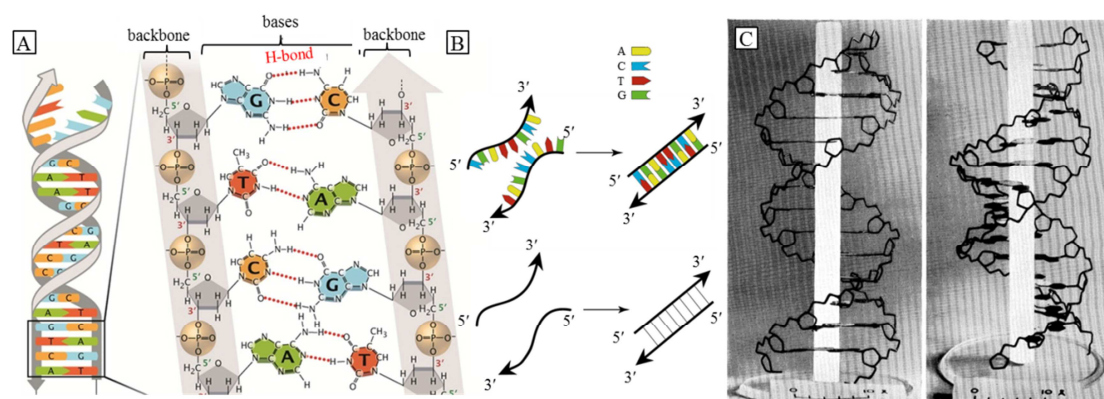


Figure 1.1: (A) Representation of a DNA double helix (left panel) and detailed structures of the base pairs (right panel) (image adapted from [13]). The atoms in the structures are colour-coded by element as indicated. (B) Schematic of two anti-parallel complementary strands hybridising. A simplified version with arrows at the 3'-end is illustrated in the bottom panel. (C) Photographs of the model representing the DNA double helix as published by Watson and Crick in 1953^[11].

By considering these specific and precise base interactions, one can consider the individual nucleotides (nt) as four, nanometer-sized bricks of Lego[®]. The arrangement of these bricks allows a tower or polymer to be built one layer at a time. The development of bioengineering^[15] and synthetic chemistry^[16] has allowed DNA to come to the front of nanotechnology. Artificial DNA sequences are now automatically synthesized by multiple providers (Annex A.2), employing solid-phase phosphoramidite chemistry^[17] first developed by Marvin Caruthers in the 1980s^[18]. Despite a relatively limited set of building blocks (A, T, C, G), DNA offers a flexible construction system with an excellent control and knowledge of its properties^[19]. Moreover, the needs of the

biotechnology industry have led not only to fast and reliable synthesis of arbitrary sequences^[16, 17], but also the production of modifications, such as biotin groups, fluorescent labels, and linking functionalities^[20]. In addition, DNA can be recognised and modified by a variety of proteins^[21, 22] that include kinases, DNA ligase, exonucleases, and restriction endonuclease. This has provided the groundwork for the development of increasingly intricate structures, in which the rigidity of the DNA helix provides an important structural support.

The rigidity of a semiflexible polymer such as DNA can be described by the wormlike chain (WLC) model^[23]. The WLC model describes the conformation of the polymer (which is considered continuous and inextensible) as a curve with a certain correlation length in the direction along the contour. This correlation length is called the persistence length^[18]. The double stranded DNA helix (dsDNA) can be considered stiff at the nanoscale, with values for persistence length between 40 and 50 nm in physiological buffer^[24, 25, 26, 27]. In comparison, single stranded DNA (ssDNA) behaves as a flexible and coiled polymer with a persistence length of only 1 nm^[28, 29]. When two parts of the same DNA polymer contain complementary sequences (called domains), they can fold up on themselves and form a particular structure known as a “hairpin”. A hairpin refers to a DNA strand that has two domains separated by a “loop” of a few unpaired bases (typically 2-6 nt) (Figure 1.2A) and was first suggested to be implicated in the DNA replication of eukaryotic cells by Cavalier-Smith in 1974^[30].

The stability of a dsDNA helix can be defined as the amount of energy required to separate two complementary strands. There are two ways of disrupting a duplex mechanically: along the axis of the molecule (shear geometry), or a gradual unzipping of DNA perpendicular to its axis (unzipping geometry)^[31] (Figure 1.2). When the two 5'-ends of a DNA duplex with thousands of base pairs (bp) are pulled apart along the molecules axis, they dissociate when the force applied exceeds a plateau of

approximately 65 pN (Figure 1.2B)^[32, 33]. When the 3'-end and the 5'-end of a DNA hairpin are pulled apart, recent studies of *in vitro* have shown that the interactions that stabilise the double helix and the elastic rigidity of single strands simply determine the sequence-dependent ≈ 12 -pN force threshold for DNA strand unzipping. (Figure 1.2C)^[32].

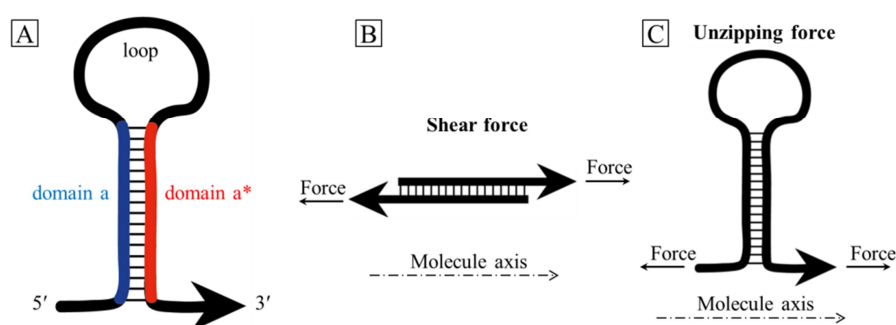


Figure 1.2: (A) A DNA hairpin created by matching bases (domain a and a*) and unmatched bases (loop). (B) A shear force is created on dsDNA by pulling the ssDNA in opposite directions along the molecular axis. (C) The shear force, when applied to a DNA hairpin, is referred to as the unzipping force.

Two complementary DNA strands hybridized together form a non-branched double helix. The observation of a DNA hairpin in a biological setting and the synthetic creation of the hairpin in the laboratory provided initial hints that DNA could adopt other structures. Branched DNA structures appear as key intermediates in DNA metabolism^[33]. A commonly observed branched motif is composed of four strands arranged into four double-helical arms (Figure 1.3). These junctions were first described by Robin Holliday and are now referred to as Holliday junctions (Figure 1.3A)^[34]. They naturally occur when two homologous strands (chromatids) meet. The sequences of pairs of arms are symmetrical, which allows the branch point to migrate (Figure 1.3B)^[35, 36]. This branch migration can be eliminated by breaking the symmetry to disable isomerisation^[37].

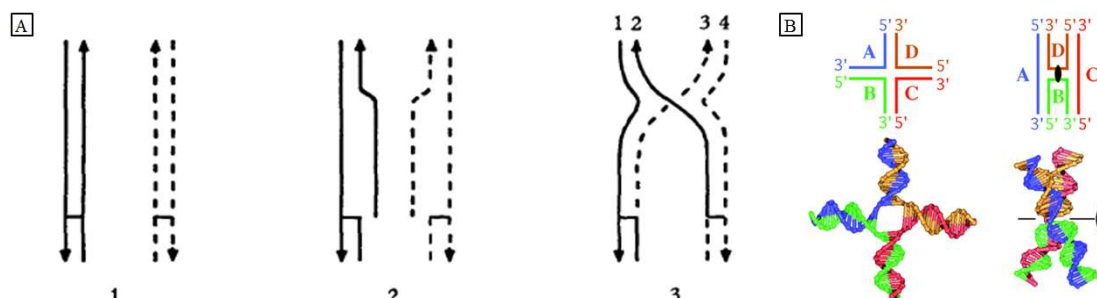


Figure 1.3: (A) Illustration of the successive stages (1 to 3) of pairing two homologous DNA strands to create a Holliday junction. Solid lines represent the DNA strands of one chromatid and broken lines represent a second chromatid. The arrows indicate the 5'-ends. Image from [34]. (B) Schematic representation of the Holliday junction in a square planar geometry (left) and a stacked-X-structure (right). Image from [36].

Inspired by these unique cross-over motifs observed in Nature that are arranged from the structural features of the DNA double helix, George Chruuch had the idea^[38] (in 1977) to use DNA to build lattices, an idea he shared with Ned Seeman and other crystallographers. A few years later, in the 1980s, Ned Seeman at the University of New York pioneered research into synthetically replicating these junctions in his laboratory. By removing DNA from its biological setting and treating it as a building material, Seeman succeeded in building nano-sized structures^[39] composed solely from the bases of DNA, thus establishing the field of DNA nanotechnology (Figure 1.4). His initial interest was the development of a method to simplify the production of pure crystals for X-ray crystallography. He realised, in 1981^[40], that DNA could be used to create periodic structures to orient target molecules, which would eliminate the difficult process of monocrystal growth. In pursuit of this goal, Seeman's laboratory published the first three-dimensional DNA cube made with ligated DNA strands (Figure 1.4)^[41, 42].

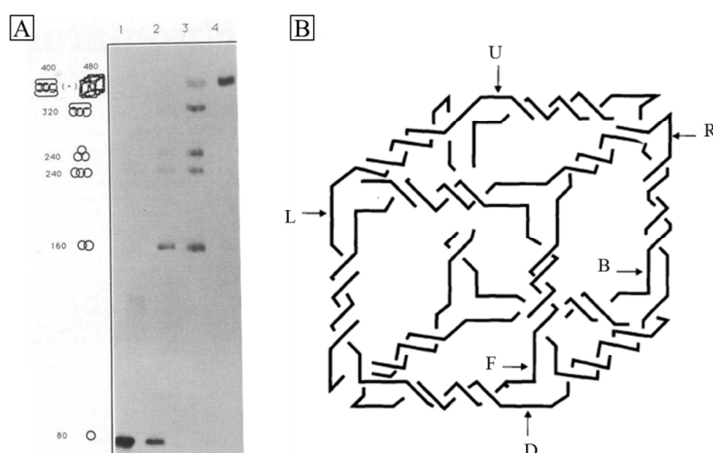


Figure 1.4: (A) denaturing gel showing the formation and analysis of the DNA cube assembled by DNA ligation. Lane 1 contains a cyclic 80-mer. Lane 2 contains markers (individually synthesized and then combined) corresponding to the intermediate products (up to four circles) shown on the left. Lane 3 illustrates the products of the final ligation. The portion of the gel containing the 80-mer circle in this lane was lost during manipulation. The top most band in this lane contains a mixture of the 6-cycle final product and the 5-cycle failure product as indicated by the (+) symbol. These materials have been separated, and lane 4 contains the purified 6-cycle final product. (B) Schematics of the DNA cube composed of six catenated strands that have been catenated together in this particular arrangement. They are labeled by the first letters of their positional designation: up (U), down (D), front (F), back (B), left (L) and right (R). Images (A) and (B) from [41].

After only a few years, relatively simple structures made of DNA were synthesized by the Seeman group^[41, 42, 43] and others^[37, 44, 44], including repeating patterns of DNA junctions known as DNA tile arrays.

1.3.1 DNA arrays

Since then, three-way DNA junctions and junctions with more than four branches have been successfully synthesised in the laboratory^[43, 46, 47]. Often, the pitch of the DNA helix is taken as a unit to express the length of a particular section. If more than 10.5 base pairs (bp) form one turn, the helix is said to be overwound, and similarly, if fewer base pairs are used, the helix is underwound^[48]. In either case, the DNA helix may remain folded; there is flexibility in the number of bases required per turn, which is an important design consideration for DNA structures.

Nanostructures formed from DNA junctions (which have also been referred to as DNA tiles^[49]) can be polymerised to form periodic DNA arrays for use in X-ray crystallography^[50, 51] and structural engineering materials^[41, 52]. Flexibility of periodic^[53] and aperiodic^[54] DNA arrays can be ensured by the insertion of extra T bases at the vertices. These additional bases remain unpaired as the system assembles to form small, flexible ssDNA loops (T-loops). He *et al.*^[55] demonstrated that regular polygons could be constructed using DNA three-point-star motifs (tiles) (Figure 1.5A). The assembly of different polygons relied on the flexibility of the DNA junction (the number of T bases in the T-loops), the concentration of the individual strands, and the meticulous arrangement and number of bases in each DNA tile. Zhang *et al.*^[54] illustrated the importance of the T-loops in the assembly of aperiodic patterns made of DNA helices to accommodate different angles (Figure 1.5B), which he used to create an impressive piece of DNA art (Figure 1.5C).

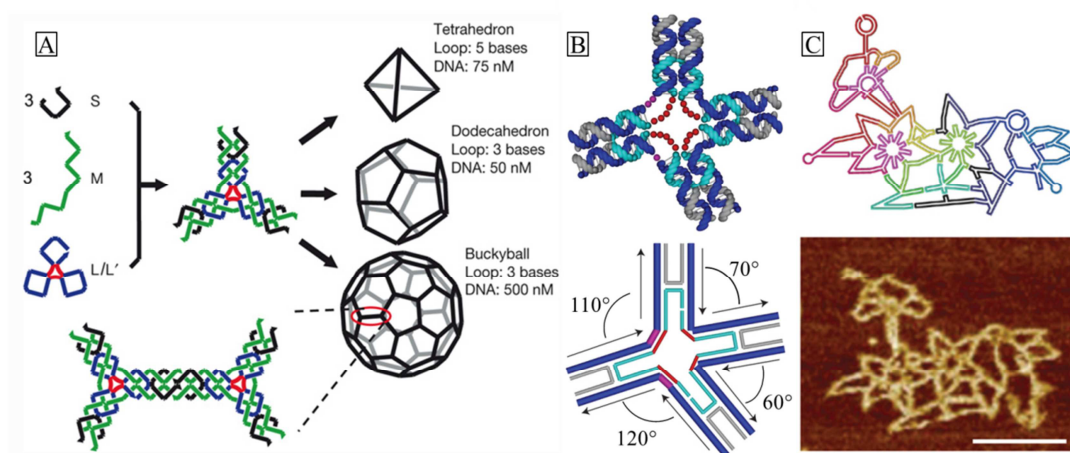


Figure 1.5: (A) Three different sequences of ssDNA assemble first into a symmetric three-point-star (DNA tile), which then assembles into polyhedra in a one-pot process. There are three single-stranded loops per tile (red) in the centre of the complex. (B) A DNA helical model of a 4x4 junction (top) and a line model of the same 4x4 junction (bottom) indicating an approximation of the angles between the helices. Unpaired bases (T-loops) are in red. (C) The design of a flower-and-bird pattern (top) and atomic force micrograph of the synthesised DNA array (bottom). Scale bar 100 nm. Image (A) from [55], images (B) and (C) from [54].

As can be observed in Figure 1.5, DNA can be used to create some amazing patterns and structure, and not just a genetic information carrier. In 2003, Yan *et al.*^[56] proposed the

implementarion of longer scaffolds strands as templates for tile assembly, and soon after Shih *et al.* reported the construction of a DNA octahedron^[57], consisting of a long single-stranded DNA. These advances^[58] improved the size and precision in the production of large DNA arrays and structures, and led to a second breakthrough in 2006 in the lab of Paul Rothemund; DNA origami.

1.4 DNA ORIGAMI

The field of DNA nanotechnology progressed significantly in 2006 when Paul Rothemund at the California Institue of Technology (Caltech) presented a building strategy to assemble much larger and more intricate DNA structures than previous methods allowed. By using a single stranded circular viral DNA strand as a scaffold, he could “fold” this strand into a desired shape based on the designed sequence and the use of hundreds of small oligonucleotides called staples^[59], which would help direct and maintain the design (Figure 1.6). He appropriately named this technique DNA origami in reference to the Japanese art. Shortly after, and with the development of computer-aided design software and simulation methods^[60, 61, 62], DNA origami is now a widespread technique to assemble nanostructures to be used in nanotechnology.

1.4.1 Synthetic considerations for DNA origami

DNA origamis are created by first designing a pathway, or folding procedure, for a single strand of circular, viral DNA in order to construct a desired shape (Figure 1.6A). The folding pathway (routing) must avoid knots and other folding dead-ends, which is often very challenging. Once the shape is outlined by the DNA scaffold, shorter complementary strands (staples) are added to the design to hybridise with the scaffold and define the shape (Figure 1.6B,C). The helices of the DNA origami are held together by regular crossovers (a DNA strands on a first DNA duplex join to a second antiparallel DNA helix) that are made by the staples or the scaffold (Figure 1.6A). A crossover

position is dictated by the relative position of the base pairs; every half turn a crossover can be made to a DNA helix above or below. A crossover is defined as the position at which strands running along one helix switch to an adjacent helix and continue from there.

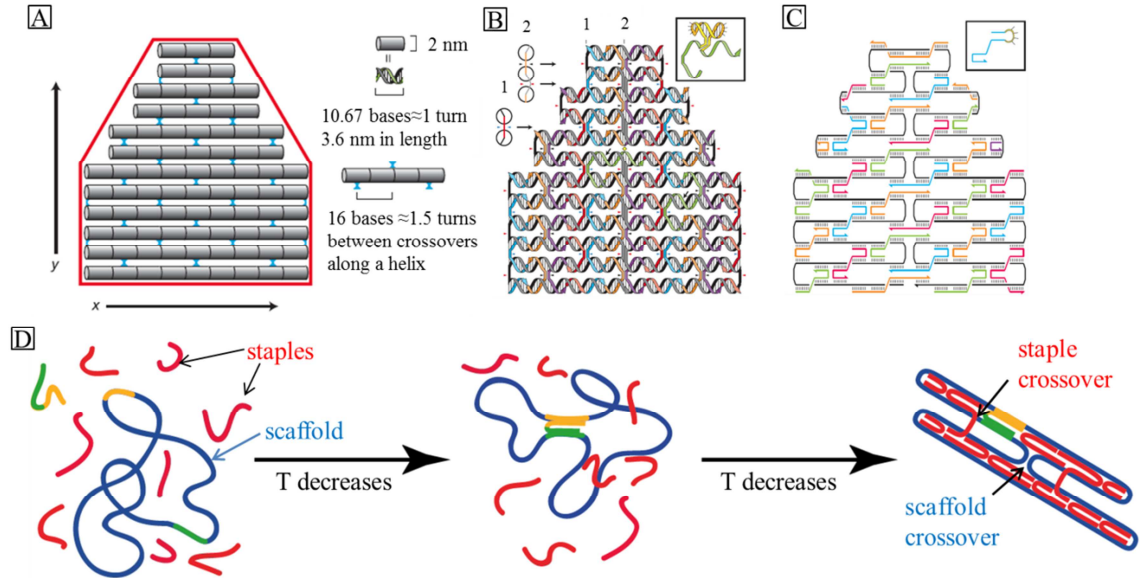


Figure 1.6: (A) A desired shape (outlined in red) made from DNA is approximated by parallel double helices joined by periodic crossovers (shown in blue). (B) The origami design is shown as double helices and staples are shown that bind to the scaffold. Positions 1 and 2, when viewed from the left, show the backbone positions of the DNA with coloured lines. (C) The same design is shown with DNA strands drawn as lines, highlighting the single strand of circular, scaffold DNA (black line) and DNA staples (coloured lines). (D) Evolution of the folding of a DNA origami as temperature decreases. The scaffold is shown in blue and the staples shown in red. One staple is highlighted in yellow and green, which binds to the complementary domains on the scaffold. Images (A)-(C) from [59].

The design of DNA origamis is aided by computer programs^[63] that help arrange staple strands and evaluate their conformational flexibility^[64, 65]. The DNA nanostructure is then folded in a one-pot reaction by allowing a heated mixture to cool down slowly (annealing) (Figure 1.6D). The blunt ends of the helices expose base pairs that can make a stacking interaction with another origami^[66]. This phenomenon can be prevented by including a few unpaired bases at the end of the dsDNA; the extra bases (often poly T) act as a protective cap^[65] and will be referred to as T-cap in this thesis.

A DNA origami can assemble into higher-order architectures by joining individual folded nanostructures^[63, 67, 68] with complementary short sequences located on different

DNA origamis (Figure 1.7). The specific pairing of these short sequences, called sticky ends, allows one to finely tune the association of the origamis (strength, periodicity, flexibility, morphology) and build large DNA superstructures^[63, 69, 70] with nanoscale precision. DNA origami extended quickly to the formation of three-dimensional shapes^[71, 72]. Douglas *et al.*^[63] demonstrated how individually flat DNA origami can be polymerised in finite number to form closed 3D wireframe polyhedra (Figure 1.7 A-C), and Liu *et al.*^[67] demonstrated the assembly of long polymers, the connectivity of which dictated the final shape of the superstructure (Figure 1.7D, E).

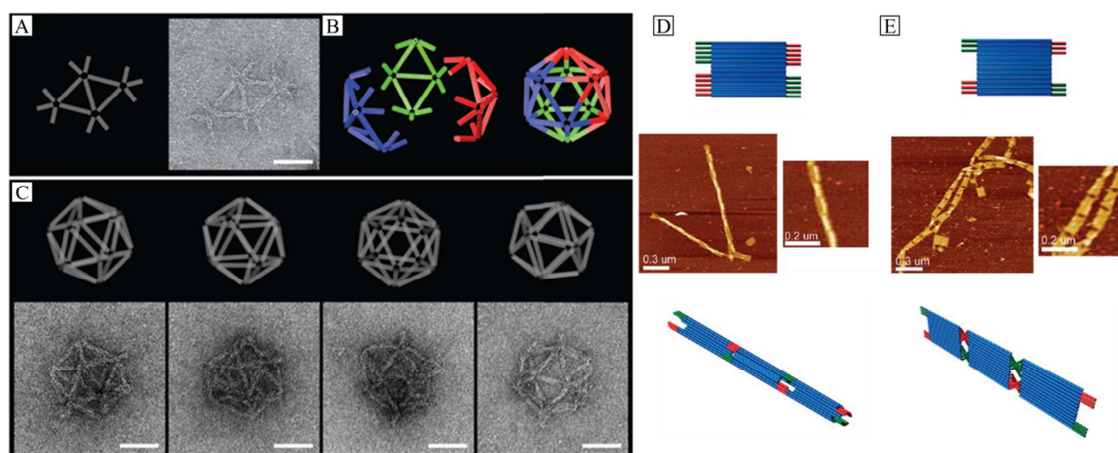


Figure 1.7: (A) Cylinder model (left) and transmission electron micrograph (right) of a double-triangle shape comprised of 20 six-helix bundle half-struts. (B) Heterotrimerisation of three double-triangles to form an icosahedron with a 1:1:1 mixture of the three monomers. (C) Orthographic projection models and TEM data of four icosahedron structures. Scale bars (A-C) 100 nm. (E) Shown top to bottom: schematic drawing of the number and the position of linkers used for the connections between origami tiles to form a DNA tube; an AFM image (scale bar 300 nm) of the synthesised structure, with magnification (scale bar 200 nm); and a schematic drawing showing the assembled structure. (F) Shown top to bottom: schematic drawing of the same tile to form a DNA ladder; an AFM image (scale bar 300 nm) of the synthesised structure, with magnification (scale bar 200 nm); and a schematic drawing showing the assembled structure. Images A-C from [63], images D, E adapted from [67].

Three-dimensional shapes can also be constructed as pleated layers of helices constrained to a square^[73] (Figure 1.8A) or a honeycomb^[63] (Figure 1.8B) lattice. In the honeycomb lattice, each double helix has three nearest neighbours and is designed to link to each with antiparallel strand crossovers. Crossover in honeycomb lattice packing are spaced in intervals of 7 bp along the helical axis^[65] (Figure 1.8C). The square lattice has up to four nearest neighbours that can also be linked every 8 bp to each helices with

antiparallel strand crossovers. A square lattice provides a more densely packed architecture, and the ability to create surfaces that are more flat than is possible with the honeycomb lattice, but display significant global twisting (Figure 1.8D) of the structures due to larger strain in the denser arrangement of the DNA helices^[73]. A honeycomb lattice do not suffer from twisting of the helices with respect to crossovers and assemble in a more porous DNA origami. A hexagonal lattice has recently been constructed by Shih *et al.*^[74]; as well as a mixture of the three lattice organisation (Figure 1.8E, F).

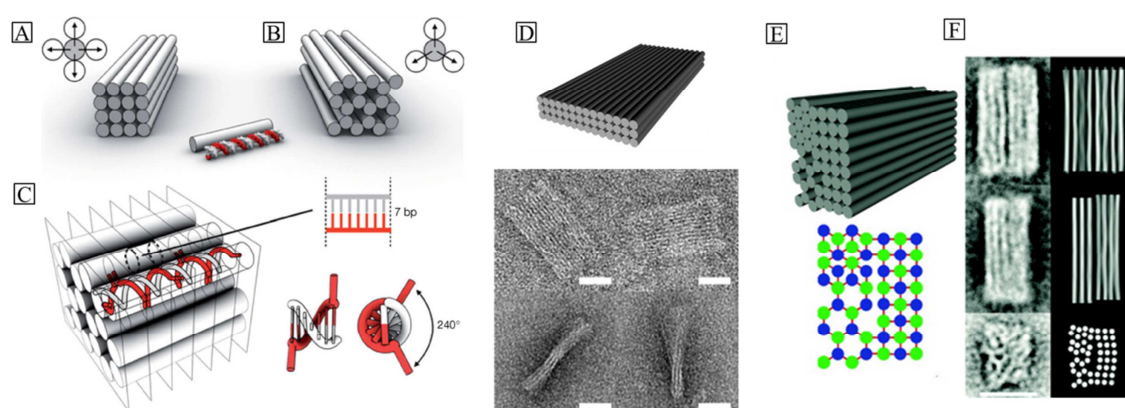


Figure 1.8: Cross-sectional view of multilayer DNA origami objects in (A) square lattice and (B) honeycomb lattice packing. (C) Cross-overs in multilayer objects with honeycomb lattice packing, spaced in constant intervals of 7 bp along the helical axis to link double-helical domains to each of three possible neighbours. The cross-over spacing of 7 bp complies with the natural B-form DNA twist density of 10.5 bp per turn. Each cylinder represents a DNA double helix. Images from [65]. (D) 3D DNA origami solid blocks with three-layers. Three-dimension perspective cylinder view (top pannel), and representative transmission electron microscope (TEM) micrographs of negatively stained particles observed showing twisting of the helices (lower pannel). Scale bars 20 nm. Images from [73]. (E) A 3D perspective cylinder view and a top view along a DNA helix axis of 52HB honeycomb/square/hexagonal lattice (F) Averaged TEM images of three different orientations of (E): XZ projection, YZ projection, and XY projection (left pannel), and images of computational simulation (right pannel). Scale bar 20 nm. Images from [74].

In contrast with the square and honeycomb lattices, it is impossible to arrange helices in a hexagonal lattice such that all nearest neighbour helices (with a crossover every 9 bp) are antiparallel. Hexagonal lattice is currently not available with computer aided programs, but offers the most densely packed DNA helices and the least susceptibility to twist amongst all three architectures.

1.4.2 Functionalisation of DNA origami

The remarkable folding precision of a DNA origami allows each staple to be positioned with nanometre precision^[59] (~6 nm). Sequence modification and chemical modification of DNA staples with a protein, dye, nanoparticle^[75, 76], lipids^[77] (discussed in section 1.7), or other molecule engenders functionalisation of the DNA to expand the chemical^[78], optical^[76] and electronic functionality of the origami^[79]. A DNA-biotin conjugate has been used to organise a streptavidin protein on a DNA tile to demonstrate protein patterning on a DNA origami template^[80, 81] (Figure 1.9A). Gold nanoparticles (AuNPs) have been attached to a DNA origami^[82] with the use of short sequences on the DNA staples that hybridise with a complementary strand attached to the surface of the AuNPs^[52, 83]. A DNA origami has also been synthesised to provide a mold for the growth of gold nanoparticle with definite shape^[83] (Figure 1.9B).

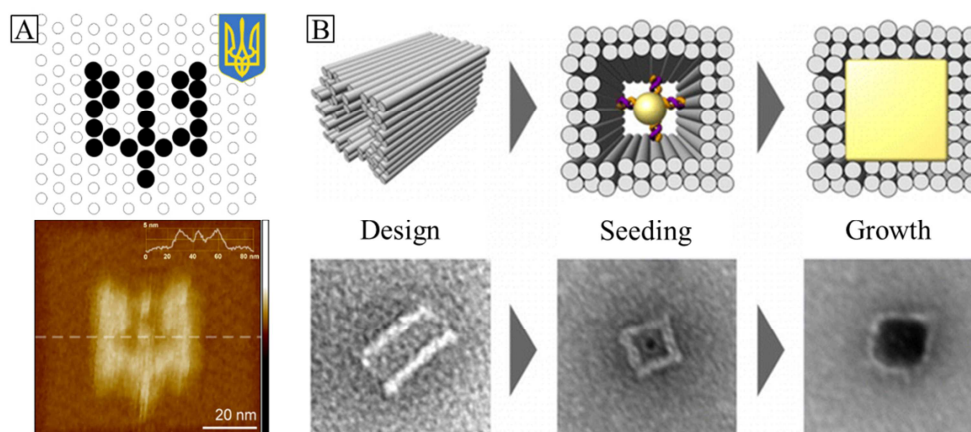


Figure 1.9: (A) Schematic diagram for patterning proteins on a surface of DNA origami (top). The filled circles represent positions of a DNA tile that have been modified with biotin to bind to streptavidin, and the unfilled circles do not have modifications. The pattern resembles the motif in the inset. A high magnification AFM image (bottom) illustrates the precision of the protein assembly. (B) Scheme (top panels) and TEM (micrograph (lower panels) of a DNA origami molds with an inner cavity (left). Capture DNA strands in the center of the cavity allow the site-specific introduction of single gold nanoparticle (AuNP) seeds carrying DNA strands with the complementary sequence (middle). In the presence of a gold precursor and a reducing agent, the growth of the nanoparticle is initiated. Further gold deposition is blocked by the mold walls, such that the particle adopts the shape that is dictated by the mold (right). Images (A) from [80] and images (B) from [83].

1.5 CELLULAR MEMBRANES

1.5.1 Biological cellular membranes

A cell is the smallest living unit and is enclosed and separated from the environment by a thin film of only a few nanometres^[84]. This thin film, the cell membrane, not only serves as the margin between life and death for a cell, but further divides the interior of a cell into organelles. This compartmentalisation is essential for cells such that they can perform a diverse and complex set of functions necessary for life. The cellular membrane is constituted of a lipid bilayer, which divides and distributes different metabolic reactions into separate compartments. The main type of lipids found in the cell membrane are phospholipids, which are molecules composed of a hydrophilic phosphorous head group and one or two hydrophobic legs of carbon chains; the lipid molecule 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) is shown in Figure 1.10A. Due to their chemical amphipathic properties, the apolar carbon chains of the phospholipids are predisposed to self-associate in water, orientating the charged phosphorous head groups to face the water molecules^[85]. This self-assembly leads toward the formation of membranes, where the hydrophobic moieties are hidden from aqueous solutions between two layers of hydrophilic phosphorous heads (Figure 1.10B).

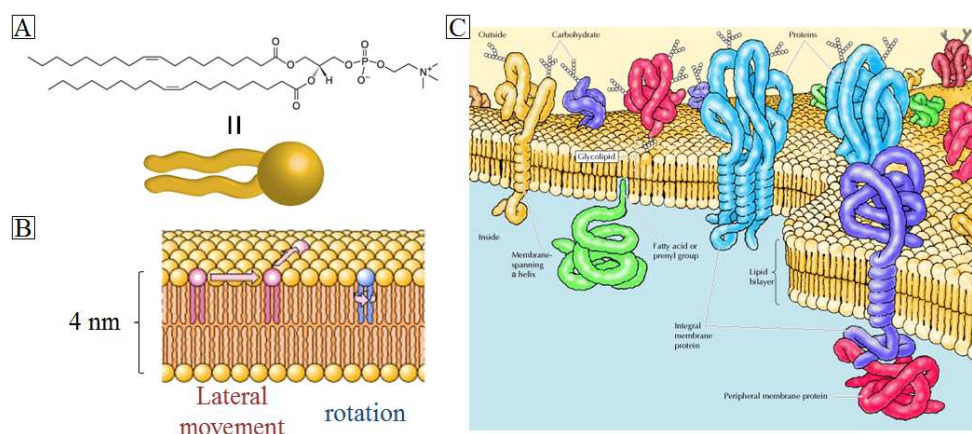


Figure 1.10: (A) Chemical structure of a phospholipid (DOPC), and (B) schematic representation of a lipid bilayer showing the mobility of the phospholipid within it. (C) Fluid mosaic model of membrane structure including membrane proteins. Image from [86].

Within the self-assembled membrane, individual phospholipids are constantly interconverting and rotating, and this fluid structure is covered with a large variety of proteins and membrane-associated proteins (Figure 1.10C)^[86]. It has been determined that these membrane-bound proteins are fundamental for biological processes including energy conversion^[87], cargo transportation^[88, 89], signal transduction^[90], and intercellular communication^[91, 92]. While compartmentalisation of the cell can be promoted by the asymmetric lipid composition of the membrane^[93, 94] and the non-equilibrium state maintained by the work of the proteins in the membrane^[95], malfunction of these integral membrane-bound proteins result in many lethal diseases^[90]. It is therefore not surprising that understanding these lipid systems and their structure-to-function relationship is of great interest for applications in therapeutic medicine, molecular biology and single molecule biophysics^[84, 96].

The structural properties resulting from different lipid chains or headgroups can favour specific membrane curvatures and biophysical properties^[97]. Lipid molecules with a large headgroup and a single carbon chain, such as detergent molecules^[98], have an overall conical shape and spontaneously form micelles in aqueous solution (Figure 1.11A, B). Cylindrical-shaped lipid molecules, such as phosphatidylcholine, arrange in a bilayer (Figure 1.11C,D). Lipid molecules with a small hydrophilic cross-section form an inverted curvature with the hydrophobic tails pointing towards the outside^[99] and form cylindrical assemblies (Figure 1.11E).

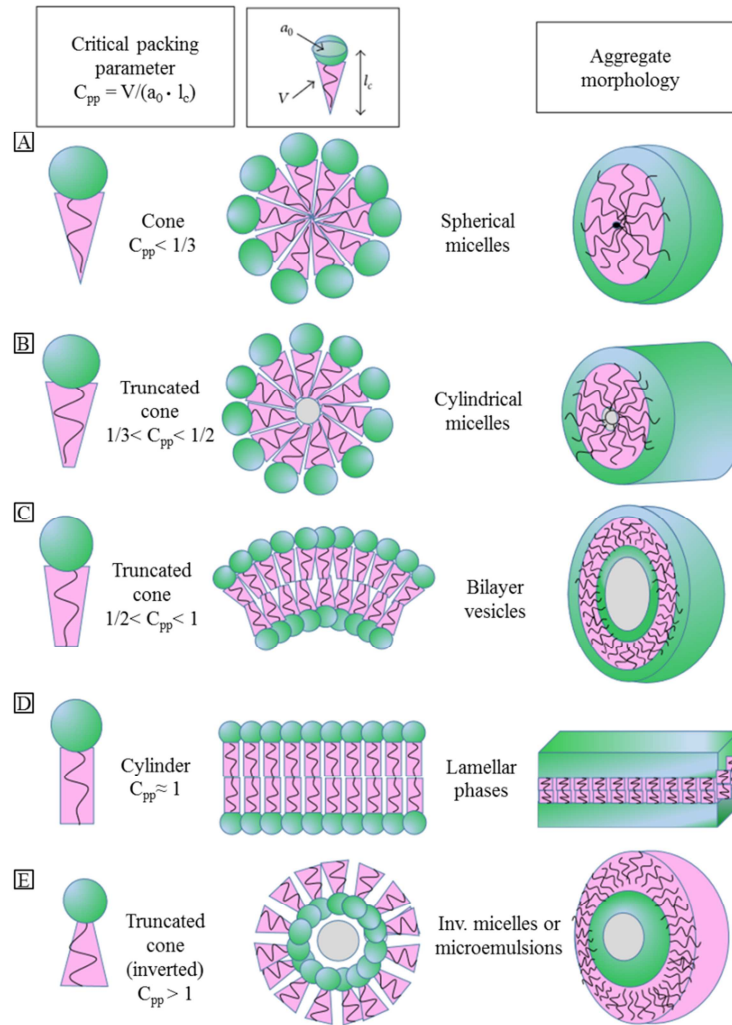


Figure 1.11: Structures formed by different lipids: Amphiphile shape factors and summary of the aggregate structures that can be predicted from the critical packing parameter C_{pp} . (A) spherical micelles, (B) cylindrical micelles, (C) bilayer vesicles, (D) lamellar phases, (E) inverted micelles. Images from [103].

It is possible to predict the structure of a lipid aggregate using the geometric properties of the lipid molecules^[100, 101]. This is defined as the critical packing parametre (C_{pp}) of lipid molecules in equation 1, where v is the molecular volume, a is the cross-sectional area of the head group, and l is the length of the molecule^[102, 103]:

$$C_{pp} = \frac{v}{al} \quad (\text{Equation 1})$$

For example, a C_{pp} of $1/3$ predicts the assembly of spherical micelles (Figure 1.11A), and a C_{pp} of 1 indicates the formation of a flat lipid bilayer (Figure 1.11D).

Although cylindrical lipids ($C_{pp} = 1$) initially form flat lipid membranes, this fluid structure can further be transformed into different shapes and sizes. For example, to form a large vesicle that is composed of a lipid bilayer, lipid molecules with the correct geometry are first aggregated. This increase in concentration of lipids with a particular geometry on one side of the bilayer is performed by the work of proteins within the cell^[104, 105]. An initially formed flat bilayer must then bend to form the vesicular shape. The approximation of the energy required to bend a flat bilayer is largely based on the classic Helfrich model^[106] which suggests that it costs approximately 200-250 kcal/mol of vesicle to create a closed spherical structure. This model assumes that the radius of curvature R is much larger than the membrane thickness d ($R \gg d$) in which case the curvature c of the membrane is considered small. However, a typical radius R for intracellular membrane vesicles is only a few tens of nanometres^[107, 108], and as the membrane thickness is approximately 4 nm, this limits the applicability of this model. To overcome this drawback and understand the kinetics of larger membrane vesicles, synthetic approaches to form abiological lipid bilayers are often pursued to investigate membrane processes, allowing scientists insight into membrane-related biological processes.

1.5.2 Model cellular membrane

A model membrane bilayer can be considered as a simplified two-dimensional structure of lipids whereby the dynamics of interacting components can be assessed. The lipid molecule DOPC, shown previously in Figure 1.10A, is one of the most common lipids in animal cell membranes and is commonly synthesized for use in biotechnology research^[109]. This phospholipid possesses physical and chemical properties that allows scientists to readily assemble model membranes^[110]. The phase transition of a lipid is defined as the temperature at which the lipid physical state changes from the ordered gel phase (hydrocarbon chains extended and closely packed forming a solid gel) to the

disordered liquid crystalline phase (hydrocarbon chains randomly oriented and fluid forming a fluid gel). The phase behaviour of a lipid refers to the relative mobility of the individual lipid molecules and how this mobility (phase) changes with temperature. DOPC and other lipids commonly found in cell membranes have a low transition temperature (-20°C for DOPC^[109]) and are in their liquid crystalline phase at 18°C and above; this feature makes them ideal to form maleable membranes. Various approaches to synthetically create a membrane bilayer have been developped to address the complexity of biological membranes^[111]. Two of these are 2D supported lipid bilayers (SLB) and 3D lipid vesicles of sizes ranging from tens of nanometre (liposomes or small unilamellar vesicles, SUVs) to tens of microns (giant unilamellar vesicles, GUVs).

Supported lipid bilayers (SLB) are most commonly formed by Langmuir transfer^[112] to a surface^[113] (Figure 1.12) or by vesicle fusion^[114]. A lipid bilayer can be deposited directly to a solid support (Figure 1.12A) or a lipid monolayer can be formed on a surface modified with hydrophobic chains (Figure 1.12B). Solid SLB offer stability, patterning^[115] and availability to a wide variety of surface sensitive techniques to characterise^[116, 117] and study basic membrane processes^[118, 119]; this also removes the requirement for fluorescent labelling, which is known to influence phase behaviour^[120]. A new emerging field in solid SLB is the developpment of air-stable lipid membranes systems for biosensors^[121].

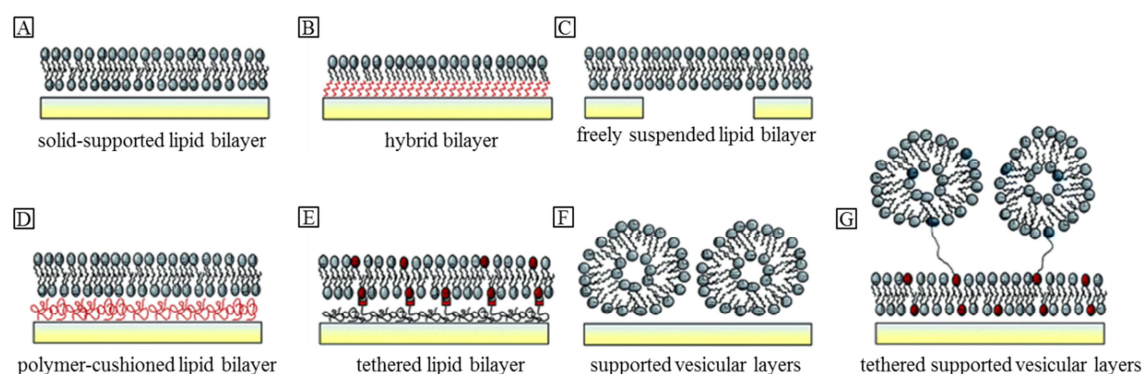


Figure 1.12: Examples of surface-confined membrane models: (A) solid-supported lipid bilayer; (B) hybrid bilayer; (C) freely suspended bilayer; (D) polymer-cushioned lipid bilayer; (E) tethered lipid bilayer; (F) supported vesicular layers; (G) tethered supported vesicular layers. Image adapted from [118].

Suspended SLB refer to lipid bilayers that are freely suspended (Figure 1.12C), polymer-cushioned (Figure 1.12D), tethered (Figure 1.12E) or that have vesicles attached (Figure 1.11F, G) to a supported surface. They have been used to study lipid-lipid and lipid-protein interactions that occur at the membrane surface^[122, 123], and to examine transmembrane proteins^[124] and membrane dynamics^[123,125].

GUVs have been successfully used to determine the phase behaviour of lipid mixtures in bilayers^[126] and as a novel architecture to study reaction dynamics in confined volumes^[127]. GUVs are typically created by electroformation using a well-defined mixture of pure lipids^[128]. A dry lipid mixture film is first rehydrated, then placed between two electrodes (Figure 1.13A). A voltage is applied (typically 5 peak-to-peak voltage [Vpp], 10 Hz) until vesicle detachment (Figure 1.13B). Giant plasma membranes, or blebs, resemble GUVs but are made from a more direct source of biological lipid material (they protrude and detach from cells in response to chemical stresses^[113]).

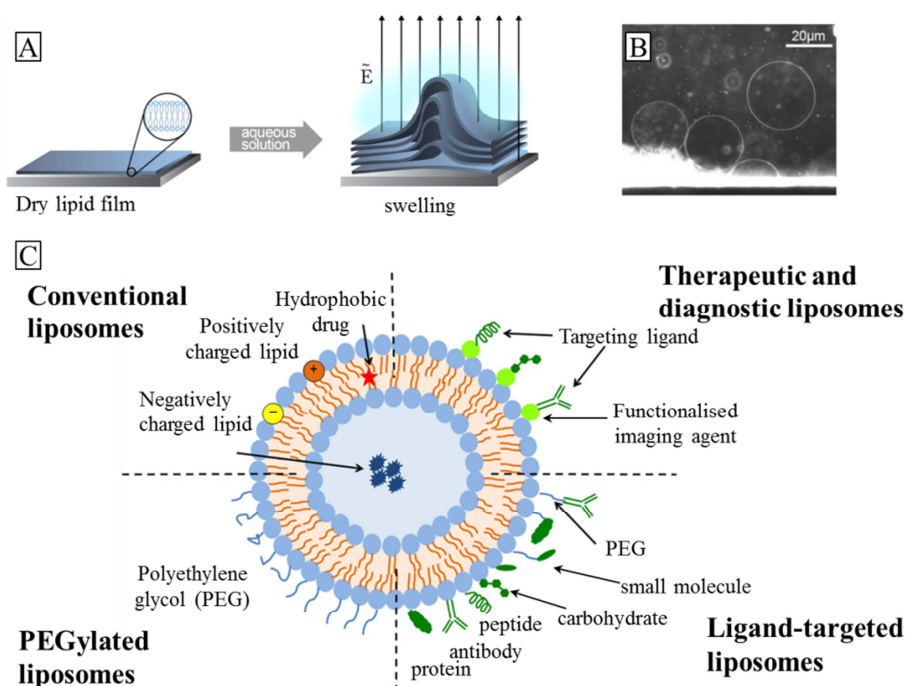


Figure 1.13: (A) Schematic of vesicle electroformation from a dry lipid bilayer. Vesicles are formed upon hydration and the application of an alternating electric field, $\tilde{E}$. (B) bright field microscopy photograph of vesicles forming, swelling and detaching along the electrode. (C) Schematic representation of different types of liposomal drug delivery systems. Images (A) and (B) from [128] and image (C) adapted from [135].

SUVs of a few tens of nanometres in diameter can also be produced similarly to GUVs from the rehydration and electroformation of a lipid film^[129]. Sonication or extrusion of the lipid solution through a porous membrane breaks the initially formed large vesicles into SUVs that are often referred to as liposomes^[130]. Because of their biocompatibility and aptitude to trap and shield both hydrophilic (in the centre of the vesicle) and hydrophobic (inside the lipid bilayer) molecules^[131], liposomes are extensively used as carriers for numerous molecules in cosmetic^[132, 4], pharmaceutical^[133, 134, 135] and food and farming industries^[136]. Liposome surfaces have been functionalised with proteins or other organic molecules (Figure 1.13C) to increase their site-specific drug delivery^[134], decrease their biotoxicity, and improve their bioavailability^[133]. Membrane-bound proteins are also integral to the shaping of the fluid lipid bilayer.

1.6 MEMBRANE-SHAPING PROTEINS

The lipid bilayer of a cell is continuously changing in shape and size^[95, 137] as a result of the interaction of proteins on the surface of the membrane^[93] or other mechanical^[138, 139], chemical or electrical stimuli^[140]. Proteins can change the membrane curvature by applying two types of forces to the bilayer: pulling and bending. The actin protein, for example, is part of a network underlying the outer membrane of the cell (cytoskeleton) and is responsible for the cell mobility and contractility^[141, 142] (Figure 1.14).

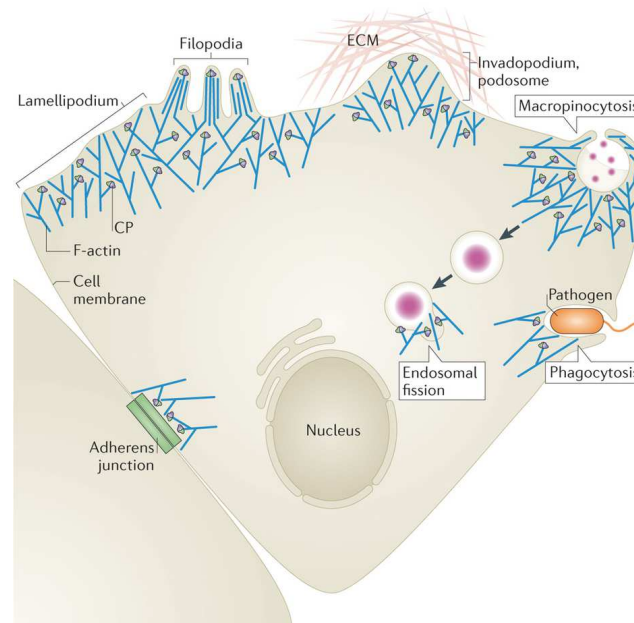


Figure 1.14: Simplified schematic of a cellular cytoskeleton illustrating actin filament (blue) deforming the membrane. Image from [142].

Polymerised actin filaments grow in the cytosol near the cell membrane and deform the membrane by applying a pulling force. As the actin filaments self-assemble, they favor the formation of tubulations (filopodia) protruding or retracting from the cell^[143], depending on the direction of growth of the actin filaments.

Specific regions (domains) of a number of proteins are sensitive to the spontaneous curvature of the cell membrane or induce a bending of the membrane (or both). The BAR domain defines a superfamily of proteins involved in membrane remodelling

through their membrane-binding surfaces (Figure 1.15)^[144, 145]. The unit responsible for membrane binding is a dimer of the BAR domain, which resembles the shape of a banana. Positively charged amino acid residues form a cluster at the curved surface of the protein^[146] and creates an electrostatic interaction with the negatively charged head groups of the lipid bilayer^[144-147]. The localisation of the positively charged amino on the curvature of the BAR domains determines the orientation of the bending of the membrane^[147].

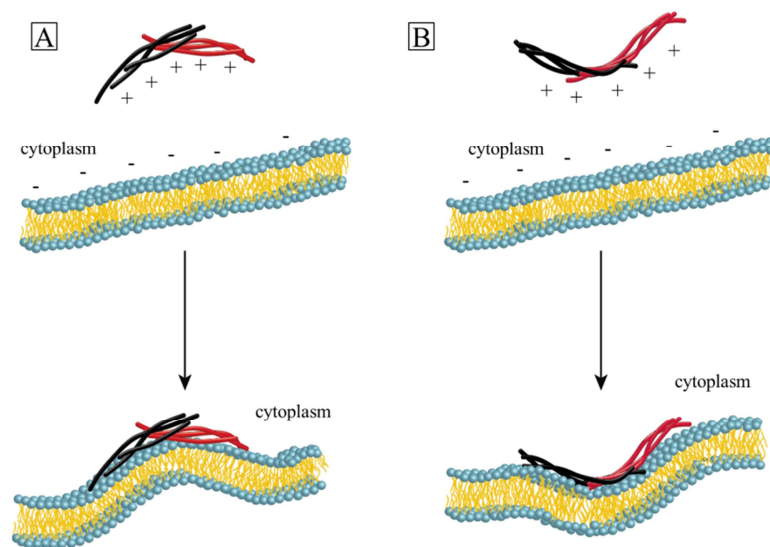


Figure 1.15: Illustration of BAR domains attaching to and bending a lipid bilayer. Positively charged amino acids are located on the (A) convex face or the (B) concave face of the BAR domains, and electrostatically attract to the negatively charged membrane to cause curvature in the membrane.

The mechanism through which BAR proteins attach to and bend a lipid bilayer is only partly understood, but it is known that the interplay between the lipid composition and the protein is believed to be a major factor that results in membrane curvature^[147].

Other membrane-bending proteins utilise transmembrane proteins present within the membrane to anchor to the lipid bilayer. For example, the clathrin protein within a cell selectively binds to the flexible region of adaptor proteins^[149] when they have bound an extracellular cargo (Figure 1.16A, B)^[150]. Clathrin is described as a trimer of three large polypeptide chains (heavy chains) that are each associated with a light chain, which are

connected at a central vertex. The bulk of the heavy chains creates an arched tripodal “leg” with a radius of 52 nm^[151], and is referred as the clathrin *triskelion* (Greek for “a three-legged structure”). Short motifs in the outer heavy chains of one triskelion can bind to the heavy chains of a neighbouring triskelion and associate into a coat-like structure, or “cage”^[152, 153], which have been observed with TEM (Figure 1.16C).

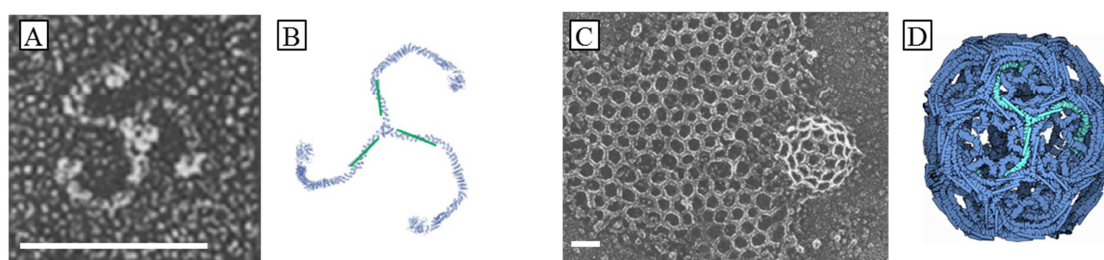


Figure 1.16: (A) Electron micrograph and (B) schematic representation of a clathrin triskelion, where the heavy chains are shaded in blue and the light chains are shaded in green. (C) Electron micrograph of a clathrin plaque and a clathrin “cage”. (D) Detailed 3D structure of a clathrin vesicle using cryo-EM reconstruction, in which one individual clathrin molecule is coloured light blue to highlight its structure. (A) and (C) images from [154], (B) image from [153] and (D) [155]. Scale bars 50 nm.

The assembled clathrins form a lattice in which the center of each triskelion sits on every lattice point^[152]. The interactions of the clathrin legs form pentagons, hexagons (most commonly), and heptagons^[156]. In addition to the 2D array of clathrin triskelia, 3D structures, or “cages”, have also been observed (Figure 1.16D). The size of a clathrin cage can vary between 35 nm and 200 nm, depending on the type of cell and the localisation of clathrin within the cell^[149, 1521, 157]. Both BAR domain proteins and clathrin proteins are involved in membrane bending and invagination, and participate in two different pathways^[158] to absorb cargo into the cell. This active transport mechanism, endocytosis, is the process through which the cell absorbs nutrients across the bilayer. BAR domain and clathrin protein can attach, assemble, and finally form a detached vesicle in seconds^[159, 160], which involves impressive protein machinery and membrane dynamics.

Clathrin-coated invaginations of various shapes^[161, 162, 163] and sizes^[157, 164] (Figure 1.17A) observed in nature in coexistence with open, flat plaques initiated discussions as

to whether an initially formed clathrin 2D plaque can transform into a 3D pit^[165]. Based on direct observations in living cells^[166, 167], it has been shown that clathrin can polymerise, bend and then invaginate a membrane (Figure 1.17B). The newly formed vesicle is then cut free from the outer membrane by scission proteins. However, many have argued that the drastic deconstruction and reconstruction of the 3D cage is energetically unfavourable^[161] and that the reduced membrane elasticity (due to an unbalanced osmotic pressure) affects and even prevents clathrin polymerisation^[168]. Thus, they propose a model in which only the 2D plaque is responsible for invagination of the membrane (Figure 1.17C). Recent methods developed in live-cell imaging has enabled a third postulate which suggests that plaques and pits rely on different dynamics and are part of two distinctive biological processes^[162].

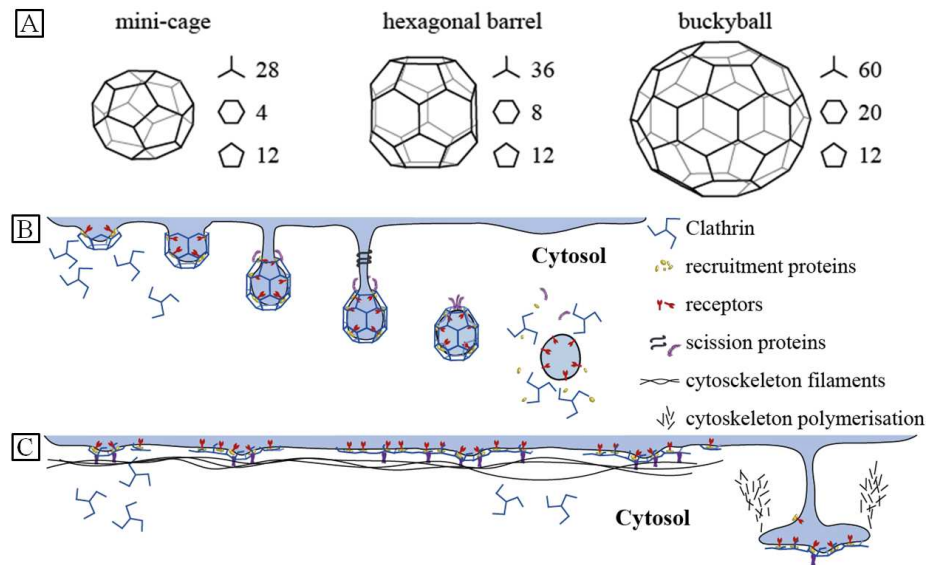


Figure 1.17: (A) Three examples of shapes that can be assembled with the clathrin triskelion. (B) Postulated stages of assembly of a clathrin-coated vesicle leading to endocytosis. Once in the cytosol, the proteins disassemble and the cycle can restart. (C) Postulated stages of assembly of a flat clathrin array leading to the formation of a plaque. (A) Adapted from [155].

The mechanism of endocytosis is still debated among the postulates mentioned above, and the forces involved in membrane bending are still uncertain. An artificial protein model based on nanotechnology has the potential to provide insight into understanding the complex interplay between proteins and lipids during endocytosis.

1.7 DNA NANOTECHNOLOGY AND LIPID MEMBRANES

As both the cellular membrane and nanostructures composed of DNA are of similar nanometer dimensions, there is considerable interest and progress made in using DNA nanotechnology as a versatile tool to mimic the mechanisms involved in regulating membrane shape and dynamics. Although negatively charged DNA molecules do not interact spontaneously with amphiphilic phospholipid membranes in biological systems, oligonucleotides have been chemically modified with hydrophobic moieties such as cholesterol, squalene or fatty acids, to enable insertion into a lipid membrane^[169, 170]. Over the last decade, lipid-DNA anchors have been developed to insert into model membranes^[118] enabling the study of membrane processes^[139, 171] and cell-to-cell junctions^[172]. The design and hydrophobic modification of a DNA nanostructure has recently lead to the formation of an ion channel mimic^[90, 173]. DNA structures anchored to membranes were also demonstrated promising results in drug-delivery systems^[6] and other DNA-lipid conjugates were shown to increase the pharmacokinetic behaviour and trans-membrane delivery of DNA into cells^[169]. A DNA origami attached to a lipid bilayer demonstrated analagous behaviour to membrane proteins such as diffusion and surface-templated polymerisation^[174]. DNA-lipid conjugates were used to disentangle the two processes (receptor binding and vesicle fusion) involved in cell infection by an enveloped virus (Figure 1.18A). DNA origami was recently used to provide control over the formation of lipid vesicles by using the DNA origami as a scaffolding template^[175, 176] (Figure 1.18B).

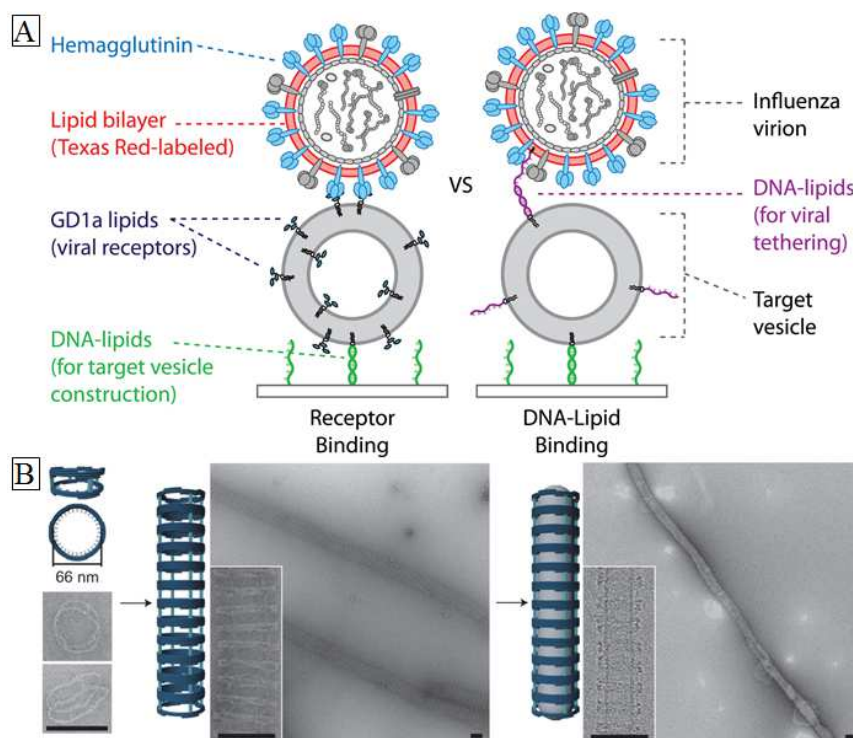


Figure 1.18: Example of DNA nanotechnology applied to lipid systems. (A) Study of cellular fusion mechanism of the influenza virion. Schematic of influenza virions bound to target DNA-tethered lipid vesicles (gray circles) by (left) natural receptors (black) in the target vesicle or (right) hybridization of complementary DNA-lipids (purple strands, as receptor mimickry) in virus and vesicle membranes. (B) Membrane tubule formation inside a 66 nm diameter DNA origami tube. Cartoon model and electron micrograph of DNA cage monomer (left), polymer (middle), and DNA-tube templated membrane tubule. Scale bare 100 nm. Image (A) from [171] and (B) from [175].

The development of DNA nanotechnology thus presents a powerful opportunity to fabricate devices on the nanometer and micrometer scales to mimic membrane-involved biological processes.

1.8 DNA ORIGAMI CHARACTERISATION

This thesis present extensive work with novel DNA origami, which required effective analysis and characterisation. The following section aim at briefly review these observation techniques, with a focus on their advantages and limitations in the context of DNA nanotechnology. Technical details are not extensively presented, as abundant literature is available elsewhere.

1.8.1 Agarose Gel Electrophoresis (AGE)

Various techniques and procedures have been developed to assess the folding of a DNA origami and directly visualise the nanostructure. Agarose gel electrophoresis (AGE) is used to purify the folded DNA origami from the excess DNA staples that are added during the folding procedure; it is also used as an indication of a successfully folded structure (see also Annex A.7). Although this characterisation allows for an early and quick indication of whether the DNA has folded into the desired origami or not, it does not provide morphological information. Thus, other techniques that provide direct visualisation of the shape of the origami are used to complement AGE.

1.8.2 Atomic Force Microscopy (AFM)

DNA origami range in sizes between 10 and 100 nm. As these nanostructures are smaller than the wavelength of visible light (400-600 nm), they cannot be imaged using light microscopy. Due to their nanometer resolution^[177], atomic force microscopy (AFM) and electron microscopy (EM, section 1.8.3) are two techniques that allow direct visualisation of the quality of the folding of the origami. AFM was developed in 1986 by Binnig, Quate and Gerber^[177] and consists of an atomically-sharp tip scanning the height of a surface on which the sample is deposited. The AFM micrograph is thus a change in height (z) as the tip scans the surface, which provides an image of the deposited sample. AFM allows imaging of thin, 2D DNA structures with great precision (e.g. DNA origami as in Figure 1.9A, or the flower-and-bird pattern in Figure 1.5C). However, it is less ideal for large, 3D DNA origami as the pressure from the tip^[178] or lateral forces^[179] can distort or drag the structures across the surface.

1.8.3 Transmission Electron Microscopy (TEM)

Additional methods that are commonly used to characterise a DNA nanostructure are transmission electron microscopy^[63] (TEM) and cryo-electron microscopy^[180] (cryo-EM). With a wavelength on the order of 1 nm^[181], the reflection and deflection of electrons to generate an image offers much higher resolution than that of an optical microscope. As DNA is quite transparent to electrons, a sample of DNA origami is often stained with a heavy metal salt commonly derived from molybdenum, uranium or tungsten that absorbs on to the sample (further details on sample preparation for TEM will be discussed in Chapter 6). These heavy metals scatter the electrons and produce a phase contrast to visualise the origami (Figure 1.19A, B). Although these staining salts provide great detail of the structure, they can sometimes interact negatively with the sample and distort the natural state of the sample^[182, 183]. Thus, biological samples can be imaged with cryo-EM to overcome these drawbacks.

For a DNA origami specimen to be imaged with cryo-EM, the aqueous sample is flash frozen (quickly after staining) into a liquid^[184] such as liquid ethane (-188°C) or liquid nitrogen (-195°C), (or a mixture of the two), thus embedding the sample in vitreous ice in a near-native state; the flash freezing also limits the interaction of the electrons with the frozen water molecules. When the specimen is homogenous (the objects are all identical), the resolution is improved by averaging multiple images at different tilt angles to increase the signal-to-noise ratio. Dietz *et al.*^[180] designed a DNA origami object (Figure 1.19C) that was imaged with cryo-EM (Figure 1.19D, E). The cryo-EM reconstruction of the image was achieved with the help of computer-aided programs to reach an atomic resolution of 3 Å (Figure 1.19F)^[185].

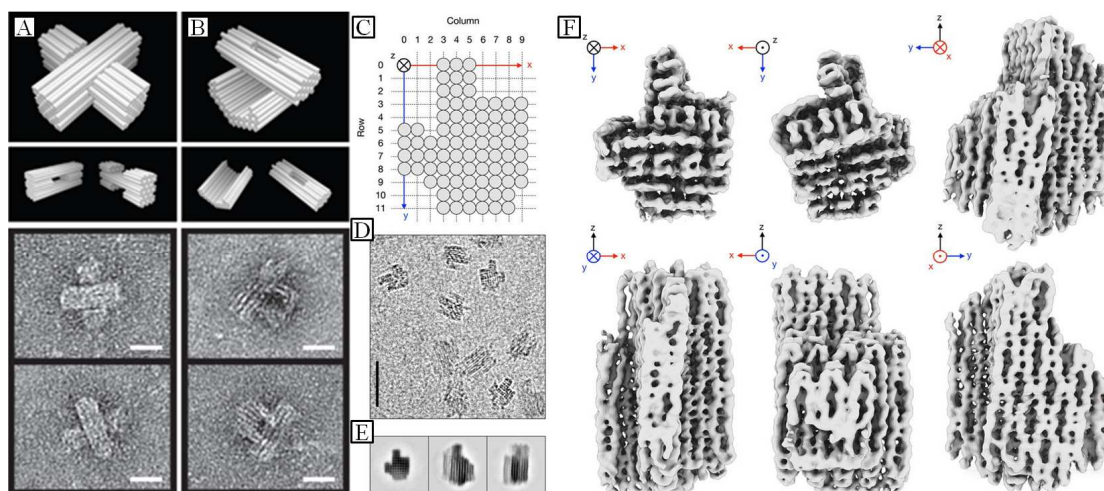


Figure 1.19: (A) and (B) illustrate the design (top panels) and electron micrographs (bottom panels) of two different DNA origamis; the samples have been stained with uranyl acetate for detection and each cylinder represents a DNA double helix. Scale bars 20 nm. (C)-(F): Cryo-reconstruction of a DNA 'object'. (C) Schematic representation of a designed rectangular lattice where each gray circle is a DNA helix. (D) Electron micrograph of the object, (E) reference-free 2D class averages, and (F) six orthogonal views of the 3D reconstruction. Images (A) and (B) from [63], images (C)-(F) from [180].

1.8.4 Fluorescence microscopy

The specific attachment of organic, fluorescent dyes at predetermined positions on a DNA origami allows for observation by fluorescence microscopy. Microscopy techniques relevant for the work presented in this thesis are epifluorescence and confocal microscopy, for the observation of DNA structure on lipid membranes. For colocalisation of a DNA structure on a membrane vesicle in solution, a standard fluorescent microscope equipped with epifluorescence is used^[186]. The specimen in solution is illuminated with light of a specific wavelength (or wavelengths) which is absorbed by fluorophores in the sample, causing them to emit light of longer wavelengths (i.e., of a *different* color than the absorbed light). The key to high resolution and high signal-to-noise (S/N) epi-fluorescence imaging is to be able to excite the sample with a specific color of relatively bright incident light, while having the capacity to separate and detect the much dimmer fluorescence being emitted from the sample^[187].

1.8.5 Confocal microscopy

Confocal microscopy is another optical imaging technique developed by Minsky between 1957 and 1961^[187] that improves the resolution and contrast of micrographs by adding a pinhole at the confocal plane to eliminate out-of-focus fluorescence from the sample^[188] (Figure 1.20A). Three-dimensional reconstruction can be made by recording a set of images taken at different focal depths (z axis, optical sectioning) and overlaying the xy scans (z-stack); this can provide topological information of a specimen. Confocal microscopy provides the capacity to observe intact specimens in a non-invasive manner and also provides 3D information of a sample, which cannot be performed with epifluorescence microscopy discussed previously.

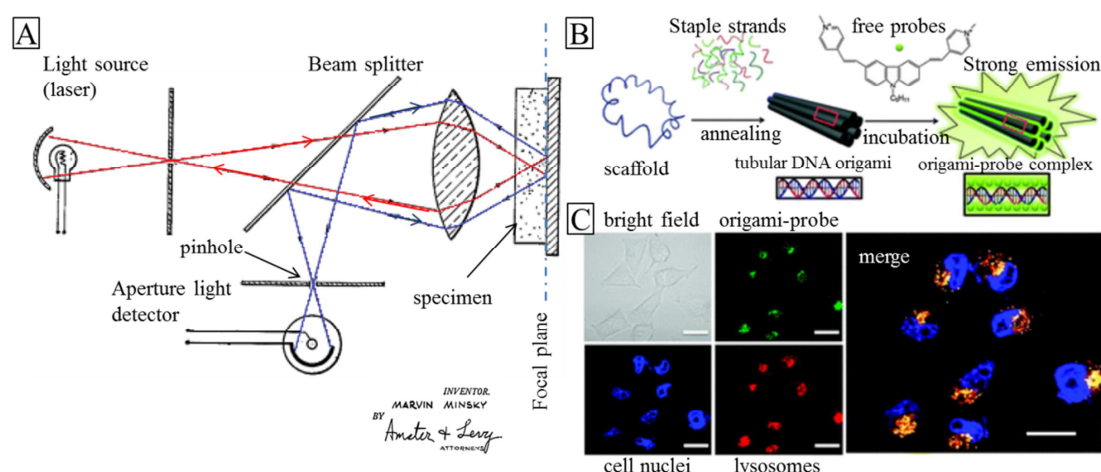


Figure 1.20: (A) Schematic of the setup for confocal microscopy; image adapted from the patent drawing of Minsky^[188]. (B) DNA origami tube synthesis and functionalisation with a fluorescent probe for cell insertion. (C) Confocal microscopy of breast cancer cells after incubation with DNA origami tubes (green). Cell nuclei are stained in blue, and lysosomes in red. Images (B) and (C) from [190].

Confocal microscopy was recently used with DNA nanotechnology for the direct observation of tubular DNA origami (Figure 1.20B) uptake into cells for drug-delivery systems^[190, 191] and for the study of cell mechanisms (Figure 1.20C)^[192]. DNA nanotechnology has been applied to study some aspects of biological processes^[193, 194], including recently to investigate the mechanical properties of the cellular membrane.

1.9 AIMS AND OBJECTIVES

This introduction highlighted recent advances in DNA nanotechnology and techniques developed for the study of model membranes. In particular, the study of biological processes happening at the cell membrane present challenges in that multiple parameters and mechanisms are involved at the same time. DNA nanotechnology offers the opportunity to construct simplified biomimetic models for the study of biological processes, but also as a starting point towards the construction of more sophisticated molecular machinery and development of drug delivery systems.

The first aim of this thesis is the development of a new DNA structure capable to interact with a membrane bilayer and to controllably bend it. The inspiration for the design of such DNA structures is the clathrin protein (introduced in section 1.6), a three-legged protein that polymerises on the cell membrane and is responsible for the endocytosis, a process through which the cell absorbs nutrients across the bilayer. Chapter 2 describes the design and study of a three-fold symmetry DNA junction made of eight short DNA strands. This structure presented challenges during folding and was therefore abandoned in favour of a second, more rigid approach. The second version of a three-armed DNA structure is described in Chapter 3 and uses a particular strategy called DNA origami for the formation of a more rigid and functionalisable nanostructure. The successful design and synthesis of a new DNA origami will be discussed, as well as the optimisation of a polymerisation strategy resulting in the formation of large DNA arrays. The shape of the structure can be modulated to vary the intrinsic curvature of the DNA origami and apply a bending force on a lipid membrane. Chapter 4 describes the insertion of the DNA origami into lipid vesicles using DNA-cholesterol conjugates as anchors, and fluorophores for localisation of the structure during fluorescent microscopy experiments. The polymerised DNA origami showed excellent insertion into the lipid bilayer and deformed the lipid membrane upon polymerisation. The second aim of this thesis is presented in Chapter 5, which explores

the development of a technique to couple the interaction specificity of the fluorescent molecule used in super-resolution microscopy and the high-resolution output of electron microscopy. It is postulated that if a cell contains the genetic blueprints that assemble oligonucleotides to produce a 3D, DNA nanostructure, the resulting structure would be recognised but not degraded by the immune system of the cell. The initial test towards this objective is the construction of a small DNA structure for EM imaging in cell lysate in order to identify the optimal shape and size required for the development of an endogenous RNA tag for cryo-EM imaging. Chapter 6 discusses and presents the various techniques and procedures for TEM that required optimisation in order to correctly visualise DNA origami and their interactions, and provides recommendations for TEM imaging of DNA and lipid structures. Finally, Chapter 7 is a conclusive chapter that summarises and offers a reflection on the whole work presented in this thesis.

CHAPTER 2

CLATHRIN-INSPIRED DNA STRUCTURES

Dynamic compartmentalisation by means of lipid bilayers is essential for cells such that they can perform a diverse and complex set of functions necessary for life. Clathrin-driven endocytosis is the first identified process through which the cell absorbs nutrients across the bilayer. This pathway also regulates the internalisation and thus activity of many plasma membrane receptors involved in the cell signalling cascades^[195, 196]. Precise details of the membrane-budding mechanism and dynamics are yet to be determined, despite being extensively studied^[156-168]. Chapter 2 describes the initial design and construction of DNA structures to synthetically mimic the clathrin protein and investigate controlled membrane bending.

2.1 DESIGN OF CLATHRIN-INSPIRED DNA MODEL

2.1.1 General considerations from the clathrin protein

Clathrin forms cages of different sizes in nature that often resemble symmetric Archimedean solids (Figure 2.1 and Introduction section 1.6).



Figure 2.1: sequence of formation of a clathrin cage. Animation by A. Bruce and T. Kirchhausen.

For example, a buckyball (truncated icosahedron) can be constructed from individual clathrin proteins assembling into regularly arranged hexagonal and pentagonal faces. This requires flexibility in the structure and the capacity for clathrin to polymerise into coats, or arrays. The tight interaction between the cell membrane and clathrin arrays bends and stabilises curved membrane structures. Therefore, the synthetic version must be equipped with membrane anchors that will guarantee such closeness between the membrane and the clathrin mimic. The synthetic clathrin and its adaptors also must have the capacity to accommodate small dihedral angles as well as small variations in the lengths of the edges^[163, 197]. The more the vesicle invaginates, the smaller the average dihedral angles emerging from each clathrin vertex^[198]. (Figure 2.2C) Indeed, equatorial angles of pentagons and hexagons in mini-coats or hexagonal barrels coats deviate from the respective 108° and 120° , and have various edges lengths. A buckyball always has a 566 type vertex, where one pentagon and two hexagons meet (Figure 2.2B), which determines angles and length to consider when deciding the dimension of the structure.

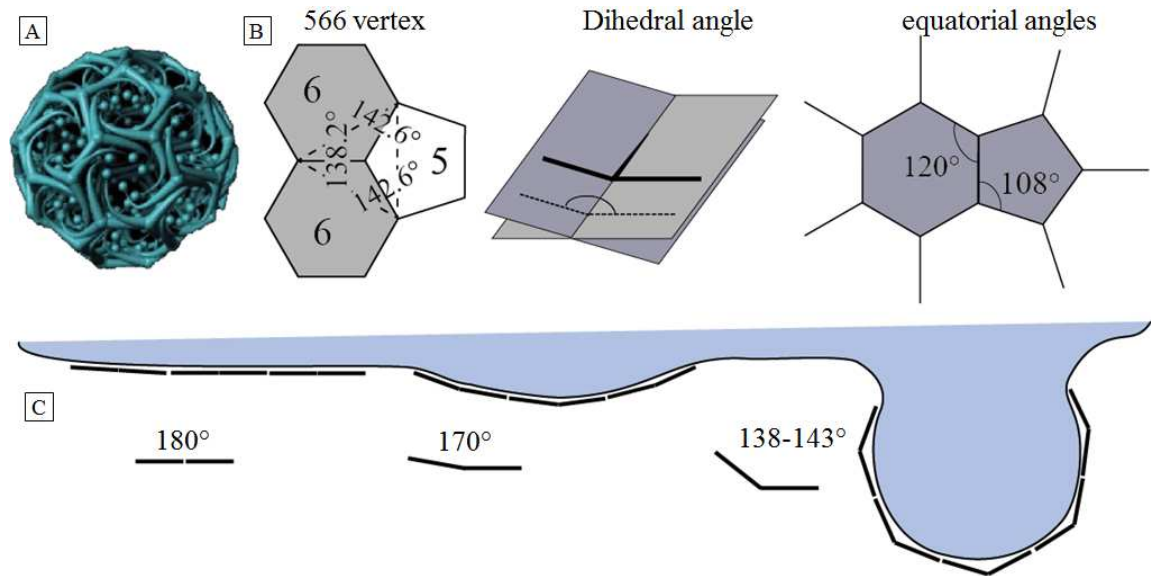


Figure 2.2: Illustrations of angles involved in a clathrin coat (A) model of a clathrin-coated buckyball. (Image from Kirschhausen *et al.*) (B) The buckyball (truncated icosahedron) is a regular polygon with only one edge's length and two different equatorial angles, 120° and 108° . All vertices are of the type 566, with dihedral angles either 142.6° or 138.2° . (C) Illustration of how the dihedral angle decreases upon invagination of the vesicle.

A three-fold symmetry construct becomes unmistakably the starting point of any clathrin-inspired DNA structure.

Both DNA strand assembly (section 1.3) and DNA origami (section 1.4) are suitable for this work. The first option was initially chosen for its simplicity.

2.1.2 Design of a DNA 3-fold array

In the design of a DNA structure, we needed to balance the flexibility of the DNA to form 3D polyhedra with enough rigidity to withstand strains and forces required to bend a membrane. In order to resist the strain occurring upon membrane bending, the free energy released by the formation of the DNA cage (in this case the association of the vertices) must be equal or bigger than the energy needed for membrane bending. We made a very broad estimation to verify that DNA provides enough energy: If we consider that a DNA double helix organises around a lipid vesicle with the pattern of a buckyball, each of the vertex is made of the meeting of three DNA duplexes. These three-fold symmetry DNA constructs have 80 bases pairs (approximatel 27 nm^[10-12]) between two vertices (Figure 2.3A, approximation of distance between the centre of two DNA tiles, discussed below in this section), which give a radius of 65 nm approximately. The persistence length of DNA is defined as the length over which correlations in the direction of the double helix are lost. Under the persistence length (which is approximately 50 nm, see section 1.3), the DNA can be considered as a stiff rod. It is the case in this estimation since the distance between two vertices is only 27 nm. Using the classic Canham, Helfrich, and Evans theory^[106, 199], the energy cost per membrane area of creating a curved sphere has been estimated by Stachowiak *et al.*^[97] as

$$G_{bending} = \frac{8\pi\kappa}{(4\pi R^2)} \quad (\text{Equation 2})$$

Where κ is the bending rigidity of the membrane and R is the vesicle radius (Figure 2.3B).

2.1 Design of clathrin-inspired DNA model

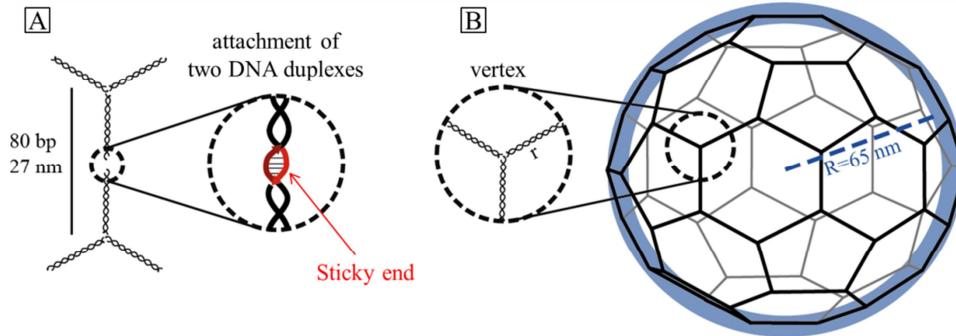


Figure 2.3: Schematic of the dimensions considered in the estimation of the size of the vesicle and the DNA construct. (A) schematics of an edge and of a vertex made of three DNA duplexes of 40 bp long (radius r). circular inset: two vertices are attached together by a sticky end of a few nucleotides linking two DNA duplexes. (B) Illustration of a buckyball of radius R formed with this particular size of DNA vertex.

Calculations^[200] were performed for soft fluid membranes (such as DOPC membranes) for which

$$\kappa \sim 10 k_B T \quad (\text{Equation 3})$$

Where k_B is the Boltzmann constant in [J/K] and T is the temperature in Kelvin.

The finite tension of biological membranes opposes changes in shape and is therefore added to the total energy cost $G_{bending}$. The membrane tension, which has units of energy per area, is approximately the energy required per membrane area to form a spherical vesicle from an initially flat membrane surface. A typical value of

$$0.02 k_B T \text{ nm}^{-2} \quad (\text{Equation 4})$$

was estimated^[200] based on micropipette aspiration measurements of membrane tension before significant straining. We therefore estimated the energy required to bend the membrane of a 65 nm radius vesicle at 293°K (= 20°C) to be the sum of equation (2) and (4):

$$\frac{8\pi\kappa}{(4\pi R^2)} + 0.02 k_B T \text{ nm}^{-2} = \sim 1.0 * 10^{-22} \frac{J}{\text{nm}^2} \quad (\text{Equation 5})$$

2.1 Design of clathrin-inspired DNA model

We then compared this energy cost with the energy released upon the attachment of the DNA vertices together (Figure 2.3A), assuming that each DNA vertex is an inseparable unit. As we are planning on using sticky ends of a few bases to assemble each DNA vertex into a DNA array (see below), we considered the energy released by the assembly of sticky ends made of 4 bases. Computer simulation using NUPACK^[201, 202] indicated that the free energy released by the attachment of a 4 bases duplex at 20°C made of complementary sticky end is -10 kcal/mol, or:

$$\frac{10 \times 4.18}{N_A} 1000 = 6.9 \times 10^{-20} \text{ J per sticky ends} \quad (\text{Equation 6})$$

Where N_A is the Avogadro number. In this estimation, there are 90 sticky ends that attach two vertices together and form an edge (i.e: there are 90 edges per buckyball). The surface of the considered vesicle is 53000 nm² approximately (that is, 1 sticky end per 590 nm²), so we divide equation 6 by 590 to obtain the result:

$$\sim 1.2 \times 10^{-22} \frac{\text{J}}{\text{nm}^2} \quad (\text{Equation 7})$$

Therefore, and according to this simplified estimation, the energy provided by the attachment of the DNA vertex with 4 bases sticky ends provides roughly the same energy required for membrane bending. It is thus reasonable to consider bending a membrane into a vesicle of a few tens of nanometres using DNA strands arranged in a buckyball pattern as depicted in Figure 2.3B. We considered examples of 3-fold DNA junctions (or vertex) formed with more than one DNA duplex per edge to help the future DNA array resist the force that it would likely endure during membrane bending.

Junctions with three-fold symmetry resembling the one discussed above have been constructed by DNA strand-assembly^[49-55]. Previous studies by Mao *et al.*^[55, 203, 204] have shown that supramolecular polyhedra can be constructed from DNA strands with extra T bases at the intersections (T-loops) provided flexibility, rigidity and concentrations of

2.1 Design of clathrin-inspired DNA model

the individual components are given special consideration in their 3D-assembly (Figure 2.4A). The work of LaBean *et al.*^[205, 206] (Figure 2.4B) on robust four-helix DNA junctions provided the blue prints to design a strong structure likely to endure strains and forces resulting from bending a membrane. We have adapted these design strategies for our own work.

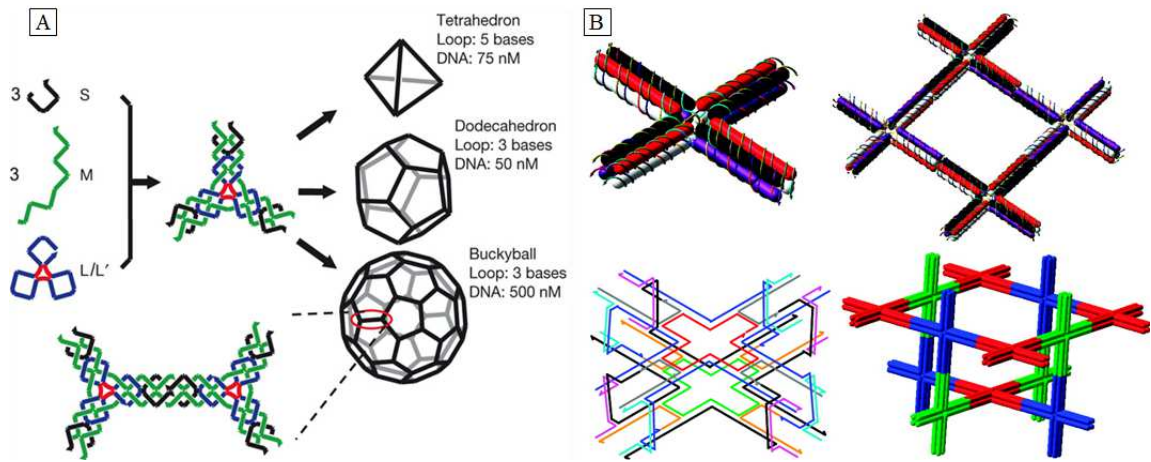


Figure 2.4: (A) Mao *et al.* self-assembled polyhedra^[55]. (B) LaBean *et al.* double-decked tile^[206].

The general shape of our clathrin-inspired DNA lattice has a 3-fold symmetry, and a repeating unit will be referred to as a “star” in the following thesis (Figure 2.5). The sequence is adapted from Majumder *et al.*^[206], where rigidity is provided by the double-decker nature of the system. The 2x2 double helix bundles of each arm are closely packed together by crossovers between the left and right duplexes (left-right crossover) and another between the upper tile and the bottom one (up-down crossover). Flexibility is provided by T-loops at the vertex.

The tile consists of two layers, each layer is a three-way junction, each arm of the junction consists of two double-stranded DNAs linked by a single crossover between them (Figure 2.5A, B). The design consists of eight strands (S1-S8). Each layer contains three long strands in a 3:3:1 stoichiometry. The two layers are joined by introducing two

crossovers per arm (six crossovers in total) positioned such that they link the bottom surface of the top layer to the top surface of the bottom layer (Figure 2.5C).

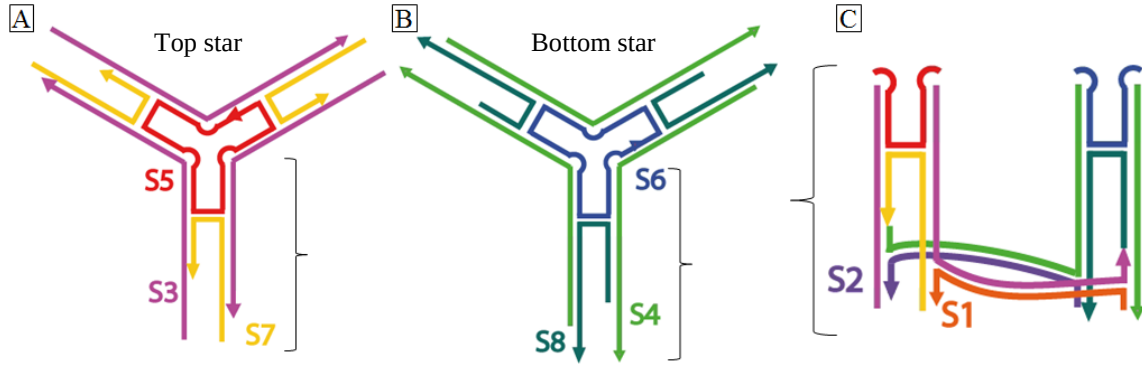


Figure 2.5: Schematic representation of the 3 main DNA strands forming the DNA junction with three fold symmetry, top (A) and bottom (B). (C) Representation of the up-down crossovers including strands S1 and S2, and the longer strands S3 and S4. The T-loop on the top tile is made of 5T (T_5), and on the bottom tile is made of 3T (T_3).

The DNA junctions of LaBean (Figure 2.4B) are designed to cover a flat surface. Because the 2x2 helix bundles introduce some inherent curvature within the tile^[203], their lattice was designed such that adjacent tiles are flipped, which, in turn, requires every vertices to be spaced by an odd number of half turns. In the context of our system, each star needs to be in the same orientation to guarantee that the angle of curvature of each star will sum up. The arms of our DNA construct have thus been shortened by three base pairs to have an even number of half turns (14 half turns, Figure 2.6D) separating two adjacent tile centres.

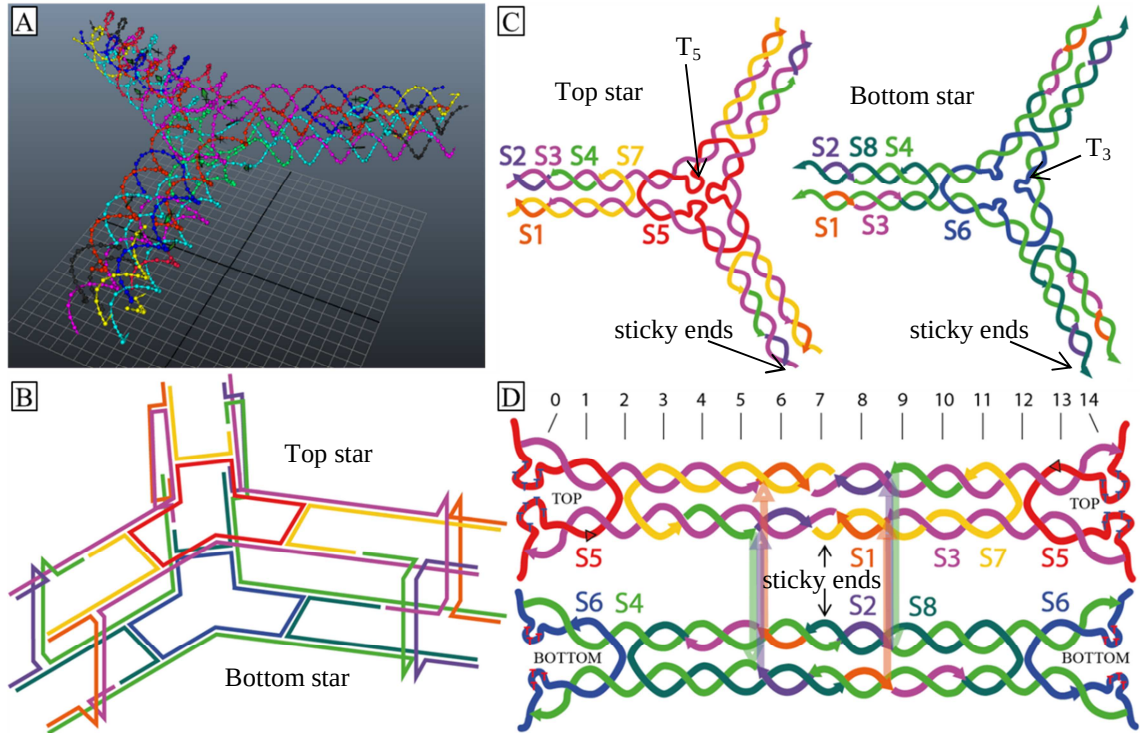


Figure 2.6: double-decked star lattice models. (A) 3D representation in Maya (vHelix plugin^[207]) (B) 3D representation of the strand trace through the tile as straight lines (C) model of the two layers with allocated strand number (S1 to S8) as helices. (D) Sticky end interface showing the crossovers between top and bottom tile (straight perpendicular arrows), and number of half turns between the joined vertices.

The lattice also has to accommodate a range of both dihedral and equatorial angles arising from the coating of a non-planar surface with a mixture of hexagonal and pentagonal faces. (Figure 2.2) This double flexibility can be addressed both in one step, which is the meeting position of the three-armed tile. Flexibility of the DNA assembly is guaranteed by T-loops at these vertices. As the final structure designed here should form an approximating spherical shape, the lower DNA helices (designed to be next to the membrane) must accommodate an angle slightly smaller than the ones located farther away from the centre of the sphere. More unpaired Ts on a DNA strand provide increased flexibility due to the enhanced degree of freedom of the loop^[208]. Consequently, the top and bottom layers are similar except that the strands have different sequences and the top layer has a T₃ linker at the vertices whereas the bottom layer has a T₅ linker. It is important to note that the presence of T-loops is insufficient to promote any membrane bending; it only contributes to the pliability of the tile.

Polymerisation of the tile is made possible by the use of sticky ends located at the extremities of each arm (Figure 2.6C, D). Their length was included in the calculation of the distances between two vertices such that the arms maintain 14 half turns irrespective of the length of the sticky ends. In addition, the few bases that form the sticky ends have non-palindromic sequences to guarantee a correct attachment. In other words, it ensures that the inherent curvature of the double-decked tile always bend in the same direction as it polymerises.

2.1.3 Interaction of DNA arrays with a lipid membrane

After addressing rigidity and flexibility in our clathrin-inspired 3-fold symmetric DNA nanostructures, we next considered how to integrate this star into a membrane and how to controllably increase the curvature of the membrane. As is the case of the clathrin protein, it is important that the DNA structure is held tightly to the membrane bilayer. As highlighted in the introduction, the DNA structures can dock to membrane bilayers if functionalised with lipophilic moieties^[173, 174, 209]. Often, commercially available 3'-end cholesterol-modified oligonucleotides are used as lipid-DNA anchors^[6, 171, 174]. Furthermore, cholesterol is believed to be more easily inserted into the membrane than lipids, due to its conical shape and strong interaction with sphingolipids^[210]. It was first argued that cholesterol moiety at the 3'-end of an oligonucleotide directly involved in the star construct would maximise the proximity between the anchor and the membrane better than hybridization of a Cholesterol-DNA conjugate to a 5'-end handle (Figure 2.7).

Nevertheless, this approach has a flaw in that the cholesterol moiety of cholesterol-DNA conjugates can easily form micelles in solution^[211] (Figure 2.7A), which would inhibit the assembly of the star. Including the cholesterol-DNA conjugate inside the membrane (leaving only the corresponding handle for the Cholesterol-DNA on the star) solves this aggregation problem^[212] (Figure 2.7B). Any micellar assemblies would be washed away,

but the inserted cholesterol-DNA conjugates would remain inserted in the bilayer^[213]. All DNA, including the cholesterol-handle strand, can then be annealed in a one pot reaction, which can then be added to the separately formed supported lipid bilayer (SLB).

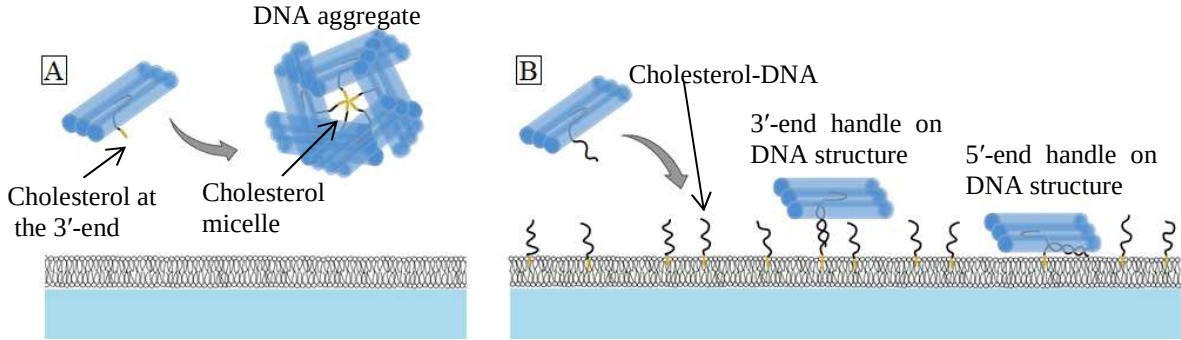


Figure 2.7: Attachment of a DNA double-helix structure to a membrane bilayer (each DNA helix is represented as a tube). (A) A cholesterol moiety included in the DNA structure can form micelles in solution. (B) Cholesterol-DNA conjugates incorporated in the lipid bilayer. The DNA handle hybridise with the cholesterol-DNA, bringing the structure in closer proximity if the 5'-end is protruding from the DNA structure.

The rational design of our system, allows a 3'-end cholesterol to protrude from the lower helices; that is, the helices programmed to be in contact with the membrane. Due to the preciseness of DNA nanotechnology in programming predictable configuration, it is possible to go further in the placement of the cholesterol docking site by looking at the angular position of each base of the lower layer. As the double helix rotates, it positions every base at a particular angle and location. The ideal base is located on the lower helices and points towards the membrane. Using LaBean's sequence^[206] and calculation of the angle of each nucleotide along with the Maya plugin vHelix^[207], this allowed identification of the nucleotides best fitted to bear the cholesterol handle on its 3'-end. It was considered that the two base pairs located at the crossover position between two helices of the same layer (on the bottom star) are horizontal. Each successive bp then rotate by approximately 34° , calculated as $\left(\frac{360^\circ \text{ for one turn}}{10.5 \text{ base per turn}}\right)$, which indicates the most likely position of the base. The strand in question (S8) was then cut into two pieces at the 13th nucleotide (renamed S8') and extended by a handle of 18 nucleotides. The cut

strand continues as S9 and bears the sticky ends. Due to its 3-fold symmetry, each star will bear three cholesterols, one in the middle of each arm. Figure 2.8 illustrates the changes described in this section.

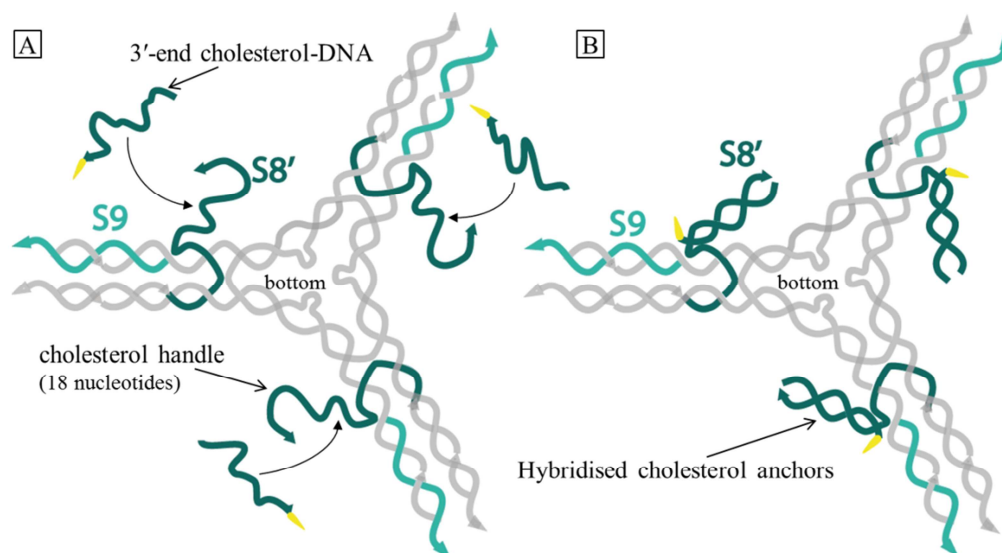


Figure 2.8: model of the two layers with added cholesterol handle (S8') and 3'-end cholesterol-DNA (A) in solution and (B) hybridised. The top star has been omitted from this scheme for clarity. The initial strand (S8) has been cut into S8' and S9.

In conclusion, cholesterol moieties should not only guarantee anchorage of the DNA to the membrane, but also determine the orientation of the structure on it. That is, each tile will have the same face bound to the membrane by the coordinated actions of sticky ends and insertion of cholesterols.

To control the intrinsic curvature of the tile, we proposed a design that would change the angles between the arms by shortening a bridge that joins the end of each arm. Because single-stranded has a much shorter persistence length than double-stranded DNA^[214], a single-stranded tether will be shorter than its double-stranded equivalent. To address this, the bridge is composed of three parts, two are part of the tile and have a fixed length, and the third is a tether whose length will be changed (Figure 2.9).

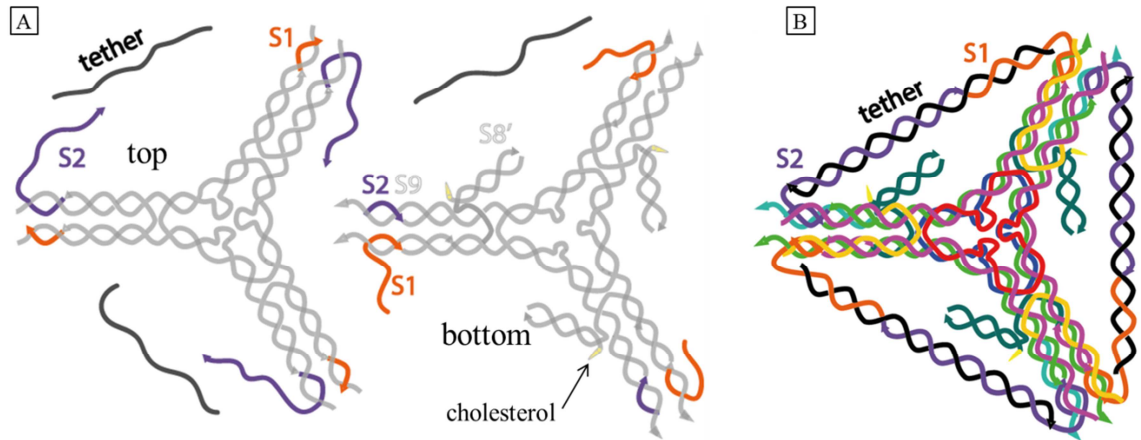


Figure 2.9: model of the star with elongated S1 and S2 and added tether. (A) View of the two layers individually and (B) superposition of the two layers with a hybridised tether that bridges the extremities of each arms.

The first part starts from the top tile as a 3'-end extension of the up-down cross over strand, S1 (orange in figure 2.9A). The second part is a 5'-end extension of S2 (purple in figure 2.9A) which is the strand forming the up-down crossover on the bottom star. Ten T bases have been added to these tethers (between the domains part of the DNA tile and the tether complement domain) to accommodate a tight curve when they reach the next arm. This number is not definitive, as it depends on the length of the bridge that we wish to achieve. Finally, a third part consists of a detached tether (Figure 2.9, black) complementary to the first two strands. We estimated the length of the bridge by considering that each base pairs between the centre of the tile and the beginning of the bridge is spaced by $0.34 \pm 0.1 \text{ nm}^{[10-12]}$ and that the two arms are at an angle of 120° . This first estimation suggests that the bridge must be roughly 65 nucleotides (including T loops) to span between the two arms when the structure is planar (Figure 2.7B).

2.2 CONSTRUCTION OF DNA ARRAYS

Unless otherwise stated, the following work was done during a secondment at the Ludwig-Maximilian Universität, München, Germany, in the group of Tim Liedl.

We first aimed to synthesise an unfunctionalised star to which we could later add complexity. To this end, the cholesterol handles on the star, and the tethers were omitted (only one T remained as a cap), corresponding to the tile illustrated in Figure 2.6. (Sequences in Annex A.16.1). The annealing programme was first chosen to be an exponential decay of temperature from 85°C to 20°C over 48 hours (Schedule in Annex A.4), which is an accelerated version of the annealing protocol that LaBean used for his double-decker tile^[206]. Stoichiometric quantities of strands were used, to achieve a final concentration of tiles between 50 to 300 nM, similarly to the work presented by Mao *et al*^[55].

DNA strands were suspended in water as received (final concentration 10 µM) and used in stoichiometric amounts. The two strands located at the vertices (S5 and S6) have to be added at one equivalent each, whilst all the other strands must be three times more concentrated to form all three arms of the structure. The strands in the DNA tile that had lengths between 18 and 38 nucleotides were purified by desalting only, but strands longer than 60 nucleotides were delivered PAGE purified. No further purification was done after folding and samples were mostly analysed by TEM and AFM (Figure 2.10). See Annex A.12 and A.13 for sample preparation.

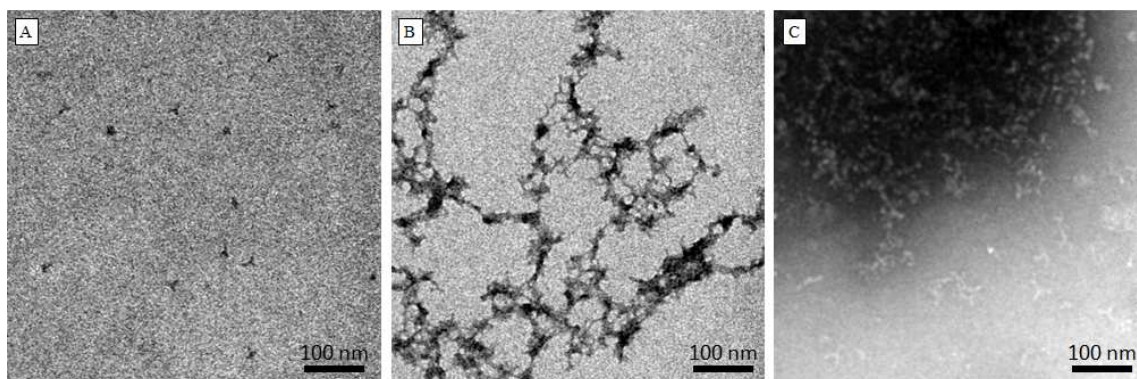


Figure 2.10: TEM micrographs of DNA star with 4 bp sticky ends after annealing. The samples were held at 4°C for 20 min before preparation of the grid. DNA concentrations of (A) 50 nM, (B) 100 nM and (C) 300 nM.

A final concentration of tiles of 50 nM showed only monomeric stars, 100 nM produced polymerised networks, and >150 nM yielded large aggregates of DNA strands.

2.2.1 Optimising the star polymerisation procedure

Sticky ends should ideally be close to a thermodynamic equilibrium such that adjacent tiles can pair with each other to grow the array in 2-dimensions, yet allow the repeating units to rearrange to generate a stable, organised array, thus avoiding kinetic traps. The longest non-palindromic sticky ends were optimised to have 4 nucleotides. Sticky ends of 3 and 2 nucleotides were also investigated, in which the hybridised extremities (S1 and S2) were elongated by 1 or 2 base pairs respectively so the total length of the arm remained the same. Figure 2.11 illustrates the TEM images with 2, 3 and 4 bp sticky ends, which demonstrates the formation of the 3-armed star in every case. Polymerisation was visible with 3 and 4 bp sticky ends and formed irregular patches up to a few hundred nanometres.

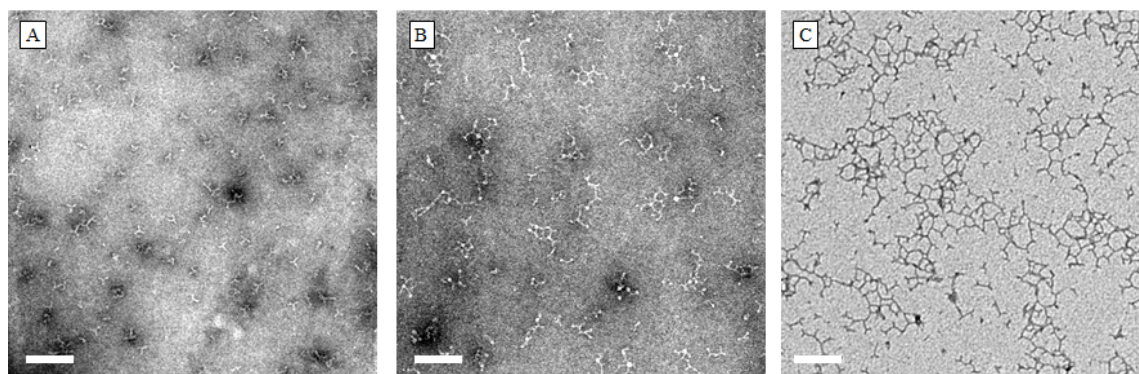


Figure 2.11: TEM micrograph of (A) 2 bp, (B) 3 bp and (C) 4bp sticky ends. Scale bars 100 nm.

Initial TEM images suggested that the assembly was not strong enough with 2 bp to form an array. Spherical aggregates were observed, after incubation at 4°C (Figure 2.12). However, a lot of the structures on the grid were degenerated into large aggregates that appear black on TEM grids (Figure 2.12A). At concentration higher than 100 nM, these aggregates form most of the observable objects, with sizes up to a few μm . Further incubation at 4°C or at room temperature reinforced the formation of these patches, although it has been noticed that if the sample is vortexed before TEM imaging, the largest aggregates disassemble into single tiles and smaller clusters of approximately 50 nm diameter.

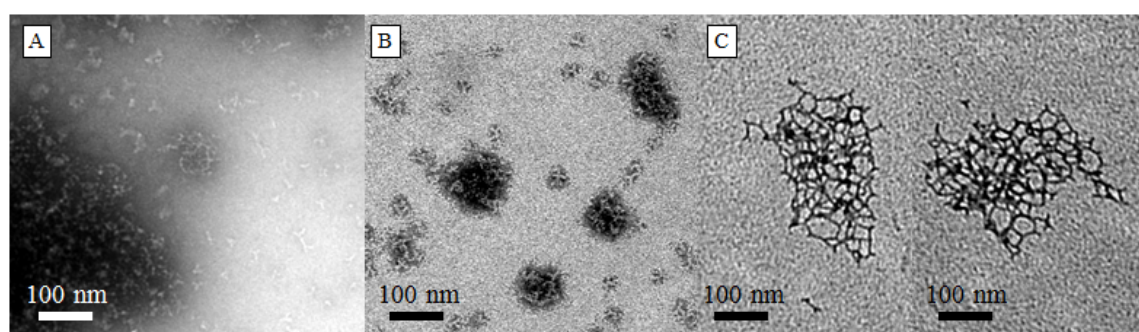


Figure 2.12: TEM micrographs of DNA after annealing and incubation at 4°C overnight, concentration of 100nM. (A) Aggregates appear in black (left side) and vesicles in light grey under positive staining, (B) negative staining showing small vesicles, and (C) negative staining of vesicles (imaged in Oxford).

It is possible that these 3D structures partially unfold when the sample is prepared for TEM, and their visibility depends greatly upon the type of staining. (Chapter 6 develops TEM techniques, see section 6.2 for staining discussion.) Thus, we tried imaging using

AFM as well, as this technique does not require harsh sample preparation, unlike the preparation for TEM, which involves drying the sample (Figure 2.13). (A detailed protocol for TEM sample preparation is discussed in Chapter 6) Indeed, samples of 100 nM showed multiple circular structures on liquid AFM micrographs.

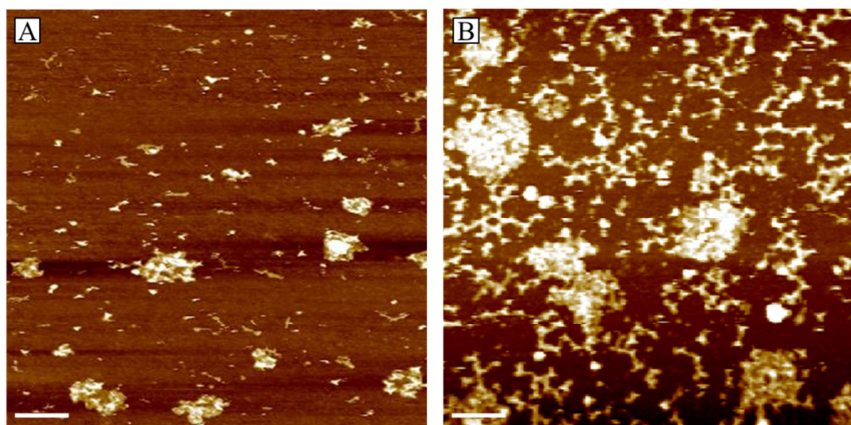


Figure 2.13: AFM micrographs of DNA after annealing and overnight incubation at 4°C at concentration of 100 nM (A) immediately after addition of the sample onto the mica disc and (B) after 20 minutes. Scale bars 100 nm.

Observation of the assemblies on TEM grids and AFM indicated that the star can assemble, but when polymerised the resulting 2D and 3D assemblies lack a defined structure. This can result from a bias in stoichiometry of the strands. Imperfect stoichiometry is capable of producing defective structures hindering the formation of the arrays. To investigate this, a titration of the four longest strands (S3, S4, S5, and S6) without sticky ends was made and analysed by PAGE after a short annealing to fix the concentration of each strand relative to the others (Figure 2.14).

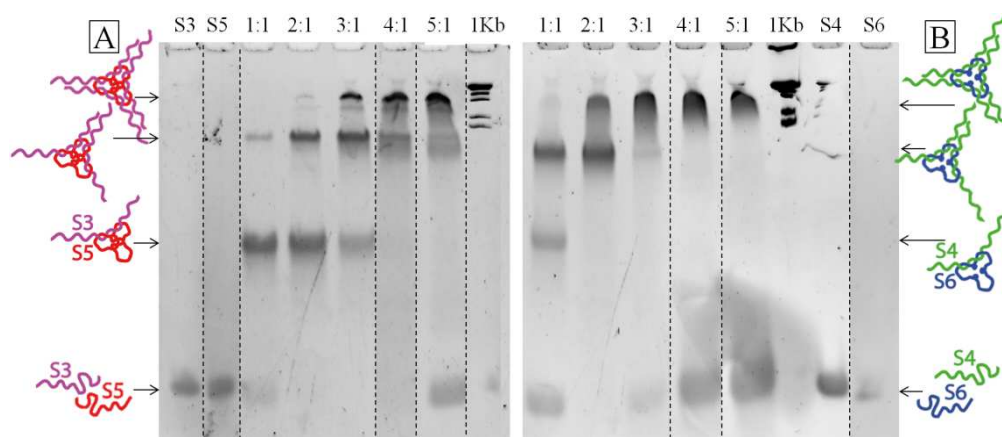


Figure 2.14: PAGE gel of S3-S5 (A) and S4-S6 (B) assembly. The number of equivalents is written above each line (S3:S5 and S4:S6). Individual strands were run alone for reference, which names appear on the adequate line, as well as a 1K bp ladder (1Kb). Interpretation of the bands is depicted by small schemes of the star next to the gels.

According to the design the relative concentration of S3:S5 and S4:S6 should be 3:1. The analysis shown in Figure 2.14 indicates that the concentration of S3 relative to S5 is under-estimated (Figure 2.14A), and that the duplex S4:S6 is at equilibrium between two different configurations (2:1 and 3:1 complexes) instead of forming a single 3:1 complex (Figure 2.14B). The former can be due to a bad estimation of the S3 concentration, whilst the latter is more likely linked to the presence of secondary structures in S4. (Because the expected structure of three S4 hybridised to one S5 was not the predominant band.) Both situations hinder the correct duplexes to form, which in turn can disturb the rest of the tile formation.

To ensure a correct strand stoichiometry, the concentration of each strand was measured with UV absorbance on the day of the experiment. Table 2.1 reflects the difference in concentration of each strand compared with the manufacturer quoted value by measuring the absorbance at 260 nm and using this value to calculate concentrations using the Beer Lambert law^[215, 216].

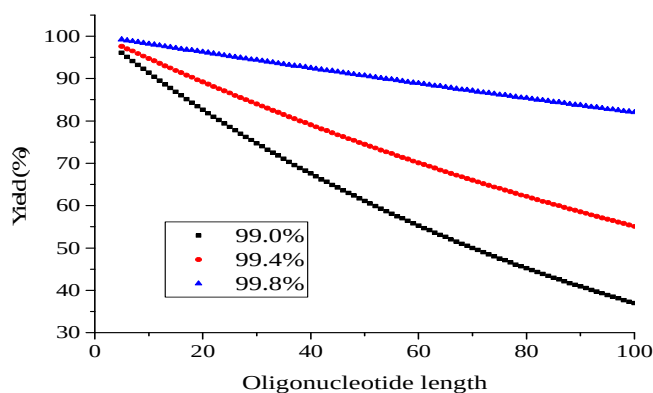
Table 2.1 Concentration of DNA strands

strand	μM^{UV}	$\mu\text{M}^{\text{suppl}}$	$\Delta\mu\text{M}$
S1	116.8 $\pm$ 0.3	100	16.8 $\pm$ 0.3
S2	104.9 $\pm$ 0.3	100	4.9 $\pm$ 0.3
S3	103.2 $\pm$ 0.3	100	3.2 $\pm$ 0.3
S4	106.6 $\pm$ 0.3	100	6.6 $\pm$ 0.3
S5	125.4 $\pm$ 0.3	100	25.4 $\pm$ 0.3
S6	109.6 $\pm$ 0.3	100	9.6 $\pm$ 0.3
S7	103.1 $\pm$ 0.3	100	3.1 $\pm$ 0.3
S8	127.4 $\pm$ 0.3	100	27.4 $\pm$ 0.3

The array construction relies on the regular and precise association of stars, which means that any error in concentration would hinder the expansion of the lattice. Imprecision arising from pipetting of the strands can also be a source of error. Since the pipettes used in these experiments were always the same and had the same accuracy, the error is constant over all the sample and was therefore omitted in this discussion. Imperfect stoichiometry (as presented in Table 2.1) is believed to be the dominant error explaining difficulties experienced in the formation of DNA arrays.

A second error arises from the synthesis of the individual DNA strands. As strands shorter than 60 nucleotides were used without purification, these strands contain errors at the rate of 99.4% $\pm$ 0.4%^[217, 218], depending on the coupling method used^[219, 220] and on the type and position of the growing nucleotide^[221]. The error is cumulative so the final strand with n nucleotides has $n-1$ coupling reactions and ($yield^{n-1}$) chance to be with the correct sequence and length (Graph 2.1).

2.2 Construction of DNA arrays



Graph 2.1: Yields of artificial DNA synthesis according to average coupling efficiency and length of oligonucleotide.

In the context of our system, this means that the strand, made of 38 nucleotides, has between 69% and 93% chance of being correctly synthesised. In order to improve the DNA strands quality, S1, S2, S7, S8, S8' and S9 were PAGE purified. (Figure 2.15)

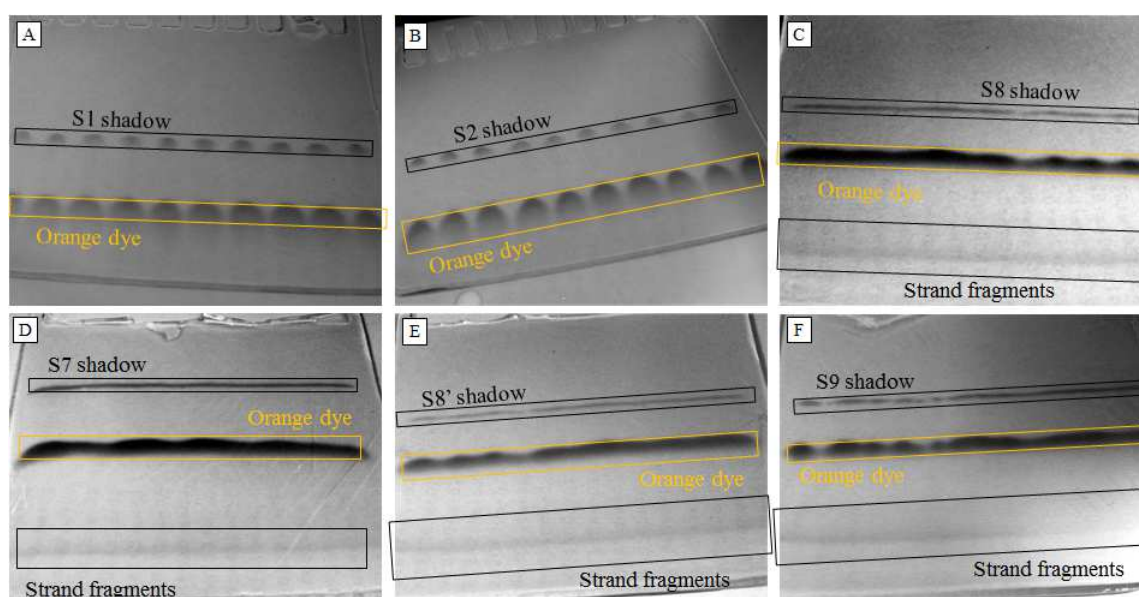


Figure 2.15: picture of PAGE gels for purification of strands (A) S1, (B) S2, (C) S7, (D) S8, (E) S8' and (F) S9 by UV shadowing.

The bands corresponding to the right DNA length were cut out of the gel and the DNA extracted as described in Annex A.3, leaving out any unwanted strand fragments at the bottom of the gel.

From the optimisation studies, we were able to ensure that a precise stoichiometry was introduced by purifying the strands and measuring their concentration with UV-Vis before each annealing.

We next investigated the possibility of other, unwanted secondary structures that could assemble, as this would limit control over the formation and polymerisation procedure. Secondary structures arise in this system when a domain (domain a) on a DNA strand matches (or partially matches) that of another strand (domain a*) than the one designed to hybridise with. The two domains a* (one on the correct strand, and one on an incorrect strands) compete to assemble with the one domain a, which leads to incorrect assembly of the DNA strands.

Majumder *et al.*^[206] designed their strands using Uniquimer, which is a software tool for generating *de novo* DNA sequences, programmed to optimise the self-assembly of their 4-fold symmetry DNA tile. It is assumed that changing from 3-fold symmetry to 4-fold symmetry would not drastically change the way each DNA strands interacts. By omitting one or more selected strand in the star, PAGE allows us, after folding, to determine if secondary structures are formed. These are formed without sticky ends to facilitate the analysis, as it is expected that fully-formed star will stay in the well and would be indistinguishable from malformed stars stuck by sticky ends which would also remain in the well.

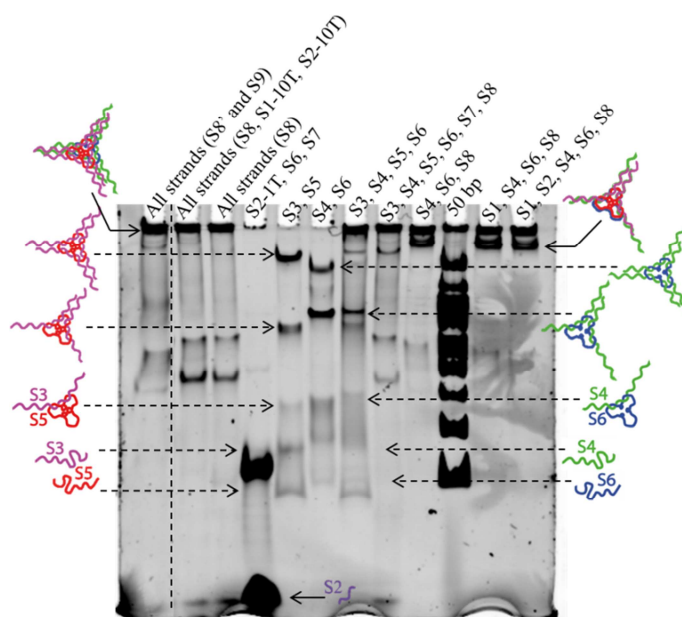


Figure 2.16: PAGE gel of tiles without sticky ends and missing selected one or multiple selected strands. Interpretation of the bands is depicted by small schemes of the star or strands next to the gels. 50 bp ladder was used for reference.

The results illustrated, unfortunately, that unintended interactions take place involving various strands, (Figure 2.16). For example, structures involving S4 produced numerous bands indicating multiple species. The current interpretation is that S4 contains short domains matching with multiple domains on different DNA strands. Arrows in Figure 2.16 indicate which structure is believed to correspond to each of the main band. Observations of uncontrolled assembly of S4 is supported by PAGE titration (Figure 2.14) in that the expected structure of three S4 hybridised to one S5 was not the predominant band. Indeed, NUPACK analysis asserted a strong intermolecular secondary structure on S4 (Annexe A.6.1) associated with the domain complementary to S1. The identified problematic domain was changed to solve this issue, as well as the complementary sequence, on S1. The two altered strands were renamed S4' and S1' respectively.

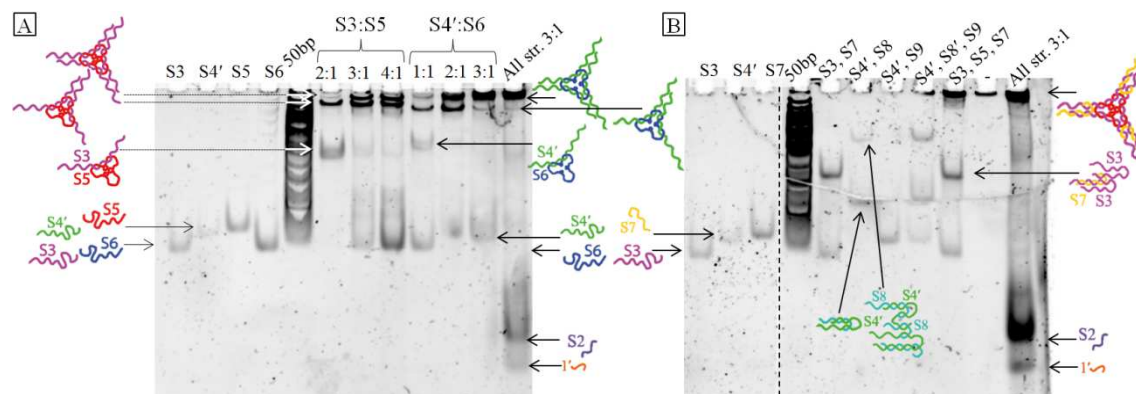


Figure 2.17: (A) PAGE gel of S3-S5 and S4'-S6 assembly. The number of equivalents is written above each line (S3:S5 and S4:S6). Strands used here have no sticky ends. Individual strands were run alone for reference, which names are written above the adequate line, as well as a 1K bp ladder (1Kb). (B) Annealing of selected strands. Interpretation of the bands is depicted by small schemes of the star or strands next to the gels. 50 bp was used for reference. The last line (All str. 3:1) on the gel is at a concentration of (A) 100 nM and (B) 500 nM.

The sequence of S4' was verified by PAGE (Figure 2.17) and showed improvement of the 3:1 duplex formation in comparison with its previous version. Due to their small size, S1' and S2 are visible on the lowest parts of the gels as their interaction with the star is reversible.

After improving bad interactions of strands which result in incomplete star assembly, we next optimised the polymerisation pathway by investigating the melting curves.

It is believed that the DNA star assembles by a hierarchical process: the centre of the tiles assembles at higher temperature, the arms fold at intermediate temperature and the weaker interactions between the arms to form arrays (or aggregates) are made at lower temperature.

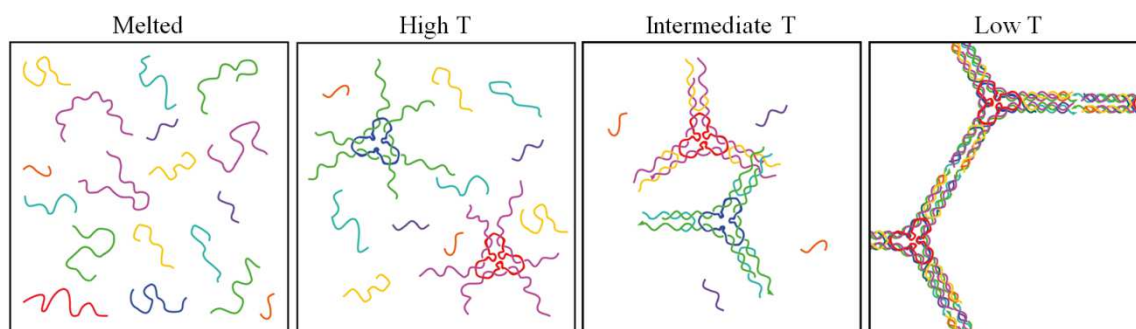
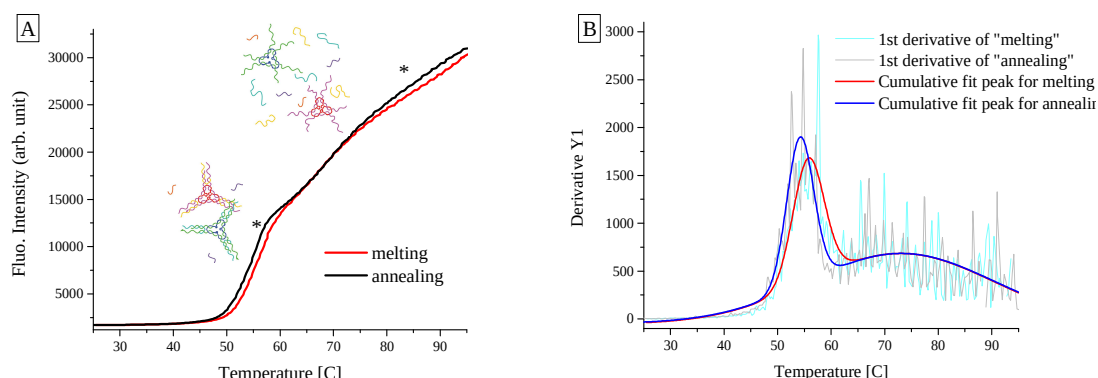


Figure 2.18: schematic representation of the DNA strands over a range of melted ($\sim 90^\circ\text{C}$), high ($\sim 70^\circ\text{C}$), intermediate ($\sim 50^\circ\text{C}$) and low ($\ll 30^\circ\text{C}$) temperatures (T).

It is to allow this type of hierarchical (Figure 2.18) assembly that DNA structures are folded over a slow cooling ramp over a few hours^[203-206, 55].

The melting temperature (T_m) of a duplex is defined as being the temperature at which 50% of the DNA is double-stranded and the other 50% is still single-stranded. T_m is therefore the best incubation temperature, providing both stability of the structure and enough energy for its rearrangement^[222, 221]. The amount of strand separation, or melting, is measured by the absorbance of a fluorescent DNA intercalator that binds preferentially to double-stranded DNA^[224, 225]; fluorescence intensity increases as single-stranded DNA hybridises (when the temperature is slowly decreased). The melting temperature T_m is then calculated as the maximum of the first derivative of the recorded intensities.

Melting and annealing analysis performed on the star (at a rate of $\pm 1^\circ/\text{min}$, 3 cycles of melting-annealing over 8 hours, see details A.14) indicate two melting transitions. The temperatures at which hysteresis happens are between 50°C and 60°C . (Graph 2.2)

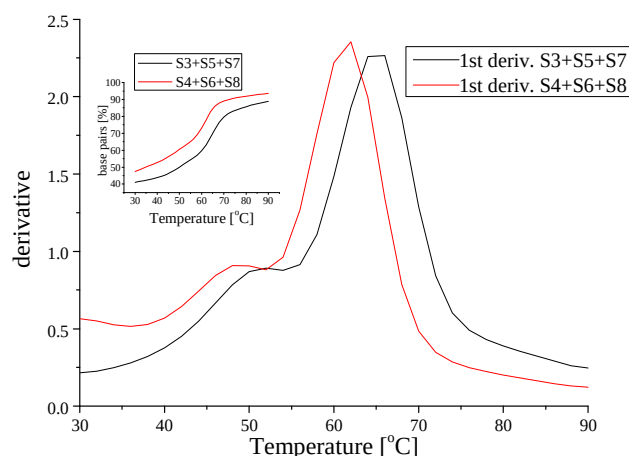


Graph 2.2: (A) Plot of Intensity vs temperature recorded during melting and cooling of star. The graph shows the averaged values over 3 melting-cooling cycles, each recording 3 values per point. Hysteresis marked by asterisks. (B) Plot of the derivative calculated from (A). The curves show a clear maximum at 54°C (annealing), and 56°C (melting), and a smooth local maximum around 72°C.

Hysteresis in DNA melting curves indicates cooperative assembly of the three-fold symmetry DNA star, a process to which the stacking interaction^[222] between the extremities of two DNA strands is likely to contribute to^[226]. This means that melting or hybridisation of DNA bases happens in a cooperative manner in these domains, resulting in a different rate and temperature-dependent assembly state depending on whether the sample is heated or cooled^[227]. It is possible that the star forms more regular arrays if the sample is incubated at the appropriate temperature (T_m). It is theorised that the second smooth maximum happening at the highest temperature (72°C) in the case of the DNA star is due to the assembly of the vertices (Graph 2.2). The sharper peaks at 54°C and 56°C are attributed to the stacking of the four helices when the arms assemble (Figure 2.18): four DNA helices per arm compose the 3-armed-star). This is consistent with the hypothesis that the vertices are made of one long domain that repeats itself three times, which would require a higher T_m . In contrast, the arms are composed of multiple shorter domains and involve shorter strands with lower T_m .

This interpretation was supported by T_m values provided by analysis *in silico* (NUPACK), which shows two maxima: $T_m = 65^\circ\text{C}$ was found for the domains located

at the vertex (S5 and S6 hybridising with S3 and S6), and $T_m = 50^\circ\text{C}$ for the domains on S3 and S4 that form the arms (Graph 2.3).



Graph 2.3: NUPACK simulation. First derivative of the melting curve for S3:S5 and S4:S6 (molar ratio 3:1, 1 equivalent correspond to 100 nM). The corresponding melting curve is showed in the small graph.

This analysis encouraged a modification of the initial thermal ramp to longer incubation time between 70°C and 50°C . The 50°C temperature range is supported by the protocol used by Sun *et al.*^[228], where they incubate a system of similar size at 50°C . It is to be noted that NUPACK suggested that individual sticky ends only assemble at temperatures below 12°C , but the PCR machine used for this experiment (See Annex A.14) does not cool below 25°C , therefore array formation cannot be observed.

2.2.2. Formation of arrays and its surface interaction

Early investigation into the successful formation of our DNA-designed star and its polymerisation indicated that a significant optimisation was required (Figure 2.10). As outlined in the previous section, we identified that strand purity and correct stoichiometry were important for assembly. The optimal star concentration was identified as being 100 nM, with each strand's concentration verified by UV-Vis prior to annealing. All strands were PAGE purified; the sequence of the strand was improved to avoid parasitic interactions during annealing. The best length of sticky ends for polymerisation is approximating 4 bp. The annealing procedure was changed to

2.2 Construction of DNA arrays

accommodate increased incubation time between 70°C and 60°C, again at 50°C for a final annealing time of three days, followed by a final long incubation at 4°C (12-36 hours).

Following our optimised annealing procedure, the samples were then imaged by TEM on glow-discharged copper grids using uranyl formate (UFo) in LMU and uranyl acetate (UAc) in Oxford for negative staining. Deposition time was adapted depending on the concentration of the sample. As in section 2.2.1, the length of sticky ends was modified to observe how it correlated to array strength and purity. Comparison between 2 bp, 3 bp and 4 bp sticky ends confirmed that 2bp sticky end interactions are not strong enough to demonstrate extended tile assembly, whilst 4 bp sticky ends produced DNA arrays with increased stability. (Figure 2.19)

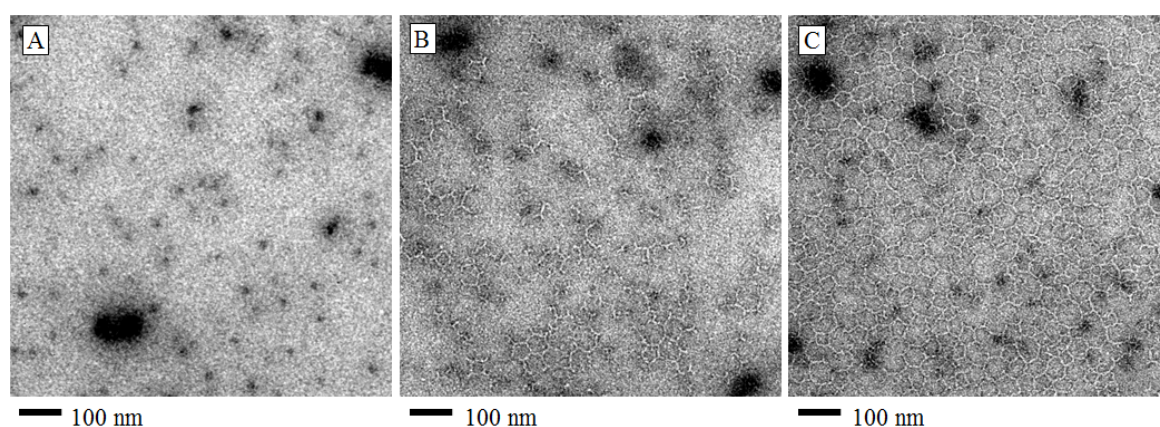


Figure 2.19: Comparison of array formation between (A) 2, (B) 3 and (C) 4 bp sticky ends. Black spots on the grids are attributed to aggregates.

TEM revealed that, in addition to the expected hexagonal shapes, larger and smaller polyhedra (squares to heptagons) were also observed on the TEM. The best results were obtained at 100 nM, which appeared to be a good compromise to allow assembly whilst avoiding massive aggregation of the DNA.

It is important to state here that S5 and S6, which are the two central strands of the star, were tested with two versions of T-loops. S5 and S6 with loops of 3T and 5T

respectively, or S5 and S6 both with 4T. For simplicity, analysis of images was made more thoroughly for 4T-loops, although both configurations showed equivalent results.

Images of Figure 2.19 were taken after 6 hours at 4°C. It was questioned whether such low temperature were indeed needed, as Majumder *et al.*^[206] obtained arrays at room temperature. Freshly made sample were applied to the same annealing ramp of 68 hours, which starts from 85°C to 20°C, then drops to 4°C when the cycle finishes. The sample was then incubated either at 4°C or on the bench at room temperature. TEM was performed at each stage of incubation, which revealed that the star is likely folded during the annealing ramp, but that formation of arrays requires overnight incubating at 4°C. At this last stage, the whole EM grid was covered with the lattice, which stays stable in solution if the sample is kept at 4°C. However, leaving the sample on the bench (20°C±2°C) causes disassembly of the arrays (Figure 2.20).

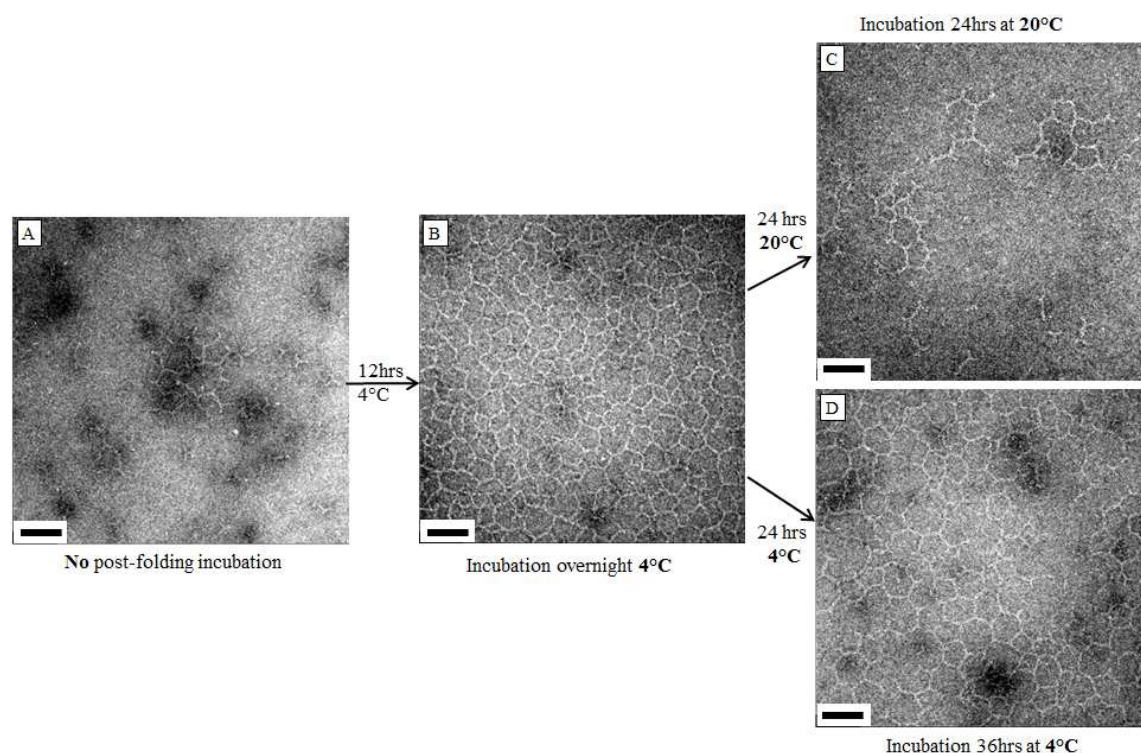


Figure 2.20: TEM images of 4bp sticky end star (A) directly after 68 hrs annealing, (B) overnight (12hrs) at 4°C, (C) incubation for one day at room temperature (~20°C), (D) incubation over 36 hrs at 4°C. Scale bars 100 nm.

Weaker interactions offered by 3 bp sticky ends were enough to cover the copper grid with the array, if not as densely. This difference was mitigated after the second incubation at 4°C, but extended observation on TEM tend to confirm that the polymerisation is even more sensitive to a rise in temperature than 4bp sticky ends. (Figure 2.21)

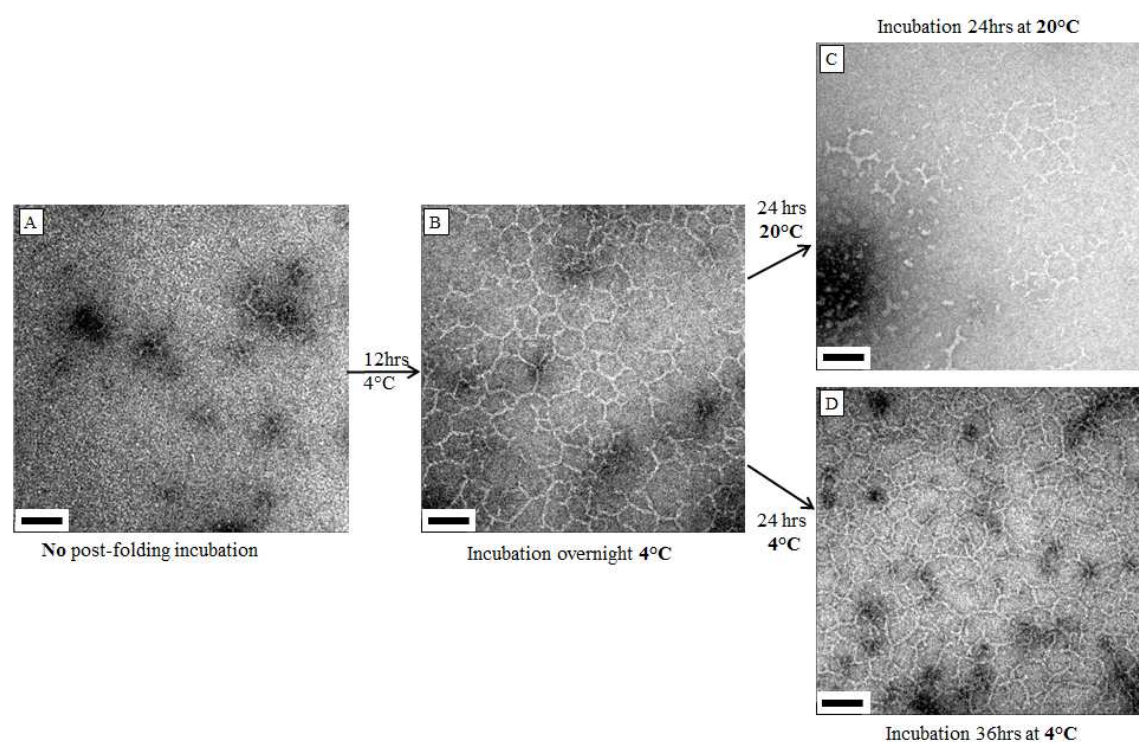


Figure 2.21: TEM images of 3 bp sticky end star, same sequence of incubation as Figure 2.20, above. Scale bars 100 nm.

As illustrated in Figures 2.20 and 2.21, 3D polyhedral structures were formed in addition to the 2D arrays upon star polymerisation and appear as black spots on the micrographs. We anticipated that a flat, 2D surface may template the formation of the array while minimising the formation of 3D structures; this effect has been observed previously (Some examples in literature discuss templated self-assembly on mica surfaces^[68, 228]). As presented in the introduction, mica is known to bind DNA when magnesium salt is present through electrostatic interaction, which is used in AFM imaging. In the absence

of a cholesterol anchor, the surface offered by a mica surface in the presence of magnesium cations can offer such a support in place of a lipid bilayer.

After a sample or array was incubated at 4°C for 36 hours, it was applied on a mica surface (see Annex A.13 for AFM protocol); the sample was applied directly to a freshly cleaved mica disc prior to adding the imaging buffer (Figure 2.22A).

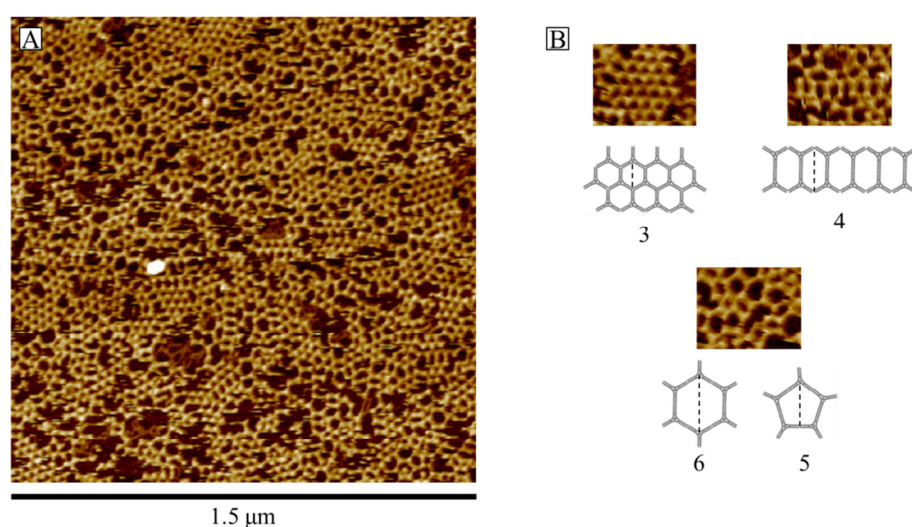


Figure 2.22: Liquid AFM micrograph after incubation of the sample at 4°C for 36 hours. (A) large scan, and (B) detail of motifs identified with corresponding schematics. The numbers below each scheme indicate the number of star involved in the formation of the opening.

Analysis of the lattice size revealed smaller and denser tile assembly than the corresponding sample imaged by TEM. It is believed that the DNA is locally more concentrated on the mica surface, due to the ionic interaction between the magnesium present in the buffer and the DNA backbone and the mica disc. Structures that are in solution during AFM imaging will diffuse and bind to the plane of the surface, and the DNA concentration will increase as more DNA accumulates. The construct can rearrange to accommodate an increasing number of tiles, leading to a denser organisation. In opposition to these observations, a sample imaged using TEM does not allow the construct to reorganise its assembly since the sample is dried during preparation. The lattice observed by TEM is the expected organisation where one arm

hybridizes with an arm of another star. However, analysis of the AFM micrographs reveals areas that illustrate interactions of the sticky ends (Figure 2.22B) other than the ones expected (Figure 2.23A). We believe that this is possible due to the flexibility of the four helices at each end: instead of pairing all four helices to their corresponding complements, the helices of one tile can bend and bind with two other helices on two different tiles (the two helices on the right hand side of the first tile interacting with to helices of the left hand side of a second tile) (Figure 2.23B).

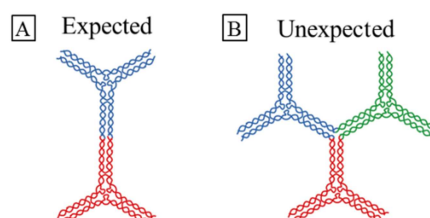


Figure 2.23: diagram showing (A) the expected association of sticky ends between two tiles, and (B) the unexpected conformation involving three DNA star.

Table 2.2 comparison between sizes of the openings on AFM (L^m) and theory (L^t)

	L^m [nm]	L^t [nm]
6	59.7 ± 0.8	47.6 ± 0.3
5	47.9 ± 0.5	36.6 ± 0.3
4	42.3 ± 0.3	37.2 ± 0.3
3	31.7 ± 0.1	23.8 ± 0.3

The measured size of the openings (L^m , showed as dotted line in Figure 2.22B) increases as the structure formed involves more star. Similarly, the measured size difference increases as the structure formed increases in flexibility, due to the range of angle that the three-way junction can adopt at the central vertex; the more flexible the lattice, the bigger the average size difference. Simple calculations give smaller theoretical values than measurements, because the length estimation does not account for the angle (and dimension) of the vertices, which are hard to evaluate.

Analysis of the height profile of an AFM micrograph identified two height regimes. (Figure 2.24). It is possible for a tile to be lacking one of the two layers of the double-decked DNA star, as the two double-helices are fixed with each other only by one crossover per arm and involve only 9 base pairs. Most structures have heights of approximately 4 nm (Figure 2.24B), the height of two stacked helices. Heights of only 2 nm are consistent with one single DNA helix.

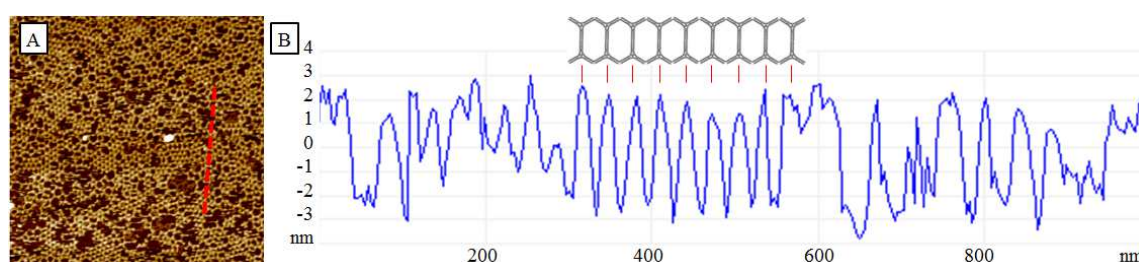


Figure 2.24: (A) AFM micrograph and (B) height profile corresponding to the dashed line of star without cholesterol handle. A recognisable motif is illustrated above the graph.

Section 2.1.3 described how strand S8 can be divided in two parts to support a cholesterol handle (of 18 nt) on one of the moieties. We tested whether the star formed arrays with the cholesterol handle attached to it following the same annealing protocol as before. After 36 hours at 4°C, the sample was imaged with TEM (Figure 2.25A) and AFM (Figure 2.25B).

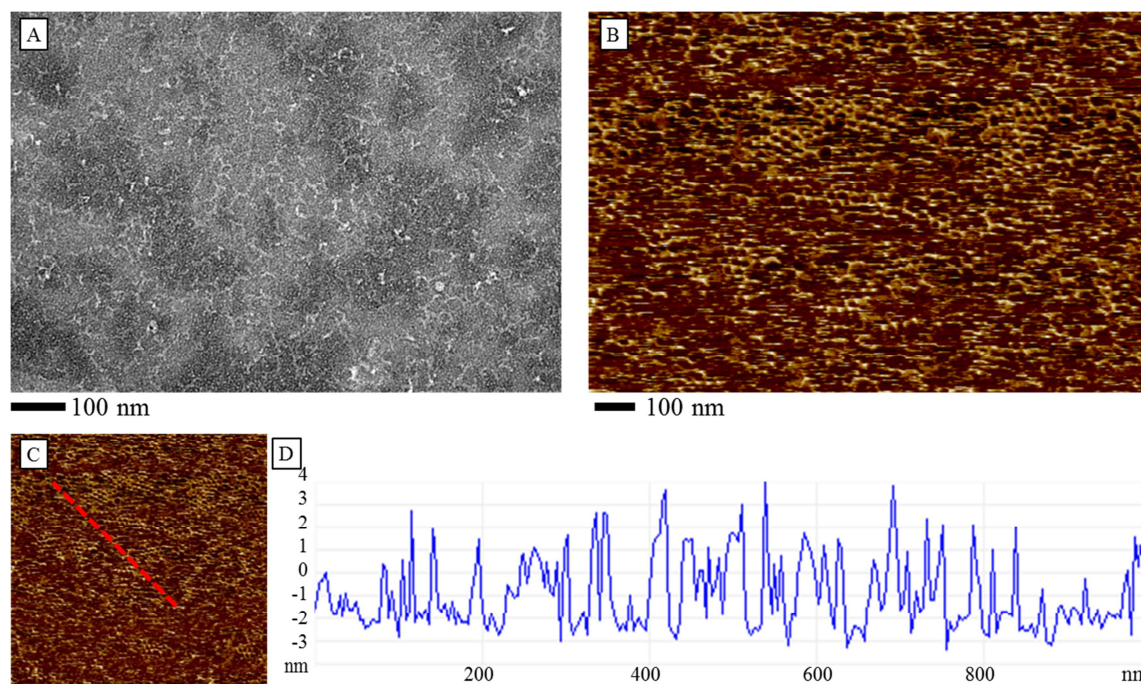


Figure 2.25: micrographs of star with cholesterol handles (one per arm, on S8') imaged with (A) TEM (imaged in Oxford), (B) AFM. (C) AFM micrograph and (D) height profile corresponding to the dashed line of star with the cholesterol handle on S8'.

The results obtained with this design showed fewer and smaller arrays on the mica, an effect that is likely linked to the presence of additional strain (by the addition of DNA handles) on the tile, as well as shortened strands (S8' and S9 are shorter than S8).

Despite difficulties encountered during the folding, the structure was applied to a supported bilayer containing cholesterol anchors to test whether some structures insert into it. In order to give optical contrast in fluorescent microscopy, the DNA star must include a dye as well as the lipid bilayer. It was proposed to chemically add a dye ATTO-488 (blue) on the 3'-end of one of the strands. After consideration of the design, S5 was selected because of its favourably localised 3'-end, on the top vertex. (See Annex A.2.2 for functionalisation protocol). The bilayer includes a fluorescent lipophilic carbocyanine DiD and the Chol-DNA complementary to the handle on S8' protruding from the DNA structure. After functionalisation of the 3'-end and purification of the strand, the star was folded and added to a 30 μ L channel on a microscope glass slide

(Ibidi, sticky-slide VI 0.4), with a formed supported lipid bilayer (SLB) for incubation overnight at 4°C (Figure 2.26A). The handle from each formed tile should hybridise with the one from the cholesterol anchor and excess DNA is washed away.

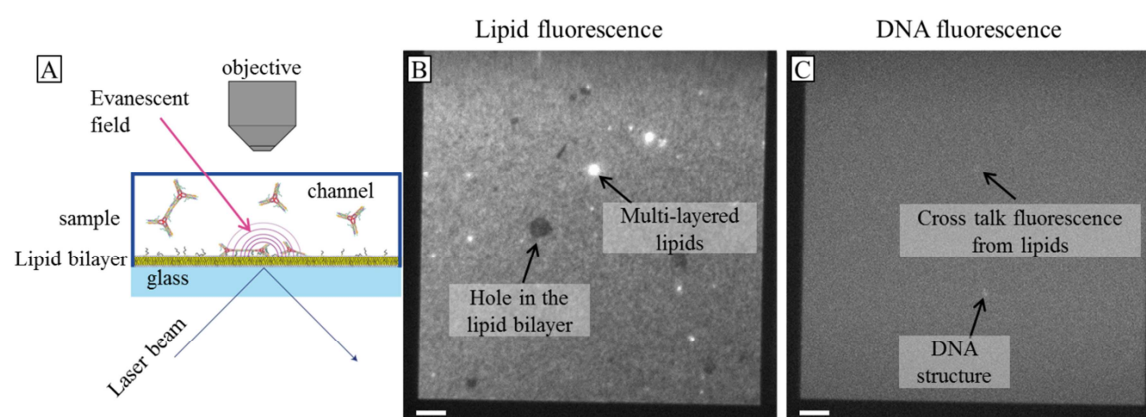


Figure 2.26: TIRF microscopy of a SLB with DNA star. (A) Illustration of the TIRF setup. The sample is imaged by (B) lipid fluorescence (lipophilic carbocyanine DID), and (C) DNA fluorescence (DOP-ATTO-488). The lipid bilayer shows brighter and darker spots; the latter are multi-layered lipid and the former are holes in the bilayer. DNA is identified (C) when the fluorescence coming from DNA corresponds to a plain lipid bilayer and not a bright multi bilayer. Scale bars approx. 1µm.

DNA and lipid fluorescence was recorded using a home-build TIRF in LMU, and it has been established that the position of the signal coming from ATTO-488 corresponds to DNA on a lipid-covered surface and not on a hole or a lipid protuberance (Figure 2.26C). Since the dye and the cholesterol-handle are on two different strands, any DNA fluorescence should be coming from polymerised DNA structure. Although it is probable that the limited signal observed in this experiment corresponds to aggregated DNA tile, this experiment confirms that a cholesterol-handle inserted in the SLB can anchor the DNA to it.

2.3 SUMMARY

This chapter presented the design of a polymerisable three-fold DNA star, and how the system was optimised to lead to its successful synthesis and polymerisation. The arrays were characterised by AFM and TEM. Similar to the model proposed by Majumder et al^[206], 4 bp sticky ends are judged optimal for polymerisation. However, three stars (or tiles) were observed to join during AFM analysis contrary to the initial expectation of the array motif (which involved only two DNA stars). It is believed that this altered organisation is concentration-dependent as the motif arises upon surface-enhanced, high local concentration of the DNA on mica.

Addition of a cholesterol-handle to the bottom of the tile appears to successfully anchor the DNA to the membrane, but the resulting signal observed on the SLB was poorly reproducible and likely arising from an amorphous structure, as shown by TEM and AFM images. It has been discussed whether having the cholesterol chemically linked to the star itself would increase the risk of aggregation. This problem was solved by inserting the cholesterol-DNA directly on the SLB, and placing its corresponding handle on the DNA star. Stars with such a handle (and two shorter strands) revealed smaller arrays, and it is theorised that this is because these add strains and destabilise the star.

Although our design of a DNA-star that could polymerise into an array has been successfully implemented, as evidenced by TEM and AFM, the star was too flexible to direct the formation of a regularly repeating array, which is necessary for controlled membrane bending. We thus altered our strategy to synthesise a star with DNA origami as this approach has the potential to circumvent the weaknesses of our initial approach.

CHAPTER 3

CLATHRIN-INSPIRED DNA ORIGAMI

DNA origami is a molecular engineering technique in which one long scaffold of viral DNA is folded into the desired shape with the help of multiple, smaller, artificial DNA staples (Introduction section 1.4.1). This chapter covers the design and synthesis of a synthetic analogue of the clathrin protein using DNA origami. We developed a polymerisation strategy that allowed this novel nanostructure to polymerise into large array mimicking the pattern of a clathrin coat. This three-fold symmetric DNA nanostructure was designed to be able to adopt both a flat and a bent conformation, with the aim to observe difference in behaviour when polymerised on a lipid membrane. This DNA origami was functionalised with cholesterol to anchor the structure into the lipid membrane, and we observed their interactions first by transmission electron microscopy (TEM). We observed that the lipid membrane offered direction and support for polymerisation into extended array and that curved origami nanostructures formed spherical features consistent with a budding of the membrane. The structure was further functionalised with a dye and imaged on a large lipid vesicle in solution using fluorescence microscopy. In these experiments, our novel DNA origami in its flat conformation formed arrays that did not deform the membrane of the lipid vesicle, observed as bright patches during fluorescent microscopy, but the curved conformation produced budding of the malleable vesicles with localisation of the DNA origami at the base of the bud.

3.1 DESIGN OF A CLATHRIN-INSPIRED DNA MODEL

DNA origami overcomes many of the problems encountered in Chapter 2: first, as assembly occurs spontaneously upon slow annealing of the mixture, the structures can be purified from excess staples post folding; this resolves the stoichiometry issues. Secondly, the larger scale of the folded DNA origami facilitates the identification of each repeating unit in TEM analysis, compared with the three-way junction from Chapter 2. Finally, The structure of DNA origami facilitates its functionalisation with cholesterol or a dye handle^[77]. DNA origami is thus a suitable strategy to replicate the shape of a clathrin protein, with the ability to control and precisely functionalise its structure.

3.1.1 Design of a 3-fold DNA origami

The design of our DNA-origami to mimic clathrin took into account the following necessary features: (1) the structure must be capable of interacting with a membrane bilayer; (2) it must be able to polymerise and cover a defined surface (in particular, to coat a vesicle); and (3) it should accommodate a controlled change of the curvature of the structure. A three-fold DNA origami structure was proposed, capable of performing these functions, following the strategy defined for the previous DNA-assembly.

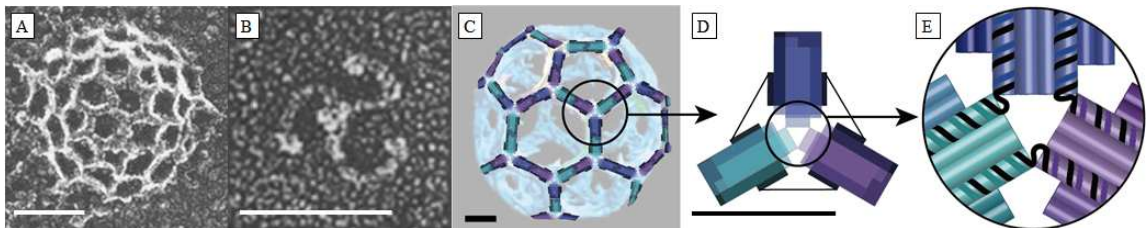


Figure 3.1: Electron micrographs of (A) a clathrin protein pit and (B) a single clathrin triskelion. Images (A) and (B) are from reference^[154]. Schematics of (C) a vesicle coated with DNA origami, (D) detail of one monomer, and (E) detail of the centre of the monomer, with the scaffold crossing from one arm to the next one in black. Scale bars 50 nm.

The previous approach of using only short DNA strands assembled into four double helices, described in Chapter 2, suggested that the arms were too fragile to induce membrane bending. We thus decided to keep the arms of this new three-fold symmetry nanostructure relatively short and bulky. This led to the construction of a structure of similar size to the biological clathrin triskelion (Figure 3.1). The resulting monomer triskelion is composed of the phage-derived scaffold M13mp18 (7249 bases) and 179 staples. To balance the rigidity of the thick arms, a small loop of 5 bases created by the scaffold at the centre of the tripod provided the flexibility required for the formation of hexagonal and pentagonal faces (Figure 3.1 C, D, E). This central loop also ensures the attachment of each arm with its neighbours. The new structure was named “triskelion” as it is inspired from the shape of the clathrin monomer. The routing of the scaffold successively runs through the three arms, forming first the helices that make the bottom layer of an arm before crossing to the next arm by forming a bridge at mid-length (See Annex 17.1 for CaDNAno model). The routing then completes the rest of the origami, crossing at the centre of the nanostructure (which is the meeting point of the three arms) (Figure 3.2 A and C). To induce controllable membrane curvature, the scaffold that makes the bridge at the midpoint of each arm can be hybridised with two staples, and has a loop of single stranded DNA scaffold located in the middle (Figure 3.2 B). The resulting bridge is then one DNA double-helix with the scaffold forming a loop of a few unpaired bases in the centre, and two DNA staples on each side. The length variation of the bridge is made by changing the number of nucleotides (nt) involved in the scaffold loop. It is postulated that the extent of curvature of the structure can be modulated from $180^\circ \pm 4^\circ$ to $118^\circ \pm 4^\circ$ by changing the bridge length between 90 and 60 base pairs.

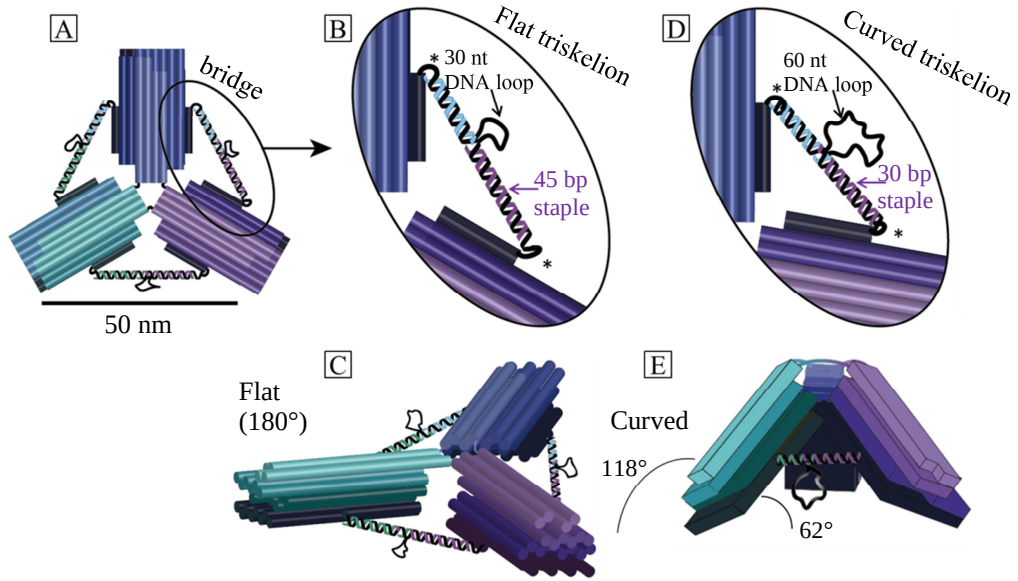


Figure 3.2: Illustrations of a DNA bridge of various lengths. (A) Schematic of the origami triskelion, top view. (B) Details of the 90 bp-long bridge and (B) side view; (D) details of the 60 bp-long bridge and (E) side view. A star (*) indicate 5 unpaired bases of the scaffold.

The triskelion is flat ($180^\circ \pm 4^\circ$) when two staples of 45 nt each are hybridised, leaving a DNA loop of 30 nt (Figure 3.2B, C). Two shorter strands of 30 nt each force the triskelion to curve and form an angle of $118^\circ \pm 4^\circ$ and leave a 60 nt-long loop (Figure 3.2D, E). As discussed in Chapter 2, artificial synthesis of oligonucleotides suffers from higher risks of mismatches as the DNA chain extends. Therefore, suppliers advise not to exceed 60 nucleotides or else recommend purifying the DNA strand post-synthesis. The staples used to hybridise with the bridge are therefore between 40 and 60 nt and are used without further purification. We saw in the introduction (section 1.3) that the persistence length of one DNA double helix is approximately $50 \text{ nm}^{[229-231]}$. Since the estimated length of the bridge is approximately 30 nm, it is expected that a DNA double helix can be considered as a straight and rigid unit.

Five bases from the scaffold are left unpaired on both sides of the bridges where the DNA makes a tight angle (see * Figure 3.2B and D). This is to allow enough flexibility for the double helix coming from the body of the arm to change orientation and become the double helix of the bridge.

3.1 Design of a clathrin-inspired DNA model

The construct has four layers of DNA double helices packed in honeycomb fashion, approximately 13 nm in height and each arm 33 nm long (Figure 3.3 A-D). To ensure that the triskelion will bend always on the same direction, the central loops are positioned on the highest layer of the DNA origami, and the mid-length bridges are localised as close as possible to where the membrane will be, on the lowest part (Figure 3.3 E, F). The shapes of the inner faces of each arm were also designed specifically towards one bending orientation: the central end finishes as an upside-down stair, a higher layer going further than the one underneath and so on (Figure 3.3 C, D). The structure can carry various handles (staple extensions) to which cholesterol-DNA (positioned at the bottom layer) and fluorophore-DNA (positioned on top of the triskelion) conjugates are attached. There are 3 precisely defined positions for cholesterol anchors on each arm (9 total), which will dock the triskelion to the lipid bilayer, and 2 positions for fluorescent dye per arm (6 total) that will help for visualisation and localisation.

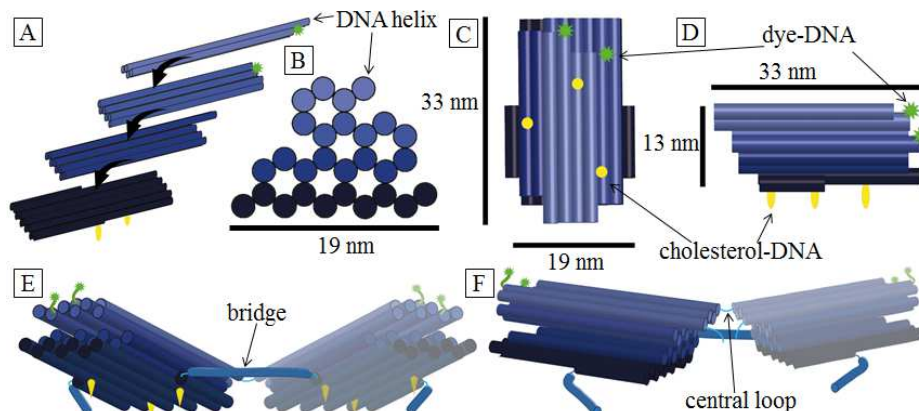


Figure 3.3: schematics of one arm of the triskelion showing (A) four layers of DNA double-helices stacking (B) perpendicular cut through one arm showing the honey-comb arrangement of the helices, then dimension and localisation of cholesterol-DNA and dye-DNA (C) top view, (D) side view. 3D illustration of two arms joined by their bridge and central loop, view from (E) outside and (F) the centre of the triskelion.

The height of the central scaffold crossings and low location of the bridges, along with the stair-like shape of the inner faces ensure that when the triskelion will bend, it will preferably curve in one direction. The cholesterol anchors will be on the face of the arms

that get closer as the triskelion bends, and should force the membrane in which it is inserted to follow the curvature of the origami.

3.1.2 Design of a polymerisation method

To polymerise individual triskelia into a regular array of hexagons and pentagons, we designed sticky ends on each arm of the triskelion, such that each arm would pair with a complementary arm of another triskelion. Sticky ends protrude from the DNA helices of the horizontal outer extremity of each arm (Figure 3.4 A). Alternate layers of helices are slightly extended to allow the pairing of the sticky ends, which are elongations of the staples forming the ends of the double helices. Sticky ends with 2, 4 and 6 bp were investigated to study the stability of the resulting array. In order to hybridize, a sticky end protruding from a double helix end have to face a sticky end of opposite direction coming from another triskelion (5'-end faces 5'-end indicated as arrows, and 3'-end faces 3'-end) (Figure 3.4 B).

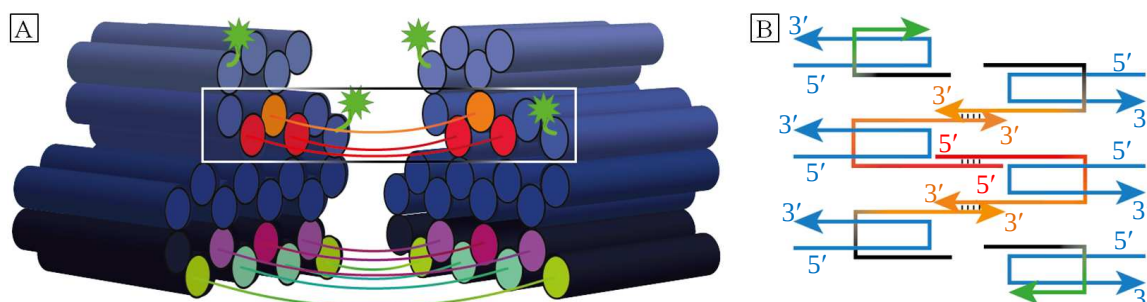


Figure 3.4: Illustration of the interface between two arms of two triskelia showing (A) 3D representation and localisation of the sticky ends. Each pair of sticky ends is shown by a different colour and dyes are shown by green stars. (B) Schematics of the interface corresponding to the rectangle in (A). 5'-ends are represented by arrows. The scaffold is shown in blue. In this case, both green 3'-ends correspond to the beginning of a dye handle.

A total of 10 sticky ends per arm were designed with a unique matching sequence localised on the corresponding arm. The 3D proprieties of the triskelion allowed us to distribute the sequences of the sticky ends so that two triskelia create a continuous plane on the membrane (Figure 3.5) without risk of twist between them.

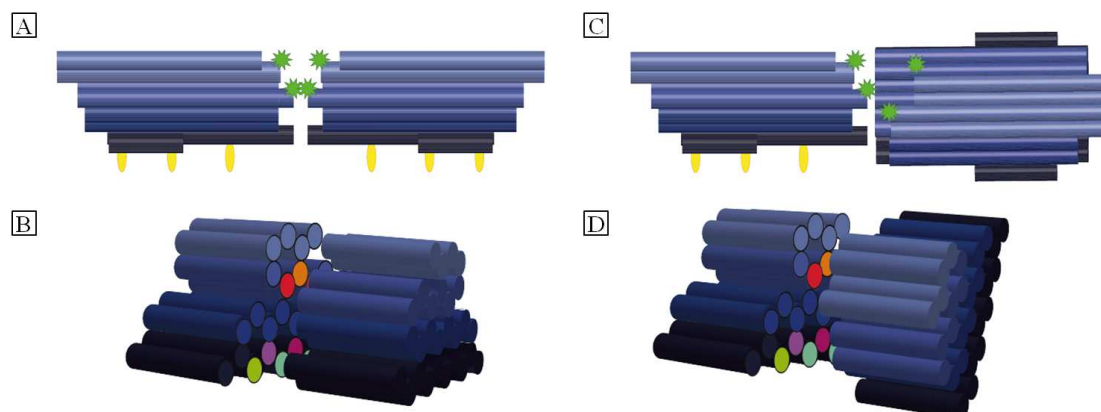


Figure 3.5: Illustration of (A), (B) correct and (C), (D) incorrect alignment of the extremities of two arms. Only the correct alignment is possible when the sticky ends hybridise.

Finally, any DNA double helix extremities not involved in sticky ends or loops that could make undesired stacking interactions were capped with single-stranded extremities of 5T at the centre of the triskelion and 3T at the arm extremities.

Two patterns of sticky ends complementarity were used (Figure 3.6). The first allows linkage of any pair of arms and is therefore referred as “all $\leftrightarrow$ all”. The second, called “1 $\leftrightarrow$ 1, 2 $\leftrightarrow$ 3”, distinguishes the arms and forces “arm 1” to hybridise with another identified arm 1 of a second triskelion. The two other interfaces (arm 2 and arm 3) are complementary to one another and complete the polymerisation. The 1 $\leftrightarrow$ 1, 2 $\leftrightarrow$ 3 interactions are perfectly satisfied by the formation of a truncated icosahedron enclosing a sphere of approximately 340 nm which is illustrated in Figure 3.1C. we expect the all $\leftrightarrow$ all pattern to be less selective in the formation of hexagons and pentagons than the 1 $\leftrightarrow$ 1, 2 $\leftrightarrow$ 3 pattern, but will facilitate the association of two arms and will therefore be faster. The pattern 1 $\leftrightarrow$ 1, 2 $\leftrightarrow$ 3 is more likely to form a truncated icosahedron, which is a targeted assembly.

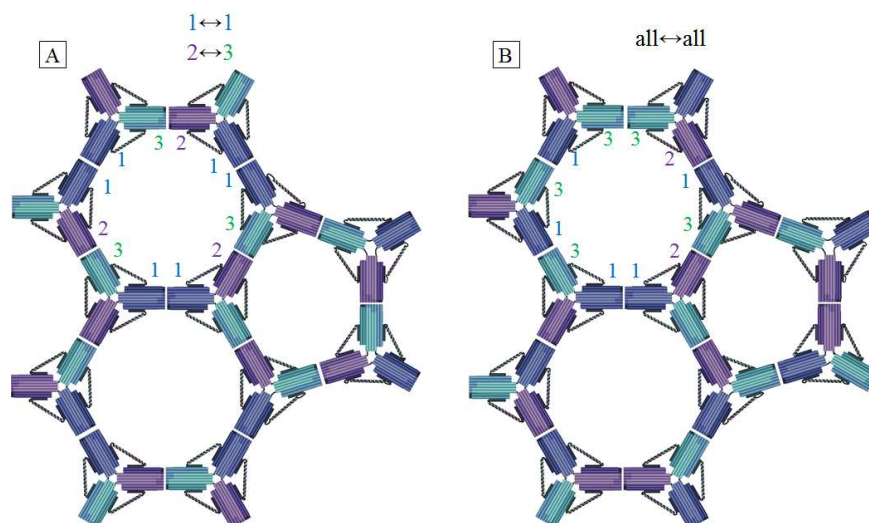


Figure 3.6: Illustration of polymerisation arising from the two patterns. (A) Ordered $1 \leftrightarrow 1$, $2 \leftrightarrow 3$ and (B) random $\text{all} \leftrightarrow \text{all}$.

When the triskelia polymerise, the length of the bridges between the arms will dictate the curvature of the resulting array. It is anticipated that when the array embeds itself into a lipid bilayer, through cholesterol insertion, it will deform the membrane by the angle prescribed by the origami. If applied to a vesicle, this action will mimic early stage of clathrin-driven endocytosis (Figure 3.7).

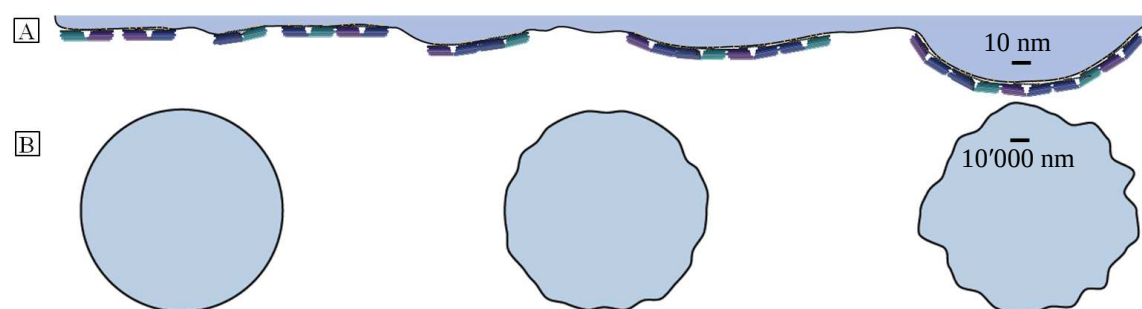


Figure 3.7: Illustration of a membrane bending due to the presence of triskelia, views (A) at the nanoscale and (B) at the macroscale on a giant unilamellar vesicle. Scale bars are approximations.

3.2 SYNTHESIS OF DNA ORIGAMI

3.2.1 Optimising the triskelion folding procedure

The triskelion was successfully folded using an annealing ramp of 20 hours, and analysed by agarose gel electrophoresis (AGE) and by TEM (Figure 3.8). The analysis of the results of the folding conditions were made with a DNA origami design with 90 bp bridges, which would form a flat triskelion, omitting the handles for cholesterol and dye conjugates, and with no sticky ends. The extremities of the arms are protected from unspecific interactions by a cap made of 6T added at the end of every DNA helix.

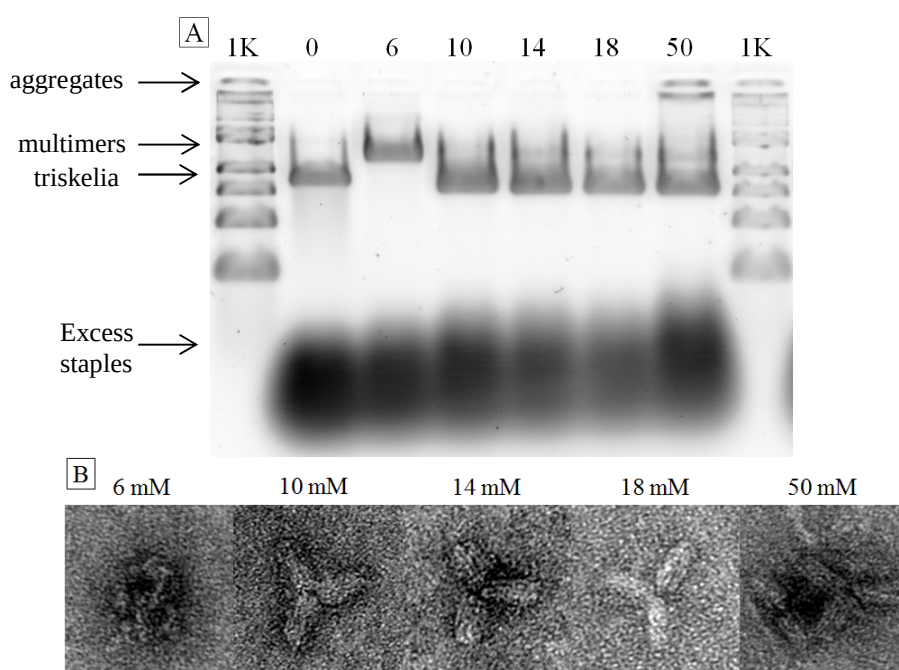


Figure 3.8: (A) 1.5% agarose gel of triskelia at different $MgCl_2$ concentrations. 1K indicates the DNA ladder and the number above the wells refers to the concentration of $MgCl_2$ in mM. (B) TEM micrographs of the triskelion at each salt concentrations. Scale bar 50 nm.

At concentrations of $MgCl_2$ between 10 and 18 mM, the successfully folded triskelia were observed by TEM analysis and visualised as a predominant band in the agarose gel. If $MgCl_2$ was not present during the folding procedure, the structure did not form properly and the band migrated slightly slower than the correctly folded triskelion (Figure 3.8A). High salt concentration (50 mM) creates aggregates that remained in the

well in addition to the triskelia. A stable, irregular intermediate is reached when the magnesium concentration is at 6 mM as observed by TEM (Figure 3.8B), but this secondary product disappears when an adequate MgCl_2 concentration is reached. The optimal salt concentration for correct folding was determined to be 14 mM MgCl_2 . Although misfolded DNA scaffold is present at all concentration of MgCl_2 investigated, observed as a dimeric band in the gel (Figure 3.8A), these secondary structures were removed during purification (see later) and are considered negligible as they were rarely observed.

After optimising the concentration of MgCl_2 for triskelia folding, we investigated the effect of the scaffold concentration on the triskelia formation.

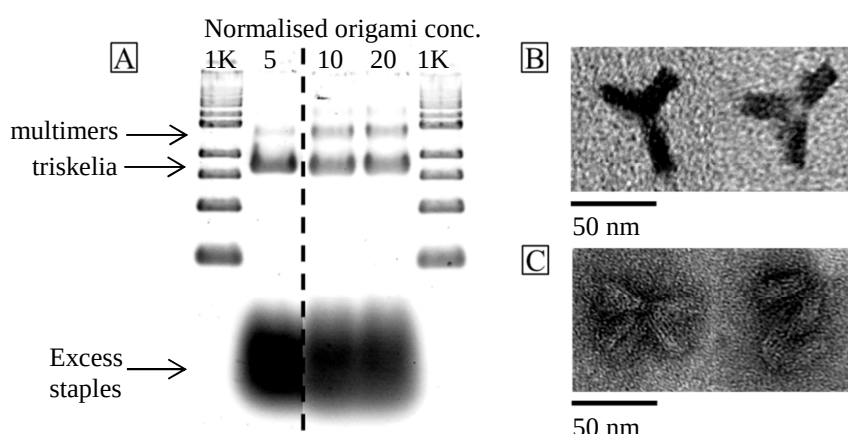


Figure 3.9: (A) 1.5% agarose gels of triskelia folded at different scaffold concentration as written above the wells. Typical electron micrographs of origami recovered from the main band of the gel: (B) origami monomer and (C) coupled triskelia (20 nM only).

Triskelia were folded at a concentration of 5-20 nM and then loaded in the gel in Figure 3.9A so that the concentration of triskelia per well is constant. When the triskelion is folded with 5 or 10 nM scaffold, the vast majority of structures are the monomeric triskelia (Figure 3.9B), as observed as the single band in the gel (Figure 3.9A). At a concentration of 20 nM, however, TEM analysis observed structures made of two scaffolds sharing some staples (Figure 3.9C). These coupled scaffolds represented only 10% of the structures in this and were not observed at a concentration of 5 or 10 nM. Therefore, triskelia in the following discussion were always folded at a concentration of 5

or 10 nM. Similarly to Chapter 2, TEM images show either positive staining (Figure 3.9B) or negative staining (Figure 3.9C). Staining methods and results will be discussed in Chapter 6, although it is important to precise for this chapter that both stains can occur on the same grid and depends on the sample preparation and hydrophobicity of the surface. Therefore, positive and negative staining will be treated interchangeably.

After optimising the salt and scaffold concentration required for triskelia folding, we then looked into the functionalisation of the triskelion monomers with cholesterol and dye. As many DNA nanostructures presented in literature are folded with their handles and functionalised; post-annealing, with their functional DNA-conjugates^[68, 75-78, 82], we followed this procedure to decorate our triskelion. After folding the origami with the handle for the 9 cholesterol and 6 dyes (Alexa-647, Figure 3.3), we incubate the origami with complementary DNA conjugated to cholesterol and dye for 2 hours at 30°C. The successful hybridisation of the DNA conjugates to the handles on the triskelion was identified by AGE (Figure 3.10).

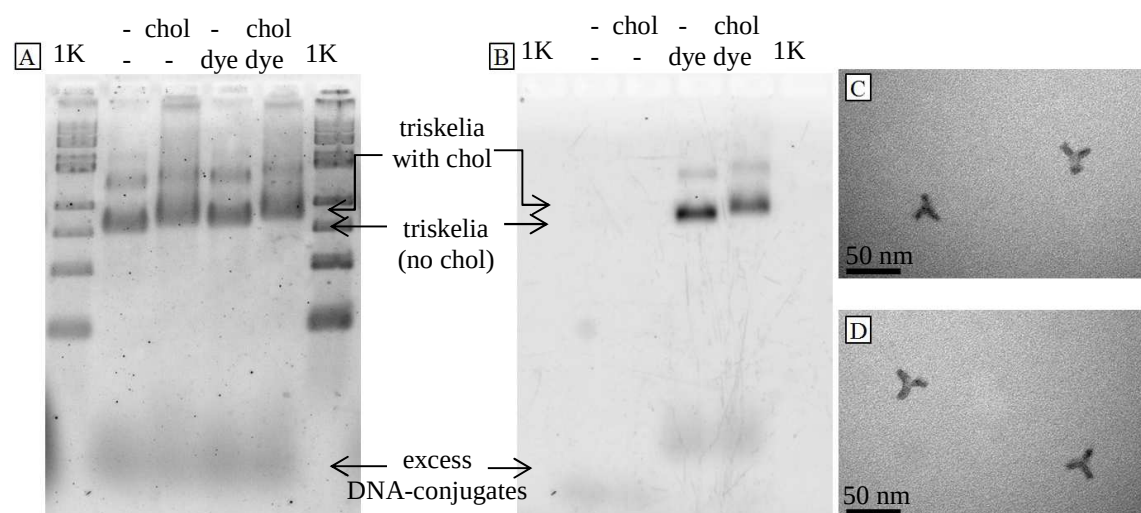


Figure 3.10: 1.5% agarose gel of folded triskelia with DNA conjugates after incubation at 30°C. The type of handle used during the folding of the triskelion is written above the well, and a – indicates the absence of handle. The gel was scanned (A) after staining with SYBR gold, and (B) for fluorescence from the dye. Electron micrographs of triskelia (C) with dye and (D) with cholesterol and dye.

The fully functionalised origami with both cholesterol and dye moieties displayed a smaller migration in the agarose gel, which is attributed to a weak, unspecific interaction between triskelia due to the hydrophobic effect of the cholesterol. The dye was easily

identified by fluorescence and confirmed that the DNA-Alexa647 can only attach to the origami in the presence of the handle^[232, 233].

Fluorescence analysis was used to verify the efficiency of the handle incorporation when the DNA-Alexa647 is added without pre-folding of the triskelion, as it would simplify the production of functionalised triskelia by removing one purification step. In this experiment (Figure 3.11), we titrated increasing concentrations of DNA-Alexa647 to the origami, and the equivalents were calculated with respect to the number of handles present (and not to the number of origami). These triskelia were extracted from the agarose gel for TEM imaging (Figure 3.11D) for direct observation of the folding quality.

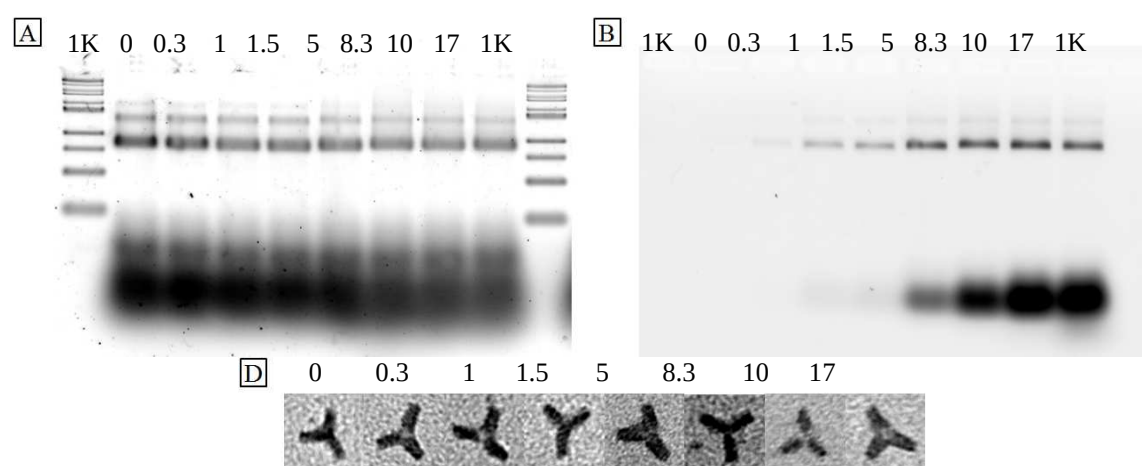


Figure 3.11: Analysis of equivalent of dye needed for total site occupancy. Numbers above each well correspond to the amount of dye equivalent added during folding with respect to the number of handles. Gel scanned for (A) SYBR gold fluorescence and (B) Alexa-647 fluorescence. (D) Electron micrographs showing typical triskelion recovered from the gel for each line. Scale bar 50 nm.

The triskelion folded correctly over the titration experiment as confirmed by AGE (Figure 3.11A, B) and EM micrographs (Figure 3.11D). Analysis of the gel using ImageJ^[234] (Table 3.1) determined that five equivalents were sufficient to have a full occupancy of the dye handles. We could thus prepare dye- and cholesterol-functionalised triskelia in a one-pot folding procedure with good yields. We assumed that the insertion of cholesterol would follow the same incorporation efficiency as the dye^[76].

Table 3.1: Image intensity values [%] of the gel from dye fluorescence calculated with ImageJ.

Dye equivalent:	0	1/3	1	1.5	5	8.3	10	17
Dimer [%]	0	0	7.0	5.5	1.8	1.2	1.1	0.9
Monomer [%]	0	57.0	45.4	40.8	21.3	13.0	10.7	9.0
Staples [%]	0	43.0	47.5	53.7	77.0	85.7	88.3	90.1

Although the correctly folded, fully functionalised triskelia could be recovered from the agarose gel to allow visualisation by TEM analysis, we also observed residual agarose polymer in the micrographs (Figure 3.12A, B). As we intend to investigate the interaction of the polymerised triskelia with lipid vesicles, and knowing that agarose can change the mechanical properties of a lipid bilayer^[235], we required a purification method to isolate the triskelia from agarose.

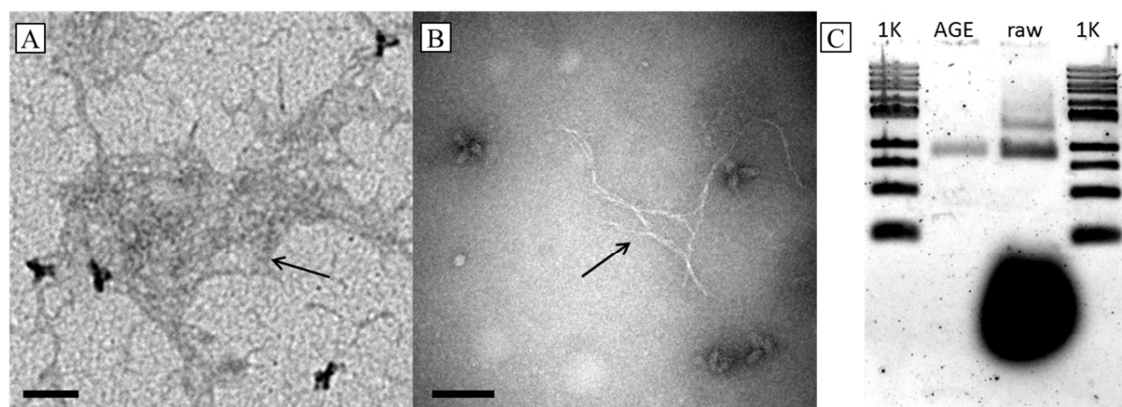


Figure 3.12: Agarose purification of triskelion. (A) Positive and (B) negative staining electron micrographs showing triskelia and pieces of agarose (arrows). (C) AGE for comparison of sample before (raw) and after (AGE) purification by gel extraction. Scale bars 100 nm.

Size-exclusion and concentration methods such as sephacryl resin S300^[223] (Figure 3.13A, B), PEG purification^[236] (Figure 3.13C, D), Amicon filter (Figure 3.13E, F) or ultracentrifugation^[237, 238] (Figure 3.13G, H) provided concentrations of the triskelia between 4 and 20 nM, but produced aggregated or misfolded triskelia that could sometimes be seen on AGE analysis in the well or as a slower and smeared band (Figure 3.13D, F) or leaked contaminants in the sample (Figure 3.13, arrows). PEG precipitation, for example, was particularly unreliable as the sample could sometimes be removed by

mistake with the supernatant (Figure 3.13D right hand-side) and produced aggregates visible on the agarose gel and on electron micrographs.

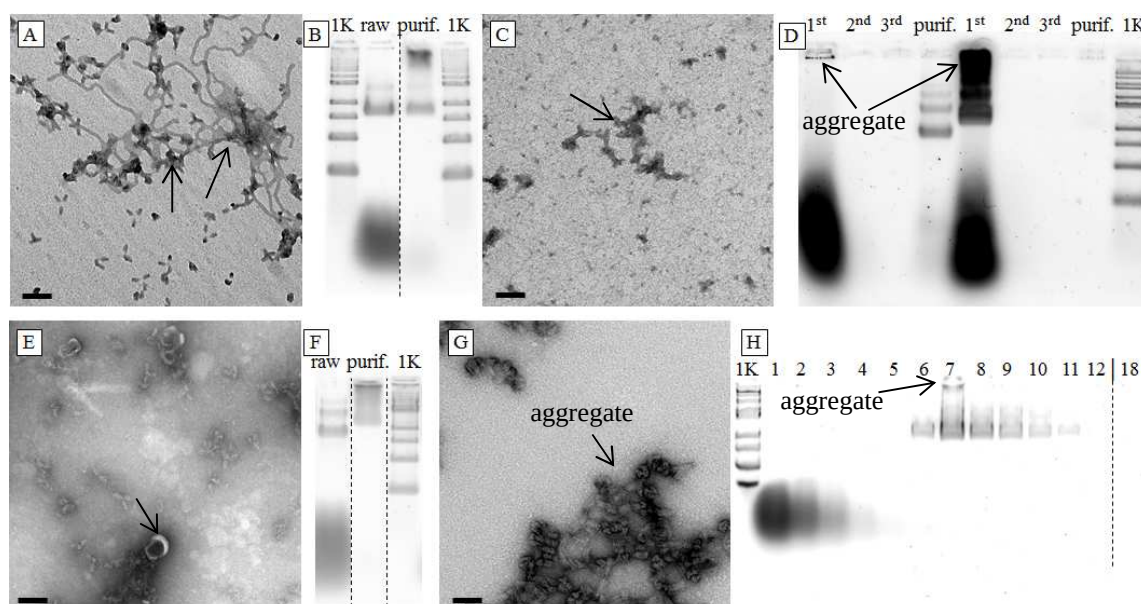


Figure 3.13: TEM and AGE scans comparing the raw sample (raw) and the sample after purification (purify.) made by (A), (B) resin S300 spin-column; (C), (D) PEG precipitation (two different samples are shown, the number of pellet is written above the well); (E), (F) Amicon filter and (G), (H) ultracentrifugation (number above the well indicate the fraction as recovered). Arrows on the electron micrographs indicate contaminant arising from the purification step. Scale bars 100 nm.

It is believed that the radial arrangement of the three arms of the triskelion is particularly sensitive to shear forces such as centrifugation as the bridges can entangle the structure and break the 3-fold symmetry. Deformed monomers are thought to be more susceptible to aggregation than correctly-folded structures. We therefore required a gentle purification method that would yield a high concentration of origami while preserving the integrity of the structure. As gravity-driven size-exclusion columns are a standard purification method for biomolecules and lipid vesicles^[7, 239], we were able to purify folded triskelia from their excess staples (50-850 μ L sample volume) by passing through a PD-10 column (GE Healthcare) with a bed volume of 8.5 mL (Sephacryl S300) (Figure 3.14).

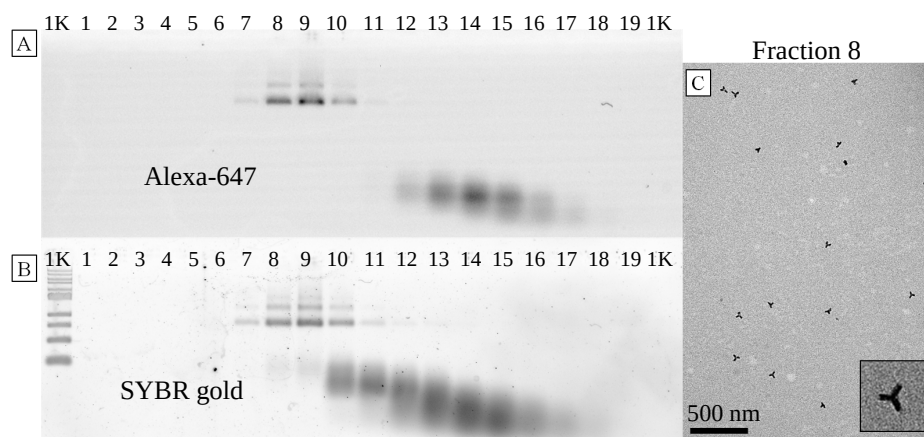


Figure 3.14: AGE of gravity-driven size-exclusion columns (A) scan of Alexa-647 fluorescence and (B) same gel after SYBR gold staining. The number above the well correspond to the fractions' number. (C) TEM micrograph of fraction 8. Inset: detail of one triskelion.

To ensure that the selected triskelion-containing fractions are staple-free, the collected volumes were analysed by SYBR gold-stained AGE, as most staples are not fluorescent (Figure 3.14A, B). A typical 200 μ L raw sample at 10 nM concentration gave an acceptable separation and concentration of 500 μ L at 3 nM (75% yield) without contaminant or aggregates. The triskelia remained well-folded after purification (Figure 3.14C).

3.2.2 Optimising the triskelion polymerisation strength

With the purified, folded and functionalised triskelia in hand, we proceeded to assemble the individual flat triskelia (90 bp-long bridge) in the absence of cholesterol and dye, to form nanometre-sized arrays capable of covering a large surface area.

As two DNA helices can interact at their extremities by end-to-end stacking interactions^[240], triskelia were initially folded with a set of extremity staples without the protective 6T-ends called blunt ends to examine if unprotected double helices ends can polymerise solely from stacking interactions at the interface of the arms. Triskelia missing these extremity staples were also folded as a control experiment to determine that unspecific polymerisation does not occurs from dangling ssDNA. The results were analysed by AGE and TEM (Figure 3.15).

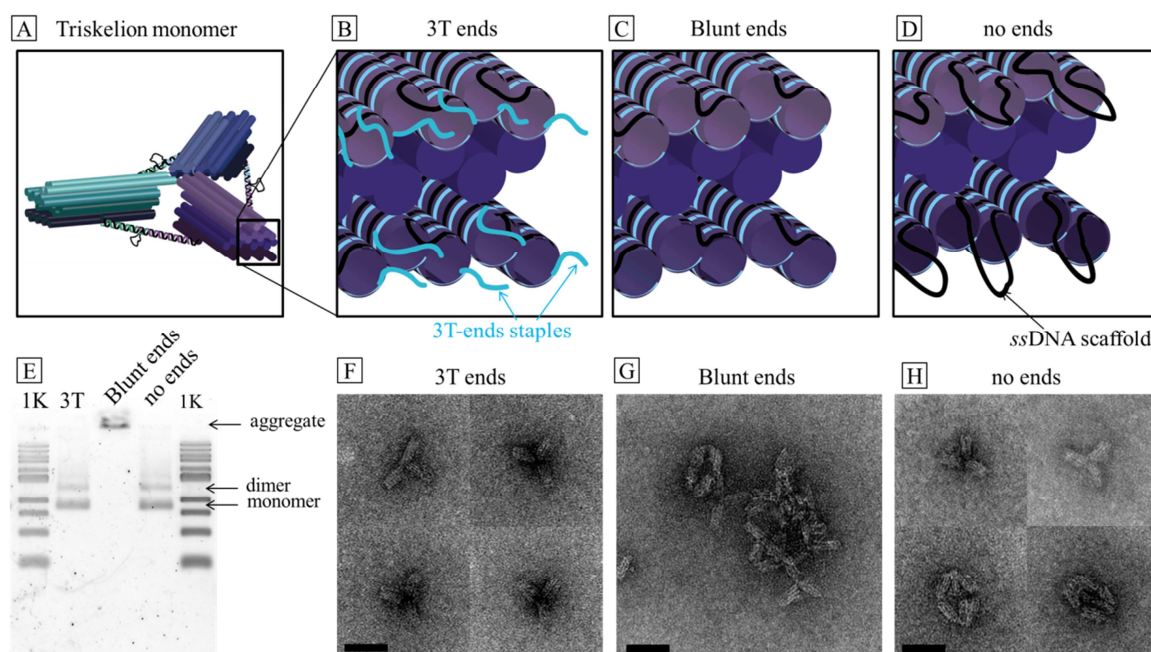


Figure 3.15: (A) Representation of a triskelion monomer and zoom in the detail of the extremity of an arm finishing with (B) 3T ends (light blue), (C) blunt ends and (D) missing the staples of the arms extremities: “no ends”, leaving the scaffold single stranded (black). (E) Analysis of triskelia monomers by AGE and (F, G, H) TEM. Triskelion monomers capped (F) with 3T ends, (G) with blunt ends and (H) with “no ends”, corresponding to the schematic above it. Dimers in (H) are observed because of the stacking interactions. Scale bars 50 nm.

As expected, capped triskelia displayed no interactions due to the extra T bases and the monomer was clearly identified by AGE and TEM (Figure 3.15E, F and schematics B). Analysis of the blunt-ended triskelia by AGE indicated that the DNA remained in the well, suggestive of polymerised triskelia. Corresponding TEM revealed that the blunt ends at the arm extremities are capable of stacking interactions forming small aggregates of several origamis (Figure 3.15G and schematics C). However, due to the lack of specificity of the interactions between the arm interfaces, a precise orientation and alignment of two triskelia were not observed. In addition, these aggregates are too small to sufficiently coat a large vesicle (which suggests that these interactions are not strong enough to produce extensively polymerised triskelia). Interestingly, the triskelia that did not contain extremity staples (“no ends”, Figure 3.15D) displayed correctly folded arms as it migrated through the agarose gel (Figure 3.15E), and well-formed triskelia were observed on the EM grid (Figure 3.15H). These triskelia displayed minor unspecific

interactions at the extremity of the arms as observed as dimers (approximately <5%) on the EM grid (lower panel of Figure 3.15H), that involved stacking of all three arms between the two structures.

Triskelia with no ends were then used to test polymerisation procedures using sticky ends with different number of base pairs. For simplicity, the triskelion was not purified before addition of polymerisation staples when we first tested different length of sticky ends. The staples of each arm extremity were omitted from the folding mix and replaced, after folding, by new sets of strands with sticky ends of 2, 4 or 6 bp and the two patterns ($1 \leftrightarrow 1$, $2 \leftrightarrow 3$ or $\text{all} \leftrightarrow \text{all}$) described earlier (Figure 3.16A). The sequence of every sticky end is unique and carefully chosen to ensure that each sticky end will bind with only one other sticky end from another arm, which will control the alignment of each arm and their connectivity (Figure 3.5A, B). The successful hybridisation was analysed by AGE (Figure 3.16B).

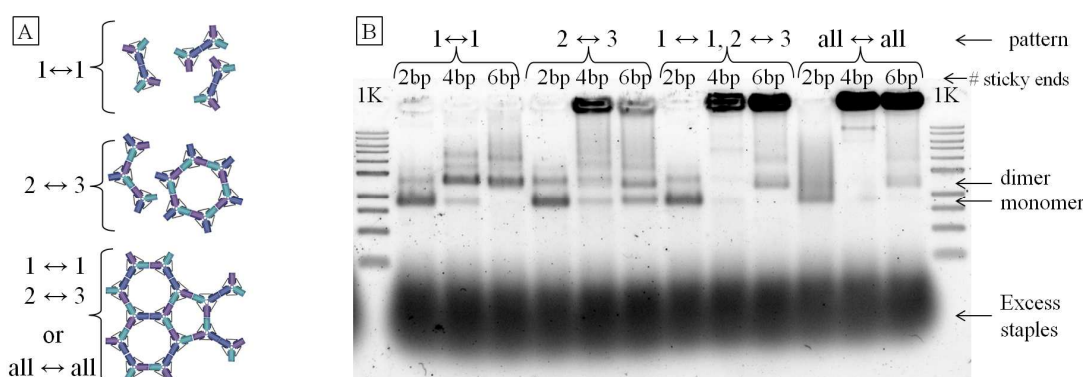


Figure 3.16: AGE of *in situ* polymerised triskelia stained with SYBR gold for comparison between the number of base pairs of sticky ends (2bp, 4bp and 6bp) and patterns ($1 \leftrightarrow 1$ only, $2 \leftrightarrow 3$ only, $1 \leftrightarrow 1, 2 \leftrightarrow 3$ or $\text{all} \leftrightarrow \text{all}$).

It is anticipated that upon polymerisation, triskelia will remain in the well of an agarose gel as the formed assemblies cannot penetrate the agarose^[233]. Such assemblies were not observed when 2bp sticky ends were used in all patterns investigated, which suggests that these sticky ends are too weak to pair origamis. Both 4 and 6 bp sticky ends formed dimers as confirmed by a clear shift of the band when only strands for the $1 \leftrightarrow 1$

connection were added (pattern $1 \leftrightarrow 1$ Figure 3.16), which can only form dimeric triskelia. In the presence of $2 \leftrightarrow 3$ staples, a mixture of dimer and larger assemblies resulted in multiple bands in the gel as well as structure large enough to stay in the well, which is consistent with the formation of chains of various lengths due to the $2 \leftrightarrow 3$ staples. Both 4 and 6 bp sticky ends indicated large aggregates when $1 \leftrightarrow 1$, $2 \leftrightarrow 3$ and $\text{all} \leftrightarrow \text{all}$ patterns were used. A band consistent with the individual triskelia was not observed when 4 and 6 bp sticky ends were used, but a band corresponding to dimeric triskelia was observed weakly with 6 bp sticky ends.

To confirm the above analysis, we prepared samples for TEM imaging (Figure 3.17). Triskelia with missing ends were folded and then purified by size-exclusion gravity column. Each sample of a specified interaction was mixed with a ten-fold excess of sticky ends staples of 2, 4 and 6 bp. The samples were then polymerised by incubation at 30°C for 8 hours.

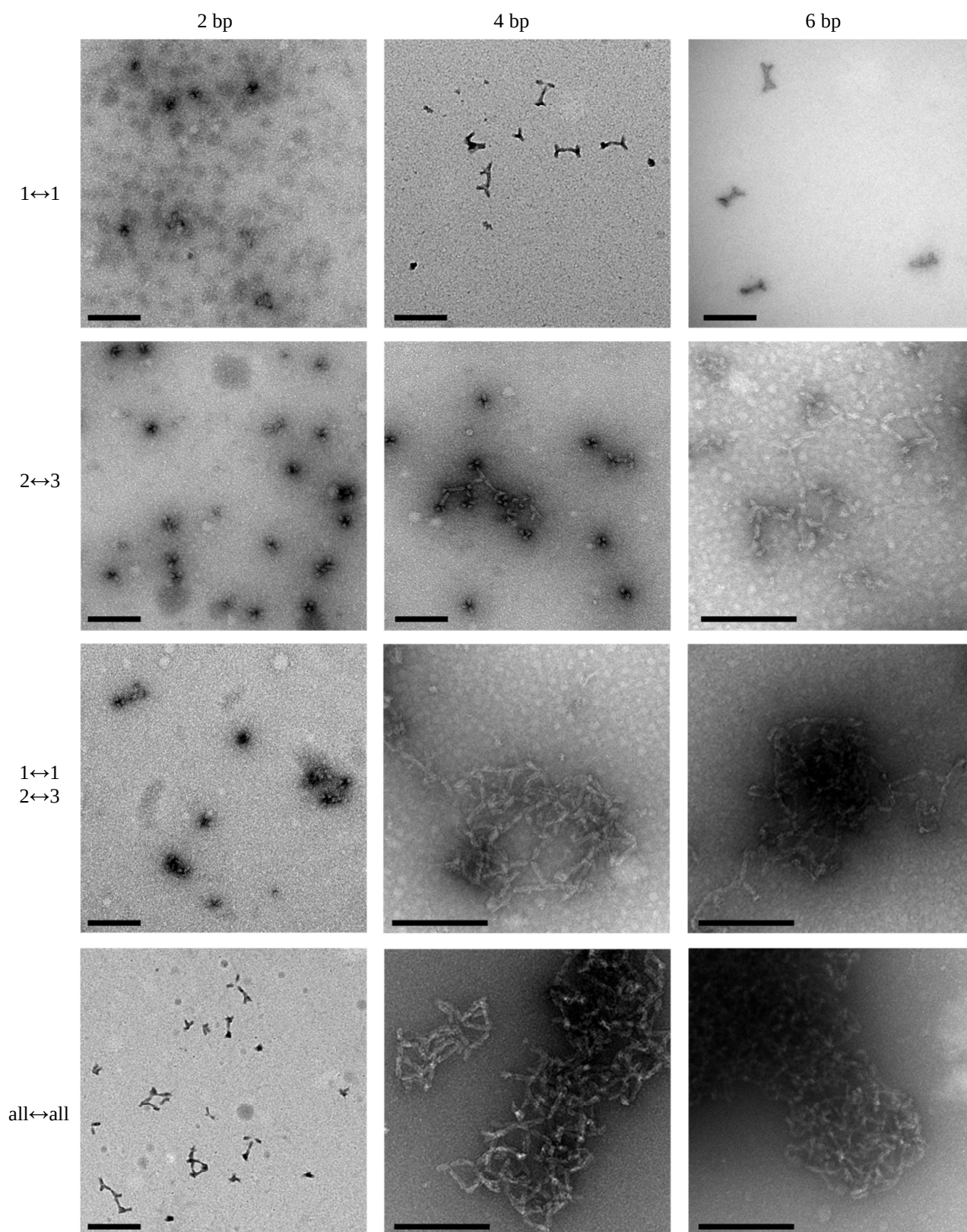


Figure 3.17: Electron micrographs of polymerised triskelia. Images in columns are made with 2, 4, or 6 bp, respectively; rows correspond to the pattern (1↔1 only, 2↔3 only, 1↔1, 2↔3 and all↔all) used as indicated on the left hand-side. Scale bars 200 nm.

Consistent with AGE analysis, samples prepared with only 2 bp sticky ends showed no efficient polymerisation, strongly indicating that the sticky ends are too weak to stabilise two arm interfaces. The successful hybridisation of triskelia with 4 bp and 6 bp sticky ends were observed by TEM, with only little difference of the resulting structures observed when the connectivity patterns $1 \leftrightarrow 1$, $2 \leftrightarrow 3$ or $\text{all} \leftrightarrow \text{all}$ were used. Although extended arrays of triskelia were observed, the 3D features of the polymers were often lost as they collapsed on the grid during sample preparation (see chapter 6 for TEM imaging discussion). The images obtained by EM and AGE analysis suggested that the polymers made using 6 bp sticky ends were strongly linked together, in contrast to the weak interactions provided by 2 bp sticky ends. However, reversible sticky ends hybridisation is required to error-correct while the triskelia polymerise, forming well-ordered, thermodynamic minima structures. Therefore, we selected the length of 4 bp for the sticky ends for our polymerisation procedure, balancing the interactions necessary for triskelia pairing and organised polymer growth.

3.2.3 Optimising the triskelion polymerisation temperature

We next investigated the effect of temperature on the polymerisation of triskelia with 4 bp sticky ends, aiming to optimise a protocol for controlled, repeatable polymerisation. Purified triskelia without extremity staples were mixed with a ten-fold excess polymerisation staples with 4bp $1 \leftrightarrow 1$, $2 \leftrightarrow 3$ and 4 bp $\text{all} \leftrightarrow \text{all}$ (Figure 3.18). The samples were allowed to polymerise using different incubation temperatures: room temperature ($20 \pm 2^\circ\text{C}$), 25°C , 30°C , 45°C for 18 hours, or by slowly cooling down the sample from 44°C to 10°C over 18 hours (anneal). The annealing ramp was chosen based on a protocol developed in parallel with Robert Schreiber for polymerisation of DNA origami nanoflowers^[241] (44°C to 20°C over 24 hours). We chose a shorter protocol with a lower final temperature to accommodate our structure with a shorter length of sticky ends (4 bp on triskelia, 6 bp on nanoflowers) that do not have a spacer between the end of the DNA

helix and the sticky end (the nanoflower has a 10 bases spacer). We also verified that the triskelion remained stable at temperatures up to 45°C by mixing T-capped triskelia with a ten-fold excess of 4 bp all $\leftrightarrow$ all staples and annealing it from 44°C to 10°C or by leaving it at room temperature for 18 hours. Capped-triskelia with 6Ts were also folded and mixed with extremity staples with sticky ends (all $\leftrightarrow$ all 4 bp as described earlier), one sample at room temperature and a second annealed, as a control experiment to see if the staples bearing the sticky ends can displace the ones with T-caps. If the triskelia remain stable, the staples with T-caps already on the origami should not be displaced by the ones with sticky ends in solution. If the triskelion starts to unfold, the excess polymerisation staples will take the place of the T-caped DNA and polymerise the origami which will then remain in the well.

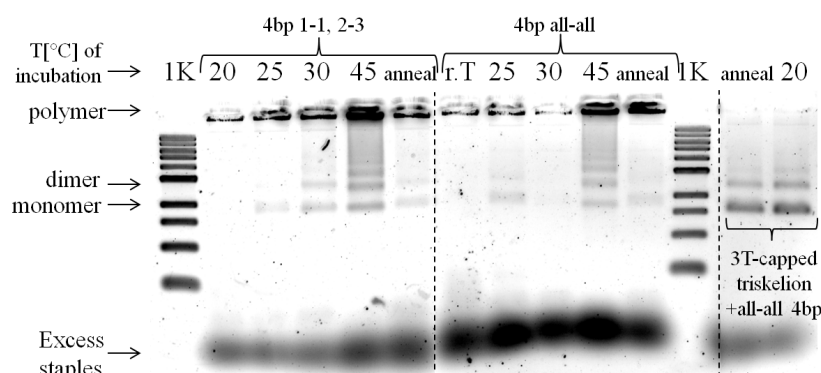


Figure 3.18: Agarose gel stained with SYBR gold. Triskelia with 4 bp 1 $\leftrightarrow$ 1, 2 $\leftrightarrow$ 3 (left) or 4 bp all $\leftrightarrow$ all (right) were incubated at room temperature (20), 25, 30, 45°C, or annealed from 45°C to 10°C (anneal) over 18 hours. A 1 kbp (1K) ladder is shown as reference. The two lanes to the right of the gel are 3T-capped triskelia annealed from 45°C to 10°C (anneal) or left at room temperature (20) for 18 hours.

AGE showed that the polymerisation occurs at room temperature and is stable for at least 18 hours at temperatures between 18°C and 45°C. The 1 $\leftrightarrow$ 1, 2 $\leftrightarrow$ 3 and all $\leftrightarrow$ all pattern and indicate that the DNA remains in the well, which corresponds to polymerised triskelia. Between 30°C and 45°C, a band corresponding to triskelion monomer appears, suggesting that the sticky ends can disassemble as the temperature increases. The signal intensity in the well decreases as temperature does; which is attributed to a loss of DNA material from the well, suggesting that the polymers are getting too big to penetrate the agarose gel. The origami did not show any signs of disassembly at temperatures used for

annealing, as both T-capped triskelion samples (incubated at room temperature and annealed from 44°C to 10°C, displayed bands corresponding to the usual monomer and secondary dimer structures.

Finally, we investigated the stability of the polymerised material when the samples were kept at 4°C over an extended period of time. The purified triskelia that were incubated at different temperatures with their polymerisation staples, as described above, were stored at 4°C for 48 hours before re-analysis by AGE (Figure 3.19).

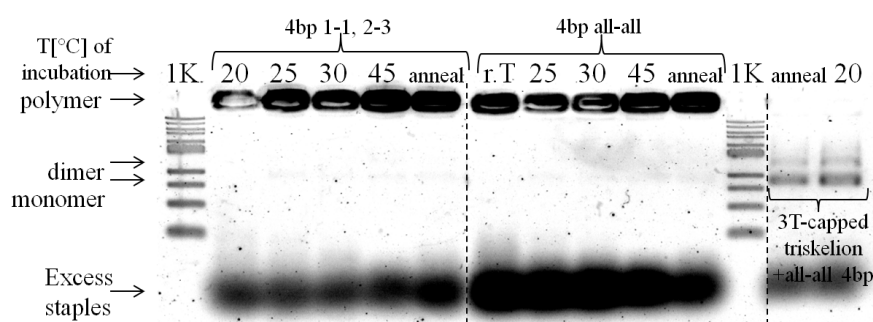


Figure 3.19: Agarose gel stained with SYBR gold. The samples that were analysed by AGE directly after polymerisation (Figure 3.18) were stored kept at 4°C 48 hours, and then re-analysed.

After storage at 4°C for 48 hours, AGE analysis indicated that the material continued to hybridise, as indicated by the darker bands of the polymerised triskelia, and reduction in intensity of the monomer and dimer bands. We further analysed these samples by TEM (Figure 3.20).

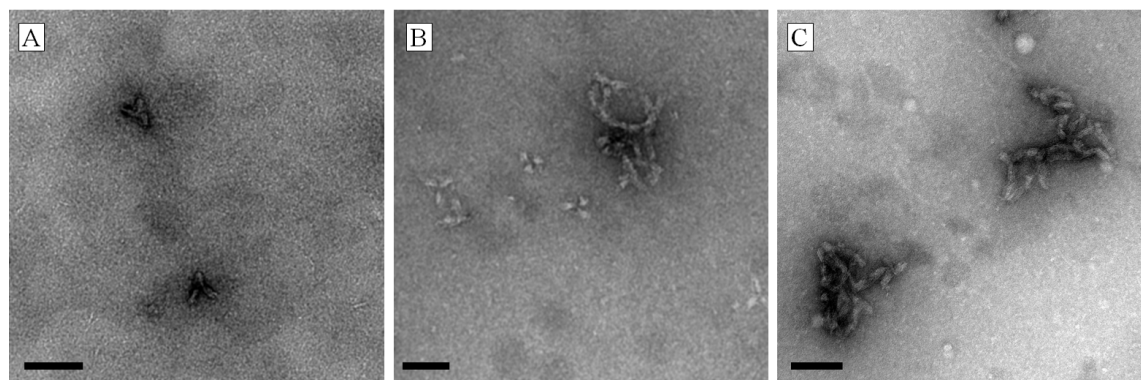


Figure 3.20: Electron micrographs of (A) 3T-capped triskelion mixed with all↔all 4bp staples and annealed from 44°C to 10°C, (B) polymerised triskelia with 4 bp all↔all incubated at 20°C for 18 hours and (C) polymerised triskelia with 4 bp all↔all incubated at 20°C and then stored at 4°C. Scale bars 100 nm.

Electron micrographs of the 3T-capped triskelion showed folded monomers without polymerisation, despite the presence of the polymerisation staples and the 44°C-10°C overnight annealing ramp (Figure 3.20A), indicating that the DNA staples with 3T-caps at the extremity of the arms remain on the origami, hence preventing polymerisation. Polymerised triskelia with polymerisation staples 4 bp all↔all for 18 hours (without incubation at 4°C) showed little clusters of polymerised triskelia and monomers in solution (Figure 3.20B). When the triskelia of the same sample were imaged after 48 hours at 4°C, TEM revealed bigger polymers, as indicated by the agarose gel (Figure 3.19). Therefore, at low temperature, hybridisation of the polymerisation staples to the arms of the triskelia can occur, but a correction of mismatched hybridisation does not, which results in disorganised clusters (Figure 3.20C). The indication that triskelia polymerise at 20°C is an important information as the project aim toward polymerisation of the triskelia and bending of a lipid membrane at temperature approaching those of a healthy cell (homeostasis temperatures), say between 20°C and 37°C (physiological temperature). Although polymerisation of the triskelia was observed at temperatures between 4 and 45°C, the arrays were irregular, and we sought to further improve the polymerisation procedure.

3.2.4 A new polymerisation strategy

To improve the regularity of the polymers, a new approach was considered that relies on triskelia dimers as “seeds” for polymerisation. It was discussed previously that for a 2D array to fold into a spherical shape, the repeating units must produce both pentagonal and hexagonal rings. Although our initial triskelia design allowed for this, we can also consider a dimer, attached via 1↔1 hybridisation that can self-assemble into regular, repeating 5- and 6-membered rings (Figure 3.21).

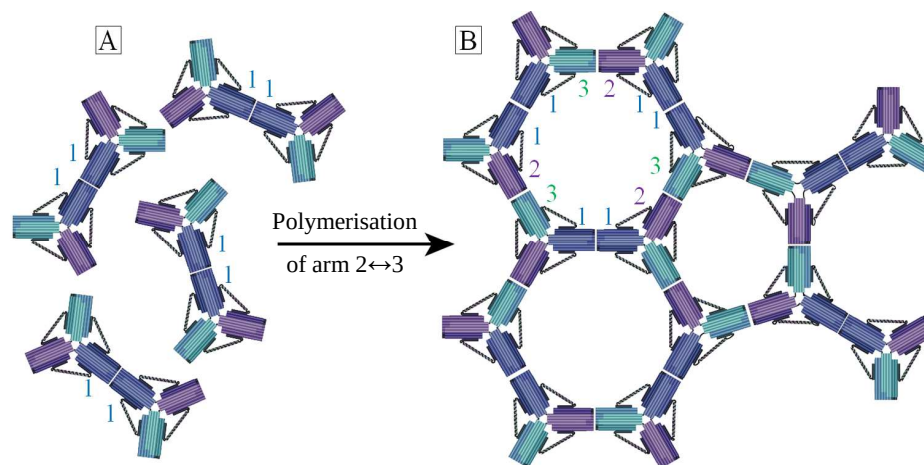


Figure 3.21: Illustration of polymerisation arising from pre-formed triskelia dimers. (A) Dimers ($1 \leftrightarrow 1$) and (B) polymerised dimers linked upon addition of the staples for the remaining arms ($2 \leftrightarrow 3$).

A dimer will provide the building block to form pentagons and hexagons, leaving the only pairing interaction necessary for array formation to $2 \leftrightarrow 3$. This strategy thus provides more direction to the formation of regular arrays.

To guarantee the stable attachment of two triskelia into a dimer, we redesigned the sticky ends as explained below. The extremity staples of arm 1 (designed to hybridize with the same arm 1 of a second origami, $1 \leftrightarrow 1$) were exchanged for a new set of oligos that we called “invading strands”. These invading strands hybridize with two origamis: 20 nucleotides in arm 1 on an initial origami are followed by several bases (between 4 and 8 bases), hybridising with the arm 1 of a second triskelion (Figure 3.22). When discussing the polymerisation staples, we highlighted how the staples at the extremities of two triskelia have to face ssDNA strands of opposite direction (3'-end faces 3'-end, and 5'-end faces 5'-end, Figure 3.22C) to allow hybridisation of the sticky ends. The situation is different for the design of our invading strands as they require, on the contrary, that a 3'-end faces a 5'-end from another arm. The new arrangement of the arm extremities is therefore shifted by one more DNA helix compared with the sticky ends arrangement as illustrated in Figure 3.22B and C.

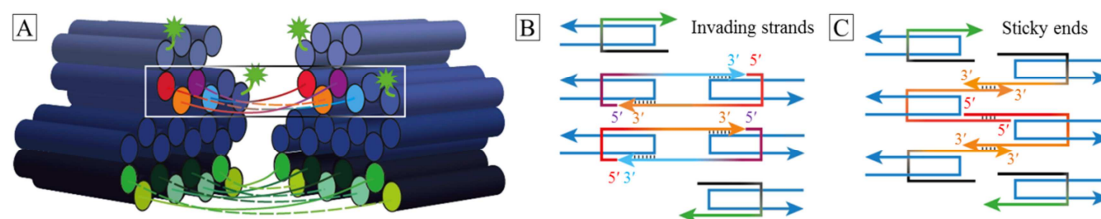


Figure 3.22: Illustration of the interface between two arms of two triskelion showing (A) 3D representation and localisation of invading strands. Colour code indicates the same helix extremity on both arms. (B) Schematics of the invading strand interface corresponding to the rectangle in (A). 5'-ends are represented by arrows. The scaffold is shown in dark blue. Both green 5'-ends correspond to the beginning of a dye handle. (C) Sticky ends interface for comparison.

Due to the honeycomb organisation of the arm extremities, this new connectivity creates an off-set in the heights of the helices by half a DNA helix, approximately 1 nm. Thus, as an invading strand protrudes from a first arm to attach the second arm, the end of the DNA strand must extend the height of half a DNA helix to hybridise. To accommodate this offset, invading strands with a short spacer between the two interfaces were investigated. This spacer is made of 3T and was inserted between the 20 nt-long domain on arm 1 and the 4 to 8 invading bases (Figure 3.23)

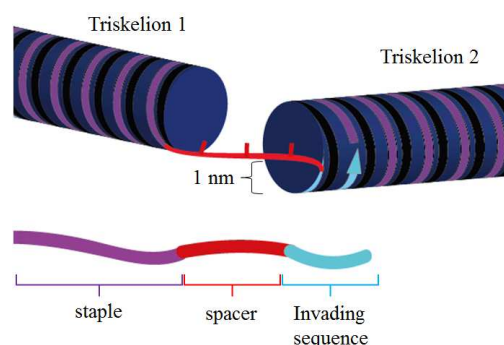


Figure 3.23: illustration of the interface between two helices of two triskelia showing the 3T spacer between the two staples domains that hybridise with the scaffolds of the two triskelia.

Dimers assembled using these new invading strands are anticipated to be rigid and irreversible in comparison to dimers formed with sticky ends, as the invading strands that connect the two triskelia insert into the arm (rather than being an extension of it), and also pair more bases between the two origamis (4-6 bp vs 8-16 bp). Invading sequences of 4, 6 and 8 bases were investigated and the new sets of staples, either with or without a 3T spacer, were added to the one-pot folding (Figure 3.24).

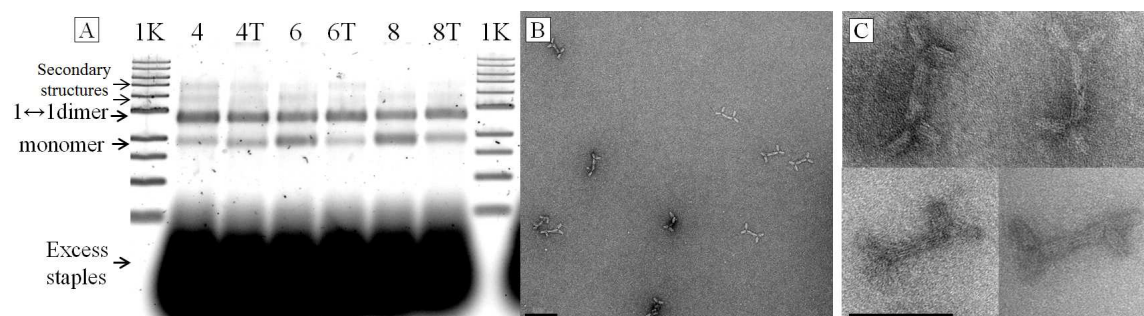


Figure 3.24: Analysis of dimers by (A) AGE and (B, C) TEM. (A) The number above the wells indicates the number of bases that invade into the arm of the second triskelion, and a T signifies that a spacer of three T is added at the interface. Electron micrographs (B) large view and (C) detail of dimers obtained during this folding. Scale bars 100 nm.

Analysis of the gel from AGE indicated that dimers were formed with all of the different lengths of spacer under investigation (Figure 3.24 A), and dimers were observed in TEM micrographs (Figure 3.24B, C). Analysis of the gel using ImageJ determined that the best yield dimer (70%) was obtained with an invading sequence length of 6 bases and the 3T spacer (column 6T Table 3.2)

Table 3.2: Yield [%] of dimer, monomer and secondary structures with six sets of invading strands.

Set of inv. str:	4	4T	6	6T	8	8T
Dimer [%]	66.9	64.6	46.4	70.3	41.2	58.9
Monomer [%]	23.6	28.2	48.9	25.2	56.8	37.5
2 nd struct. [%]	9.5	7.2	4.7	5.9	2.0	3.7

We compared the yield of dimer production using the 1↔1 6 bp sticky ends (6 bp) staples and the new invading strands with 6 nt and 3T spacer (6T). Dimers were made by adding the dimerisation staples in the mixture either prior to or after folding of the triskelion.

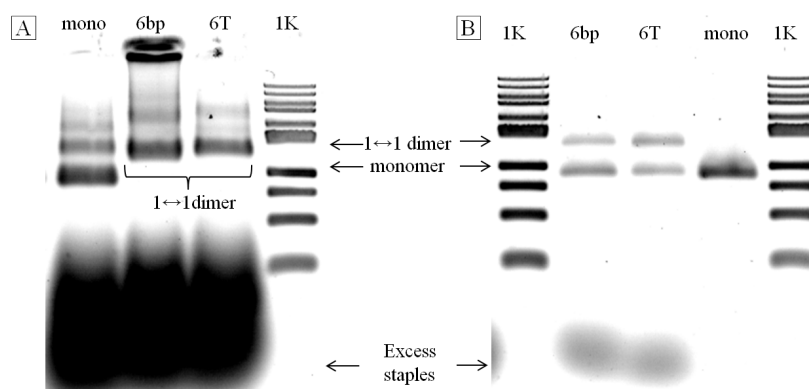


Figure 3.25: AGE analysis of dimer production using 6 bp sticky ends (indicated as 6bp) or 6 nt invading strands with 3T spacer (indicated as 6T). A monomer is used as reference (mono) in addition to the usual 1kbp ladder (1K). (A) Staples 6 bp and 6T added prior to folding, and (B) staples 6 bp and 6T added after folding and purification of the triskelion.

When added to the folding mixture, 6 bp sticky ends produced dimers, but aggregates were also assembled, as indicated by a band that remained in the well (Figure 3.25A) and suggesting uncontrolled assembly. When invading strand 6T were added to the folding mixture, only dimers were observed. When the triskelion monomers were folded and purified prior to the addition of dimerisation staples 6 bp and 6T and incubation at 30°C for 2 hours, it produced dimers with 40% and 54% yields, respectively. (Figure 3.25B). The staples for dimerization were added in ten-fold excess in both cases and can be detected at the end of the gels. The production of dimer using 6T invading strand was therefore the most efficient method to produce triskelia dimers and the one selected for further investigation of triskelia polymerisation.

The stability of the dimers assembled with the invading strand 6T (and 6 bp sticky ends for comparison) was also investigated. We therefore extracted the dimers from the agarose gel to eliminate the 1↔1 excess staples and remaining monomers (Figure 3.25B). The purified dimers were then applied to temperatures between 20°C and 45°C for 18 hours and analysed by AGE and TEM (Figure 3.26).

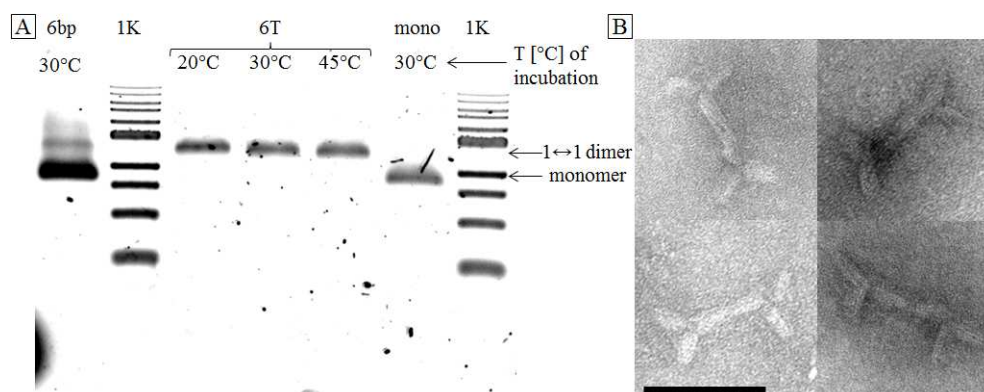


Figure 3.26: (A) AGE of purified 6T dimers incubated at 20, 30 and 45°C for 18 hours. For comparison, 6 bp dimers and triskelia monomer incubated at 30°C are also investigated. (B) Electron micrographs of dimer incubated at 45°C for 18 hours. Scale bare 100 nm.

Purified dimers assembled with the invading strands 6T were stable at temperatures from 20 to 45°C, as indicated by a single band in the gel (Figure 3.26A). When left at 30°C. The dimer assembled with 6 bp sticky ends indicated unpairing when incubated at 30°C, and observed as a monomer band. We therefore continued our investigation with dimers prepared with invading strands with 6 bases and a 3T spacer (6T).

Following the success of the formation of the triskelia dimer in a one-pot reaction with invading strands, we investigated if the two remaining arms (namely arm 2 and arm 3) would assemble into chains following the new procedure. Invading strands with 6 bases (marked as 6) and with a 3T spacer (marked as 6T) spacers were designed for these two arms and added to the folding mixture of triskelion monomers. Analysis of the agarose gel indicated that the triskelia form aggregates that remain in the well, as well as smaller assemblies such as dimer and trimers (Figure 3.27).

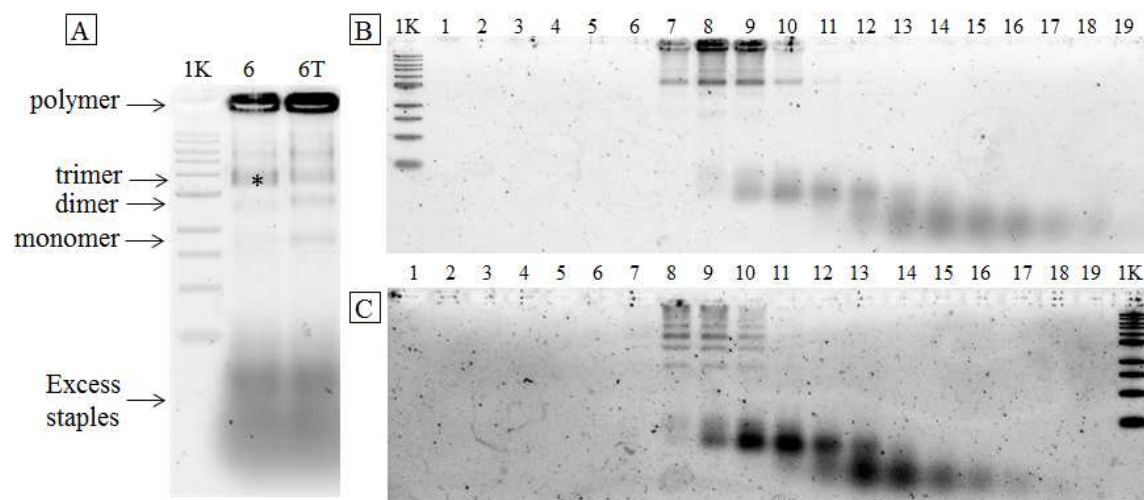


Figure 3.27 AGE of chain polymerisation of the arms 2↔3. (A) Unpurified sample after folding with 6 bases invading the opposite arm (indicated as 6), and 6 invading bases with a 3T spacer (6T). The asterisk indicates that the line was extracted and imaged by TEM (Figure 3.28). (B) Fractions from the size-exclusion column of the sample with 6 invading bases, and (C) 6 invading bases with 3T spacer.

Analysis by TEM of the product of polymerisation, eluted from a size-exclusion column to remove excess staples, revealed that if the 2↔3 invading strands are added during the folding, the main products are closed circles comprising three triskelia and long chains of triskelia (Figure 3.28). Such closed arrangement is unsuited for further polymerisation and is unlikely to reorganise when exposed to heat, since we already observed that triskelia dimers were stable up to 45°C (Figure 3.26). Extraction and imaging of the trimer band (marked as * in Figure 3.27) confirmed that the triskelia of this band are arranged in a closed triangle (Figure 3.28B, C).

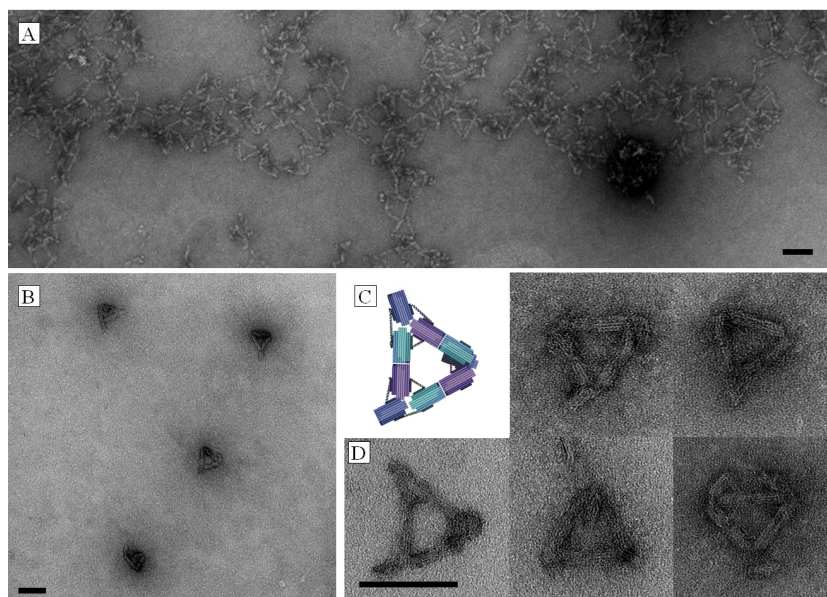


Figure 3.28: (A) Electron micrograph of chain polymerisation using the set of invading strand (6T) from fraction 8 of the size-exclusion column of Figure 3.27B. (B) Trimer observed from the band extracted from agarose gel labelled with an asterisk on Figure 3.27A: extended and (C) model of a triskelion trimer and (D) magnified view of the triangles. Scale bars 100 nm.

To avoid these smaller, triskelia trimers during polymerisation, we anticipated adding the $2 \leftrightarrow 3$ staples post-folding of the triskelia dimers.

3.2.5 Study of the polymerisation kinetics of triskelia

Before optimising the protocol for polymerising the dimeric triskelia with invading strands, we sought to investigate the kinetics of polymerisation of the invading strands. It is expected that polymerisation of the triskelia using invading staples will take longer than the sticky ends, as the invading strands have to insert into the DNA origami in order to access the complementary domain. Furthermore, the loops of unpaired bases at the extremities of the arms may form short matching domains and stacking interactions that the polymerising DNA strands may have to compete with. We investigated the kinetics of hybridisation (of all the arms) by comparing the rate of polymerisation of the invading strands with the equivalent pattern of 4 bp sticky ends ($1 \leftrightarrow 1$, $2 \leftrightarrow 3$). Starting from purified flat triskelia monomers (with no cholesterol and no dye), we added the two sets of staples (6T, and 4 bp) in ten-fold excess. Small aliquots were taken from the

polymerising samples over the course of the experiments (12 hours), and frozen before analysing by AGE (Figure 3.29) and TEM (Figure 3.30). We also polymerised the triskelion following the annealing ramp discussed previously (44-10°C) for comparison to the room temperature experiments.

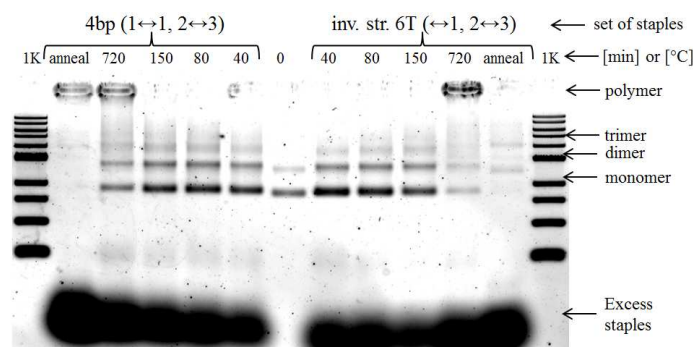


Figure 3.29: AGE of polymerised triskelia using the set of staples 4 bp sticky ends ($1 \leftrightarrow 1$, $2 \leftrightarrow 3$) or 6 bases invading strands (inv. str. 6T) with spacer. Evolution over time of the polymerisation made at $20 \pm 2^\circ\text{C}$ (room temperature) after 40, 80, 150 and 720 min (12 hours); and annealed from 44°C to 10°C (anneal) over 16 hours. The well indicated by 0 indicates purified triskelion monomer without any staples run as a control.

The agarose gel presented in Figure 3.29 suggests that for both type of polymerisation strands, triskelion polymerisation was only observed after an extended (12hrs) incubation time at room temperature, as observed by the material that remains in the well, indicating of large assemblies. After the 12 hours incubation period, a darker band from the well of the triskelia polymerised with 6T invading strands was observed in comparison to the 4 bp sticky ends, and fewer bands corresponding to monomers or dimers in the gel, suggesting that the polymerisation is more efficient with invading strands. The addition of polymerisation staples to either sample triggers the formation of dimers which can be observed after 40 minutes. Both samples showed efficient polymerisation upon the 44 - 10°C annealing ramp, as no dimers or monomers are observed. The absence of signal coming from the well 44 - 10 for the invading strands is attributed to a loss of the DNA present in the well during staining, as the polymerised triskelion were observed in TEM (Figure 3.30).

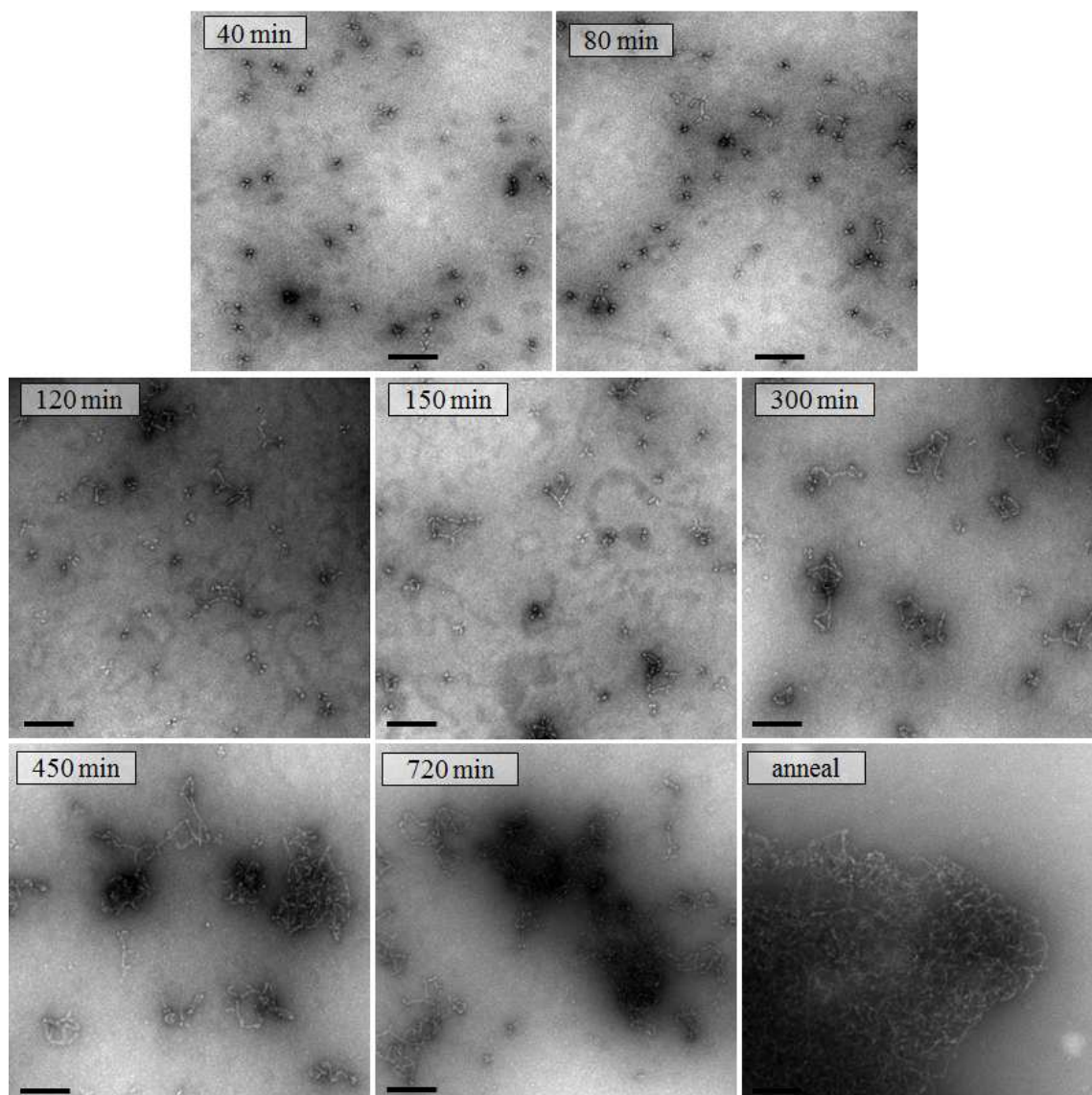


Figure 3.30: Electron micrographs showing polymerisation using 6T invading strand ($1 \leftrightarrow 1$, $2 \leftrightarrow 3$) from monomers. The main series of images illustrate the evolution over 12 hours (720 min) at room temperature ($20 \pm 2^\circ\text{C}$), and the last image (anneal) illustrates the final result after an annealing ramp from 44°C o 10°C over 16 hours. Scale bars 200 nm.

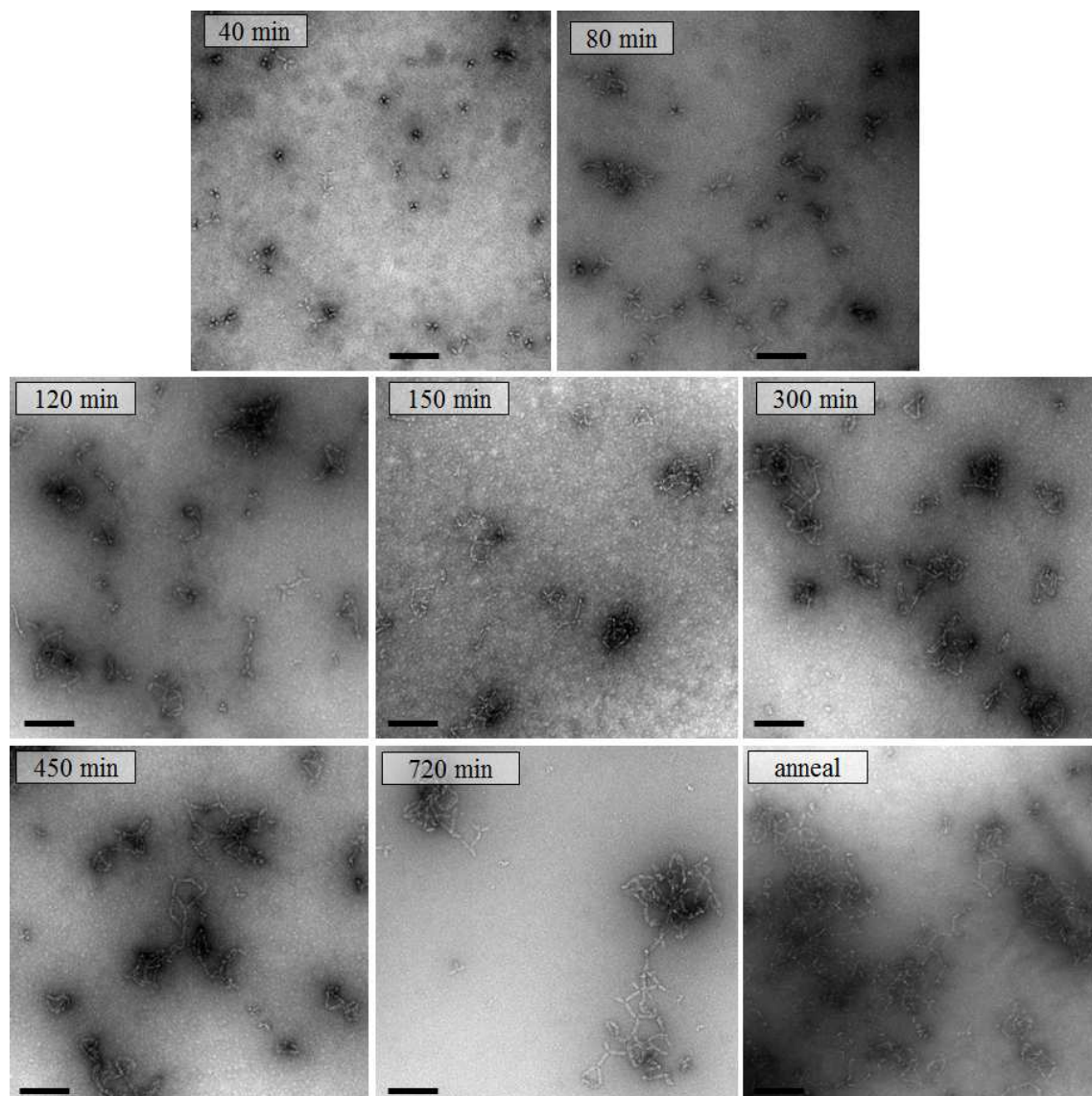


Figure 3.31: Electron micrographs showing polymerisation using 4 bp sticky ends ($1 \leftrightarrow 1$, $2 \leftrightarrow 3$) from monomers. The main series of images illustrate the evolution over time (12 hours) at room temperature ($20 \pm 2^\circ\text{C}$), and the last image (anneal) illustrates the final result after an annealing ramp from 44°C to 10°C over 16 hours. Scale bars 200 nm.

We then compared the TEM images of the polymerisation using 6T invading strands (Figure 3.30) and polymers made with 4 bp sticky ends (Figure 3.31) (both samples use the pattern $1 \leftrightarrow 1$, $2 \leftrightarrow 3$). The time lapse TEM micrographs indicate that the polymerisation using the invading strands takes longer to happen in comparison to the polymerisation with 4 bp sticky ends, but forms larger assemblies over 12 hours. When annealed from 44°C to 10°C , both samples produced large aggregates. The triskelia polymerised with the 6T invading strands formed large aggregates that appeared to

better maintain their 3D features in the TEM grids, as evidenced by the accumulation of stain that appeared darker on the micrographs. We therefore decided to continue to work with the strength of a dimer made using 6T invading staples, coupled with the faster polymerisation provided by the 4 bp sticky ends for the arms 2↔3.

The clathrin protein deforms lipid bilayers by polymerising at a rate of seconds^[160]. In an attempt to reduce the time required for polymerisation of our synthetic triskelion, we desired to have a time scale as short as possible. A short time frame of the experiments also minimises the effect of lipid oxidation^[242] and limits the decrease of vesicle quality as they undergo physical changes such as bursting, fusion, tubulation and lipid oxidation^[243]. We showed earlier that flat dimers (with no cholesterol and no dye) form more regular assemblies than monomers, that 6T invading staples form stronger interactions than 4 bp sticky ends staples, and that 4 bp sticky ends polymerise triskelia faster than 6T invading strands. By amalgamating these results, we decided to polymerise the triskelia starting from a rigid and stable dimer with 6T invading strands (1↔1) with 4 bp sticky ends to polymerise 2↔3. To examine this, we compared the polymerisation of dimers formed with 6T 1↔1 invading strands using either invading strands 2↔3 (Figure 3.32A), or 4 bp sticky ends 2↔3 (Figure 3.32B).

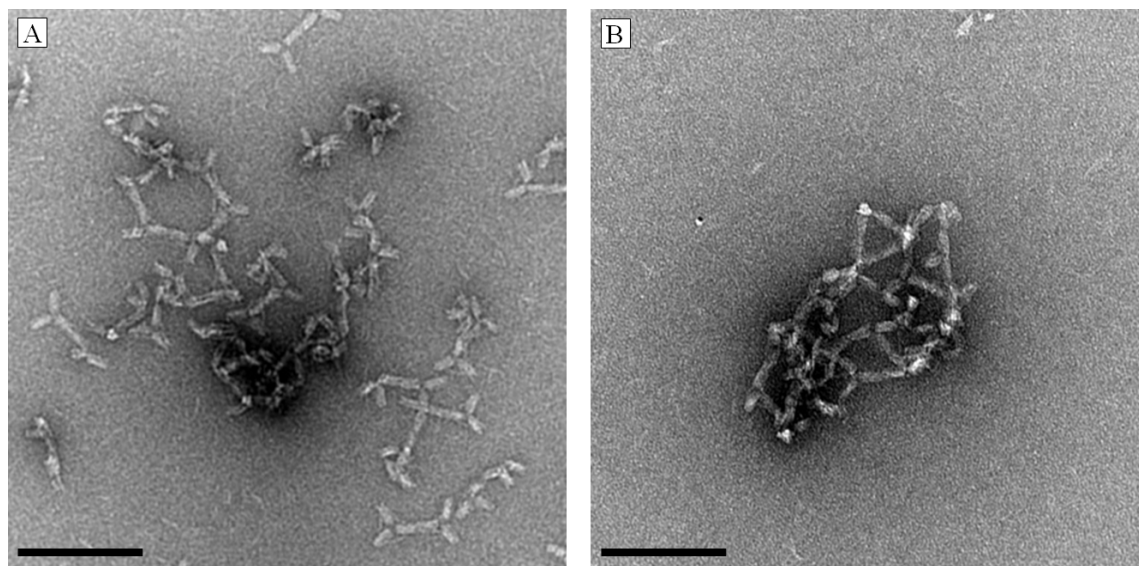


Figure 3.32: Electron micrographs of polymerised dimers using (A) $2 \leftrightarrow 3$ 6T invading strands, and (B) $2 \leftrightarrow 3$ 4 bp sticky ends. scale bars 200 nm.

Polymers made using the 4 bp sticky ends appeared to consume the entirety of the 6T dimer in solution within an incubation time of 4 hours. These results encouraged us to use 6T dimers with 4 bp sticky ends for polymerisation in later experiments involving giant unilamellar vesicles (GUVs).

3.2.6 Variation of the bridge length of a triskelion monomer

We also investigated how modifying the bridge length between the arms would affect the polymerised product, applying our optimised procedure. The length of the bridges on the triskelion investigated this far has 90 bp and was designed to hold the three arms in a flat configuration ($180 \pm 10^\circ$). To be able to force the membrane to bend, we also designed shorter bridges (Figure 3.2 and 3.3) as described at the beginning of this chapter. We then next investigated the effect of shorter bridge on the opening of the arms. As mentioned in section 3.1.1, bridge lengths of 30, 60 bp were also designed for the triskelion, with the expectation that the dihedral angle would reach $102 \pm 2^\circ$ for a 30 bp bridge and $118 \pm 4^\circ$ with a 60 bp bridge. The staples for these alternative bridges were added to the folding mixtures and analysed by AGE and TEM (Figure 3.33).

3.2 Synthesis of DNA origami

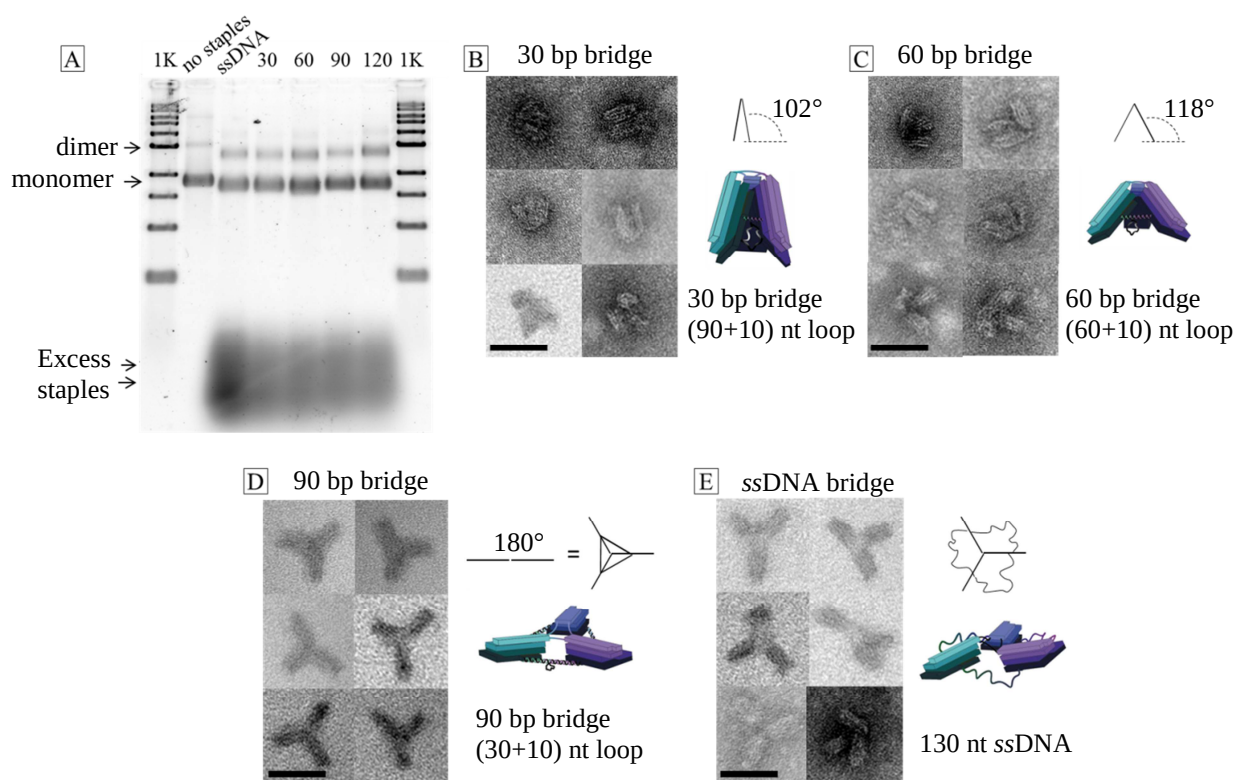


Figure 3.33: Analysis of triskelia with different bridge length. (A) AGE run for 5 hours to reveal differences in the triskelia migration, “no staples” indicate that the scaffold was annealed in the absence of staples. ssDNA refers to a triskelion with a ssDNA bridge, and the numbers (30, 60, and 90) refer to the number of bp that form a double helix on the bridge. Electron micrographs and schemes of the corresponding triskelion: (B) 30 bp bridge for an expected dihedral angle of 102°, (C) 60 bp bridge, expected dihedral angle of 118°, (D) 90 bp bridge, dihedral angle of 180° (flat, conformation for reference, and (E) triskelion with ssDNA-only bridge. Scale bars 50 nm.

The shortened bridges of 30 and 60 bp indeed produced structures with their arms forced into a closer configuration than the flat triskelia formed with a 90 bp bridge, as observed in TEM micrographs (Figure 3.33B, C respectively). The agarose gel displays a band slightly faster when the triskelion is folded with the 60 bp bridge, which can be attributed to a correctly folded structure that is more compact in comparison to the other versions. Surprisingly (and despite a geometry even closer than triskelia with 60 bp bridge), triskelia with 30 bp bridge did not indicate a faster migration. This difference in migration is however extremely small. The last triskelia folded with a single stranded bridge leaves the scaffold part of the bridge single stranded and flexible^[244, 245] (Figure 3.33E). Electron micrographs of this ssDNA bridged-triskelion indicated that the

structure can be folded without the complementary strands for the formation of the structure and appeared to preferentially deposit on the EM grid in an open configuration.

Despite the presence of 30, 60 or 90 bp bridges between the triskelion arms, irregular arm positions were observed on the EM grid (see Annex 4.14). A bridge composed of one DNA double helix may therefore lack rigidity and strength to maintain the arms at their expected 120° angles due to the presence of weak points on the DNA double helix that are the beginning and end of a staples, or the location of the scaffold loop at the centre of the bridge (Figure 3.2). In these positions, it is theorised that the DNA helix can bend where a staple stops, as they endure a lot of strains from the arms on both sides. It was therefore questioned whether a single DNA double helix can resist the tensegrity strain of the structure. Indeed, the bridge must endure both the fluctuation of the positions of the arms in solution, and maintain a long reservoir of unpaired nucleotides.

We theorised that a stiffer bridge would impose a more rigid constraint of the angle between the arms, which would also provide strength in future membrane bending investigations. Consequently, bridges of length of 60 and 90 bp were designed that contained three DNA double helices. Bridges of 30 bp was not investigated as we did not expect them to be used in experiments involving the deformation of a lipid bilayer due to their extremely tight configuration. Indeed, the angle previously obtained with the shorter bridge of 30 bp (Figure 3.33B) seemed to close the triskelion in a configuration believed to be unfavourable for a membrane interaction, as the cholesterol are hidden inside the structure. The scaffold still contains a loop whose length can be varied, but the hybridized part of the scaffold is now coupled with two extra helices formed by five more staples. The sequences for the new strands were designed using Nupack^[200] to prevent parasite hybridisation within the new staples. The staples for these bridges were added to the folding mixtures and analysed by TEM (Figure 3.34).

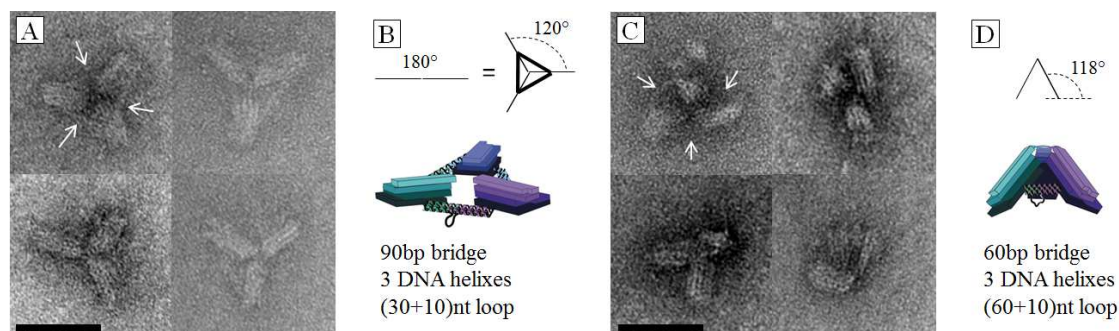


Figure 3.34: (A) Electron micrographs and (B) schematic depiction of triskelia with stiff 90 bp-long bridges. (C) Electron micrographs and (D) schematic depiction of triskelia with stiff 60 bp-long bridges. The stiff bridges are visible on the micrographs (white arrows on top left pane). Scale bars 50 nm.

The thickness of the bridges allowed their visualisation on EM micrographs, as indicated by white arrows in Figure 3.34A and C. The two bridges improved the reproducibility of the angle between the arms; >95% of the stiff 90 bp bridge maintained a 120° angle between the arms (Figure 3.34A, B) and the stiff 60 bp bridge brought the arms closer while maintaining a constant angle (Figure 3.34C, D). Analysis of the triskelia structure with stiff 90 bp bridges in TEM images determined that, out of 400 counts, 95% (382/400) of the triskelia folded in the expected configuration with their arms spaced by $120 \pm 5^\circ$, while only 60% (243/400) maintained this angle when folded with the single bridge and imaged on TEM. (As this change of bridge occurred during the development of the polymerisation procedure, TEM micrographs of Figure 3.32 present triskelia with stiff 90 bp bridges.)

Triskelia folded with a 60 bp bridge were observed to orientate on the EM grid with their arms extremities pointing out of plane, thus preventing clear identification of the angle between the arms and preventing the analysis of the angle distribution. However, the upward position of the arms allowed us to image the end of the arms of the triskelia revealing the honeycomb arrangement of the helices, precisely as we had designed (Figure 3.35).

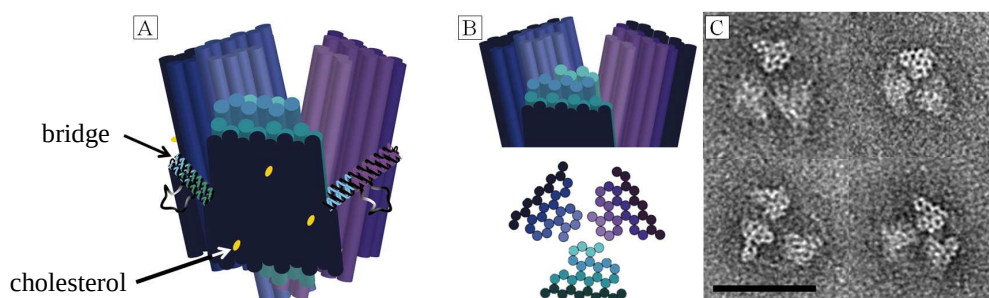


Figure 3.35: (A) Illustrations of the triskelion with arms raised out-of plane by a stiff 60 bp bridge. (B) Electron micrographs of triskelion with stiff 60 bp long bridges showing the same honeycomb lattice as the model. Scale bar 50 nm.

Surprisingly, the orientation of the honeycomb indicated a triskelion closed in the opposite direction than the one expected. This implies that the initial positions designed to attach cholesterol anchors now face the outside of the bent triskelion (Figure 3.36A) and not the inner space between the arms of the triskelion as initially planned (Figure 3.36B).

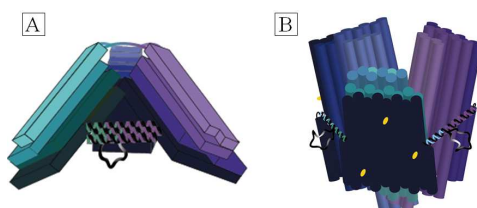


Figure 3.36: Schematics of triskelion with cholesterol anchors bent with stiff 60 bp bridges: (A) initial planned conformation with the cholesterol located towards the inner space of the structure and (B) conformation as observed by TEM, which will expose the cholesterol anchors (yellow) towards the outer space.

This unexpected conformation of the bent triskelia signifies that the membrane pinching we ought to observe would be of opposite direction than the one we initially expected. This alternative conformation is likely to have happened with bridges of a single DNA helix (Figure 3.33C) and could be the reason for the faster migration in the agarose gel (Figure 3.33A) of the triskelion with 60 bp bridge in comparison with the other bridge lengths. By bending the other way around, the bridge is then localised at the outer side of the structure and makes the triskelion more densely packed than expected (Figure 3.36). However, this conformation still fulfils our aim of controllably bending a lipid membrane, and so the project was pursued.

3.2.7 Improved triskelia polymerisation strategy observed by TEM

In view of the improved control over the dihedral angle of the triskelion using the bridge with three double helices, we applied this improved structure and polymerisation strategy (triskelia dimers with 6T invading strands $1 \leftrightarrow 1$, and 4 bp sticky end for the connection $2 \leftrightarrow 3$) for the production of polymers (Figure 3.37).

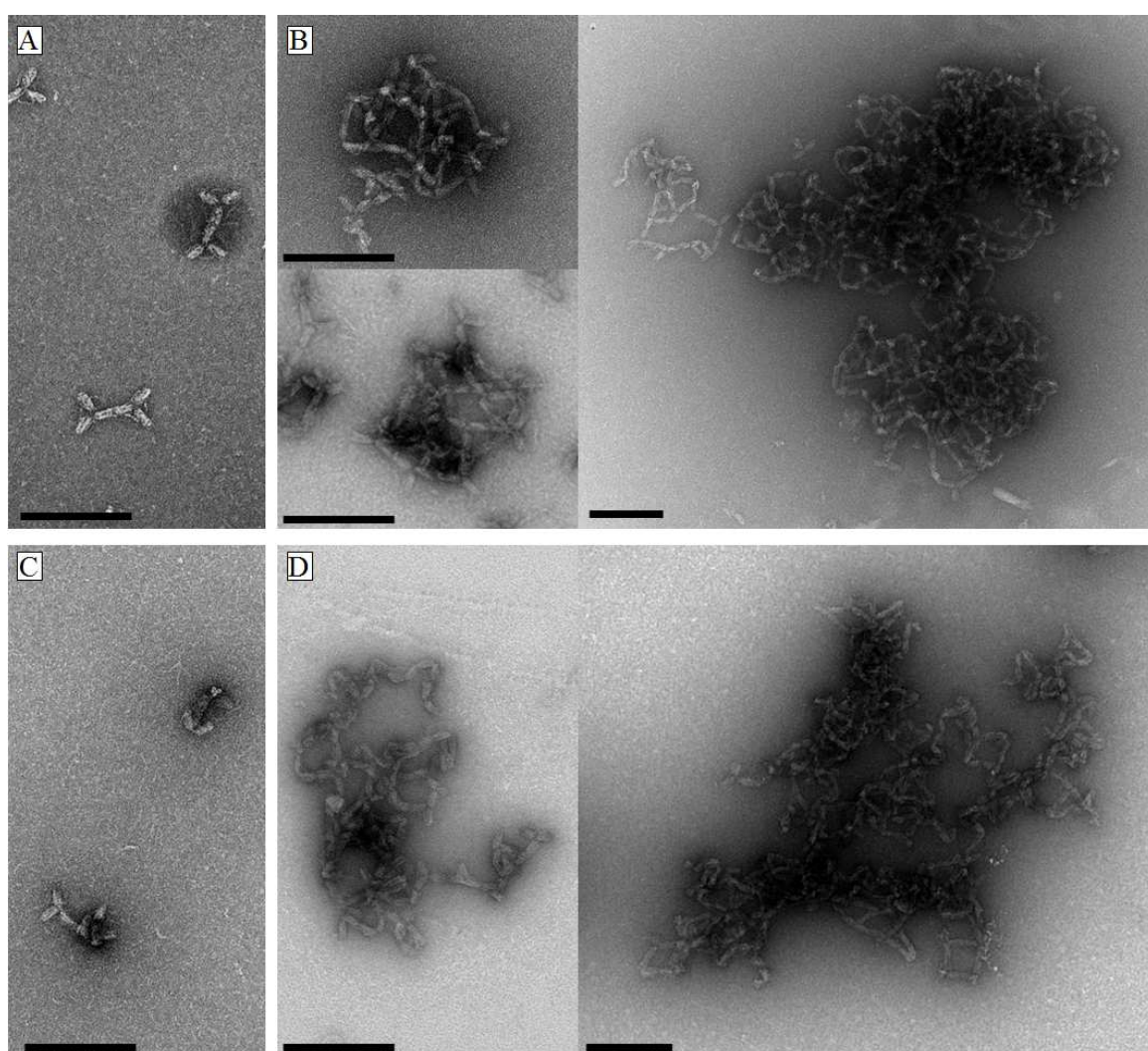


Figure 3.37: Electron micrographs of (A) triskelia dimers (6T invading strands) with stiff 90 bp bridge, and (B) polymerised with 4 bp $2 \leftrightarrow 3$ sticky ends. (C) Triskelia dimers (6T) with stiff 60 bp bridges and (D) polymerised with 4 bp $2 \leftrightarrow 3$ sticky ends. Scale bars 200 nm.

Triskelia dimerised with 6T invading strands and with stiff 90 bp bridges (Figure 3.37A) and then polymerised with 4 bp $2 \leftrightarrow 3$ sticky ends (Figure 3.37B) showed large polymers

that overall presented better features and opening than the polymers obtained before optimisation of the bridges and polymerisation procedure. The equivalent triskelia dimers with stiff 60 bp bridges were observed on TEM with the arms closer to each other as expected (Figure 3.37C). These curved triskelia formed polymers observed with a tendency to form 3D superstructures with sharper edges than the ones with stiff 90 bp bridges (Figure 3.37D) due to the forced closed conformation of the arms by the stiff 60 bp bridges.

In addition to the polymerised material the triskelia produced with 6T invading strands and stiff 90 bp bridges, smaller regular polyhedra were also imaged, which were not observed with 4 bp sticky ends (Figure 3.38).

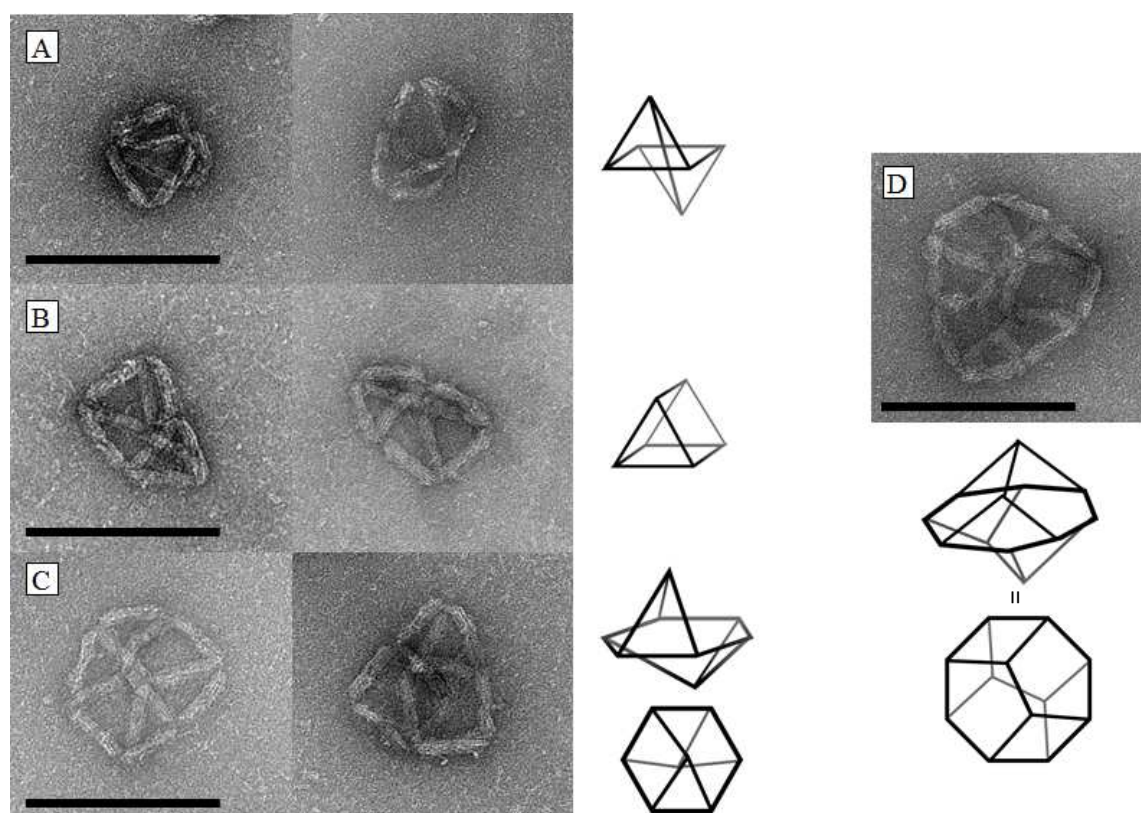


Figure 3.38: Electron micrographs and schematic representation of small closed polyhedral triskelion assemblies made with 6T invading strands. (A) Deformed prism, (B) triangular prism, (C) hexagonal axis 3D polygons and (D) octagonal 3D polygon. Scale bars 200 nm.

These regular 3D shapes satisfy all of the connections of the arm extremities. Although the formation of these polyhedral structures indicated that our synthetically designed triskelia monomer can indeed form 3D cages, reminiscent of the pinched bilayer vesicle coated by clathrin, their sporadic and uncontrolled formation prevented us from investigating their assembly further. Large, planar arrays of flat (stiff 90 bp bridges) were not observed and we believe that our system required a surface templation in order to assemble into a planar array, due to the remaining flexibility of the arms. A lipid bilayer would offer a surface for the triskelia with cholesterol anchors to insert into and would thus provide a planar, spherical template for the assembly of triskelia array. We therefore investigated the effect of polymerised dimers on lipid bilayer (see Chapter 4).

3.3 GOLD NANOPARTICLE FUNCTIONALISATION OF THE TRISKELION

In addition to the purification procedure developed to isolate the triskelia that involved a size-exclusion column, we also developed a procedure that relied upon gold nanoparticle (AuNP) functionalisation of the triskelion (Figure 3.39). Functionalising the triskelion with an AuNP enables it to be purified by AGE and then concentrated by centrifugation, a procedure that is not possible with the unmodified triskelion. The high contrast of the NP-functionalised origami also enables identification on a TEM grid, which allowed determination of the yield and quality of the polymerised structures.

Attachment of a AuNP to the triskelion was made following a literature procedure^[241,246]. Phosphine ligands on AuNPs can be exchange with thiolated ssDNA in the presence of salt (NaCl). The sequence of this thiol-ssDNA can then hybridize with a complementary sequence incorporated in an origami. We thus used poly T (10 thymine bases) ssDNA sequences attached on a AuNP to hybridise with poly A (six adenines bases) functionalised at the centre of the triskelion (Figure 3.39)

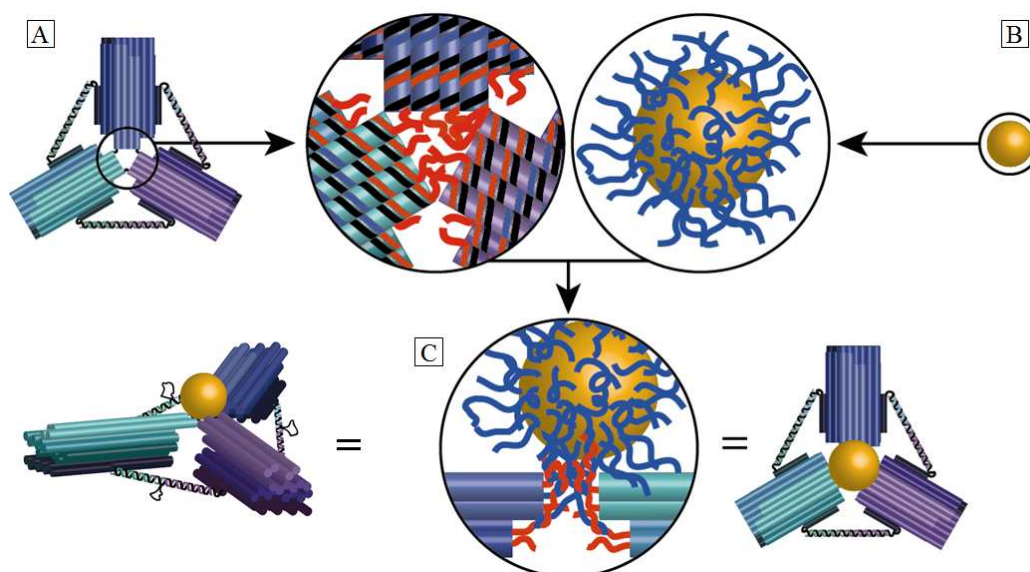


Figure 3.39: schematics for AuNP functionalisation of triskelion. (A) triskelion decorated with 6A in the centre (red staples), (B) Gold nanoparticle (AuNP) functionalised with poly T (10T, in blue) on its surface, and (C) AuNP fixed on the triskelion by the hybridisation of the red and blue ssDNA.

3.3.1 Synthesis of AuNP-triskelia

AuNP with diameter from 5 nm to 40 nm were added to the folding mixture of triskelion monomers (one DNA helix, 90 bp bridge, and handles for cholesterol and dye omitted) before annealing and analysed by AGE and TEM (Figure 3.40).

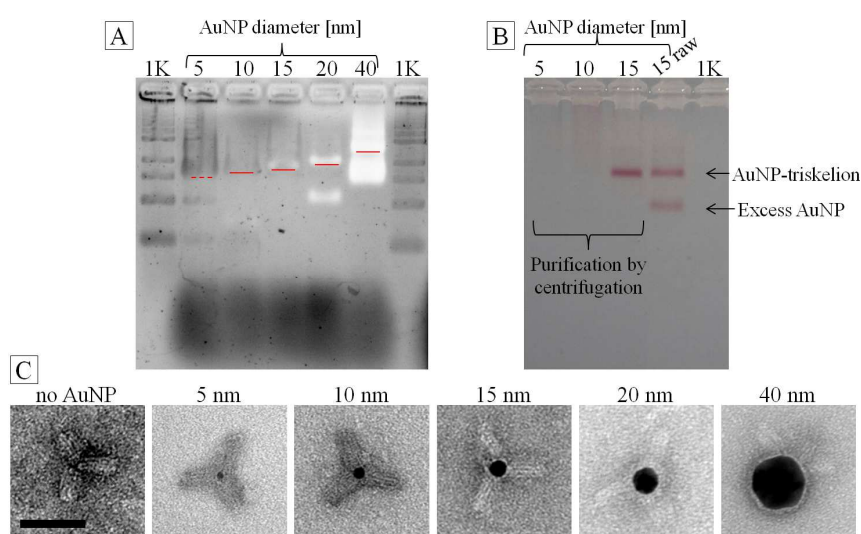


Figure 3.40. (A) Agarose gel of various AuNP sizes scanned with SYBR gold dye. Numbers above the well refer to the dimension in nm of the AuNP diameter. Red lines highlight the position of the AuNP-triskelion; other bands are attributed to excess AuNP. (C) AGE of purified AuNP-triskelion by centrifugation. The well “15 row” indicate an unpurified sample run as a reference. (D) TEM of triskelion decorated with AuNP in their centre. Scale bar 50 nm.

The gold-decorated structures were successfully folded and purified by AGE. Structures modified with smaller AuNPs migrated quickly in the gel and were less fluorescent (Figure 3.40A); any other bands located under these are excess AuNP, which are added as 1.3 equivalents. Triskelia functionalised with AuNP between 15 nm and 40 nm were pelleted without damaging the origami, as observed by a single, red band in a gel (Figure 3.40B). Triskelia with smaller AuNP (5 and 10 nm) did not pellet at rates of centrifugation that would not damage the structure. Electron micrographs of the functionalised triskelion monomers revealed that the 40 nm AuNP deformed the triskelion (Figure 3.40C). We thus continued our polymerisation investigations with the triskelia modified with a 15 nm diameter AuNP, as the monomer could be successfully purified and the AuNP did not alter the triskelion structure.

As this investigation with AuNP functionalised triskelia was performed before our development of the procedure for polymerising with dimers and invading strands, we used sticky ends (and bridges of 90 bp with a single DNA double helix) for polymerisation. The 15 nm-diameter AuNP-functionalised triskelion was mixed with the two patterns of polymerisation staples (all $\leftrightarrow$ all and 1 $\leftrightarrow$ 1, 2 $\leftrightarrow$ 3). We examined if the AuNP would change the conditions required for polymerisation, by using both 4 bp and 6 bp sticky end staples. After annealing from 44°C to 10°C over 16 hours, we imaged the arrays with TEM (Figure 3.41).

3.3 Gold nanoparticle functionalisation of the triskelia

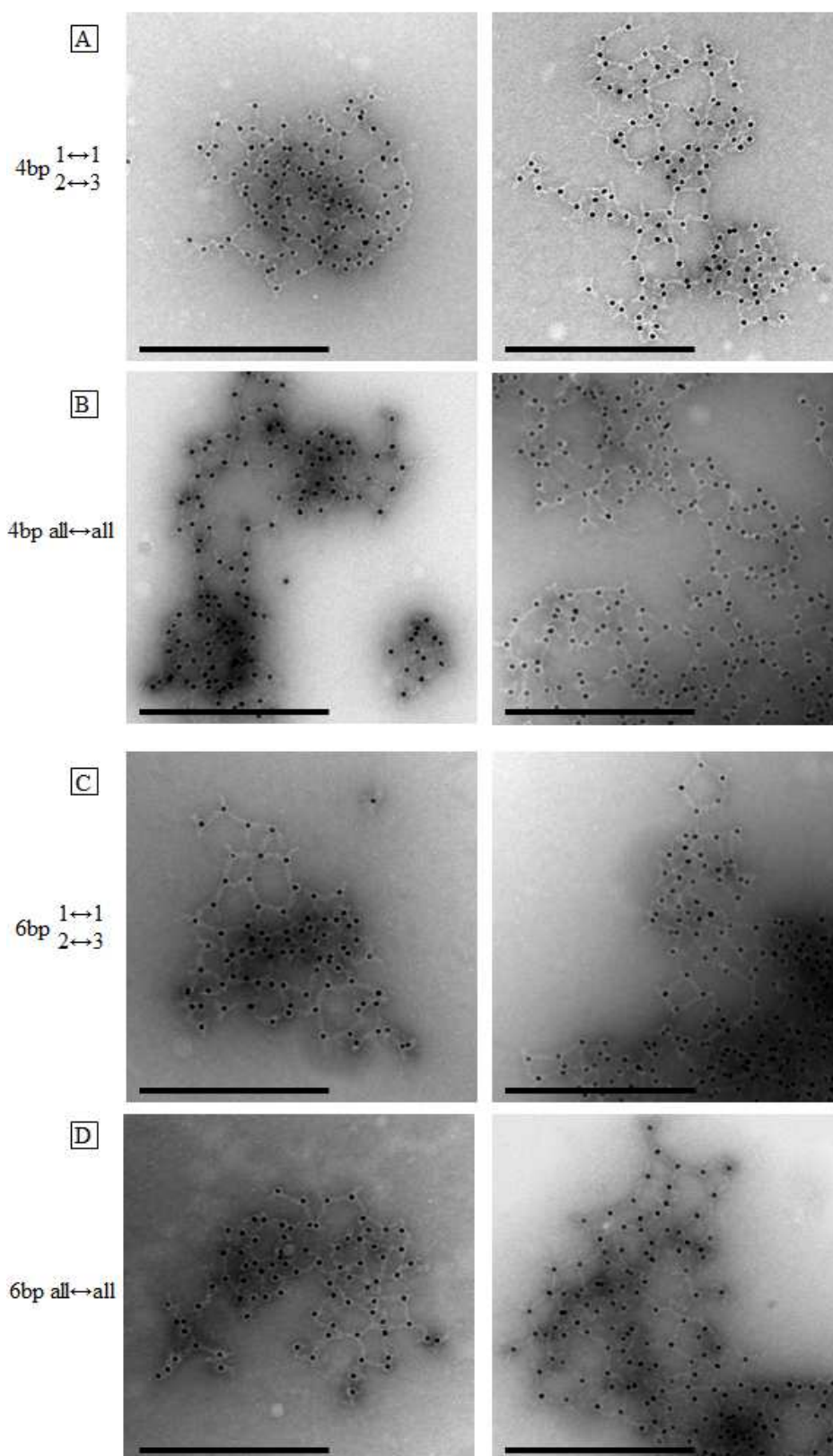


Figure 3.41: TEM micrographs of triskelion arrays modified with 15 nm diameter AuNP (observed as black dots). The polymerisation staples were added after purification by centrifugation. (A) 4 bp, 1↔1, 2↔3, (B) 4 bp, all↔all, (C) 6 bp, 1↔1, 2↔3, (D) 6 bp, all↔all. Scale bare 500 nm.

Polymerisation was observed for both 4 bp and 6 bp sticky ends and for the two patterns of attachment, as previously observed with the triskelia without AuNP (Figure 3.17). The presence of the AuNP on the triskelion did not appear to change the polymerisation efficiency in comparison with polymers formed in the absence of the nanoparticle; therefore we proceeded to use the 4 bp sticky ends for future experiments. As the arrays formed successfully with AuNP functionalisation (and with higher purity), we also investigated templating strategy using liposomes to direct and optimise the formation of the desired spherical structures as described in Chapter 4, section 4.1.

3.3.2 Variation of the bridge length of a AuNP-triskelion

We next investigated if TEM imaging would allow the observation of controllably deformed liposomes with AuNP-functionalised triskelia using the stiff 3-helices bridges described in section 3.2.6 (See also Figure 3.33). As the contrast offered by the AuNP enhances observation of the triskelion by TEM, we investigated if the presence of the AuNP would interfere with the constricted triskelia with shorter bridge. AuNP-triskelia were folded with the two stiffer bridges; 60 and 90 bp and analysed by AGE (Figure 3.42) and TEM (Figure 3.43).

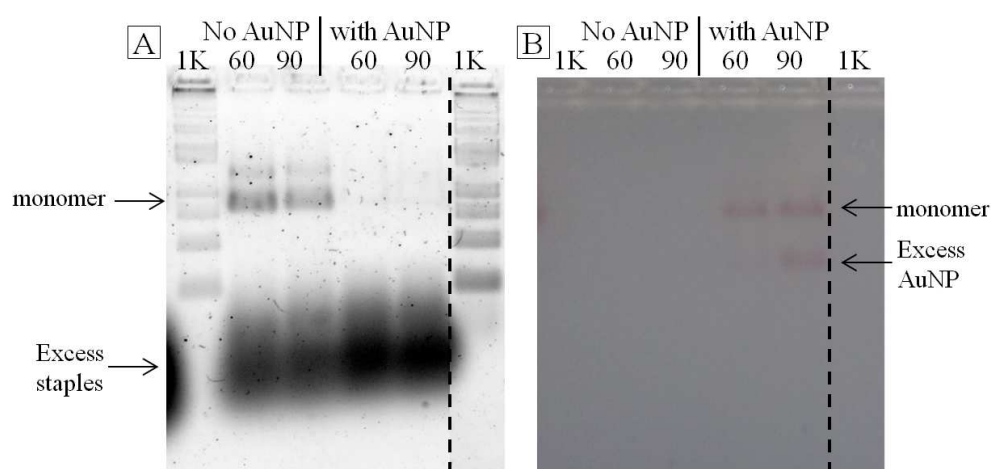


Figure 3.42: AGE of triskelion folded with stiff bridges of 60 bp and 90 bp long. The number of bp of the bridge is indicated above the well of the gel. No AuNP above the gel indicate that the triskelion of the well do not have AuNP attached to it. With AuNP marks AuNP-decorated triskelion. (A) Gel stained with SYBR gold, and (B) photography of the same gel.

For comparison, triskelion with and without AuNP were analysed. Triskelia without AuNP functionalisation requires staining with SYBR gold for analysis, but this dye bleaches the signal from the AuNP-triskelia. Therefore, a photograph of the gel was made to show the red band of the AuNP (Figure 3.42 B); the band under the monomers on the gel is the excess AuNP, added in 1.3 equivalents. The gel indicates correct folding of the structure with incorporation of a AuNP. Triskelion folded with and without AuNP presented similar mobility in agarose gel suggesting that the two structures are similar. The band corresponding to the monomer AuNP-triskelion with 60 and 90 bp-long bridges were extracted and imaged by TEM (Figure 3.43).

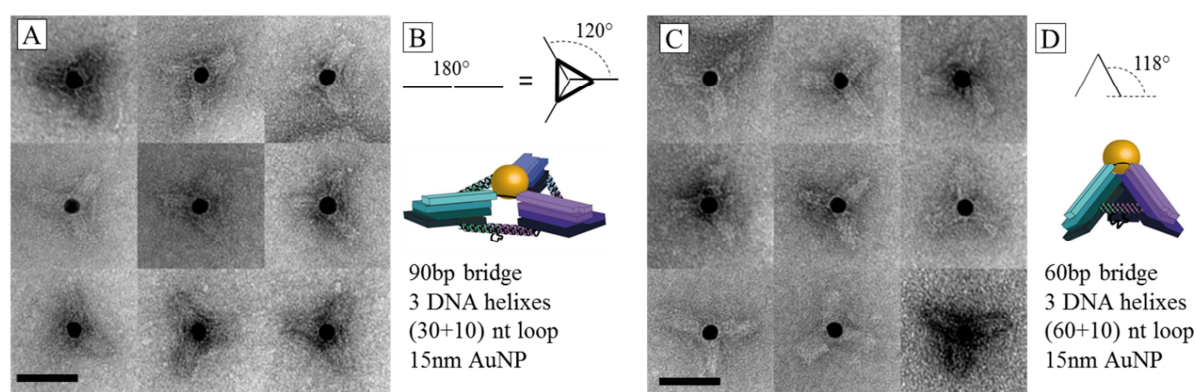


Figure 3.43: Electron micrographs of AuNP-triskelion extracted from the gel in Figure 3.42. (A) AuNP-triskelia with stiff 90 bp-long bridges and (B) schematics. (C) AuNP-triskelia with stiff 60 bp-long bridge and (D) schematics. Scale bars 50 nm.

Electron micrographs revealed that the presence of the AuNP on the triskelion fixed the planar angle between the arms at $120^\circ \pm 2^\circ$ (Figure 3.43A, B). However, it also prevents the triskelion from constructing to an angle of 118° with a short, 60 bp stiff bridges; the dihedral angle was maintained at 180° (Figure 3.43C, D). Therefore, the AuNP-triskelion was not used further in the investigation of the controllable deformation of a membrane bilayer.

3.4 SUMMARY

This chapter presented the successful design and synthesis of a clathrin-inspired structure using DNA origami with the purpose of controllably bending a lipid membrane. The new structure (triskelion) was designed for polymerisation into a pattern resembling that of a buckyball, and it was determined that the optimal strategy was to use triskelia dimers (using 1 $\leftrightarrow$ 1 6T invading strands) and 4 bp sticky ends (arms 2 $\leftrightarrow$ 3). Each triskelion can be functionalised with 9 cholesterol for attachment to lipid membranes, and 6 dyes for fluorescence visualisation; the study of triskelia on lipid membrane will be presented in chapter 4.

Triskelia can be folded with bridges of two different lengths; 90 bp (flat) and 60 bp (curved) and purified from excess DNA staples. We determined that a bridge comprising 3 DNA double helices was able to force the triskelion to maintain a fixed angle between the arm of 120°, and a dihedral angle of either 180° or 118°, using bridges of 90 bp or 60 bp respectively. We discovered that the 60 bp bridge forced the triskelia in an unexpected conformation, which orientates the position of the cholesterol anchors towards the outside of the structure.

A triskelion functionalised with a 15 nm AuNP was also studied, and helped in the development of the polymerisation and purification procedure. AuNP-triskelia will be used to determine optimal coverage of lipid vesicle in chapter 4. However, the presence of the AuNP also prevents the triskelion from constructing to an angle of 118° with a short, 60 bp stiff bridges. Therefore, the AuNP-triskelion will not be used in the investigation of the controllable deformation of a membrane bilayer.

CHAPTER 4

CLATHRIN-INSPIRED DNA ORIGAMI IN INTERACTION WITH LIPID MEMBRANES

The previous chapter described the design and synthesis of a DNA origami which shape and function were inspired from the clathrin protein. We named this new nanostructure triskelion to refer to the three-armed shape of the DNA origami. Chapter 3 also described the improvement of the triskelia polymerisation method; we determined that triskelia formed extended arrays when a $1 \leftrightarrow 1$ dimer (attached using 6T invading strands) was mixed with 4 bp sticky ends connecting arms $2 \leftrightarrow 3$. The following chapter presents first the study of the optimal concentration of AuNP-triskelia required to efficiently anchor the DNA origami with cholesterol anchors to the lipid membrane of liposomes. We then consider triskelia dimers (without AuNP) anchored to lipid membranes by cholesterol anchors and the effect of the formation of triskelia arrays for membrane bending.

4.1 OPTIMISING COVERAGE OF LIPID VESICLES

BY AuNP-TRISKELIA

We hypothesized that the surface offered by a lipid bilayer would template the organisation of triskelia into a spherical shape. DOPC liposomes of approximately 100 nm diameter were added to purified flat monomers of AuNP-triskelion bearing cholesterol anchors, concurrently with the polymerisation staples. The samples were allowed to incubate at $20 \pm 2^\circ$ for 12 hours before EM imaging (Figure 4.1). This method has the advantage of limiting the damage endured by the lipid vesicles and reduces the

risk of the AuNP falling off the triskelion^[247] (Figure 4.1 B), which was observe to happen after 24 hours.

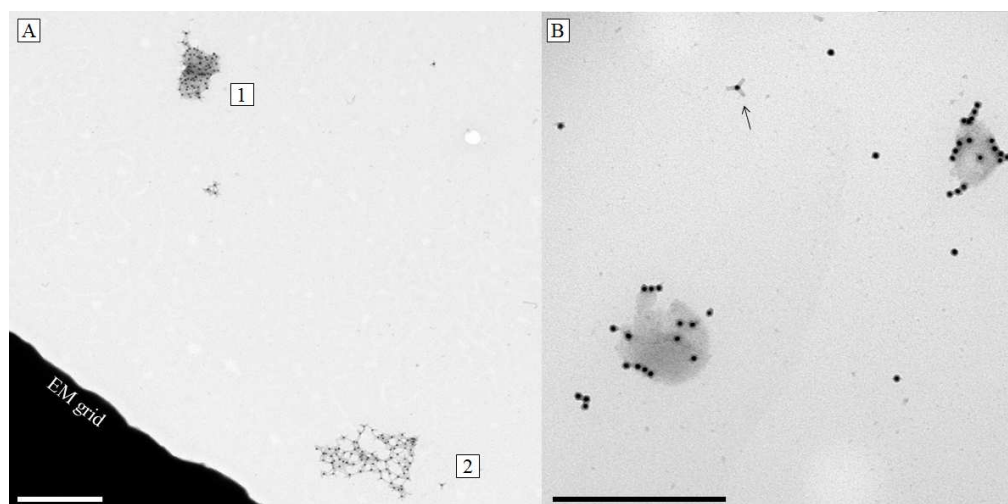


Figure 4.1: Electron micrograph (A) showing two polymerised assemblies of AuNP-triskelion (1) with a lipid vesicle and (2) without lipids interacting. (B) Sample after 24 hrs showing AuNP without triskelion interacting with liposomes. Arrow indicates one AuNP-triskelia remaining. Scale bars 500nm.

The presence or absence of a lipid vesicle surrounded by a polymerised assembly can be observed by a contrast difference; as lipid vesicle appear darker on the TEM micrograph for negative staining (Figure 4.1).

Excitingly, the structures observed in the TEM micrographs indicate that the triskelia assemble around a lipid vesicle and form an array (Figure 4.1A). To further examine this result, titration of AuNP-triskelion with respect to the number of lipid in solution was performed (Figure 4.2). The concentration of lipid refers to the initial quantity of lipids used for the production of liposomes (through a 0.2 μm pore size membrane) and is therefore an estimate of the final lipid concentration. The concentration of purified AuNP-triskelion was calculated according to the absorbance of the 15 nm AuNP at 520 nm, as we assumed one triskelion per AuNP.

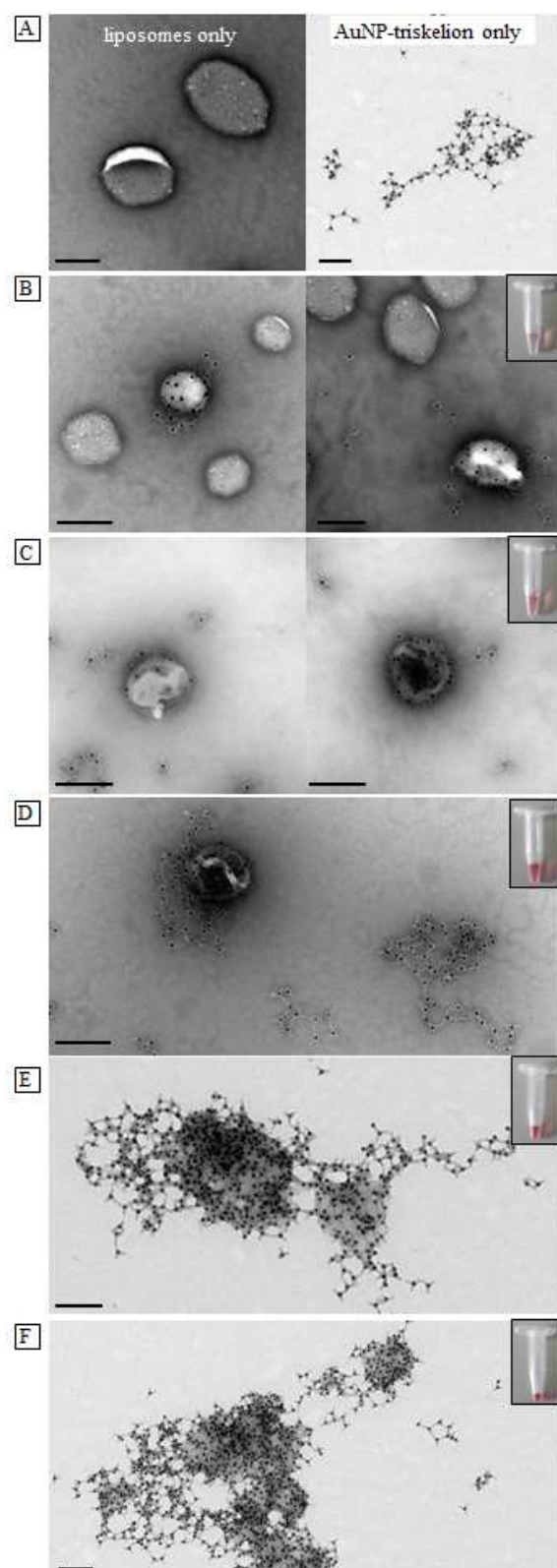


Figure 4.2: Electron micrographs of AuNP-triskelion of (A) control samples: 0 nM (left) and 3 nM (right), and mixed with a constant concentration of 0.1mg/mL liposomes. Concentrations of AuNP-triskelia are (B) 0.5nM, (C) 2.3nM, (D) 5.5nM, (E) 8.2nM, (F) 14.6nM. Insets: Photograph of the tubes containing the samples. Scale bars 200nm.

When the concentration of the AuNP-triskelion was 2.3 nM and added to a lipid concentration of 0.1 mg/mL, we observed complete coverage of all of the lipid vesicles by the polymerised triskelia (Figure 4.2C); at concentration below 1 nM, liposomes without AuNP-triskelia could be observed (Figure 4.2B). The liposomes were either entirely covered by the AuNP-triskelia, or not at all. This suggests that once polymerisation begins at a point on the vesicle, it will continue until the vesicle is completely surrounded. At concentration higher than 5nM, the surface of the liposomes became saturated and excess polymerised AuNP-triskelia were observed to be incorporated into the covered liposomes, or remain in solution (Figure 4.2D). These over-saturated AuNP-triskelia liposome eventually attach to other polymerised vesicles (Figure 4.2E, F). Therefore, we believe that an excess triskelion should be avoided in order to obtain individual vesicle coating. We could see no evidence that the AuNP-triskelion disturbs the lipid bilayer.

We next examined if polymerised triskelia would also efficiently surround vesicles, to compare against the polymerisation procedure that occurs on the surface of the vesicles. Pre-polymerised AuNP-triskelion at 5nM monomer concentration with a ten-fold excess of staples (4bp 1↔1, 2↔3) were added to liposomes (Figure 4.3A). We also added 5nM monomers AuNP-triskelia to a solution of liposomes for comparison (Figure 4.3B). Both samples were then left at room temperature for 24 hours before EM imaging.

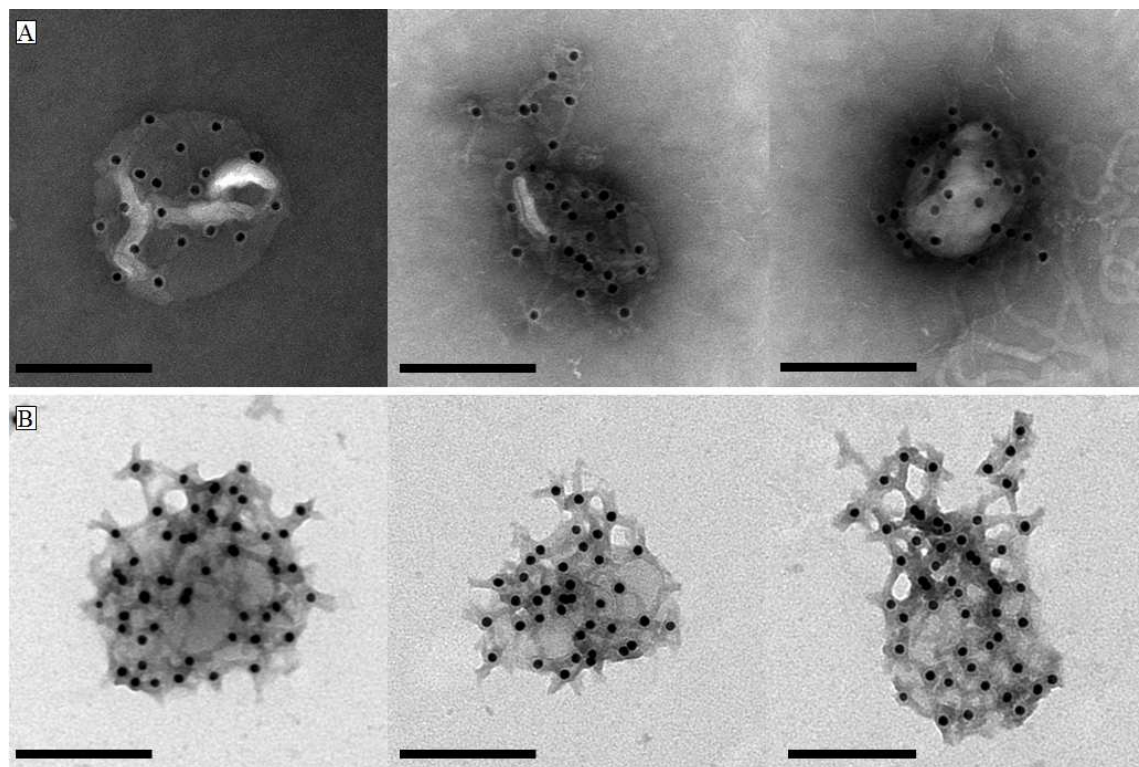


Figure 4.3: TEM of polymerised AuNP-triskelion on DOPC liposomes. (A) Triskelia polymerised before mixing with liposomes, and (B) Triskelion polymerised after addition of the liposomes. Scale bars 200 nm.

The images obtained by TEM indicated no clear difference between the addition of monomer or pre-polymerised AuNP-triskelia; the liposomes showed dense coverage by the polymerised origami.

Perrault *et al.*^[6] and Yang *et al.*^[175] suggested that lipid vesicles form gradually, nucleated by the cholesterol anchors on origami structures, using the liposomes as a lipid reservoir. In these studies, the lipid membrane was altered by the presence of surfactant to observe these results. As our triskelion samples do not contain membrane disrupting molecules such as surfactants, it was inferred that this nucleation theory does not apply in our system. In rare occasions, we observed small lipid clusters around the cholesterol anchors of the triskelion (Figure 4.4) which would support the first theory that the liposomes grow from a reservoir of lipid.

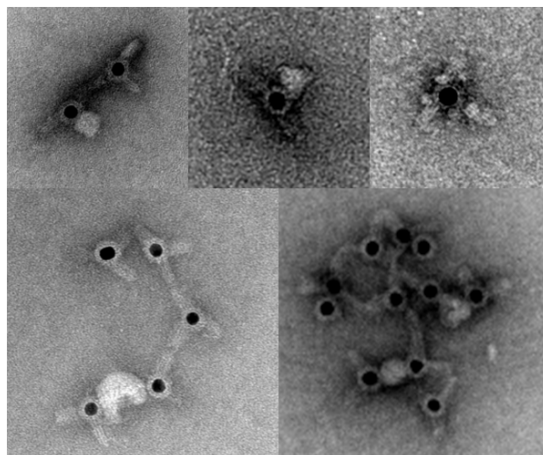


Figure 4.4: Electron micrograph of lipid nuclei on the cholesterol localised on the arms of the triskelia. Scale bar 50 nm.

However, as these lipid nuclei were not observed to form larger vesicles, and indicate only a solution interaction between the cholesterol and DOPC lipid, we suggest that the triskelia insert gradually into existing liposomes and build a polymer around the lipid vesicle. Smaller liposomes may occur from the preparation of the liposomes, which yields a distribution of sizes.

Despite a high risk of deformation due to the staining process for EM, liposomes with only partial coverage by DNA structures (Figure 4.2B and C left panel) tend to look more spherical and regular than liposomes with a high coverage of triskelion (Figure 4.2C right panel, D and Figure 4.3). Observations like this must be interpreted with care, as many parameters can create distortions and misleading conclusions. However, due to the numerous experiments done and because this phenomenon was regularly observed, it is believed (after carefully elimination of any suspicious images) that these deformations are the results of the triskelion insertion and polymerisation. The observation of liposomes only partially covered by triskelia also reinforce our theory by which the triskelion monomers insert into pre-existing liposomes and gradually builds a polymer around it. We thus concluded that the addition of the polymerisation staples post-insertion of the triskelia into the lipid membrane is a promising protocol for vesicle

coating, and that a partial coverage of the liposomes surface is acceptable in order to study a membrane deformation.

Although the use of a AuNP was successful in helping us to identify the optimal concentration of triskelion required for coating a liposome, and in the development of early polymerisation procedures and purification of the triskelia (Chapter 3 section 3.3), the incapacity of the triskelion to close in the presence of the AuNP and the shorter, stiff 60 bp bridge did not allow us to continue our study of AuNP-triskelia systems. The rest of the study was pursued with triskelion in the absence of gold nanoparticle.

4.2 LIPOSOMES DEFORMATION BY TRISKELIA OBSERVED BY TEM

Cholesterol-functionalised triskelia with stiff bridges of 60 or 90 bp were folded as dimers using 6T invading strands connecting arms 1 ($1 \leftrightarrow 1$) from two origamis. The dimers were then purified by size-exclusion column and mixed with liposomes made of the phospholipid 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), before the addition of the 4 bp sticky ends ($2 \leftrightarrow 3$) polymerisation staples. After 8 hours incubation at $20 \pm 2^\circ$ (room temperature), the resulting samples were prepared for TEM imaging following a staining protocol that helps to prevent bursting of the liposomes on the TEM grid. This protocol is discussed in Chapter 6 and was developed at the Dunn School of Pathology, Oxford. It was hoped that despite the deformation generated during EM grid preparation, changes in the general appearance of the coated liposomes would give preliminary results regarding membrane bending (Figure 4.5).

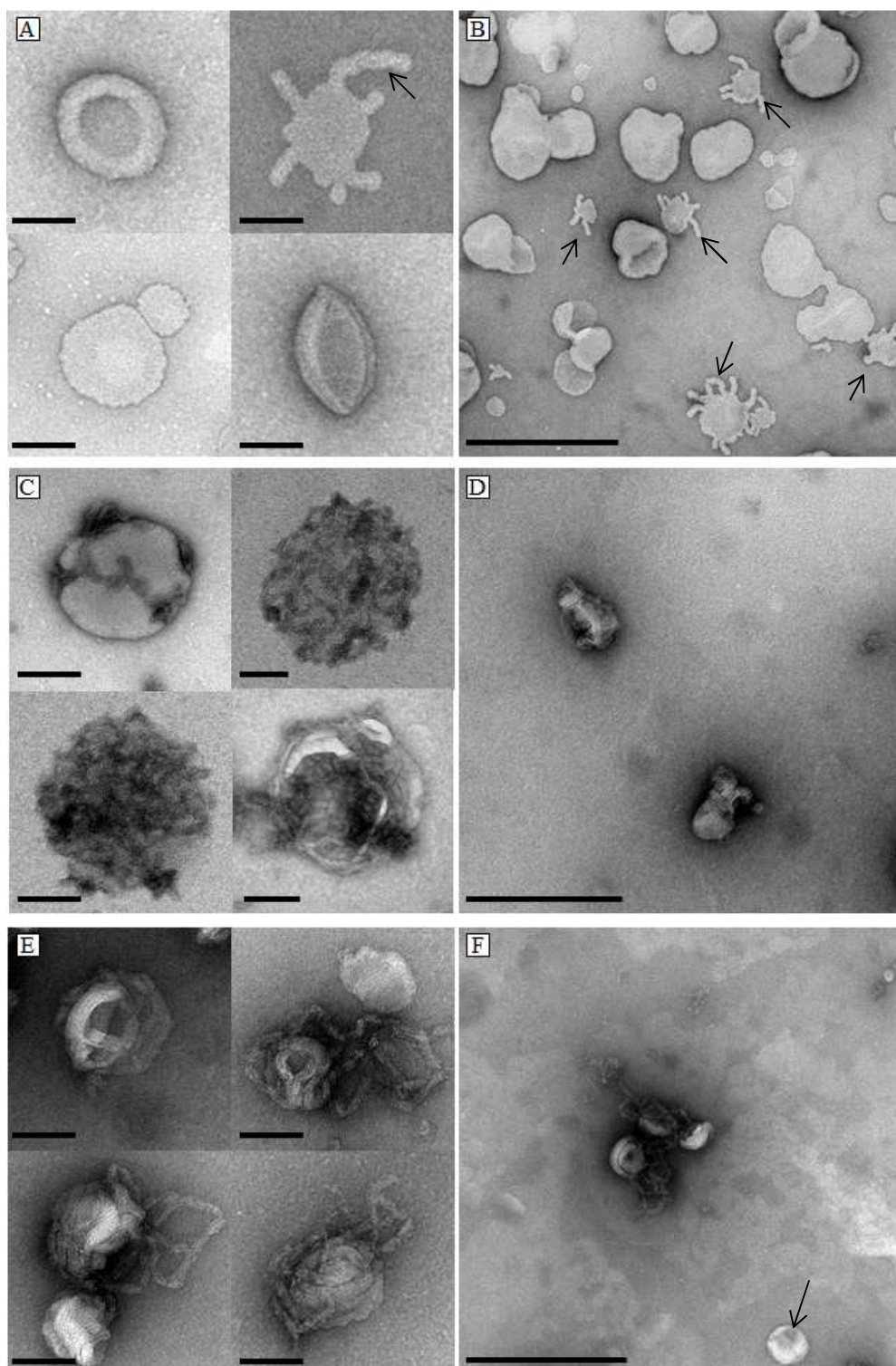


Figure 4.5: Electron micrographs of triskelia polymerised on liposomes. (A), (B) liposomes without triskelion (control), some of which showed tubulations (arrows) (C), (D) Triskelia with stiff 90 bp bridges on liposomes. The liposomes appear surrounded by a darker surrounding, and small darker spots on the background are local differences in staining on the EM grid. (E), (F) triskelia with stiff 60 bp bridges on liposomes, stained in the same conditions. An uncovered liposome in (F) is indicated by a black arrow. Scale bars for (A), (C) and (E) 100 nm; scale bars for large views (B), (D) and (F) 500 nm.

Liposomes that did not have triskelia added were observed to burst and to show tubulation on the EM grid (Figure 4.5A, B). The addition of triskelia was observed to increase the rigidity of the liposomes, as the stain accumulates on the edges of the vesicle, and appears darker on the electron micrograph without tubulation or bursting (Figure 4.5C, D). Liposomes with bent triskelia of stiff 60 bp bridges (Figure 4.5E, F) were deformed and showed coverage of their surface by the polymerised structures. Bent, 60 bp bridged triskelia were observed to bring multiple vesicles in close proximity, and flat 90 bp triskelia coated individual liposomes. This difference between bent and flat triskelia is attributed to the presence of the cholesterol: oriented towards the inside of the polymer for 90 bp triskelia, and towards the outside of the structure for bent 60 bp triskelia. However, we could not observe drastic differences in shape between liposomes covered with 60 bp bridges and liposomes covered with 90 bp bridges triskelia; there was not sufficient evidence to conclude the deformations from a spherical shape of the liposomes are resulting from the effect of bent or flat triskelia. More importantly, the sample preparation for TEM imaging does not allow us to draw a clear conclusion on topology, as the vesicles are flattened during sample preparation. In order to more efficiently investigate membrane bending, we moved towards fluorescent microscopy to visualise deformation in solution.

4.3 TRISKELIA AND LIPID MEMBRANE ON EM GRID

The formation of a lipid monolayers on a TEM grid has been used previously as a template for the formation of two-dimensional crystals of soluble^[248] and membrane^[249] proteins. McMahon and his research group demonstrated that a hydrophobic electron microscope grid will orientate the hydrophobic tails of lipids and form a monolayer on the surface of the grid that can be studied in the electron microscope^[250, 251]. When proteins are added, they interact with the exposed head group of the arranged lipids. The lipid monolayer simulated the inner leaflet of the plasma membrane, and was used

to reconstitute early stages of endocytosis. They observed the formation of ordered clathrin assemblies using negative stain electron microscopy (Figure 4.6), and platinum shadowing to reveal the invagination of these coats^[252].

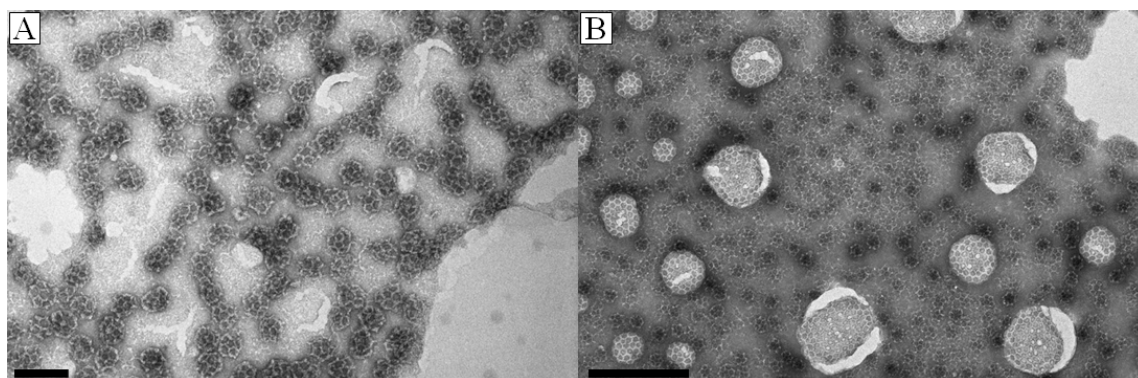


Figure 4.6: Electron micrographs of (A) clathrin pits obtained with a lipid monolayer assay developed by McMahon. Scale bare 100 nm. (B) large budding of the monolayer due to the assembly of clathrins. Scale bare 300 nm. Images from the Epsin 1 gallery^[253].

It was discussed in Section 4.2 that negative staining for TEM imaging deformed the lipid vesicles and hindered the identification of membrane deformation by the polymerised triskelia. The promising imaging technique of the monolayer assay was thus pursued with the hope that it would allow us to observe and identify triskelion-driven membrane deformation on EM. We designed and machined a chamber to allow preparation of a lipid monolayer on a TEM grid, similar to the one described in the work of Higgins *et al.*^[254]. This technical project was started at LMU in Germany and pursued at the University of Oxford, in the UK. The samples were prepared in the EM lab at the Dunn School of Pathology, Oxford.

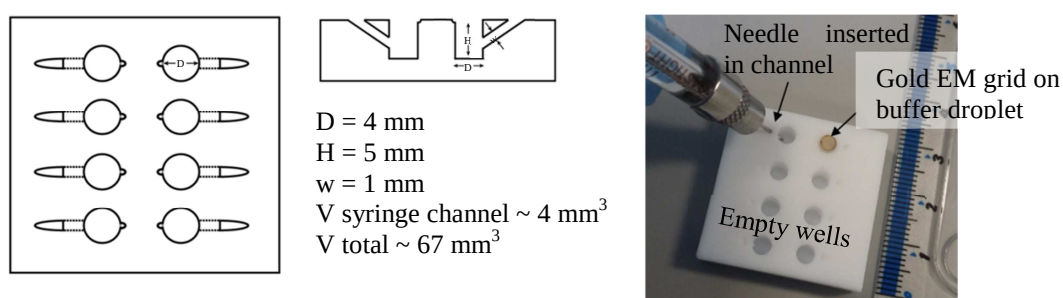


Figure 4.7: (left) Plan and dimensions of the Teflon block as given to the workshop in LMU for production. (right): picture of the final apparatus (3 cm long) with one gold grid on top of a buffer droplet in a well, and a syringe inserted in the top left channel for demonstration.

The apparatus contains 8 wells of approximately 70 μL volume, that can be used independently to treat 8 TEM grids (Figure 4.7). To prepare a monolayer on a TEM grid, first a droplet of lipid in organic solvent (hexane) was pipetted on top of the well filled with a buffer solution (Figure 4.8A). When the solvent evaporates, a lipid monolayer should form at the air-liquid interface (Figure 4.8B). A gold EM grid, not glow-discharged, is then placed on top of the well in contact with the lipid monolayer (Figure 4.8C). Triskelia bearing cholesterol (1 μL , 1-2 nM) are injected through a channel located on the side of the well, so as to not disturb the monolayer (Figure 4.8D). The cholesterol anchors of the triskelia will progressively insert into the monolayer formed on the grid. After 30 mins, polymerisation staples were injected in the solution (Figure 4.8E). The Teflon block is then incubated in a humid chamber for 12 hours at 20°C to allow the triskelia to polymerise before carefully removing the grid for staining and imaging (Figure 4.8F).

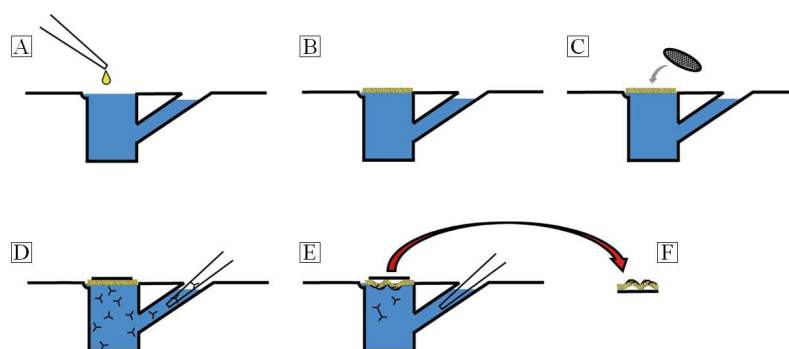


Figure 4.8: Schematics of the protocol for the lipid monolayer assay. (A, B) A lipid monolayer is first formed at the surface of the buffer and (C) an EM grid is placed on top. (D) Triskelia are then injected under the grid and (E) polymerise with the addition of staples. (F) The grid is then removed for staining and imaging.

Incubation in TE or TBE buffer longer than a few minutes can damage copper grids due to oxidation^[182, 255]; the presence of the support film is not sufficient to protect the entire surface. Gold EM grids do not undergo chemical reactions in contact with buffers, therefore formvar and carbon-coated gold grids were used to prepare monolayer lipids. A higher current was applied to the carbon rod during carbon-coating of the grid to make the carbon film thicker and more resistant. We investigated the appearance of a lipid

monolayer formed using this technique with TEM; uniform lipid layer was expected to be undistinguishable under the electron micrograph if the stain is evenly distributed. A lipid layer was formed by placing a 1 μ L droplet of lipid in hexane (DOPC, 0.1 mg/mL) at the surface of the well filled with TBE buffer and 10-14 mM MgCl₂. A gold grid that was not glow-discharged was then placed on top of the well, and incubated at 20°C for 12 hours (Figure 4.9). TEM imaging indicated that without addition of lipids, the background showed little contrast (Figure 4.9A). When added, the contrast of the grid changed, which was attributed to the presence of the lipids that covered the entire grid, and an excess of DOPC formed multi-layered surfaces observed as lighter dots (indicated by arrows) on the EM grid of approximately 100 nm (Figure 4.9). The distinction between a mono-layer or a higher-order layer of lipid was not possible, but it is suspected that the grid is covered by a bilayer (rather than a monolayer claimed by McMahon^[250, 254]) because the amount of lipid added was larger than the amount required to cover the surfaces of the well (walls and EM grid), as well as the spontaneous bilayer arrangement of DOPC in buffer.

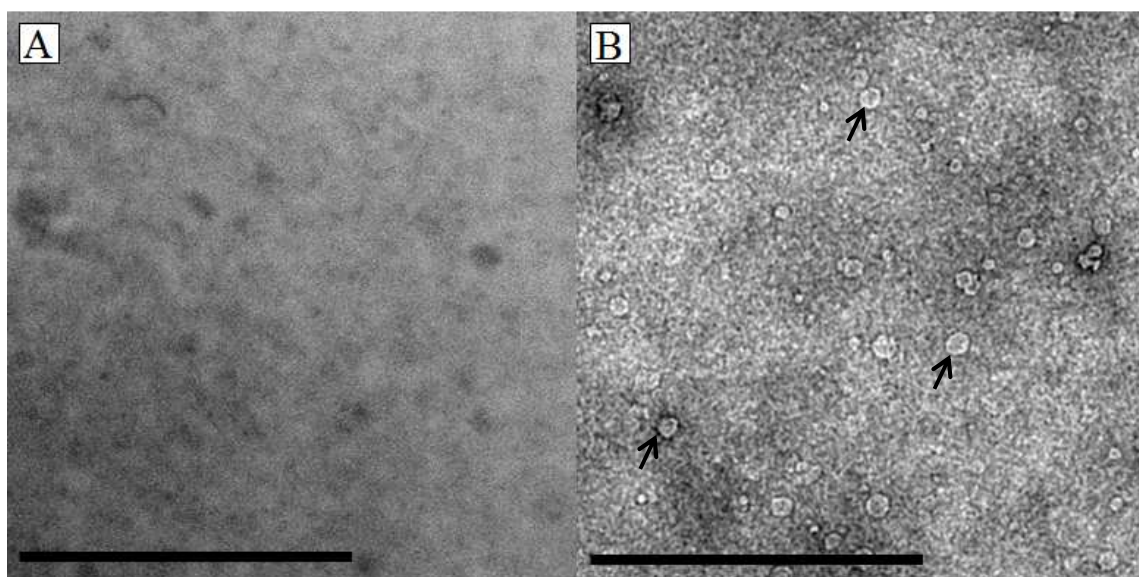


Figure 4.9: Electron micrographs of UAc stained gold grids. (A) Empty grid, (B) grid with a layer of lipid. Arrows indicate small surfaces where a lipid multi-layer is formed due to an excess of lipid in the well. Scale bars 1 μ m.

We then compared the deposition of flat cholesterol-triskelia dimers on an untreated support film (Figure 4.10A) and on a lipid-layered one (Figure 4.10B). Triskelia dimers were observed flatly deposited on the grid in both cases. The “naked” support film showed a better contrast between the triskelia and the support film than the one with the lipid layer (Figure 4.10C). This distinction in the stain adsorption of the two grids was attributed to the presence of the lipid on the surface of the grid.

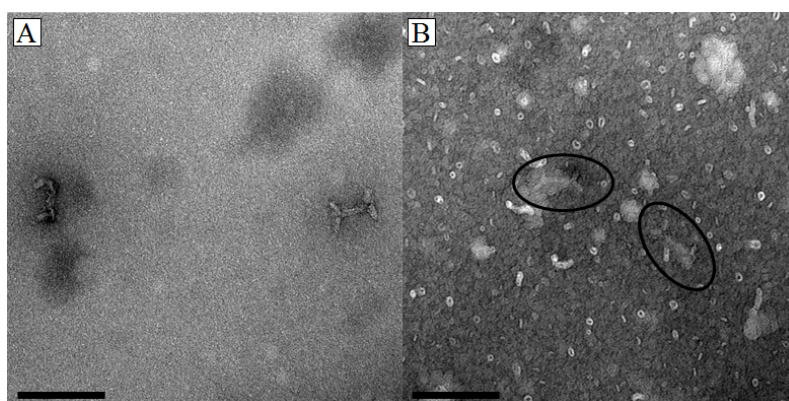


Figure 4.10: Flat triskelia dimers injected into the well under a grid (A) without lipid and (B) with a lipid layer. Triskelia dimer on the lipid layer are highlighted by the black circles. Scale bars 200 nm.

We next compared the result of polymerisation of cholesterol-triskelia with either flat (stiff 90 bp bridges, Figure 4.11A) or curved (stiff 60 bp bridges, Figure 4.1B) dimers. After the cholesterol-triskelia were added to the chamber with a lipid-covered EM grid and incubated for 1 hour at 18°C in a humid chamber, polymerisation staples 1↔1, 2↔3 4 bp were injected in excess and the sample allowed to polymerise for 12 hours before imaging.

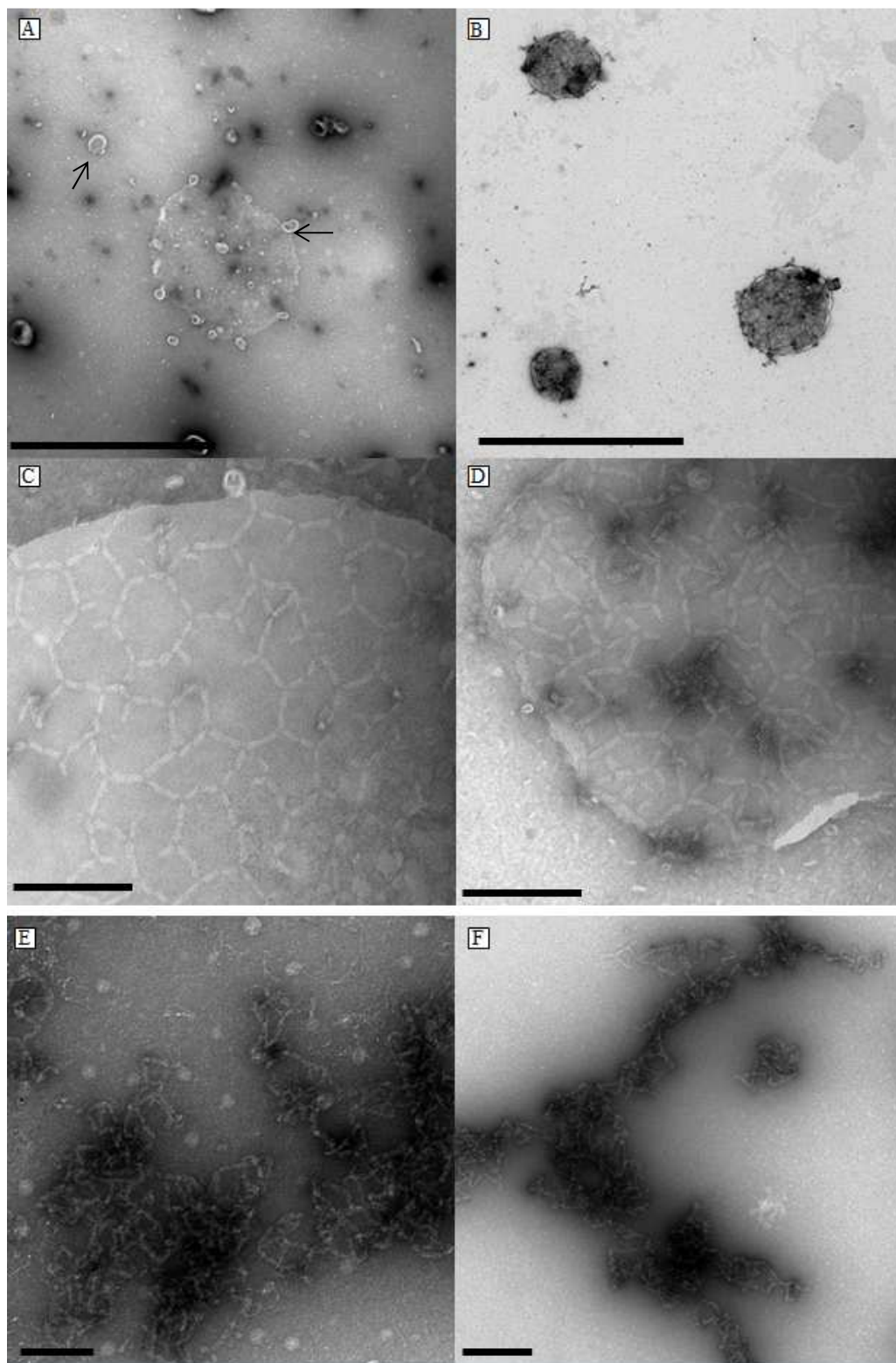


Figure 4.11: Electron micrographs of (A) flat and (B) curved triskelia polymerised on a lipid layer, large view. Arrows indicate liposomes due to an excess of lipid. Scale bars (A-B) 2 μ m. Detail of (C) flat and (D) curved triskelia arrays on a monolayer. (E) flat and (F) curved triskelia polymerised in the absence of the lipid layer. Scale bars (C-F) 200 nm.

Polymerised triskelia from flat dimers covered patches of lipid with arrays of hexagons and pentagons (Figure 4.11A, C). The extent of the planar polymerised flat triskelia arrays were not observed with triskelia that polymerised in solution (Figure 4.11E). Polymerised triskelia from curved dimers indicated special circular features on the lipid-layered grid (Figure 4.11B, D) which resembles spherical lipid vesicle coated with polymerised, curved triskelia and is similar to the electron micrographs of clathrin (Figure 4.6). The flat triskelion dimers were added to the well under the lipid-coated EM grid and allowed to first insert into the membrane before adding the polymerisation staple. The observation of large, regular arrays then results from the diffusion of the dimers on the membrane, the meeting of two arms and hybridisation of the polymerisation staples. These results indicate that a mobile lipid layer can be formed on an EM grid using the custom-made apparatus, and that it offers support and direction for the polymerisation of triskelia. In the absence of the lipid layer (Figure 4.11E, F), polymerisation of the dimers resulted in disorganised polymers as previously observed (Section 3.2.7).

4.4 GUV DEFORMATION OBSERVED BY FLUORESCENCE MICROSCOPY

The interaction of giant unilamellar vesicles (GUVs) with triskelia in aqueous solution was investigated in collaboration with Vivek Ramakrishna and Prof. Mark Wallace at King's College London. Dual-colour epifluorescence microscopy and total internal reflection fluorescence (TIRF) microscopy was utilised to observe the interaction of the polymerising DNA on the surface of GUVs. Triskelia monomers with cholesterol anchors and stiff 90 bp bridges were further functionalised with a red dye, Alexa-647, (as described in section 3.2.1), and allowed to interact with fluorescent GUVs made of DOPC and 0.01 mol% of a blue dye-conjugated lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (ATTO488-DOPE). GUVs were prepared in 100 mM sucrose to

increase their density and mixed with TE buffer with 14 mM MgCl_2 . The GUV in this experiment was at hypertonic conditions that forces the GUVs to adopt a taut sphere shape^[256] which is optimal to observe initial interactions between the origami and GUVs. The sample was placed onto a microscopic slide protected by a lipid bilayer to prevent the GUVs from bursting and imaged using a laser set at 474 nm (blue, lipids) and 644 nm (red, triskelion) wavelengths. We observed fluorescence co-localisation between the triskelion monomers and the GUV (Figure 4.12), which confirmed that the origami interacts with the lipid membrane, consistent with our observations with TEM.

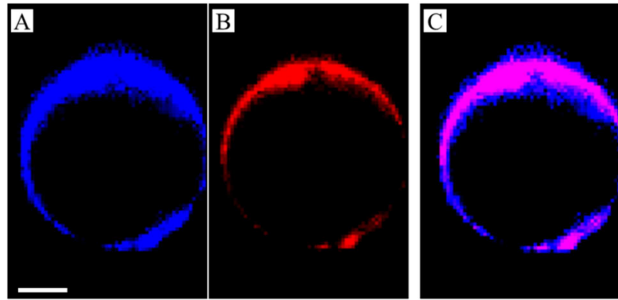


Figure 4.12: Fluorescence microscopy of a GUV coated with triskelion monomers anchored by their cholesterol anchors. (A) fluorescence from lipids (ATTO488-DPPE), (B) fluorescence from triskelia (Alexa647), and (C) merged channels. Scale bar approximately 10 μm .

For the triskelion to bend the membrane of a GUV, hypotonic conditions (osmolality inside the GUV lower than the osmolality outside) are required. We thus investigated different conditions that would allow the DNA origami to maintain its folded structure and give the GUVs malleable membranes. The triskelion requires a divalent cation such as Mg^{2+} to stabilise the multiple cross-overs^[257, 258] within the structure, but the bilayers of GUVs (and cells) are known to adhere in the presence of this salt^[259, 260] which would prevent studying membrane bending. This phenomenon was observed when isotonic DOPC GUVs in TE buffer were imaged on bright field microscope with 50 mM MgCl_2 (Figure 4.13).

4.4 GUV deformation observed by fluorescence microscopy

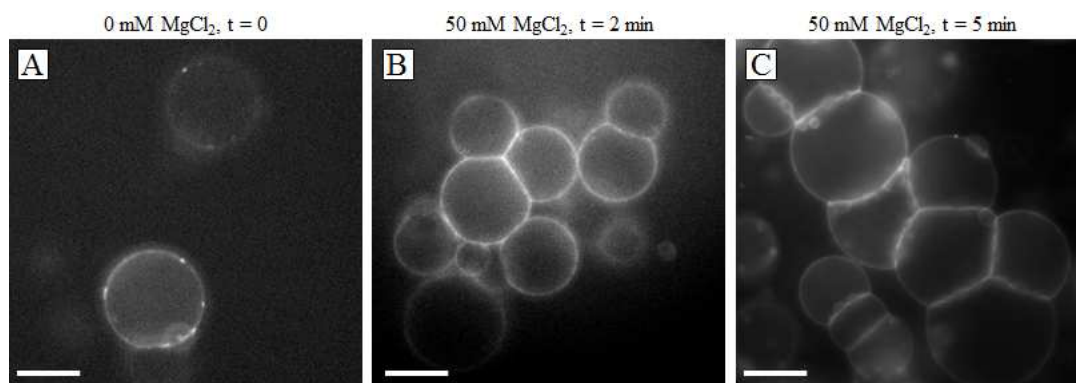


Figure 4.13: bright field microscopy of GUV (DOPC, 100mM sucrose) over addition of MgCl₂ (approximately 50 mM final concentration). Scale bars approximately 20 μ m.

Moreover, MgCl₂ allows unspecific interactions to occur between the bilayer and the DNA backbone^[261, 262] of the triskelion, an effect observed as well in our system (Figure 4.14). In this situation, flat triskelion monomers to which the cholesterol handles are omitted were mixed with GUVs under the same condition as above.

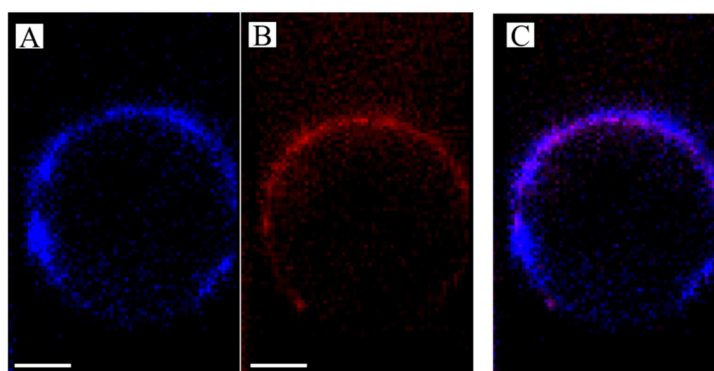


Figure 4.14: Fluorescence microscopy of a GUV showing unspecific interaction with triskelia in the absence of their cholesterol anchors. (A) Fluorescence from blue lipids (ATTO488-DPPE), (B) from red triskelia (Alexa-647) and (C) merged channels. Scale bar approximately 10 μ m.

Without magnesium, cholesterol anchors have been shown to be solely responsible for DNA origami docking onto the membrane^[223] and unspecific adhesion of GUVs would be prevented. We therefore studied the folding of our triskelion in the absence of MgCl₂. Only recently was a DNA origami assembled by Martin *et al.*^[263] in the absence of MgCl₂ by using a specific routing of the DNA scaffold and a very high concentration of monovalent ions (1.2 - 4.3 M NaCl). Inspired by this result, we folded the triskelion monomers in TE buffer with concentrations of MgCl₂ between 14 and 0 mM, and NaCl

4.4 GUV deformation observed by fluorescence microscopy

concentrations from 0 to 3 M. Two common buffers used for lipid systems^[264, 265] were also investigated: HEPES (10 mM HEPES, 10 mM MgCl_2 , 100 mM NaCl) and PBS (pH 7.4; provided by Life Technology). After folding, the samples were incubated at room temperature for 2 days and analysed by AGE and TEM (Figure 4.15).

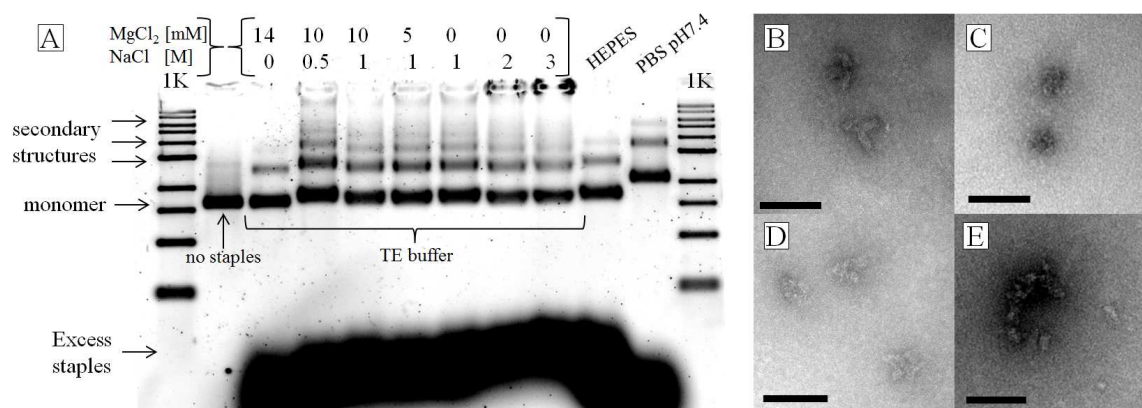


Figure 4.15: AGE of flat triskelion monomers folded in different salt concentrations and buffer. The samples are in 1x TE with various MgCl_2 and NaCl concentration, or in HEPES and PBS buffer, as indicated above the wells. Electron micrographs of triskelia monomers after 2 days in various buffers: (B) HEPES, (C) TE + 1 M NaCl (D) TE + 2M NaCl and (E) PBS pH 7.4. Scale bars 100 nm.

The agarose gel showed that the triskelion folds correctly in HEPES buffer. However, in TE buffer with NaCl (all concentrations) and PBS buffer, aggregated structures were observed as a band that remained in the well (Figure 4.15A). Imaging of these samples by TEM allowed us to observe that the triskelia were folded correctly only in HEPES buffer (which contains 10 mM MgCl_2) or in TE buffer with 14 mM MgCl_2 (Figure 4.15B-E). Triskelia thus require MgCl_2 to remain folded and polymerise, so we reduced the number of GUV added to the well during TIRF experiment to decrease the chance for two GUVs to meet and adhere due to MgCl_2 in solution.

GUV's uncontrolled tubulation and invagination due to an unbalanced osmotic pressure^[266] must be controlled in order to differentiate between morphological changes due to the flow of water^[267, 268] from those due to the DNA origami only. We used fluorescent GUV prepared with 50 mM sucrose in TE buffer + 14 mM MgCl_2 to place

the GUV in hypotonic condition, and observed inwards and outwards tubulations of the GUV in the absence of triskelia (Figure 4.16).

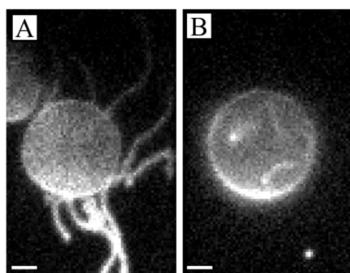


Figure 4.16: Fluorescence microscopy of GUV with blue fluorescent lipid (ATTO-488) in hypotonic condition. GUV showed tubulation (A) outwards and (B) inwards. Scale bar approximately 10 μm .

In order to explore controlled bending of a membrane by the triskelia polymer, we ought to find a compromise between taut, rigid GUVs (hypertonic) and excessively floppy GUVs (hypotonic). GUV prepared in 50 mM sucrose placed in 0.5 TBE buffer + 12 mM MgCl_2 provided ideal (slightly hypotonic) condition to observe the effect of the triskelia on large lipid vesicles with malleable membranes.

Triskelion dimers with stiff bridges (90 bp or 60 bp) functionalised with a total of 18 cholesterol and 12 red dyes (Alexa-647) were allowed to interact with GUV prepared with 50 mM sucrose and placed in slightly hypotonic condition described above. Excess polymerisation staples (4 bp sticky ends $2 \leftrightarrow 3$) were then added to polymerise the dimers on the surface of the GUVs. The blue GUV was not observed to be deformed by the polymerisation of the triskelia on its surface (Figure 4.17A), but preliminary results showed that the flat dimers form patches on the GUV observed by a bright red signal in TIRF (Figure 4.17B), which indicate that the triskelia polymerise when the 4bp sticky ends are added ($2 \leftrightarrow 3$).

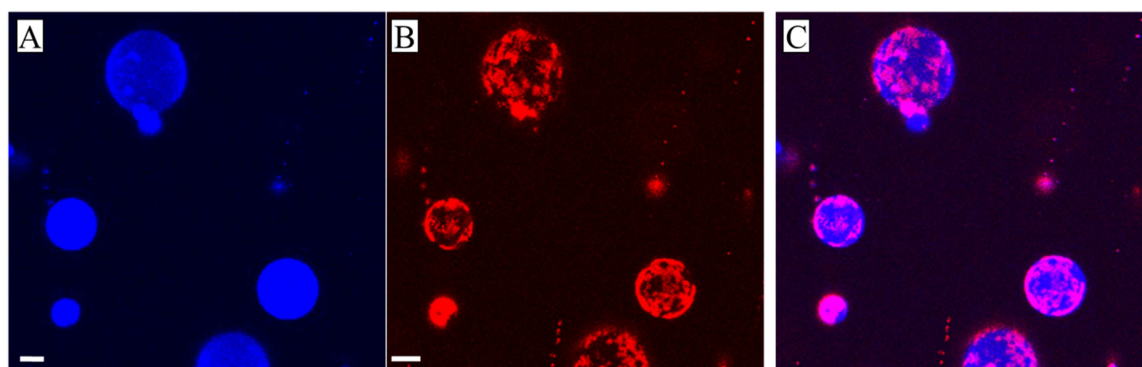


Figure 4.17: Confocal fluorescence microscopy of (A) GUV with blue fluorescent lipid (ATTO-488) and (B) polymerised flat triskelia dimers with stiff 90 bp bridges, cholesterol anchors and red dye (Alexa-647), and (C) merged channels. Scale bar approximately 10 μm .

Curved dimers formed polymerised structures that trigger budding of the GUV as observed on the blue channel during fluorescence microscopy (Figure 4.17A, C). Patches of origami were clearly identified at the base of these protruding bumps (Figure 4.18B, D) on the membrane of the large vesicles, indicating that the curved triskelia accumulate where the membrane has a big curvature satisfying the shape of the triskelion inserted into the membrane by cholesterol anchors.

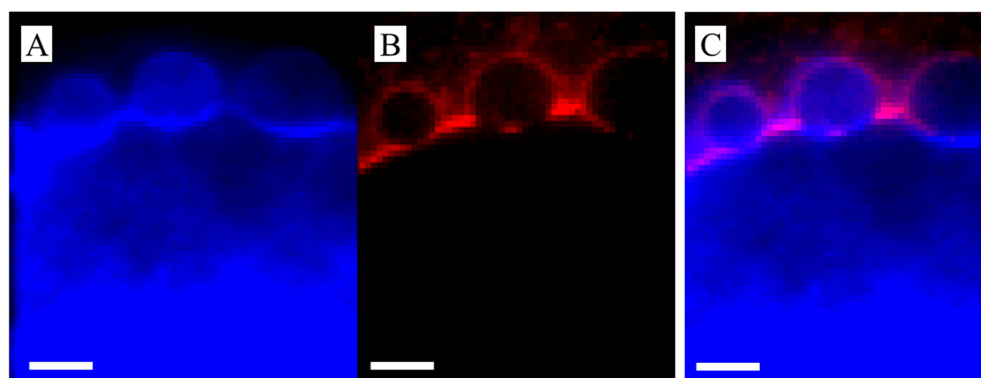


Figure 4.18: Fluorescence microscopy of (A) GUV with blue fluorescent lipid (ATTO-488), (B) polymerised curved triskelia dimers with stiff 60 bp bridges, cholesterol anchors and red dye (Alexa-647), and (C) merged channels. Images were averaged (ImageJ) over 20 frames to show the edges of the budding GUV. Scale bar approximately 10 μm .

It is theorised that flat triskelia, when put in contact with a membrane surface, polymerise into an array similar to the one observed in the experiment involving a lipid membrane on an EM grid (Figure 4.11C). The flexibility of the membrane bilayer of the GUVs accommodate the formation of hexagons and pentagons, thus showing no

detectable deformation by fluorescence microscopy as the triskelia dimers assemble into patches on the membrane (Figure 4.19A). Curved triskelia dimers gradually accumulate at the base of a budding, maintaining the membrane deformation as polymerisation occurs until it become visible by fluorescence microscopy (Figure 4.19B).

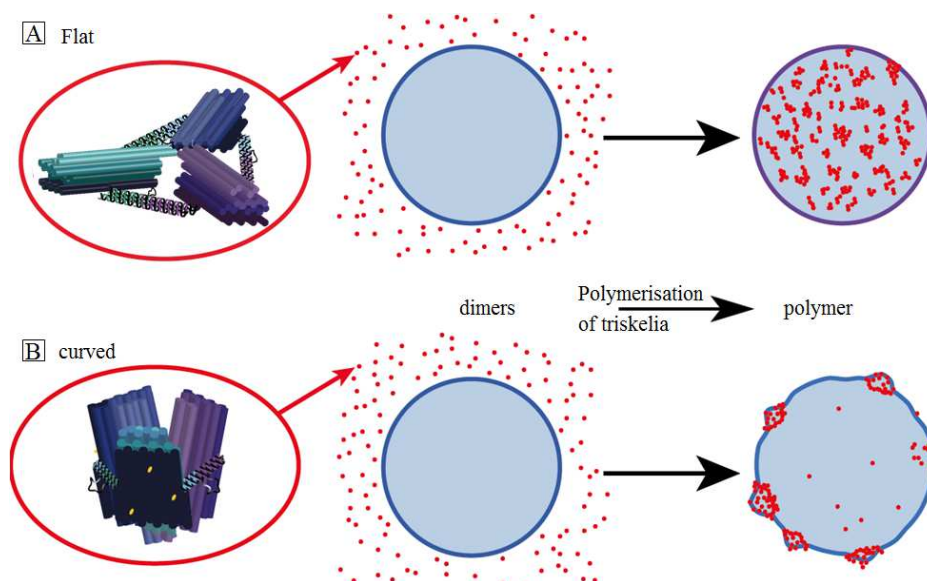


Figure 4.19: Schematic of proposed mechanism for GUV deformation by (A) flat triskelia dimers, and (B) curved triskelia dimers as the structures polymerise on the surface of a GUV.

Our Triskelion is not expected to be able to detach a vesicle from the main GUV, as the system does not drastically pinch or stretch the membrane at the base of the budding. Nature successfully uses actin and other proteins to cut the newly formed vesicle from the cell membrane (see Introduction section 1.6), a process omitted in this simplified, artificial system.

4.5 SUMMARY

Chapter 4 presented the successful interaction of triskelia dimers with lipid bilayers. We used electron microscopy to study the interaction between the triskelia and the lipid bilayers of liposomes, and using a custom-made apparatus for the formation of a lipid membrane on an EM grid. We observed that flat triskelia formed arrays with the expected hexagons and pentagons openings in the presence of a lipid membrane, which acted as a surface template. Curved triskelia produced shapes on the EM grid resembling budding made by the biological analogue, clathrin. This budding behaviour of curved triskelia was again observed by fluorescence microscopy, indicating that our polymerised, curved DNA origami forces the membrane to bend. This work is a first step towards the development of a bio-inspired system that will help us understand the mechanisms and forces involved in deforming lipid membranes. A second polymerisation strategy was presented in chapter 3, using triskelia functionalised with AuNP of 15 nm diameter. This approach was discarded because the AuNP prevented curvature of the triskelion, but provided information regarding optimal triskelion and lipid concentrations required to obtain efficient coverage of lipid vesicles.

A possible extension of this work to further explore and understand the kinetics and conditions that allow for the budding process would be to functionalise the curved triskelia with a third fluorescent dye and observe partitioning of the two colours on the membrane of a GUV as the triskelia polymerises: curved triskelia at the base of the budding, and flat triskelia array at the top. This would demonstrate that the triskelia localise on the membrane in order to satisfy the configuration of the arms maintained in place by the 90 and 60 bp bridges. An extension of the work on the lipid monolayer on an EM grid would be the imaging of a grid by SEM (with a monolayer and polymerised triskelia on it) with a tilt angle may reveal 3D profiles would be expected to support the observation revealed with TEM imaging.

Biomimetic systems such as the one presented here can serve as simplified models for the study of biological ones, but also as a starting point towards the construction of more sophisticated molecular machinery. One such machine is a drug-delivery system, with a drug protected within a lipid-DNA vesicle. The unprecedented control over DNA nanostructure allows us to address the surface with multiple ligands, which would mediate targeting to and passage through endothelial barriers to diseased tissues, as well as to accelerate cellular uptake at the desired target, while escaping expulsion and destruction by the immune system. This ultimately would maximize potency and minimize side effects of toxic cargoes. As the field is progressing rapidly, it is believed that exciting new directions will emerge.

CHAPTER 5

DNA ORIGAMI AS CRYO-EM TAG

This chapter presents an initial step in the development of a technique to couple the labelling specificity of the fluorescent molecule used in super-resolution microscopy and the high-resolution of electron microscopy. Instead of the exogenous tags currently in use^[269, 270] for electron microscopy, structure constructed with DNA origami would be genetically encoded to be recognisable solely from its shape in the cell native state.

5.1 CELLULAR IMAGING

Much of our understanding of cellular biology has developed from our capacity to directly visualise cellular functions. Advancements in structural biology and imaging techniques (fluorescence microscopy and electron microscopy) have provided detailed insight into the structure and functions of cellular processes^[271].

For example, the detection of specific and multiple structures within cells has been granted by labelling with exogenous markers, such as peptide affinity tags^[272] (strongly attaches a fluorescent dye to the target) or colloidal gold particle conjugated to antibodies^[273] (detection of the nanoparticle on electron micrographs). These tags allow the illumination and detection of multiple structures *in situ*, but despite these successes, many important biological questions remain unanswered. This is partly because current methods for electron microscopy often require isolation of the structures or heavy sample preparation techniques that denature the system. Exogenous labelling for fluorescent microscopy is limited by penetration^[274] and labelling efficiency^[275] in

addition to the loss of resolution from invasive sample preparation and light diffraction^[276].

The development of the green fluorescent protein (GFP) has allowed the observation of proteins and their interactions in their native environment^[277] with unprecedented details using fluorescent microscopy. The ubiquity of this technique was recognised in 2008 as a Nobel prize in chemistry^[278]. Similarly, the stunning technique of super-resolution microscopy (Nobel Prize in chemistry 2014^[279]) “brought optical microscopy into the nanodimension” with a resolution of 20-50 nm^[280] (Figure 5.1).

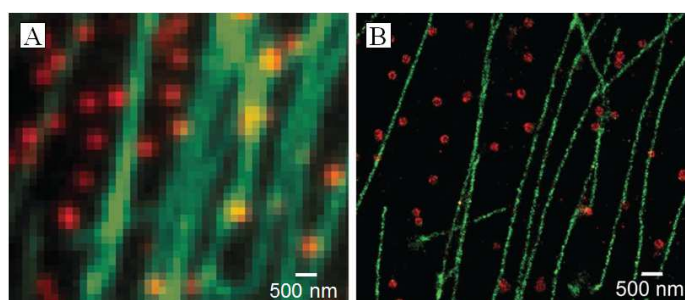


Figure 5.1: Two-color fluorescent microscopy of immunostained microtubule (*green*) and clathrin-coated pits (*red*) (images from [281]). Microtubules overlapped in the conventional fluorescence image (A) are resolved in the super-resolution image (B). Clathrin-coated pits and microtubules with substantial colocalisation in the conventional fluorescence image are clearly separated.

The crowded environment of a cell presents great challenges in data interpretation, forestalling the identification of most structures and functions with fluorescent imaging^[276]. Photobleaching of the probe remains an issue for live cell imaging^[282], as well as problems of cytotoxicity due to the laser exposure and the fluorescence emitted by the dye itself^[283].

A second revolution in bio-imaging began with the development of electron microscopy. From the first electron microscope in 1931^[284] to the development of cryogenic electron tomography (cryo-ET) in 1990^[284, 285], the quality of image has been improved to a resolution as little as 0.4 nm^[280], for the Zika virus. Cryo-ET is now another technique commonly used in modern cell biology to study the cell at the nanoscale, and relies on fast-freezing of the sample to “capture” specimens in a near-native state for imaging.

Images obtained by transmission electron microscopy rely on variation in refractive index of the specimen, which gives rise to phase contrasts, and are therefore in grey scale, although colour can be added to the image afterwards and “false-colour” electron micrographs can be beautiful^[286] (Figure 5.2). However, the lack of contrast makes it difficult to identify structures of the cell in the crowded cytoplasm. Computational methods have been developed to recognise known structures in 3D, but do not work well in congested cellular environments. Unless specifically tagged, only large and distinct structures such as viruses, ribosomes and organelles can be identified by computer-aided analysis (Figure 5.2).

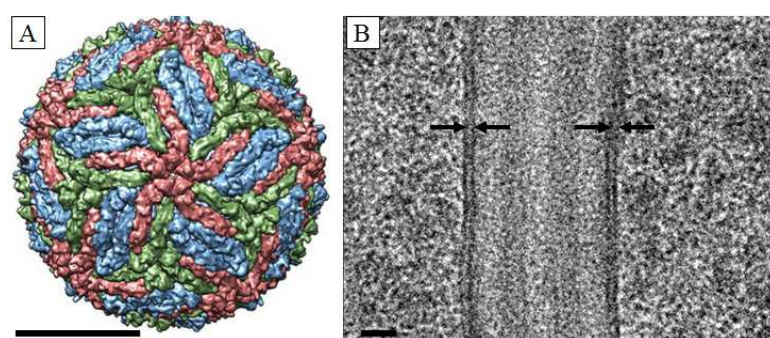


Figure 5.2: (A) false-coloured Cryo-EM topography of the Zika Virus. (reference [280]) (B) Identification of zones of intermediate densities between two cell membranes associated with the presence of proteins. Note that the two lipid layers are identifiable (arrows) (reference [286]). Scale bars 20 nm.

At present, there is not a standard protocol that uses molecules for endogenous tagging of cell components for electron tomography. Such a tagging technique would provide the identification of structures, analogous to the results provided by GFP in fluorescent microscopy, with the resolution provided by electron microscopy.

5.2 NUCLEIC ACID NANOSTRUCTURES IN CELLS

The elegance of self-assembly techniques in nanotechnology has allowed scientists to anchor synthetic DNA nanostructure, such as a DNA tile^[3, 287], to various proteins or other cell components to build complex hierarchical assemblies in artificial biomimicking systems. Recently, scientists from different research groups demonstrated

that DNA nanostructures can be delivered to the cytoplasm of living cells^[288-291]. For example, the DNA tetrahedron, which was first introduced by the Turberfield group^[291] was showed to remain substantially intact 72 hours after transfection. It has also been shown that a DNA nanostructure, susceptible to degradation, can be protected by encapsulation by lipids^[6] before cell delivery. The DNA tetrahedron was recently further functionalised by Keum *et al.*^[292] for carrying a single-stranded loop that caused antisense gene silencing in mice. The incorporation of synthetically designed DNA nanostructures into cells is limited by the machinery that bacteria and cells have developed to identify and eliminate foreign DNA material from the cytosol^[293]. Nucleases, among other DNA sensing devices, are proteins that selectively cut the chain of oligonucleotides to recycle or expel the degraded nucleobases.

It is postulated that if a cell contains the genetic blueprints that assemble oligonucleotides to produce a 3D, RNA nanostructure, the resulting structure would be recognised but not degraded by the immune system of the cell. This strategy is used by viruses to deceive a cell into producing their RNA genetic material. Aldaye and Silver^[294] were inspired by this approach and made a dramatic step towards the integration of synthetic nanostructures into cells: RNA components, produced by transcription from plasmid-encoded RNA strands within *E.coli*, assembled *in situ* to produce a 2D RNA array displaying aptamers that recruited two metabolic enzymes to form a high-density array with enhanced substrate turnover (Figure 5.3). The use of RNA as a structural material was pioneered by Jaeger^[295] who has shown that tertiary RNA interactions provide greater control over shape than is normally feasible with the flexible branch junctions used in DNA structures. However, nanostructures made of RNA have not been used in Cryo-ET yet.

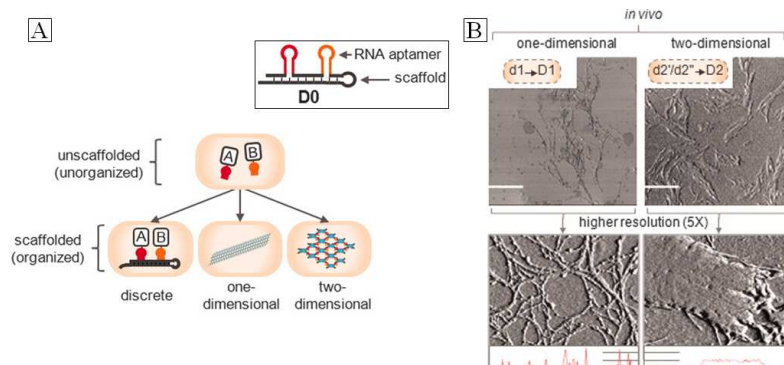


Figure 5.3: Design of RNA modules to organize proteins by Aldaye and Silver^[294]. (A) Protein A and B scaffolded onto discrete, 1D, and 2D RNA assemblies. Inset: detail of the RNA aptamer, on the scaffold, that bind to the proteins A and B. (B) AFM analysis of purified assemblies *in vivo*. Scale bars 100 nm.

5.3 DESIGN OF A CRYO-EM TAG

Inspired by the examples presented above, it was proposed that genetically engineered plasmids could be used to introduce the sequences required for the construction of an endogenous tag for EM imaging. The specific sequence of the tag will be produced by the cell, which will then self-assemble directly within the cytoplasm, avoiding issues associated with the insertion of exogenous material. This tag will bind to specific macromolecules in the cytoplasm, such as proteins, and allow their localisation with nanometre resolution by Cryo-ET. Unlike super-resolution fluorescence microscopy, the RNA nanostructure tag will localize targeted molecules in their native condition, while revealing the surrounding cell subcomponents with similar 3D resolution. Furthermore, this will allow imaging of cellular components in the absence of dyes that can photo-damage the sample^[296].

The use of RNA as a building block presents construction difficulties as secondary structures can create tertiary interactions that make folding hard to predict^[297]. The new structure tag must be recognisable when observed at any angle.

The organelles inside a cell fill the cytosolic space with a multitude of shapes, textures, and sizes which appear with different contrasts on an electron micrograph. TEM and SEM images of cellular structures inspire our imagination to observe a “smiling” cell

(Figure 5.4A), for example. However, a non-natural shape such as the DNA tetrahedron^[298], can be identified by Cryo-EM when imaged in buffer (Figure 5.4B, C).

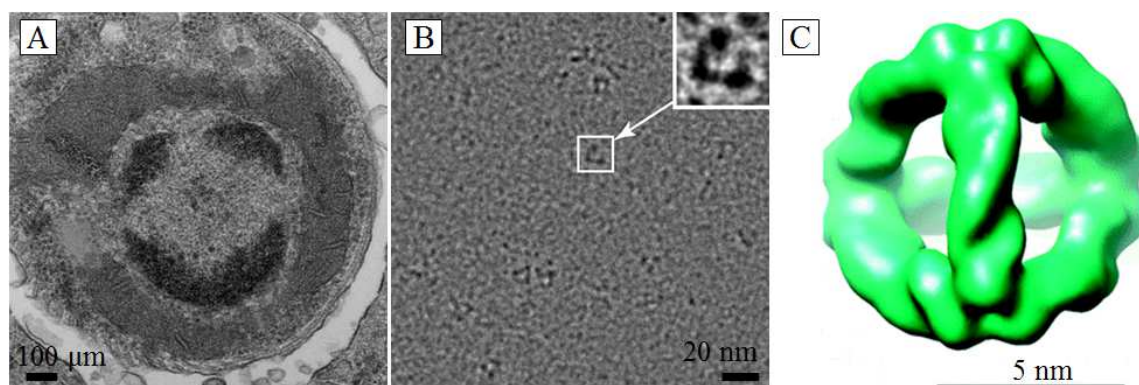


Figure 5.4: Electron micrographs showing (A) substructures in a cell appearing like a smiley on TEM. Image from the gallery of the Dunn School of Pathology. (B) Contrast enhanced cryomicrographs of a DNA tetrahedron. Inset: magnified image of a particle cut out of the micrograph. (C) 3D density map of the DNA tetrahedron of (B). Images (B) and (C) from reference [298].

As proteins and other compartments in a cell tend to appear as spheroidal shapes, we decided to design a structure with clear angles and edges to contrast with the cell surroundings. We anticipated that a hollowed, tetrahedra origami structure would allow easy recognition in EM imaging by the regular positioning of the edges independently of the angle of observation (Figure 5.5).

It was proposed to use DNA as the building block initially instead of RNA for ease of design and synthesis. Initial tests would be carried out in cell lysate (internal fluids containing the content of cells), progress towards nanostructure in interaction with lipid membrane through cholesterol interaction, and then nanostructure inserted inside cells by electroporation.

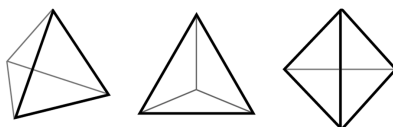


Figure 5.5: Schematics of tetrahedron view from 3 different angles.

5.4 DESIGN OF A DNA ORIGAMI

The construction of a tetrahedra DNA nanostructure required the shorter sequence of the plasmid pUC19 as the scaffold. This plasmid contains 2686 bases and is approximately one-third of the length of the previously used M13 plasmid implemented for the triskelion. The edge of the tetrahedron was designed as a 6-helix bundle of approximately 27 nm; these bundles provide rigidity to the structure. The six edges are linked together by the scaffold with 7 unpaired bases (Figure 5.6A), which provide flexibility at the vertices. The vertices of the tetrahedron are thus created by joining three 6-helix bundles together. To ensure that the rigid helix bundles do not twist along the edge, we linked together the two helices that are closest to each other in the vertex; this corresponds to linking helix 1 with helix 6 (Figure 5.6B). Staples ensure the proximity of the helices by multiple crossovers.

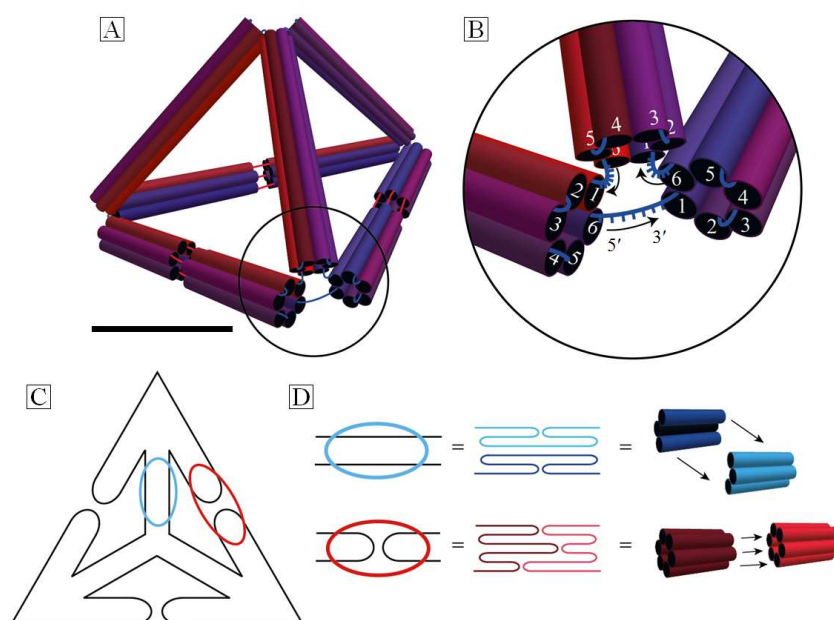


Figure 5.6: Schematics of the DNA tetrahedron. (A) 3D view of the entire structure. Each DNA double helix is shown as a tube, and the edges are 6-helix bundles. Red lines represent staples binding two helix-bundles together, and blue is the scaffold strands. Scale bar approximately 20 nm. (B) Details of the summit vertex corresponding to the black circle in (A). The scaffold routing and unpaired bases are shown in blue. A black arrow indicates the direction of the DNA; the 3'-end located at the head. The staples are not drawn for clarity. (C) Schematics of the general routing used for the construction of the tetrahedron. (D) detail of (top) lengthwise and (bottom) crosswise type of edges. Each line of the schemes on the right corresponds to the scaffold routing, which is maintained by the hybridisation of staples (not shown for simplicity).

The pUC19 plasmid, similar to the M13 scaffolds normally used for DNA origami^[59], is a circular ssDNA. As such, we had to design a routing that would pass only once over each helix and yet complete all the 6-helix bundles, while avoiding nodes. Two types of edges were designed to follow these requirements (Figure 5.6C). The first type of edge is formed when a pair of 3-helix bundles assembles and is locked in place by staple crossovers. (This can be seen as cutting the helix-bundle lengthwise (Figure 5.6D top). The second type of edge is assembled by staples linking two 6-helix bundles together. (This can be seen as cutting the helix-bundle crosswise (Figure 5.6D bottom).) The final structure is made of a total of 69 staples of 40-50 nucleotides (sequence in Annex A.16). The sequence and routing of the tetrahedron was made using Cadnano^[60] (See Annex A.17) and the stability of an individual arm was verified using the simulation provided in Cando^[65, 299]. A staple finishing at the extremity of an edge (3'-end, or 5'-end) can be elongated to offer a sticky end or a DNA handle for further functionalisation. The tetrahedron thus also offer anchoring sites at the vertices where DNA handles can be added to attach to aptamers and other cell components.

5.5 SYNTHESIS OF THE DNA TETRAHEDRON

The pUC19 plasmid required for the DNA origami tetrahedron was supplied in its double-stranded form (*dspUC19*) by New England Biolabs and was treated with nicking restriction enzymes and exonuclease (Annex A.2.4) to isolate the single stranded *sspUC19* scaffold. The purified *sspUC19* was mixed with ten-fold excess of staples in 1 x TE buffer (pH 7.5) and different MgCl₂ concentrations to determine the optimal folding conditions for the new origami. The mixture was annealed overnight using the same thermal ramp that was applied to correctly fold the triskelion (Chapter 3). The samples were then analysed by AGE and TEM (Figure 5.7 and 5.8).

5.5 Synthesis of the DNA Tetrahedron

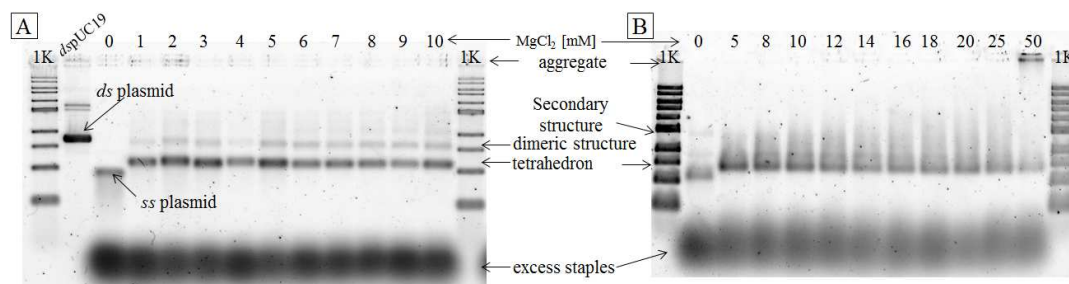


Figure 5.7: AGE of tetrahedron folded in 1 x TE buffer and various MgCl₂ concentrations. The concentration is marked above the well in [mM]. A DNA ladder is shown at both ends of the gel for reference, and the double-stranded scaffold. (A) Concentration between 0 and 10 mM, and (B) concentration between 0 and 50 mM.

The tetrahedron appeared to assemble with the lowest concentration of MgCl₂ added (1mM), as observed as a single band in the agarose gel. Above 16 mM of MgCl₂, smeared bands above the band corresponding to the tetrahedron increased in intensity. Aggregation of the DNA structures were observed in the well when 50 mM MgCl₂ concentration was reached (Figure 5.7). The bands attributed to the tetrahedron at increasing MgCl₂ content were extracted from the gel and imaged by TEM (Figure 5.8).

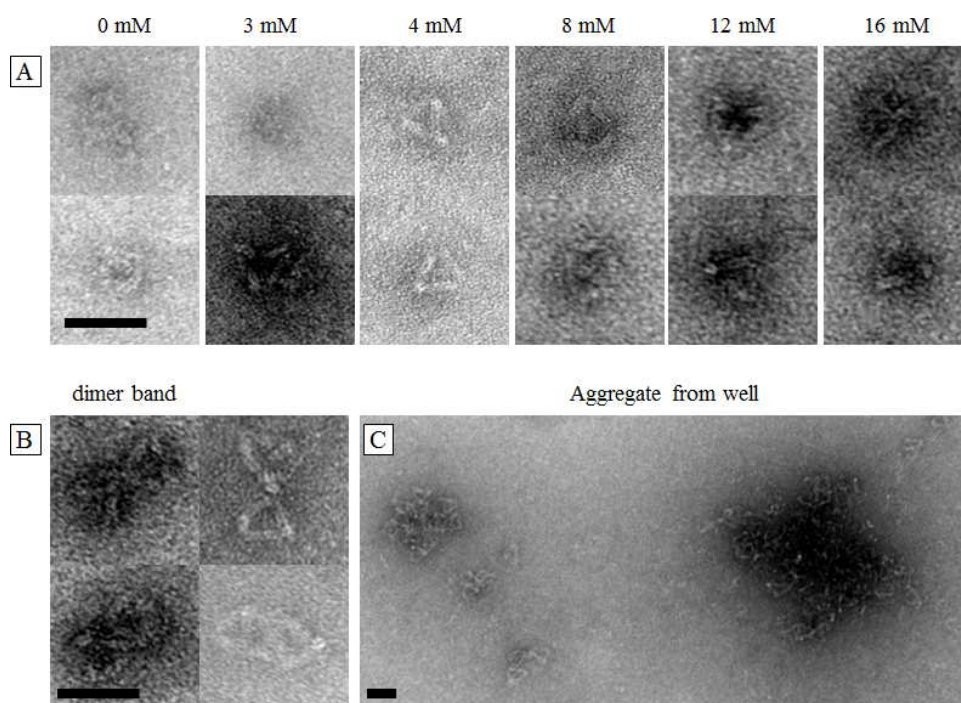


Figure 5.8: Electron micrographs of tetrahedron in 1 x TE and (A) 0-16 mM MgCl₂, as written above the image. (B) Dimers from the folding with 10 mM MgCl₂, and (C) aggregate of tetrahedron in 50 mM MgCl₂. Scale bars 50 nm.

TEM imaging revealed that the tetrahedron is correctly folded when 4-14 mM MgCl_2 is present, with a limited number of multimers. We thus used 10 mM^[6, 59, 60] of MgCl_2 in future folding experiments. At MgCl_2 concentrations of 0 or 3 mM, although some tetrahedra were observed, the majority of the structures did not show sharp edges corresponding to a well folded 3D structure (Figure 5.8A). At concentrations of MgCl_2 above 14 mM, aggregated tetrahedra were observed (Figure 5.8C). After extraction of the band attributed to dimeric tetrahedra from the agarose gel, electron micrographs revealed the presence of two tetrahedra attached at their vertices (Figure 5.8B); we believe the attachment is created by one or more shared staples. It was anticipated that a high concentration of scaffold during folding, the likelihood of interacting scaffold would increase and dimeric or aggregated tetrahedra would result. To examine this, the tetrahedron was folded at concentrations of sspUC19 between 5 and 100 nM and a ten-fold staple excess, and then analysed by AGE and TEM (Figure 5.9).

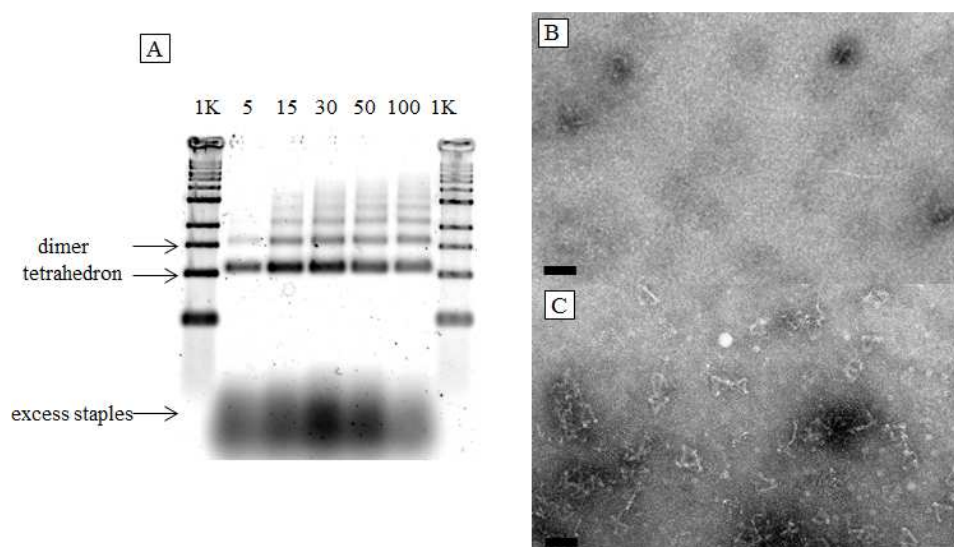


Figure 5.9: AGE (A, B) and TEM (C, D) of tetrahedron folded at different scaffold concentration. (A) Concentration in each well normalised to 5 nM post-folding. The concentration at which the tetrahedron was folded is indicated above the wells. (B) Electron micrograph of tetrahedron folded at 5 nM scaffold, and (C) 50 nM scaffold. Scale bars 50nm.

As shown by the agarose gel in Figure 5.9A, the increased concentration of scaffold resulted in an increase in dimeric and aggregated tetrahedra, relative to the monomeric

structure. We therefore determined that for optimal yield of monomeric tetrahedron, this DNA origami should be folded at 5 nM scaffold concentration.

We further developed the tetrahedra structure to accommodate a handle at each of the four vertices, by using extended staples. Such a handle can be used to hybridise various DNA-conjugates such as a DNA-cholesterol or a DNA-interacting protein. To determine if a DNA handle from the tetrahedron can indeed hybridise with a DNA conjugate, we incubated a 20 nM sample of PEG-purified tetrahedron with the dye Alexa-647 for two hours at 30°C. AGE analysis confirmed that the tetrahedron successfully inserts these handles as illustrated in Figure 5.10A and B. As cholesterol anchor are incorporated to the structure using the same handle, we assumed that cholesterol handle can be applied to the tetrahedron following the same protocol for the dye incorporation and yield similar result. Correctly folded tetrahedra with cholesterol were observed in TEM micrographs (Figure 5.10C).

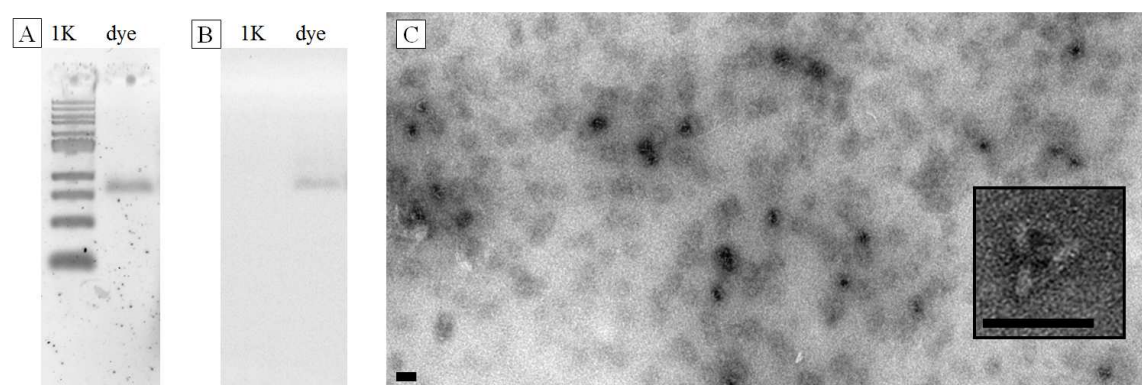


Figure 5.10: AGE of tetrahedron functionalised with Alexa647. (A) Gel stained with SYBR gold, and (B) detection of the fluorescence of the dye. (C) Electron micrograph of tetrahedron decorated with cholesterol at the vertices instead of the dye presented in (A) and (B). Inset: detail of one tetrahedron. Scale bars 50 nm.

For applications to cellular uptake, DNA origami should reach concentrations of micromolar^[6, 300, 301]. We therefore needed to find an efficient concentration method to increase the concentration of the tetrahedron post-folding.

This origami, unlike the previously discussed triskelion (Chapter 3 and 4), is a closed structure, and we could thus successfully use PEG purification, followed by removal of residual PEG using the QIAquick PCR purification kit by Qiagen (with buffers supplemented with MgCl₂ to a final concentration of 5mM). PEG molecules, as a cryo-protectant, must be thoroughly removed during sample preparation for cryo-EM to prevent ice artefacts. A final concentration of between 1.9 and 2 μ M was measured by UV-absorption. This concentration is believed to be a good starting point for investigating cell uptake and Cryo-EM^[6, 209, 300].

Tetrahedra without cholesterol were purified as described above by Emma Silvester at the Clarendon laboratory and imaged by Cryo-EM in mammalian cell lysate by Lindsay Baker at the Division of Structural Biology (Strubi), Wellcome Trust Centre for Human Genetics (Figure 5.11). Cryo-EM sample preparation and imaging was performed following the thorough protocol described in the review by Dubochet *et al.*^[302] (See also Annex A.12.2).

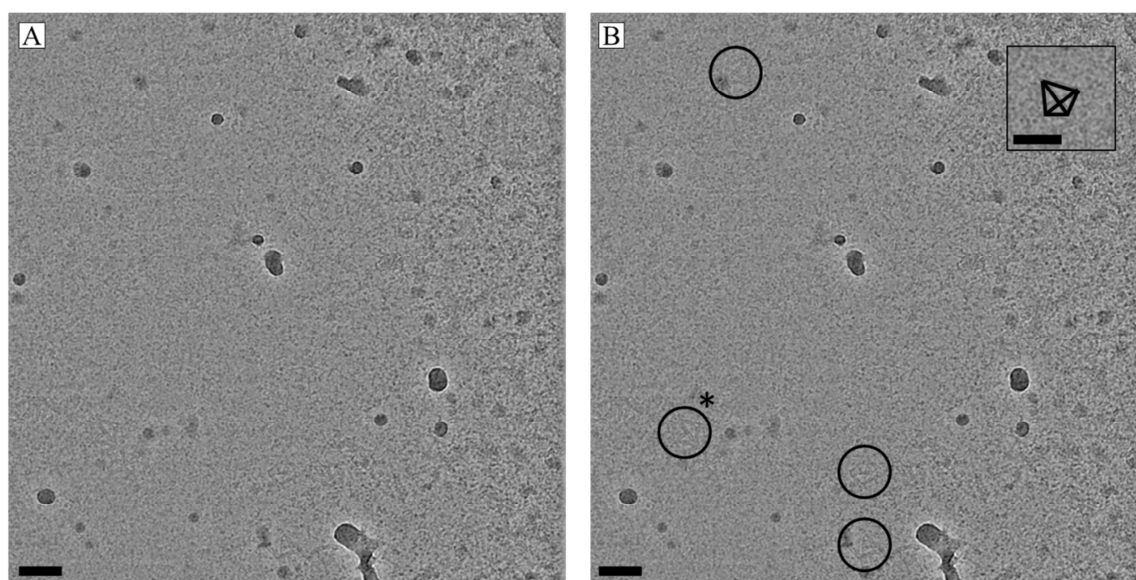


Figure 5.11: Cryo-EM micrographs of tetrahedra in cell lysate. (A) Micrograph without annotation, (B) micrograph with DNA tetrahedra circled in black. Inset: highlighted tetrahedron indicated by * on the micrograph. Scale bars 50 nm.

Samples for cryo-EM are not negatively stained, and thus present weaker contrasts on the micrographs. Correctly folded tetrahedra were successfully imaged by cryo-EM, and were clearly identified in cell lysate. This structure is thus a good reference for the design of RNA structure of similar size and shape.

5.6 SUMMARY

We have shown here the design and successful synthesis of a DNA origami tetrahedron using the plasmid pUC19. This structure folds efficiently and can be purified and concentrated for use in Cryo-EM imaging. Visualisation of the tetrahedron in cell lysate confirmed that the DNA origami can be localised straightforwardly due to its regular features. The tetrahedron described in this chapter will be an excellent candidate to help determine the optimal size and shape of a structure required for an endogenous Cryo-EM RNA tag.

It is hoped that this research will lead to the genetic engineering of a plasmid bearing the sequence required for the production and self-assembly of a 3D RNA nanostructure within the cell. This new endogenous marker could be functionalised, using the handle placed at the vertices, to target specific organelles or proteins of the cell, allowing their localisation and study by cryo-EM, while not being degraded by the immune system. This would be the first artificial endogenous marker ever designed, and the promise of new developments in synthetic biology.

CHAPTER 6

OPTIMISING PROCEDURE FOR TRANSMISSION ELECTRON MICROSCOPY

Transmission electron microscopy (TEM) has been widely used throughout this thesis to visualise and characterise a range of DNA structures, and to investigate their interaction with lipid bilayers. The appearance of liposomes, DNA or a mixture of both in electron micrographs depends greatly on a multitude of factors, including sample deposition, staining methods, type of grid, and the final placement of the grid on the sample holder for imaging. This methodology chapter presents and discusses the various techniques and procedures that required optimisation in order to correctly visualise DNA origami and their interactions, and provides recommendations for TEM imaging of DNA and lipid structures.

6.1 PREPARATION OF THE EM GRID

6.1.1 Formvar and carbon-coated grids

The nature of the sample (eg: cells, nanoparticles, DNA origamis) determines how the sample will be prepared. DNA origami and lipid vesicle are considered particulate samples, and were treated as follows. A particulate sample that is to be visualised with TEM is deposited as an aqueous droplet on a thin layer of carbon supported on a grid (Figure 6.1). This carbon surface can sit on a thin polymer support film, such as formvar (a poly(vinyl formal)), which prevents the carbon film from cracking during handling of

6.1 Preparation of the EM Grid

the grid. Non-coated copper or gold grids purchased from Alfa Aesar were coated with a support film and carbon layer as described below (see also reference [303]). Copper grids with mesh sizes between 200 or 400 (200-400 holes within 1 inch diameter) were used routinely for standard sample preparation, and gold grids for samples that required long incubation on the grid to avoid oxidation, which occurs with copper grids upon exposure to salts and would appear as black marks in the TEM.

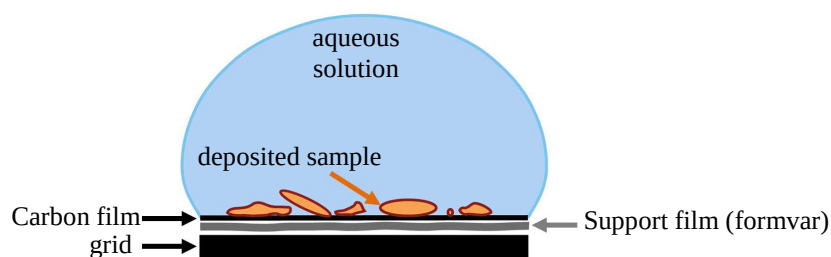


Figure 6.1: Schematic of an EM grid with a droplet of sample deposited on it.

To prepare a coated grid, a clean glass microscope slide was first dipped lengthwise in 0.2% (w/v) formvar in chloroform. The slide was dipped quickly to ensure a thin coating and allowed to dry for a few minutes sitting vertically. A razor blade was then used to score the edges of the slide with the dried film. To help the film detach from the glass, it is advised to gently breathe against the surface until droplets start to form. The slide was then immersed at a shallow angle into an ultrapure water bath (scored face up) to allow the film to detach and float on the water. Bare grids of either copper or gold were then arranged on the floating film (Figure 6.2 A).

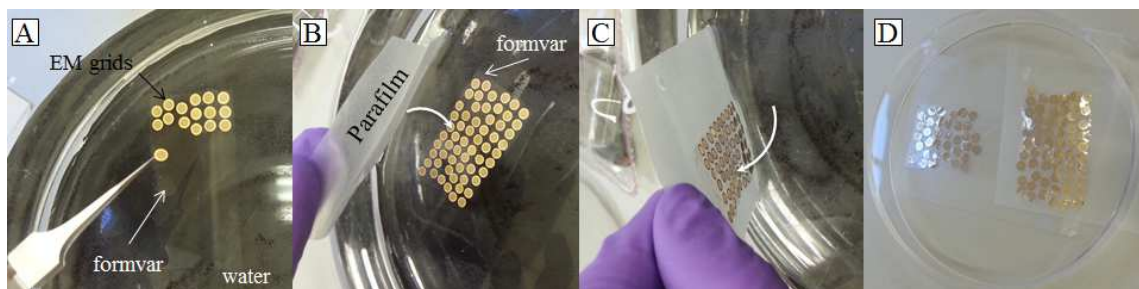


Figure 6.2: (A) EM grids are deposited onto the formvar film floating on a water bath. (B) Once filled, a piece of Parafilm is gently pressed onto the formvar film and (C) removed from the water. (D) The Parafilm with the formvar-coated grids are left to dry protected from dust before carbon coating.

These non-coated grids possess a dull face and a shiny face. (The shiny face was selected to be in contact with the formvar for this thesis.) It is recommended to arrange the grids with a consistent orientation on the formvar film; this arrangement will allow the identification of the face onto which the sample will be later deposited. Once covered with grids, the formvar film can be removed from the water bath by a confident movement using a piece of Parafilm slightly bent at the end (Figure 6.2B, C). The end of the Parafilm piece was aligned and pressed against the formvar film and into the water to adhere the polymer and the grids to the Parafilm. The Parafilm with the grids and the formvar film should then be left to dry thoroughly (~12 hours) protected from dust (Figure 6.2D) before being coated with carbon in a carbon coating unit. Formvar coated grids were then carbon coated using a Quorum 150R ES coating unit. When a vacuum of 0.25 mbar is reached in the unit, the carbon cord is heated to 2400°C by passing a high current (>30 A) into it, which will evaporate the carbon and form a thin layer (10-40 nm thick) on the Parafilm/grids placed underneath. The thickness of the carbon can be adjusted by controlling the current passing through the carbon cord.

6.1.2 Glow-discharge

Samples under investigation are often deposited as a single aqueous droplet to the top of a carbon coated TEM grid. However, the hydrophobic carbon layer can minimise the droplet contact with the carbon film. As a result, the droplet will not wet the grid uniformly and the sample will be unevenly distributed^[304]. Although samples in solvent with a low surface tension such as ethanol can easily cover the grid, most sample preparations are in aqueous medium with a high surface tension and thus form local droplets on the grid. Wetting of the support film is a complex phenomenon difficult to assess quantitatively and is extremely sensitive to contamination^[183, 305].

The process of glow-discharge is commonly^[306, 307] used to create an evenly hydrophilic surface on the carbon film such that the sample is equally distributed. Furthermore,

hydrophilicity of the film will promote a thin and regular distribution of the stain on drying, which cannot be accomplished by any other means. During a glow-discharge treatment, the carbon-coated grids are placed in a chamber and a partial vacuum is applied. A high voltage is applied between the cathode and the anode placed at either end of the chamber, which creates plasma in the chamber that can be seen as a violet glow. The remaining gas is ionised by the high voltage and the anions deposit on the surface of the carbon film, providing the grid with a thin, hydrophilic, and slightly negatively charged surface; impurities (along with some carbon) on the carbon film are simultaneously burnt away. A sample is deposited on a glow-discharged grid immediately after preparation, as the surface only remains hydrophilic for several minutes. Positive, hydrophilic macromolecules such as DNA, adhere to the grid and can be easily examined under these conditions^[308].

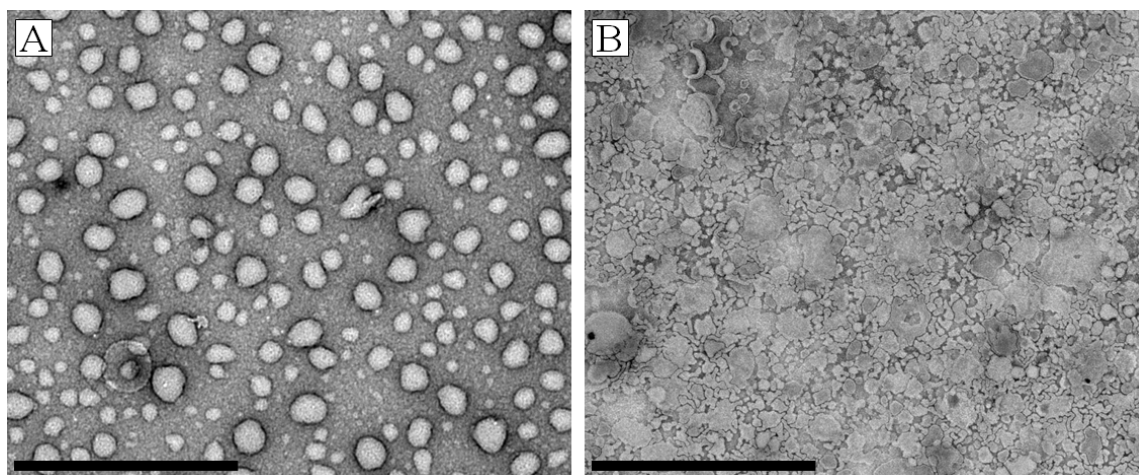


Figure 6.3: liposomes (0.1 mg/ml) deposited and stained on a grid that (A) has not been glow-discharged, and (B) has been glow-discharged. Scale bars 1 μm .

Lipids such as DOPC, DPhPC and other regularly used lipids in biotechnology have a local positively charged head groups. As such, they attach particularly well on a glow-discharged grid. Unfortunately, these efficient and strong interactions often result in vesicle bursting or create an excessively dense film of liposomes on the grid. The clear difference in the appearance of the two electron micrographs in Figure 6.3 (images representative of the entire grid) results from grids that were not (Figure 6.3A) or were

(Figure 6.3B) glow-discharged. The same sample of liposomes (0.1 mg/mL) and the same batch of grids were used for this comparison. The two grids were stained in parallel following the same procedure (5 μ L sample, 1 min deposition, then wash and 10 sec staining). The dense coverage of liposomes on a glow-discharged EM grid was a major challenge when trying to observe liposome deformation with the polymerised triskelia (chapter 4). To limit this effect, grids that were not glow-discharged were used during deposition of concentrated DNA samples (≥ 5 nM DNA) and liposome-containing samples (≥ 0.1 mg/mL lipids).

6.2 SAMPLE STAINING

A correctly stained sample for TEM imaging can mean the difference between observing and not observing a particular specimen or interaction. Too little staining will not reveal intricate details of a complex sample, and excess staining can hide important features. The image quality depends on a combination of the type of stain, the type of grid, the staining technique and the nature of the sample. The principle of negative staining relies on forming a pool of stain around the specimen and in its aqueous domains, but the sample (Figure 6.4A). Positive staining occurs when the stain binds to the specimen (rather than to the background), which will then appear dark on a lighter background (Figure 6.4B).

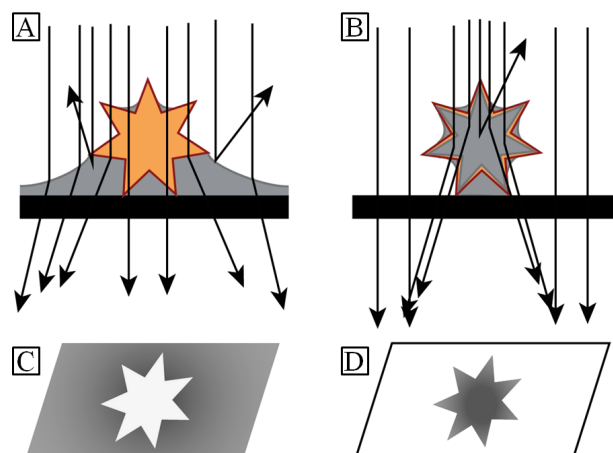


Figure 6.4: Schematic of (A) negative and (B) positive staining resulting from the interaction of the electrons with a stained sample. Image obtained from (A) positive staining and (B) negative staining.

Negative staining is believed to reduce the surface tension at the air-liquid interface that would otherwise considerably flatten the biological sample on the support film. However, a lot of water remains bound to the sample and in the stain that is pumped away in the TEM airlock vacuum, which does have a flattening effect. Positive staining produces fewer drying artefacts, because the stain is embedding the sample which is therefore less distorted than a negative stained sample would be. The stain contains heavy metal ions (such as uranium, molybdenum, tungsten) that will strongly adsorb to biological matter^[303] and scatter the electrons during imaging, hence generating a well contrasted image. These metal solutions contain complex polycations or polyanions, and their stability is highly dependent on the pH of the solution, in addition to the “ageing” and photolability^[181, 309]. Despite a limited understanding of the complex physical chemistry of negative staining, a wealth of practical experience has been accumulated^[302-311] and some important experimental details for optimal staining of DNA structures and lipid vesicles are discussed below.

6.2.1 Types of stain

Uranyl acetate (UAc) was introduced in 1960 by Van Bruggen *et al.*^[312] as a negative stain for TEM and has since become the most widely used negative stain^[182, 183, 302-314]. Uranyl acetate is generally prepared at pH 4.5 as the salt becomes unstable and precipitates at pH ≥ 6 ^[182]. A solution of 1-2% (w/v) uranyl acetate should be stored at 4°C and protected from light as it is highly photosensitive. Like many other negative stains, a solution of UAc should be filtered before use as the salt can precipitate. Recent studies demonstrated that UAc solution fixed protein structures on the millisecond timescale and thus solved problems of pH-induced conformational changes and aggregation^[313]. However, uranyl-based negative stains were also reported to create undesirable aggregation due to the presence of cations^[182, 183].

As DNA structures are not stable at pH below 5^[314, 315], the acidic UAc solution for staining was an initial concern for sample stability. Therefore, the effect of exposure time of the UAc stain on triskelia-coated liposomes was characterised (Figure 6.5).

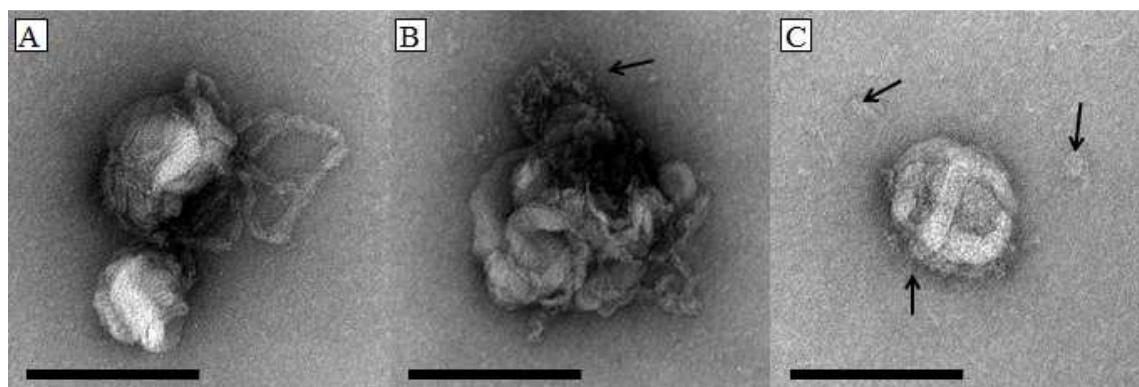


Figure 6.5: Electron micrographs of triskelion-coated liposomes when stained with UAc for (A) 10 seconds, (B) 30 s and (C) 60 s. Arrows indicate damaged triskelia. Scale bars 200 nm.

Triskelia-coated liposomes stained for 10 seconds were observed to undergo minimal 3D flattening and aggregation, and maintained excellent integrity and image resolution (Figure 6.5A). The triskelia started to appear damaged by the acidic UAc solution if the

stain was allowed to interact with the sample for longer than 30 seconds (indicated by black arrows in Figure 6.5B and C).

We also investigated other common negative stains to observe their effect on the sample quality^[316], including phosphotungstic acid (PTA) and sodium silicotungstate (SST) and different uranyl salts were also investigated (uranyl formate (UFo) and magnesium uranyl acetate (UMgAc)). Negative staining solutions were prepared as described in the literature^[182, 311].

We mainly used liposomes during these investigations as they were identified as being the most sensitive to osmotic shock and deformation during negative staining. Both tungsten-based stains, PTA and SST, showed acceptable staining for liposomes (Figure 6.6A and C). The lipid vesicles maintained a spherical shape on the EM grid and were easily identified. However, when the magnification was increased, the granularity of the SST stain was apparent, an effect also observed and reported by Harris^[182, 311]. This granularity can be compared to the size of a pixel: the smaller the pixel, the better the image resolution; and so the smaller the granularity, the better the image. Thus, this increased granularity became an issue for identifying the fine features of the origami, exacerbated when interacting with a lipid vesicle. In addition to the uneven coverage of the grid by the sample stained with PTA or SST, the main issue was the weak staining of the DNA structures (Figure 6.6B and D). When a sample of triskelion-coated liposomes was stained with PTA (Figure 6.6A and C), the details of the DNA structure were barely visible. The liposomes were well preserved, but the DNA can only be seen as a shadow, which was unsuitable for proper identification. For comparison, one uranium-based stain (UAc) is shown in Figure 6.6E and F and illustrate the finer resolution of the triskelia.

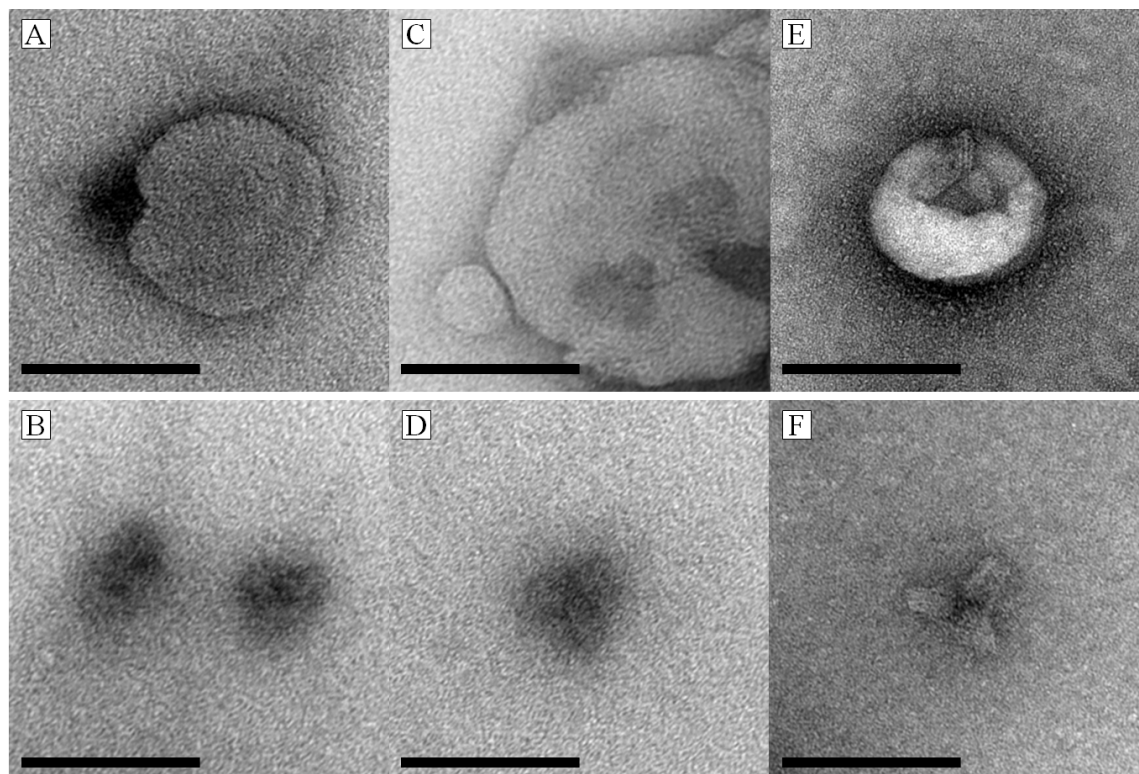


Figure 6.6: Electron micrographs of the same sample of (top panel) triskelia monomer on liposomes and (lower panel) stained with (A, B) SST, (C,D) PTA, (E, F) UAc. Samples prepared the same day using the same batch of EM grid. Scale bars 100 nm.

When samples were stained with UMgAc, the presence of the magnesium cations in the uranyl-based negative stain did not significantly help the origami to maintain its shape, as it was expected to do (Figure 6.7A). Uranyl formate was mainly used for the work on the three-way junction DNA array described in chapter 2. Very little distinction could be made between the UAc, UFo and UMgAc (Figure 6.7B and C), although the latter two were less stable in solution when stored at 4°C and were observed to precipitate more quickly.

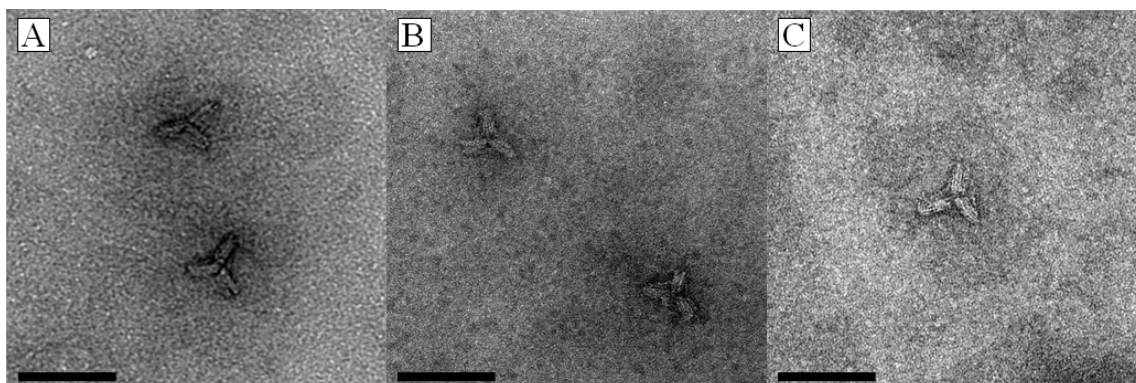


Figure 6.7: Electron micrographs of triskelia stained with (A) UMgAc, (B) UFO and (C) UAc. Scale bars 100 nm.

To summarise, although tungsten-based negative dyes were less aggressive towards the samples due to their neutral pH, they provided limited contrast and irregular stain thickness. Despite the requirement of a shorter staining time, UAc solution presented better stability and a better overall uniformity of staining than its uranyl-based analogues. Uranyl acetate was therefore the main negative stain solution used in this thesis to observe DNA nanostructure and lipid vesicles with EM.

6.2.2. Optimising staining methods

Among the various staining techniques that have been developed to observe particulate biological specimens with electron microscopy the most common method is called the droplet technique^[317, 318]. A typical sample preparation in the present study consisted of placing a 5-10 μ L droplet of 1 nM DNA on a glow-discharged grid for 3 minutes; with the grid held by a reverse action tweezer. Excess liquid was removed by carefully touching the edge of the grid with a piece of filter paper. The sample was then quickly washed with 7 μ L of stain solution and then blotted once more with filter paper. A second droplet of stain was then applied to the adsorbed sample and left on the grid for a maximum of 10 seconds before being blotted away. This protocol was applied to liposomes and triskelion-coated liposomes, but the fragility of the vesicles and

irregularity of the grid wetting required special modifications, attention and practices, as detailed below.

The adsorption time of the DNA nanostructures and liposomes onto the grids varied according to the concentration of the sample. A sample of a few pM was left for 10 minutes, where a 2 minute incubation was sufficient for samples of tens of nM before removing excess solution. This was particularly true for liposomes, as they cover the entire surface with multilayered lipid membranes if the concentration is not carefully controlled.

When a sample of DNA nanostructure prepared in a physiological buffer was applied to an EM grid, staining the sample with UAc resulted in the formation of microcrystals that contaminate the sample^[310] (particularly with a phosphate buffer) and obstruct the sample during imaging (Figure 6.8).

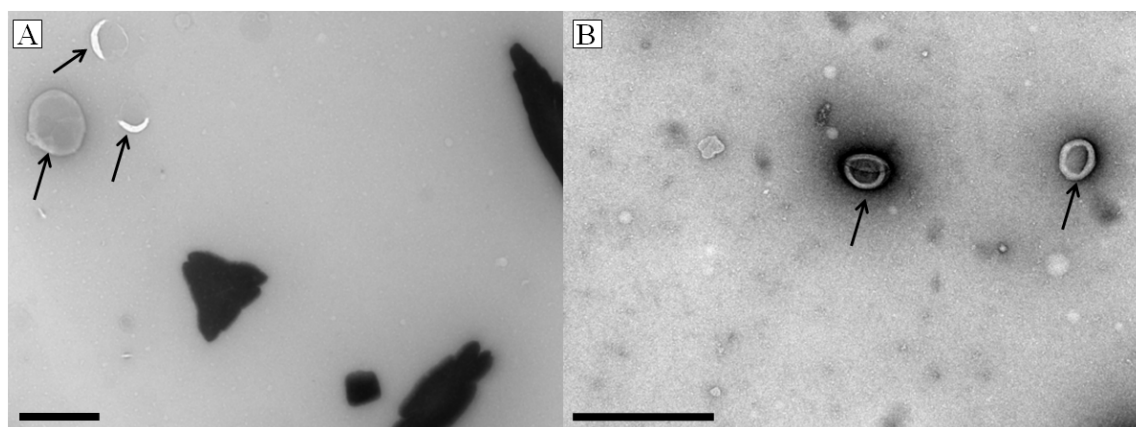


Figure 6.8: Electron micrograph of liposomes (black arrow) (A) contaminated after staining with UMgAc by salt crystals that show as black shapes. (B) Liposomes stained with UAc after one wash. Scale bar 1 μm .

In order to limit the formation of these microcrystals, the sample was first washed with one drop of stain solution, which effectively removed the majority of the buffer solution. A second drop of stain is then applied to the samples for 10 s to stain the sample. For biological samples, it is also common to briefly pass the grid over a few droplets of water before staining with UAc to remove salts and glycerol in the buffer.

Protocols often provide little or no information for removing the staining solution, which is the last step before sample imaging^[319]. A common method is to cut a circular filter paper into small triangular pieces and to absorb the solution by delicately touching the edge of the paper perpendicularly to the EM grid. However, the shape and size of the piece of filter paper influences the rapidity at which the solution is blotted away from the grid, a commonly overlooked detail. It is anticipated that if the stain solution is removed quickly, it creates a shear force susceptible of tearing the liposomes and bursting the vesicles, and can also negatively affect the fragile triskelia polymer. In an effort to limit the damage to the sample due to mechanical forces, we used small and long ribbons of filter paper (Figure 6.9).

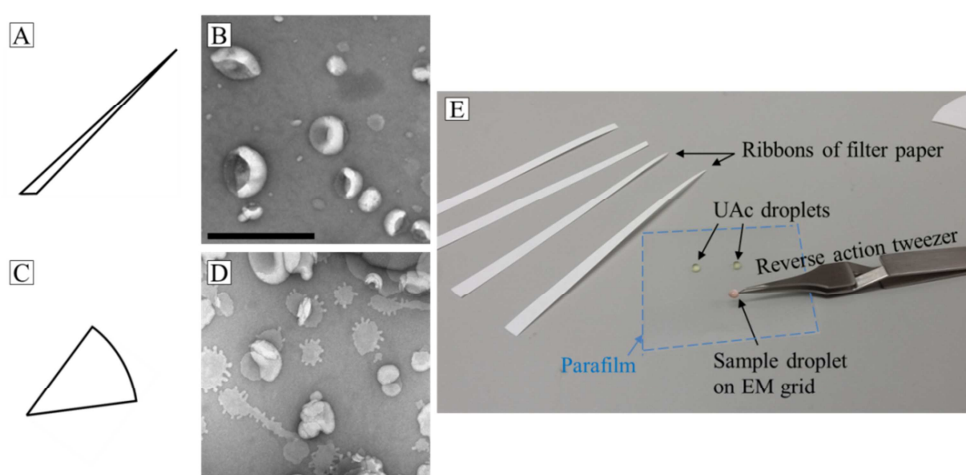


Figure 6.9: (A) Illustration of a ribbon of filter paper used to blot excess sample of liposomes from the grid before staining for (B) TEM imaging. (B) A triangular piece of filter paper used to blot excess sample of liposomes from the grid before staining for (D) TEM imaging. Scale bars 500 nm. (E) Picture of a typical setup for sample staining using ribbons. A sample droplet is deposited onto a grid held by a reverse action tweezer. Two droplets of UAc are ready for wash and staining of the sample on a parafilm piece and ribbons of filter paper are visible on the left to remove excess liquid slowly and in a controlled fashion.

By reducing the surface of the filter paper to blot the sample, the rate of dewetting was reduced and the sample experienced less strain during this staining process (Figure 6.9).

This method also allowed a better control over the amount of stain removed, which in turn changed the thickness of the stain left to dry on the deposited sample. It was found that a thicker staining provided a better support of the large assemblies deposited on the

grid and that the variable staining thickness created on the grid allowed the observation of different aspect of a sample on the same grid. Although many biological samples are fixed in resin before being imaged on an electron microscope to preserve the 3D superstructures in their native conformation^[320, 321], this resin embedding and other type of chemical fixation^[322] were not applicable to samples of DNA origami on lipid vesicles as the processes would destroy the nanostructures.

However, UAc stain thickness did affect the 3D stabilisation of liposomes and the way they collapse on the grid during staining and drying. A short staining time (10 s) was found to allow a sufficient stain thickness for visualisation while avoiding damage to or deformation of liposomes due to the osmotic shock and acidic pH (Figure 6.10A). A longer staining time (+30 s) produced a dark background of stain (Figure 6.10B) and allows penetration of the dye into the structure deposited on the grid. Some users believe that a longer staining allows a better homogeneity of the uranyl layer, although it was not rare to observe both negative and positive staining on the same grid.

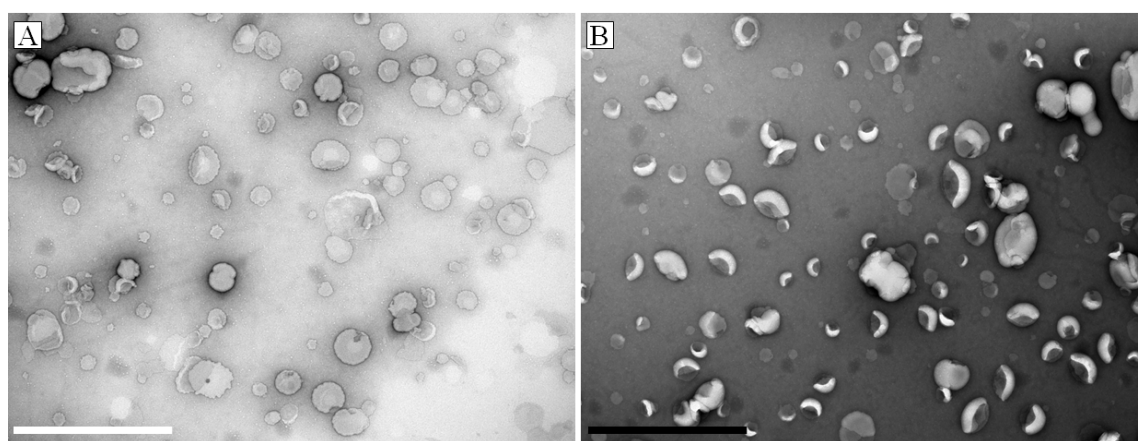


Figure 6.10: Electron micrographs showing (A) a sample of liposomes stained with UAc for 10 s, and (B) with staining for 30 s. Scale bars 1 μ m.

These liposomes have a “cup shape” as a result of the stain accumulating either on the side or in the middle of the vesicle, which appears darker.

Liposomes stained with UAc for a long period of time (30+ s, Figure 6.11A) and where UAc solution was allowed to dry on the grid produced images that presented better overall shapes, but the details were hidden by the heavy staining. Liposomes that were stained for 10 seconds and had most of the UAc solution removed provided the optimal result: vesicle stability and visualisation (Figure 6.11B, C). If too little stain was present (Figure 6.11D), the liposomes deformed and flattened, as evidenced by radial tubulations in the EM micrographs. Thus, the staining thickness depends from the amount of time that the UAc solution is allowed to penetrate the sample, and the amount of excess stain left on the grid after blotting with a ribbon of filter paper.

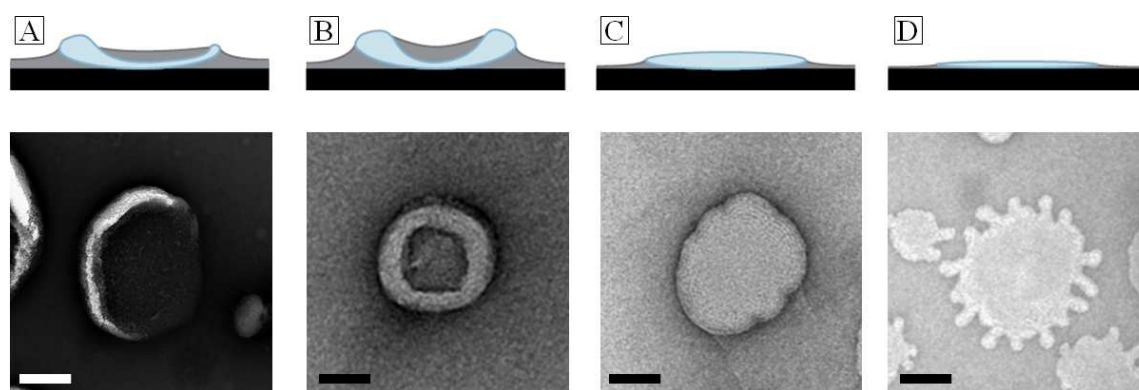


Figure 6.11: Interpretation of the profile topology of liposomes (top) as the stain thickness observed on TEM (bottom) increases from left to right. Scale bars 100 nm.

The discussion presented in this section is the result of multiple trials and comparison with references in literature^[182, 183, 323, 324]. Given the variability of liposomes appearance on an EM grid (even across the same grid), and the multiple factors involved in grid staining, it is extremely difficult to determine what process exactly is responsible for a particular result (particularly for liposome staining). The thickness of the stain depends on how quickly and firmly the stain is blotted. The concentration of sample on the grid also changes how much of the stain will pool around the liposomes as it is blotted. Longer staining times with UAc will affect how the stain binds to the sample itself; the longer it is left on, the more it becomes a positive stain rather than a negative stain, and

so the specimen will dry differently. The differences in flattening and aggregation thus depend mainly on different staining times, blotting methods, concentrations and type of stain.

6.3 SAMPLE IMAGING

Once a correctly stained sample was prepared, the grid was air dried for approximately 10 min. Remaining water is removed rapidly when the sample is inserted into the TEM and subjected to vacuum. In general, a stained sample supported by the carbon and formvar films will resist the damage induced by the electron beam during imaging, but on occasion we observed damaging effect that hindered optimal imaging.

Although a film on EM grids are known to sometimes break depending on the intensity of the electron beam, the film was observed to crack more readily with a concentrated sample (>0.3 g/mL lipids and 20 nM AuNP) that creates a dense layer on the film. However, we observed that as long as the supporting film attached to at least one of the metal mesh, this provided enough sample surface and stability for imaging.

We also encountered imaging challenges with sample prepared with staining of UAc. Thicker staining enhanced contrast and stabilised larger structures as discussed previously, but this resulted in drastically increased beam sensitivity. Samples surrounded by a dark background provided by the stain often showed quick and visible degradation under electron bombardment^[325] (Figure 6.12).

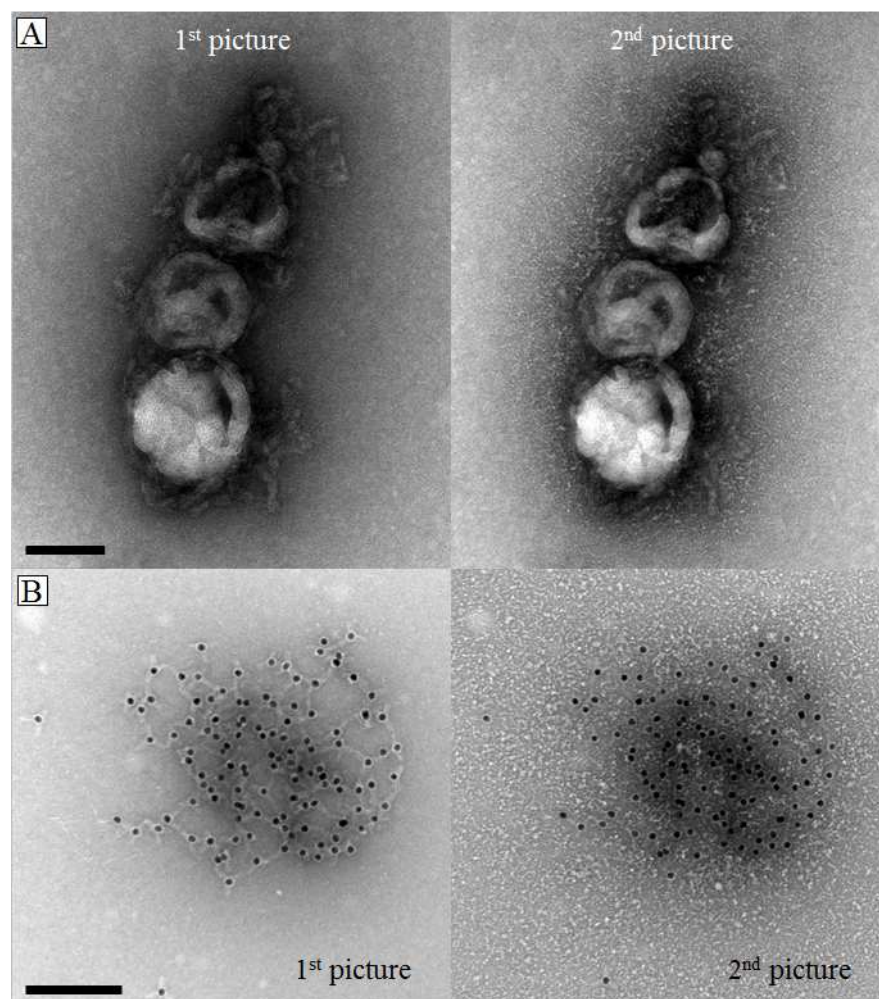


Figure 6.12: Electron micrographs showing two pictures taken sequentially with a few seconds apart showing (A) triskelion-coated liposomes and (B) polymerised AuNP-triskelia. Scale bars 200 nm.

Negative staining proved sometimes difficult to image at magnification nominally used for DNA nanostructures (approximately 16,000 x) as it starts to deteriorates as soon as the beam illuminates the sample at this magnification and exposure (120 kV, spot size 2, 200 μm condenser aperture). Prevention of excessive damage during imaging was important with thick stain, thus sample illumination was kept at a minimum by changing the configuration of the column (120 kV, spot size 4, 150 μm condenser aperture) and by recording a picture quickly to limit the exposition of the sample to the electron beam. Thus captured images presented in this thesis are the result of the superposition of multiple (25) images (recorded with a Gatan OneView camera), which reduces the

background noise and improves contrast, even with little exposure (25 times 0.04 s exposure, recorded in 1 s).

The increase of noise in the background and disappearance of the samples is the result of the UAc stain reacting with the electron beam as reported by Cremer *et al.*^[325] and Unwin^[326] who suggested that UAc was susceptible to move and degrade during electron irradiation.

6.4 POSITIVE AND NEGATIVE STAINING

The preparation of triskelia for TEM utilising the droplet technique relies on the triskelia adsorbing onto a carbon support film. As this adsorption is not specific, they will adopt multiple orientations^[316]. Staining with UAc creates characteristic “stain clouds” that surround the triskelia (Section 6.2), producing strong contrast between the background and the DNA structure that provides detailed images of the orientation of the triskelia. The resulting image is therefore a planar projection of the 3D DNA structure. Triskelion monomers can be readily identified, although the task becomes quickly difficult when the triskelia are polymerised into spherical assemblies. The irregularity of the grid wetting and staining regularly created negative and positive staining on a single grid, a known characteristic of UAc^[182, 316, 326]. Negative staining provided excellent detail of the individual DNA double-helices that compose the triskelia. Positive staining is a result of only a small layer of stain being adsorbed on the triskelia and not the grid background. Positive staining was preferred to study the size and relative angle of the arms of the triskelia (Figure 6.13). Both staining types were treated together and with no distinction throughout the thesis.

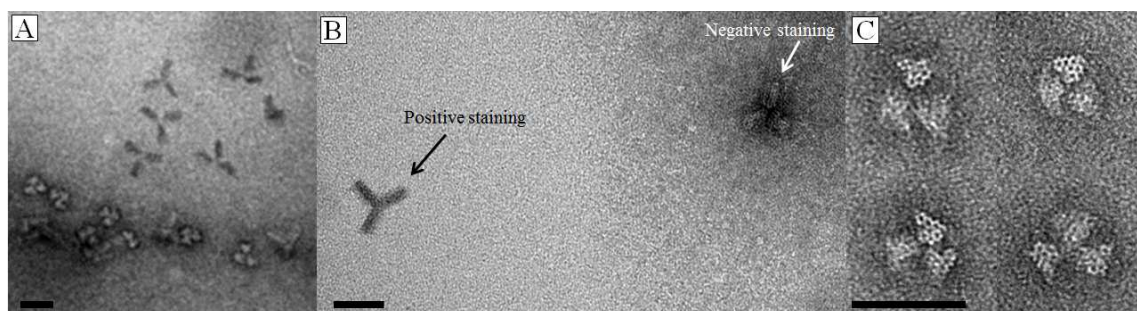


Figure 6.13: Electron micrographs showing positive and negative staining within a few nm on a grid. (A) Positive staining in this example is believed to be thinner than negative staining and allows the triskelia to adopt a non-flat configuration on the grid. (B) Negative staining of good quality can show individual DNA helices. (C) Negative staining stabilises the triskelia arms in an upward position. Scale bars 50 nm.

6.5 ADDITIONAL ARTEFACTS

An artefact in TEM is an observable phenomenon unrelated to the true nature of the sample under investigation and is a result of damage inflicted during the sample preparation or by the irradiation of the electron beam^[311, 326]. Differences in staining beam damage (sections 6.3 and 6.4) and contamination by molecules from the purification methods (Chapter 3, section 3.2.1) are the main artefacts discussed previously. In addition, other irregularities were sometimes observed in TEM images of triskelia and liposomes, which are shown in Figure 6.14. “Coffee ring” effects were observed on the grid due to the surface tension of both the sample and the stain solution^[304] (Figure 6.14A, B) and provided negative and positive staining. Double-sided stains are reported to occur if the support film is broken and the solutions can contact both sides of the grid^[182] (Figure 6.14C). EM grids coated by hand occasionally showed traces in the background under the electron beam attributed to difference in the formvar film thickness (Figure 6.14D). The irregularities observed in the backgrounds are attributed to the method of interaction and wetting between the stain and the support film

(Figure 6.14A-E) or film wetting irregularities (Figure 6.14F, G, H) that were easy to recognise as artefacts. Dust and other foreign molecules on the grid were observed due to air exposure or contaminants in buffers and reagents. (Figure 6.14I). Finally, the quantity of triskelia deposited on a grid was subject to variation, depending on the interaction between the triskelia and the support film (Figure 6.14J, K, L). Because artefacts are common in electron microscopy, it is important to be able to identify what artefacts arise from the types of sample under investigation during sample preparation and imaging, so they are not falsely attributed to the sample structure itself. Other common artefacts can be identified and minimised by referring to the extensive literature^[182, 183, , 327].

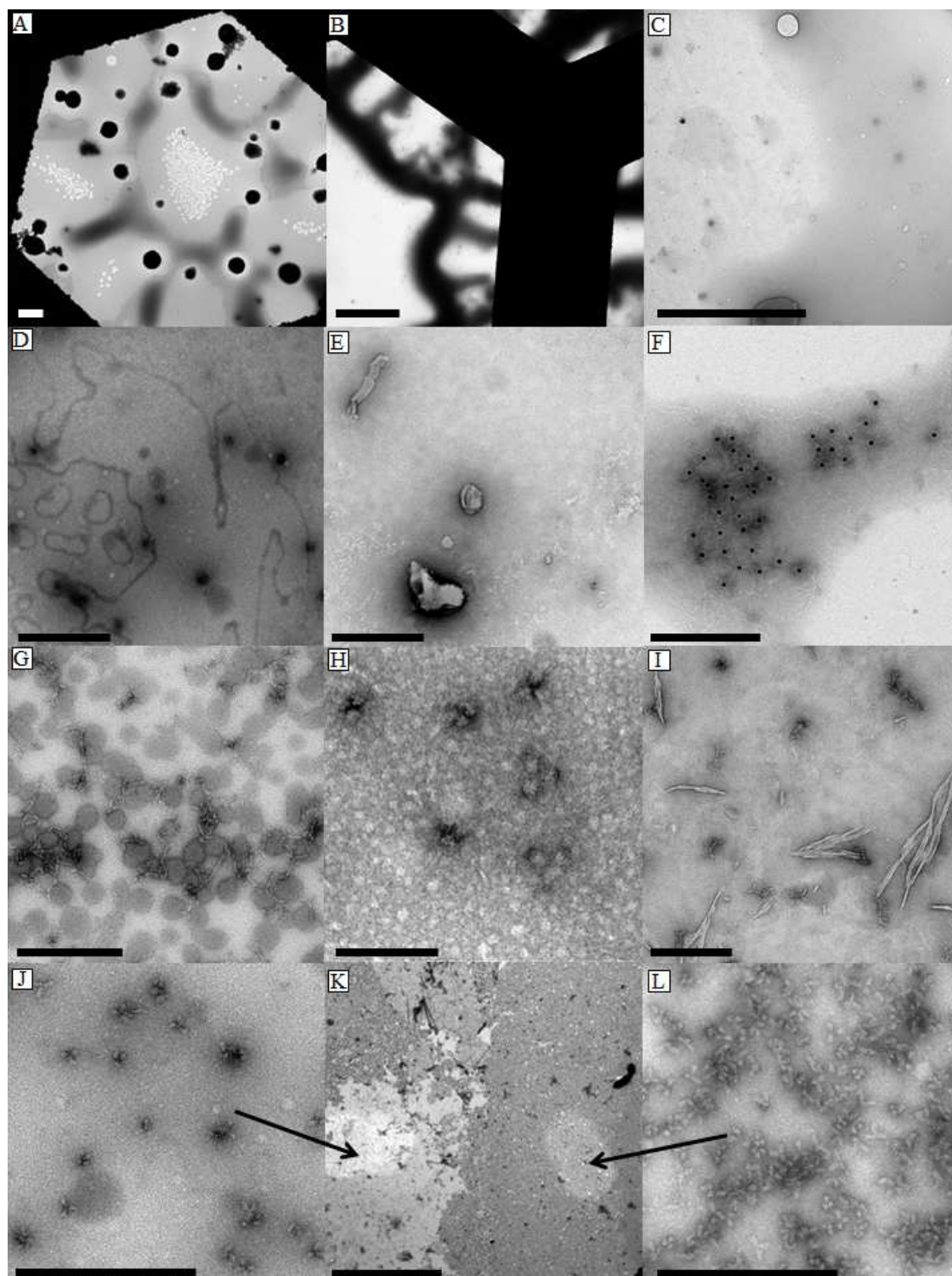


Figure 6.14: Electron micrographs of various samples of liposomes (A, C, D) or triskelia (B, E, F, G, H, I, J, K, L) showing artefact in the background arising from the grid preparation. (A) Coffee ring effect and contaminants of the sample or the grid observed as black dots, (B) coffee ring effect, (C) staining on one side (left) and both side (right) of the grid due to a broken support film, (D) variation in formvar thickness in the background, (E) deformation of liposomes and film wetting after a quick blotting, (F-H) film wetting irregularities, (I) contaminants in buffer and reagents, (J-L) variations in the amount of triskelia deposited on a same grid. Scale bars 20 μm (A, B, C and K), 500 nm (D-H, J and L) and 100 nm (I).

6.6 SUMMARY

This chapter presents discussions and methods developed during the thesis for optimal TEM imaging of DNA nanostructures and their interactions with the lipid bilayer. The preparation of a coated EM grid, the type and amount of staining, and the samples damage induced by imaging, were all very important parameters that needed to be addressed to correctly image DNA origami and lipid structures.

Electron microscopy is a technique that is broadly used in synthetic biology and nanotechnology. An in-depth knowledge of the chemical properties of a sample is required to accurately interpret EM micrographs. A large part of the artefacts observed with EM are believed to arise from sample preparation and the staining method employed. Although protocols for the preparation and imaging of biological samples are commonly found in literature, a precise description of the staining process is often overlooked or missing that is vital for optimal imaging. A wealth of literature is available that describes artefacts regularly observed with biological samples (viruses, cells, etc...) and possibly followed with advice on how to prevent them. DNA nanotechnology is no exception, as the production and behaviour of a well-folded DNA structure is very often confirmed by EM. Research investigating the interaction of DNA nanostructures with a lipid membrane has increased over the last decade, but detailed protocols regarding EM imaging has not been provided. This chapter aims at filling in these blanks and is addressed to any scientist in biotechnology seeking recommendation and interpretation of micrographs.

CHAPTER 7

CONCLUSION

7.1 SUMMARY AND CONCLUSION OF THE THESIS

This thesis presented the design and construction of three DNA nanostructures, two of which were assembled utilising DNA origami. For the first part of this thesis, two different DNA nanostructures were designed to attach to a lipid membrane using cholesterol anchors to achieve controllable deformation of a membrane. The use of a synthetic system would allow a better control and knowledge over the parameters at play during membrane bending, a fundamental process in the life of every cell. The clathrin protein, responsible for the process of endocytosis in eukaryotic cells, is the inspiration for the first part of this thesis. It led to the design of two different DNA structures, both sharing the same general ability of polymerisation and attachment to a lipid bilayer, as well as the three-fold symmetric shape of the natural clathrin model. The first nanostructure, the star, was constructed from short individual DNA strands, the sequence of which had to be adapted from the original publication to suit our new system. Following optimisation of the folding procedure, the star was successfully polymerised into arrays, as observed by TEM and AFM. When the star was decorated with cholesterol handles, the resulting polymerised arrays were smaller, and it is believed that the cholesterol handles add strain and partially unfold the star. The addition of the cholesterol handle required the use of two shorter strands. Oligonucleotides at the extremity of a short domain are more likely to be in an on-off equilibrium between hybridisation and melting, which destabilised the arm of the star. Further, the protruding

cholesterol handle from each arm of the star added strain to the system at the handle position. It is theorised that the handle was pulling on the DNA helix forming the arm, and partially melting the first few nucleotides. The functionalised star was then too flexible and fragile to direct the formation of a regularly repeating array, which is necessary for controlled membrane bending.

We thus replaced this strategy in favour of a structure constructed with DNA origami to circumvent the weaknesses of our initial star. This second, enhanced 3D DNA structure, named triskelion in reference to the shape of the clathrin, was thoroughly optimised for polymerisation into a pattern resembling that of a buckyball ($1 \leftrightarrow 1$, $2 \leftrightarrow 3$ pattern). The use of DNA origami solved the issue of stoichiometry and fragility of the arms following functionalisation with cholesterol and dye conjugates, yet remained an accurate mimic of the clathrin protein. Stiff bridges of three DNA helices with length of 60 bp or 90 bp produced either curved or flat triskelia, respectively, providing control over the intrinsic curvature of the DNA origami. A temporary strategy was also presented, using triskelia functionalised with AuNP of 15 nm diameter. Although this approach provided information regarding optimal triskelion and lipid concentrations required during folding, it was discarded because the AuNP prevented curvature of the triskelion. It was determined that the use of triskelion dimers (using $1 \leftrightarrow 1$ 6T invading strands) associated with 4 bp sticky ends (arms $2 \leftrightarrow 3$) provided optimal polymerisation to obtain efficient coverage of lipid vesicles. When decorated with cholesterol anchors, flat triskelion dimers could diffuse on a membrane-coated EM grid and assemble into large arrays (visible by TEM) of hexagons and pentagons, as was designed. Curved triskelion dimers formed the lipid membrane into circular shapes when polymerised, as observed by EM and reminiscent of the biological model. Dual-colour fluorescence microscopy of fluorescent blue GUVs and fluorescent red triskelia allowed the observation of membrane bending in solution. The insertion of triskelia into the membrane of a GUV was observed to take place within 10 minutes and observed as a homogenous and co-localised red-blue fluorescence on the GUVs. Polymerisation of flat triskelia could be

observed as bright patches on the GUVs only after the addition of the polymerisation staples, confirming that polymerisation is precisely triggered by the addition of the DNA strands. Flat dimers showed bright patches on the GUVs, but no budding. Fluorescence microscopy of curved triskelion dimers revealed spherical budding of the membrane of GUVs, and showed that the polymerised, curved DNA origami forced the membrane to bend. The fluorescence of the DNA was concentrated at the neck of each budding, suggesting that the triskelia localise preferentially at the inverted curvature of the membrane. This configuration optimally satisfied the insertion of the cholesterol (incorporated in the triskelia) into the membrane, as it was shown that the curved triskelion could adopt an unexpected “inverted” configuration. This work is the first example of an artificial membrane bending that can be triggered only by the polymerisation of DNA origami. Previous examples of membrane bending were the result of membrane crowding^[334,336] or the formation of a membrane inside a pre-assembled scaffold^[175, 176].

The second objective of the thesis was to help in the development of an endogenous EM tag made of RNA. The first step of this vast project was to create a small DNA origami with a 3D shape that would be recognisable in cryo-EM imaging. The plasmid pUC19 was designed to fold into a small tetrahedron by the addition of specific DNA staples. The DNA origami tetrahedron was successfully synthesised, concentrated and imaged in cell lysate in cryo-EM. Imaging of the tetrahedron in cell lysate confirmed that the DNA origami can be localised straightforwardly due to its regular features. The functionalisation of the tetrahedron by a DNA handle would allow the targeted attachment of the origami to particular organelles or proteins in the cell. The tetrahedron is now part of a library of DNA origamis that are being screened in order to determine the optimal structure for an endogenous cryo-EM RNA tag.

The final chapter of this thesis presented protocols and methodologies developed for TEM imaging of samples made of DNA nanostructure and lipid membranes. This technical chapter aims to fill in the blanks in the literature regarding the sample preparation and electron microscopy imaging of DNA origami, and in particular their interaction with a membrane bilayer. A large part of the artefacts observed during EM imaging were believed to arise from the sample preparation and in particular the staining method used. The same grid was observed to present drastic differences in staining, sample concentration and questionable interactions between the DNA nanostructures and the membrane. Interpretation of electron micrographs should be made only on those taken with an EM grid presenting a homogenous sample staining and appearance over the entire surface (or at least over the vast majority of it). This chapter provides advice regarding sample preparation and interpretation of the TEM images, and should be a reminder that honesty and critical analysis is important when presenting (EM) pictures. The electron micrographs of the regular array made of polymerised triskelia on a lipid membrane, discussed previously, was done with the same TEM. The successful use of the custom-made Teflon apparatus for this set of experiments is the direct result of the work and know-how presented in this last chapter.

ANNEX

MATERIALS AND METHODS

A.1 BUFFERS

The following buffers were used for the experiments presented in this thesis, they were prepared with Milli-Q-purified water and chemicals provided by Sigma-Aldrich* or ThermoFischer Scientific[†].

1 x TE Buffer

Diluted from a 20 x TE stock; final 1 x TE concentrations are:
5 mM tris(hydroxymethyl)aminomethane[†] (Tris base)
1 mM ethylenediaminetetraacetic acid* (EDTA)
pH 7.4-7.6 (pH not adjusted)

1x TAE Buffer

Diluted from a 20 x TAE stock; final 1 x TAE concentrations are:
40 mM tris(hydroxymethyl)aminomethane[†] (Tris base)
20 mM acetic acid
2 mM ethylenediaminetetraacetic acid* (EDTA)
pH 7.6 (NaOH or HCl adjusted)

0.5 x TBE Buffer

Diluted from a 10 x TBE stock; final 0.5 x TBE concentration are:
45 mM mM tris(hydroxymethyl)aminomethane[†] (Tris base)
45 mM boric acid[†]
1 mM mM ethylenediaminetetraacetic acid* (EDTA)
pH 8.2-8.3 (pH not adjusted)

1 x HEPES Buffer

Diluted from a 10 x HEPES stock; final 1 x concentrations are:
10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid[†] (HEPES)
10 mM MgCl₂[†]
100 mM NaCl[†]
pH 7.5 (NaOH or HCl adjusted)

A.2 OLIGONUCLEOTIDES AND SCAFFOLDS

The following section list the suppliers that provided DNA used in this thesis, and, if required, how particular DNA-conjugates were prepared. For protocols, see section A.3 onwards.

A.2.1 General

The reference ladders of 1 kilo base (kb) and 50 base pairs (bp) DNA were purchased from New England BioLabs Inc.

5x blue loading dye stock was prepared by mixing 0.25% $\left(\frac{w}{v}\right)$ bromophenol blue (25 mg, Sigma Aldrich), 30% $\left(\frac{v}{v}\right)$ glycerol (3 mL, Sigma Aldrich) in 10 mL batches. If needed, 0.017% $\left(\frac{w}{v}\right)$ sodium dodecyl sulfate (SDS, 1.7 mg, Acros Organics) was added to the stock. During PAGE purification in Munich, a dye with a faster migration was used to ensure that the collected DNA band contains no contaminant. The loading dye containing 5x Orange G in glycerol was provided by Alfa Aesar and used as received. Samples were mixed with loading dye for a 1x loading dye final concentration prior to being injected in an agarose gel (section A.6), or a polyacrylamide gel.

A.2.2 Chapter 2

Nucleotides were ordered from Integrated DNA technology (IDT) when ordered in Oxford, and from biomers-net when ordered in Germany. DNA strands were usually 25 nmol and shipped in RNase free water (150 μ M) at -20°C. Synthetic scale for staples longer than 60 nt were 100 nmol, shipped dried and stored in water (150 μ M) at -20°C. Staples longer than 60 nt were 100 nmol, shipped dried and stored in water (150 μ M) at -20°C. The oligonucleotides were not purified before use, unless required by the supplier (HPLC purification) or specified in the thesis.

3'-end labelling of S5 with dUTP-ATTO488

S5 labelling for use in fluorescence microscopy was performed in LMU using a kit provided by Thermo Fisher Scientific. The protocol suggested by the supplier was modified based on experience from the group of Tim Liedl to accommodate the need of the experiment. That is, we used larger quantities than that suggested and allowed the coupling reaction to react for a longer period as follows: Terminal Deoxynucleotidyl Transferase (TdT) reaction buffer (4 μ L, 5x stock as provided), S5 (1 pmol 3'-end final concentration), CoCl₂ (4 μ L, 25 mM, 5 mM final concentration), enzyme (TdT, 0.05 mM final concentration), Aminoallyl-dUTP-ATTO-488 (1 μ L, 1 mM, 400 U/reaction) and water (final volume 20 μ L) were mixed and briefly centrifuged. The mixture was incubated at 37°C for 45 minutes. Addition of NaOAc 3 M (2.5 μ L) and 57.5 μ L of EtOH (100%, -20°C) and storage in -20°C freezer precipitated the DNA. The solution was centrifuged (13'000 rpm, 30 min, 4°C) and the supernatant was discarded. EtOH (70%, 60 μ L) was added and centrifuged again for 10 minutes at the previous conditions. The second supernatant was discarded right away before the pellet dissolves again. The final product (pink solid) was suspended in water

and used without further purification. Note: dUTP was used instead of the recommended ddUTP. As opposed to ddUTP, dUTP can undergo multiple coupling on the 3'-end, leading to two or more ATTO-488 dye on one DNA strand if the solution is left at 37°C for an extended time. However, this is not an issue for the purpose of this experiment as the number of dye per star tile is not a relevant factor.

A.2.3 Chapter 3 and 4

M13mp18 (p7249) single-stranded DNA was either supplied by VWR International Ltd (1 µg/µL, in 10 mM Tris, (pH 7.5), 1 mM EDTA), or produced using the standard transformation described in box 2 of Reference^[328] using competent cells *E.coli* JM109 (New England BioLab Inc.).

5' - Thiol-C6 – 19 (T) was provided by Biomers-net, scale L, 4 aliquots, shipped and stored at room temperature in a dried state.

Synthesis scale for Cholesterol-DNA and dye-DNA conjugates were 100 nmol, shipped dried and stored in water (150 µM) at –20°C.

A.2.4 Chapter 5

pUC19 plasmid was provided by New England BioLabs Inc. The double-stranded plasmid was digested by the enzymes Nt.BspQI and Exonuclease III kits, both supplied from BioLabs Inc.

A typical pUC19 preparation is described: the double-stranded pUC19 (25 µL, 1 µg/µL, 25 U; line *dspUC19*, Figure A.1), as received by the provider, was diluted in 1 x NEB3.1 (50 µL, 2 volumes; 10x concentration NEBuffer 3.1) and mixed with the nicking enzyme Nt.BspQI (2.5 µL, 25 U). The solution was incubated at 50°C for 2 hours, then warmed to 80°C for 20 min to kill the enzyme and stop the reaction (line

Nt.BspQI, Figure A.1). The enzyme ExoIII (1.25 μ L, 5 eq., 125 U, 100x concentration) was added and the solution was incubated at 37°C for 1 hour. This step chops off the nicked strand and leaves only the ssDNA ring (line ExoIII, Figure A1). The resulting solution was mixed with sodium acetate (7.47 μ L, 3 M, $\frac{1}{10}$ volume) and ice-cold EtOH (149.5 μ L, 2 volumes, EtOH stored in -20°C freezer) and the solution was left to precipitate either overnight at -20°C, or for 1 hour at -80°C. The suspensions was then centrifuged for 10 min at 4°C and 20'000 g. (Make a mark where you plan the pellet to be in the tube.) The supernatant was carefully removed to leave the pellet in the tube, which was then resuspended in 20 μ L 1x TE (line sspUC19, Figure A.1). This yields approximately 40 μ L solution at 250 nM sspUC19,(concentration measured by UV absorbance).

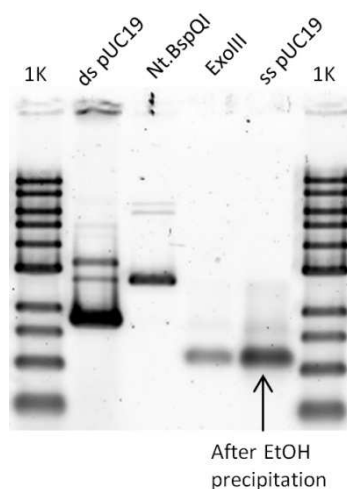


Figure A1: Analysis of plasmid preparation by AGE. The lines 1K on each side are the reference 1kb ladder.

A.3 PURIFICATION OF DNA STRANDS (DENATURING PAGE)

Polyacrylamide gel electrophoresis (PAGE) was performed for both analysis and, if required, purification of the DNA strands for the 3-armed star project (Chapter 2). For a gel of 1.5 mm thickness, 10 mL of acrylamide/bisacrylamide was needed.

For DNA purification, a denaturing gel containing urea is required to separate potential secondary structures: 10% 19:1 acrylamide/bisacrylamide (A:BA) (40% A:BA, 2.5 mL) in 0.5 x TBE + 8 M urea (5 mL, 2 x TBE + 16 M urea) and 2.5 mL water was mixed with ammonium persulfate (APS) (300 µL, 10%). Then, tetramethylethylenediamine (TEMED, 20 µL) was added and the solution was quickly poured into the gel cast for polymerisation. Denaturing gels were then pre-run for 30 min in 0.5 x TBE, 80 V at room temperature. All wells were washed with buffer using a syringe before loading the samples, and the gel ran for 40 min at 200 V and room temperature. The DNA band to be extracted was then visualised using UV-shadowing: the gel is placed on a white surface and exposed to UV light to reveal the location of the DNA band (Chapter 2, Figure 2.15).

The desired band was cut with a razor blade, chopped into fine pieces and placed in a 1.5 mL microcentrifuge tube. Eluant buffer was added (1 x TE, at least 2 x the gel volume) and the tube was shaken for a minimum of 24 hours at room temperature. Then, the sample was centrifuged (1 min, 1000 g, Eppendorf 5453, Minispin Microcentrifuge, fixed angle) to collect the supernatant. Any residual DNA was washed with small volumes of buffer, centrifuged as previously described and combined with the supernatant. Cold ethanol (EtOH, 1 mL, kept in -20°C freezer) and sodium acetate 3 M (1/5 of the volume) were added to the eluent and placed in -20°C freezer for at least 1 hour. The tube was centrifuged again (13'000 g, 30 min) and the supernatant was

discarded. The pellet was mixed with 70% EtOH and centrifuged again for 7 minutes. The pellet was recovered and suspended in water or 1x TE buffer. Finally the concentration was measured by Absorbance at 260 nm using the extinction coefficient given by the supplier for each DNA strand.

A.4 ANNEALING OF DNA STAR

The optimal DNA array obtained in chapter 2 was folded using the following procedure:

85°C for 15 min

Decrease to 75°C at $-0.5^{\circ}\text{C}/20\text{ min}$

Decrease to 60°C at $-0.2^{\circ}\text{C}/5\text{ min}$

Decrease to 45°C at $-0.2^{\circ}\text{C}/10\text{ min}$

Decrease to 30°C at $-0.2^{\circ}\text{C}/25\text{ min}$

Decrease to 20°C at $-0.2^{\circ}\text{C}/20\text{ min}$

Decrease to 10°C at $-0.5^{\circ}\text{C}/5\text{ min}$

Hold at 4°C as required

Incubation at 4°C was maintained for 24 or 36 hours in order to obtain the arrays presented in section 2.2. Samples folded following this procedure were analysed by AFM or TEM without further purification.

The first folding program (mentioned in section 2.2) was adapted from the LaBean protocol^[206] and followed the following schedule:

85°C for 15 min

Decrease to 75°C at $-1^{\circ}\text{C}/10\text{ min}$, +1 min/iteration

Hold at 20°C for 1 hour

Hold at 4°C until analysed

A.5 ANALYSIS OF dsDNA HYBRIDISATION (NATIVE PAGE)

A polyacrylamide native gel allows the observation of the DNA strands hybridised with their complementary domains. The gel was prepared with: 15% 29:1 A:BA (30% A:BA, 5 mL) in 1 x TAE (0.5 mL, 20 x TAE) and 4.5 mL water was mixed with APS (300 µL 10%). Then, tetramethylethylenediamine (TEMED, 20 µL) was added and the solution was quickly poured into the gel cast for polymerisation. Native gels were run in 1 x TAE + 12 mM MgCl₂ between 70 and 100 min, 200 V on ice. The gel was stained for 15 min with SYBR Gold, and destained in 1 x TAE + 12 mM MgCl₂ for 2 min before scanning.

A.6 NUPACK ANALYSIS

A.6.1 NUPACK analysis of S4 secondary structure (Chapter 2)

The secondary structure of each individual strand composing the DNA star was analysed using NUPACK. The sequence was entered and the parameters were fixed to be 100 nM, 20°C, and a maximum complex size of 4 strands. Analysis of the strand S4 revealed that two strands S4 can hybridise together by a complementary domain of 16 bp (Figure A.2A), which bind the two strands together, preventing the formation of the DNA tile (discussion Section 2.2.1).

The sequence of S4 that is responsible for the secondary structure contains the domain complementary to S1. We thus randomly changed the domain S1-S4 to minimise the interaction of S4 with itself, while limiting the number of sequence changed in the STAR. The two strands were renamed S1' and S4'. The new sequence was then

analysed with NUPACK to verify that no secondary structure was made between S4' and itself (Figure A.2B), or S4' and the other strands of the DNA star (not shown).

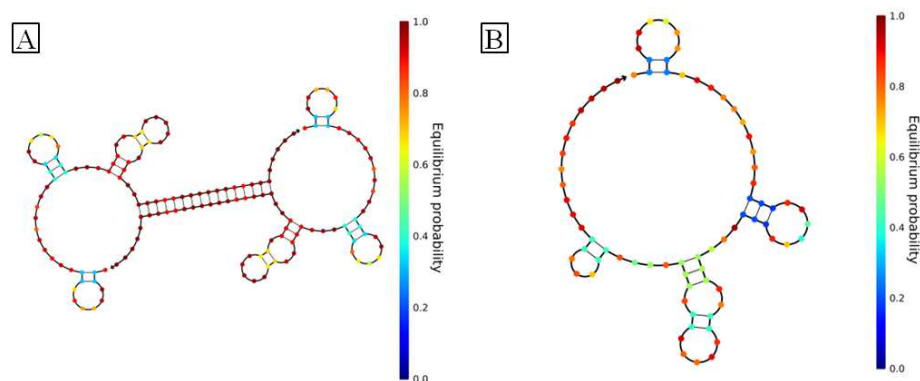


Figure A.2: Results of NUPACK analysis at 20°C, 100 nM DNA concentration and a maximum of 4 strands per complex. (A) Main secondary structure formed by S4-S4, and (B) main secondary structure made by S4'-S4'.

A.6.2 NUPACK design and analysis of stiff bridges (Chapter 3)

The new sequences of the DNA staples forming the stiff bridges were designed such that they did not form secondary structure with themselves, or interact with the part of the scaffold involved in the loop at the centre of the bridge. The staples for the assembly of a bridge for the flat triskelion (90 bp long) and the curved triskelion (60 bp long) were first designed using CaDNAno to determine the domain lengths and crossovers (Figure A.3A, B), and then by using the design section of NUPACK. As NUPACK does not accommodate hybridised DNA that forms a closed circle (an example of such a conformation is shown by a blue arrow in Figure A.3A), we virtually cut the bridges into domains and DNA strands to avoid the formation of structures not supported by NUPACK algorithms. These virtual domains, their name, and connectivity are depicted in Figure A.3C.

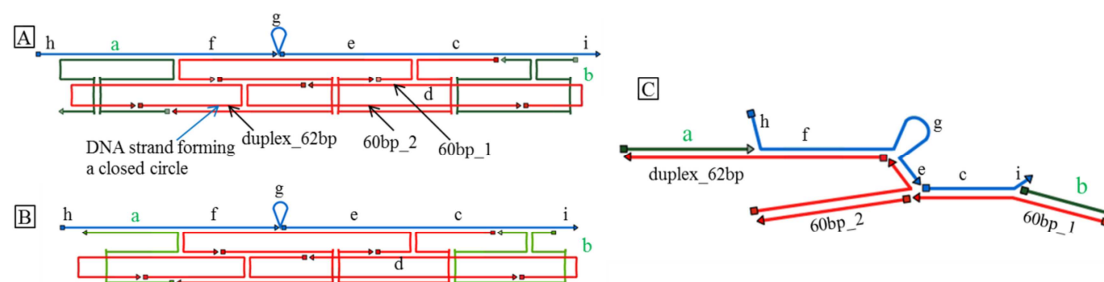


Figure A.3: Schematics from CaDNAno representing (A) the flat stiff bridges (90 bp-long) and (B) the curved stiff bridges (60 bp-long). The scaffold is in blue, strands common to the two length of bridges (flat and curved) are in red, and strands of which the sequence changes are in green. (C) Diagram of the DNA domains (virtually cut DNA) to accommodate NUPACK design and analysis.

We then wrote an algorithm to generate these virtual domains, and selected the sequences with the smaller normalised ensemble defect offered by NUPACK (algorithm below). We generated the real sequences of the DNA strands by matching the sequences of the virtual domains used in NUPACK simulation with the CaDNAno file. The same method was used to generate the three flat, stiff bridges. As some of the strands forming the flat and curved stiff bridges are the same (in red in FigureA.3A, B), we generated the additional strands sequences (required for the curved, stiff bridges only) manually by writing the complementary sequences of the green strands depicted in Figure A.3. We defined the sequences for the three different flat, stiff bridges; between arm 1&2 (HB87 in CaDNAno file: see Annex A.17), 2&3 (HB84) and 3&1 (HB85). The example of algorithm given below was used to generate the sequences of the flat, stiff bridge between arms 1 and 2.

```
#      HB87 FILE
# design of the three strands common to both file II and III for reinforced bridges. These
# strands are in blue in the CaDNAno file. They are meant to be designed such that they
# do not interact with the scaffold. This should guarantee the rigid bridge to form easily.
#
material = dna
temperature[C] = 20.0 # optional units: C (default) or K
trials = 3          # will show us the three best results
sodium[mM] = 50.0    # optional units: M (default), mM, uM, nM, pM
dangles = some

#
# target structures
structure duplex_62bp = D62(+)
structure duplex_60bp_1 = D60 (+)
structure duplex_60bp_2 = D60 (+)
structure attached_60bp_1 = D29 + U4
structure attached_60bp_2 = U29 D24 +
structure start_scaffold = U4 D39 +
#
# sequence domains
# domains h,f,g,e,c,i are the scaffold, g is the loop on the scaffold, h and I are the 4bp
# loop at the end
domain a = N62
domain b = N60
domain c = AAAAAATCGTTTCTTATTTGGATTGGGAT      #29 nt
domain d = N60
domain e = CTATTTTCATTTTGGACGTAAAC            #24 nt
domain f = GGGTGTTTCAGTTAATTCTCCCGTCTAATGCGCTTCCCTG #39 nt
domain g = TTTTATGTTATTCTCTCTGTAAAGGCTG      #29 nt (reservoir)
domain h = TTCA                                 #4 nt
domain i = AAAT                                 #4 nt

# strands (optional, used for threading sequence information
# and for displaying results)
strand duplex_62 = a
strand duplex_62* = a*
strand bp60_1 = b
strand bp60_1* = b*
strand attached_60_1* = c i
```

```
strand attached_60_1 = c*
strand bp60_2 = d
strand bp60_2* = d*
strand attached_60_2* = e*
strand attached_60_2 = g e
strand start_scaf = h f
strand start_scaf* = f*

# thread strand sequence information onto target structures
duplex_62bp.seq = duplex_62 duplex_62*
duplex_60bp_1.seq = bp60_1 bp60_1*
duplex_60bp_2.seq = bp60_2 bp60_2*
attached_60bp_1.seq = attached_60_1 attached_60_1*
attached_60bp_2.seq = attached_60_2 attached_60_2*
start_scaffold.seq = start_scaf start_scaf*
#
# specify stop conditions for normalized ensemble defect
# default: 1.0 percent for each target structure
# duplex.stop[%] = 0.1

# prevent sequence patterns
prevent = AAA, TTT, CCC, GGG, AAAA, TTTT, CCCC, GGGG, AAAAA, TTTTT,
GGGGG, CCCCC, AAAAAA, TTTTTT, GGGGGG, CCCCCC
```

A.7 FOLDING AND ANALYSIS OF DNA ORIGAMI

Both the triskelion (with and without AuNP) and the tetrahedron were folded by thermal annealing ramp on a Thermocycler (a programmable, temperature-controlled Peltier heat block, Eppendorf Mastercycler DNA Engine Thermal Cycler PCR) with the following schedule:

65°C for 15 min

Decrease to 58°C at -1°C/5 min

Decrease to 40°C at -0.5°C/30 min

Decrease to 20°C at -1°C/5 min

After a solution of the DNA scaffold and staples has been annealed to yield the desired origami, a few microlitre (μL) of the sample is loaded in a well of an 1.5% agarose gel with 0.5 x TBE buffer + 12 mM MgCl_2 . A potential (50-60 V) is then applied along the length of the gel. As the backbone of DNA is negatively charged, the DNA molecules will migrate towards the cathode and through the agarose gel. Smaller DNA molecules will migrate faster than large DNA assemblies, which allow a size-separation of the species in the sample over a few hours (2-5 hours). A DNA origami is often more compactly arranged than an unfolded DNA scaffold of the same number of base pairs, which results in the origami migrating slightly faster through the gel^[238]. For long electrophoresis, the buffer was replaced half way such that the current is maintained at approximately 12 mA. The bands of interest were cut out with a scalpel, finely chopped and pressed between two Parafilm-protected microscope slides to extract the solution. This recovered volume was then imaged by TEM if a quality check was necessary.

A.8 PURIFICATION OF DNA ORIGAMI

The method of purification depends on the volume and concentration required for downstream experiments. If only a small volume is required (5-100 μL), then the samples (triskelia and tetrahedra origami) were purified using AGE as described in section A.7. For larger volumes (100-800 μL) and a concentration between 2 and 8 nM, the DNA origami triskelia were purified using an alternative method developed using size-exclusion chromatography driven solely by gravity (Description of this purification method can be found in section A.8.1-2). Large quantities of DNA origami tetrahedra for cryo-TEM were purified by PEG purification (section A.8.3), followed by removal of residual PEG using the QIAquick PCR purification kit by Qiagen (with buffers supplemented with MgCl_2 to a final concentration of 5 mM).

A.8.1 Sephacryl stock

1. 25 mL of Sephacryl S-300 resin (GE Healthcare Life Science, S-300, high resolution) as received by the supplier was shaken before use and mixed in a 50 mL tube with 25 mL of ddH₂O and centrifuged for 2 min at 1'000 g (Eppendorf 5810 R, rotor F-34-6-38, fixed angle).
2. The supernatant was discarded, the volume brought to 50 mL with the needed buffer, and the mixture shaken.
3. The solution was centrifuged for an additional 2 min at 1'000 g.
4. Repeat steps 2 and 3 for a total of 6 centrifugation.
5. After the last centrifugation, the pellet was resuspended in buffer to a final volume 40 mL.
6. This Sephacry stock can be stored at 4°C for up to three month. Mix well before use.

A.8.2 Size-exclusion columns

This type of size-exclusion method foregoes centrifugation and relies on gravity to elute the buffer through the resin. The resin stock used here is the one described in section A.8.1. A bed volume of 8 mL is needed to separate the origami from excess staples. Disposable columns (GE Healthcare Life Science, PD-10) were hand-packed with a bottom frit circle followed by the resin stock in appropriate buffer (typically 1 x TE + 14 mM MgCl₂). The column was rinsed with buffer of twice the bed volume to sediment and equilibrate the column before placing an upper frit circle that protects the resin from drying out. A sample from 50-800 μ L was then applied to the top of the frit. To reduce dilution, it is important to wait until the sample has been totally absorbed in the resin, and the first fraction collected before adding more buffer to the reservoir. Typically, 19 fractions of approximately 250 μ L are collected per sample and analysed by AGE (Figure A.4). Once the sample has been purified, the column can be reused by washing with buffer (minimum twice the resin volume) to remove any remaining staples. Cleaned columns can be stored at 4°C for one month. A 50 μ L sample of 20 nM usually yields one 250 μ L fraction clear of staples at a concentration of 2-3 nM. This method works for triskelion monomer and dimers, as their migration in the resin is similar.

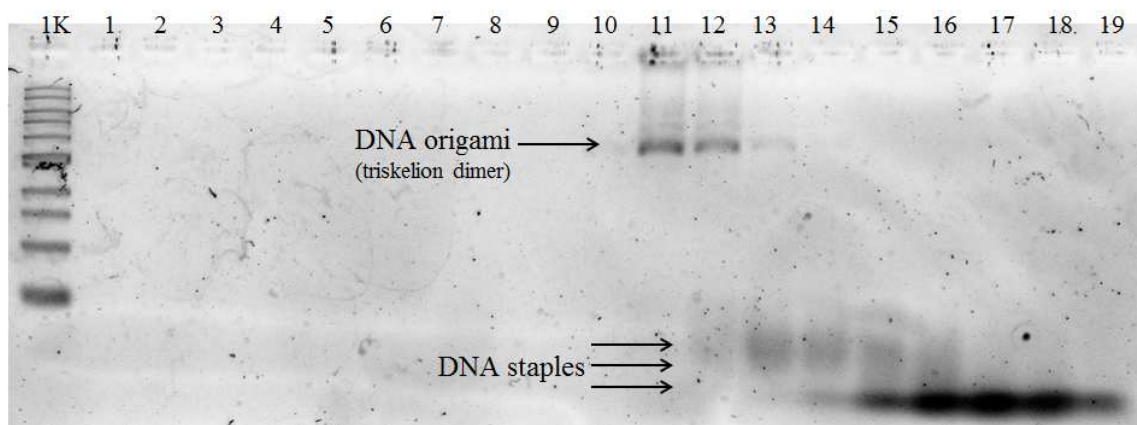


Figure A.4: AGE of 19 fractions collected from a 8mL size-exclusion column filled with Sephacryl S300. 1K ladder confirms that the migration of the DNA in the gel ran as expected. Numbers above each line correspond to the fraction number. In this case, fraction 11 was used for further experiments.

A.8.3 PEG precipitation

The folded DNA origami tetrahedra solution was mixed 1:1 (v/v) with folding buffer (1 x TE + 14 mM MgCl₂ for triskelion and 1 x TE + 12 mM MgCl₂ for tetrahedron) + 500 mM NaCl containing 15% PEG 8000 (w/v) (Fisher Scientific). The solution was mixed by tube inversion and spun at 16'000 g, at room temperature for 30 min using a microcentrifuge ((Eppendorf 5810 R, rotor, fixed angle) and the supernatant was removed using a pipette. If this method is used for purification as well as concentration of the sample, the sample was topped up to twice the initial volume with the PEG-containing buffer and centrifuged again under the same conditions as above. This step was repeated once more. After the last spin, the pellet was thoroughly dissolved in target buffer as indicated for each set of experiments and the concentration was measured using UV absorbance at 260 nm wavelength.

A.9 GOLD NANOPARTICLE ON DNA ORIGAMI

A.9.1 Gold nanoparticle (AuNP) preparation

DNA functionalisation with AuNP mainly follows the protocols from Ding *et al.*^[329] and Schreiber *et al.*^[330]. Citrate-stabilized colloidal 15 nm AuNPs (25 mL, BBI™ Solutions) were mixed with BSPP (bis(*p*-sulfonatophenyl)phenylphosphine dihydrate dipotassium salt, 10 mg, 750 μM, Sigma-Aldrich) and incubated for three days on a stirring plate at room temperature. AuNPs were precipitated by addition of NaCl (5 M, ~5% (v/v), or until colour change occurred) and pelleted by centrifugation at 1600 g for 30 min at 8°C. The supernatant was removed immediately (~1% volume was left in the tube at this stage) and then the AuNPs were resuspended in BSPP solution (2.5 mM, 0.4 mL) and methanol (0.4 mL). The mixture was centrifuged again (1600 g, 30 min, 8°C) and the supernatant was removed. BSPP solution (2.5 mM, 0.4 mL) was added and the concentration of the AuNP was measured with UV-Vis spectroscopy, according to the

equation $\frac{Abs_{520} \cdot dil.factor \cdot l}{ext.coef} = M_{AuNP}$. Where Abs_{520} = measured absorbance at 520 nm, $dil.factor$ = dilution factor of the measured sample, l = length of the light path [mm] and $ext.coef$ = extinction coefficient of the AuNP (3.67×10^8 for 15 nm AuNP).

AuNPs were then conjugated with thiolated ssDNA strands at a ration of AuNP:DNA that was dependent on the diameter of the AuNP: For 10nm diameter AuNP, a ratio of 1:300 was used, the ratio was then increased by a factor of Δd^2 with increasing AuNP diameter, d . (1:675 for 15 nm, 1:1200 for 20 nm, etc...) The disulphide bond in the thiol-modified oligonucleotides was reduced to monothiol using TCEP (20mM, 1 h) in water prior to mixing with the AuNP. The AuNP-thiol-DNA mixture was left at room temperature overnight before salt aging. Starting from 25mM NaCl, salt aging consists of doubling the salt concentration every hour until it reaches 700mM NaCl. The AuNP-DNA solution must remain red; if it turns blue the AuNP are aggregated and the sample is no longer viable. The AuNP-DNA mixture was left to sit at room temperature overnight. Excess thiol-DNA and small molecules were removed by washing 8 times with 400 μ L of salt-free buffer (1 x TE) using a centrifugal size-exclusion filters (10 min spin, 10000g, Amicon Ultra-0.5, 100MWCO, Merck Millipore). The concentration of these AuNP-DNA conjugates was estimated from the optical absorbance at 520nm as described above. Freshly prepared, fully covered AuNPs did not precipitate in 1 $\times$ TE + 100 mM $MgCl_2$, which is preferred for the formation of DNA origami. This high salt resistance property of fully covered AuNPs enables their assembly on the DNA origami template.

A.9.2 Folding and purification of DNA origami with AuNP

The AuNP-decorated DNA nanostructures (AuNP-triskelion) were folded in a one-pot reaction with 10 nM M13 scaffold, 1.2 equivalent of AuNP and ten-fold excess of DNA staples, following the same protocol as was used for the DNA origami without AuNP.

After folding, the DNA origami structures were purified from excess AuNP and staples by AGE (1.5% agarose, 0.5 x TBE + 10 mM MgCl₂, minimum 3 hours at 55 V). The band was cut from the gel and the AuNP-origamis were extracted by first cutting the gel into pieces, and then squeezing the pieces between a hard surface protected with clean Parafilm and a Parafilm-coated thick microscope slide. The solution that escaped from the gel was collected and centrifuged at 5'000 g for 30 min and 8°C to pellet the AuNP-triskelion. The supernatant was carefully removed and applied to a second centrifugation step (5'000 g, 30 min, 8°C), to obtain a second pellet of AuNP-triskelion. Both pellets were resuspended in 10 µL of origami buffer (1 x TE + 14 mM MgCl₂). This second centrifugation allows AuNP-triskelion to be obtained with less residual gel fragments. Although we used the first pellet for control experiments to measure the quality of folding, and fixation of the AuNP-triskelion, the second pellet was used preferably for delicate lipid vesicle coating experiments.

A.10 LIPIDS

DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine), 10 mg/2.5 mL in chloroform, from Avanti Polar Lipid for experiments made in Oxford. DOPC was provided by Lipoid GmbH in powder form (then resuspended in chloroform) for experiments made in KCL, London.

ATTO488-DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine) was obtained from ATTO-TEC GmbH in powder form.

A.10.1 Formation of liposomes

Liposomes were formed by rehydrating a lipid film. A DOPC lipid stock solution (100µL, 10 mg/mL in chloroform) was added to a glass vial and dried under vacuum overnight (or a minimum of two hours) to remove traces of solvent. The dried lipid film

was then rehydrated in 1 mL of buffer and left at room temperature for one hour without stirring. This facilitates the detachment of the film from the bottom of the vial, and forms a suspension of polydisperse multilamellar vesicles. Then the glass vial was vortexed to produce a homogenous turbid solution. The turbid lipid solution was then either sonicated with a diving tip or applied to cycles of freeze-thaw to break the large multilamellar vesicles into smaller liposomes. If sonicated, the glass vial was placed in a cold water beaker and sonicated (0.5 sec every second for 5 x 60 sec, 80% power). The sonication tip was then washed by starting a 1-min cycle of sonication in a mixture of 1:1 EtOH:isopropanol followed by a 1-min cycle in water. If the vial was applied to freeze-thaw cycles, the vial was first frozen by plunging it into liquid nitrogen, followed by a hot water bath. The freeze-thaw cycle was repeated five times or until the turbidity of the solution was reduced. Lipid aggregates and larger vesicles were removed by extrusion through a 0.2 μm polycarbonate membrane (Whatman) with the volume passed through Mini-Extruder (Avanti Polar Lipids) 21-times. Liposomes were stored at 4°C under argon atmosphere and used within one week. When mixed with triskelion and imaged by TEM, the liposome solution was diluted ten-fold, assuming a yield of 80%.

A.10.2 Electroformation of GUV

Giant unilamellar vesicles (GUVs) were produced by the electroformation technique^[331] described by Angelova *et al.*^[332] and Dimova *et al.*^[333]. Lipids in chloroform (50 μL , 1 mM DOPC, or 0.01% mol ATTO488-DOPE, 99.99% mol DOPC) were spread on two clean, ITO-coated conductive glass slides and dried in a vacuum desiccator for 30-60 min. A chamber of ~ 1 ml volume was formed between the two ITO slides by sealing with a rectangular PDMS spacer. Electrodes were connected to the two conducting sides of the slides (Figure A.X). A 1 mL solution of sucrose (50-100 mM) was injected into the chamber between the two electrodes and a square wave potential was applied with

an amplitude of 1.3 V and a frequency of 10 Hz for 90 minutes. (Figure A.5A). GUVs in sucrose solution form inside the chamber (Figure A.5B), and are collected using a wide bore needle (19G) to prevent shearing and used for epifluorescence and confocal microscopy experiments.

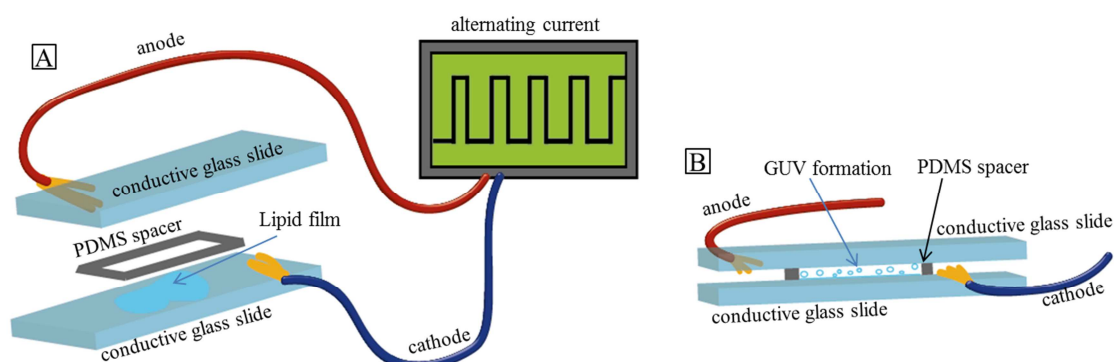


Figure A.5: Schematic of the chamber for electroformation of GUVs. (A) General view showing the electrode linked to an alternative current generator. (B) Side view showing the GUV formation when an alternating current is applied.

A.11 FLUORESCENCE MICROSCOPY

Epifluorescence and confocal experiments were done at King's College, London, UK in collaboration with Vivek Ramakrishna and Prof. Mark Wallace.

A.11.1 Supported Lipid Bilayer (SLB) formation

A small ring of approximately 1 cm in diameter was formed on a hydrophilized glass cover slip with Corning vacuum grease, and filled with a mixture of 50 μ l of vesicle solution and dilution buffer (PBS). Upon incubation for 15 minutes, a lipid bilayer was formed on the coverslip. The vesicles burst from the osmotic shock and hydrophilicity of the glass and forms a bilayer. Excess vesicles were removed by rinsing the bilayer with 2 ml of degassed deionised water (200 μ l per rinse), followed by rinsing with the dilution buffer. Finally, the dilution buffer was similarly exchanged for the experiment buffer (0.5 x TBE with 14 mM MgCl_2 , or 1 x TE with 14 mM MgCl_2).

A.11.2 Imaging GUVs in fluorescence microscopy

Fresh GUVs (<2 h old, 50 or 100 mM sucrose, 10 μ L, 0.01% mol ATTO488-DOPE, 99.99% mol DOPC) were added to the well containing the SLB. The higher density of sucrose trapped in the GUVs causes them to sink to the coverslip. The SLB acts as a passivating surface. This allows for the GUVs to be observed using epifluorescence microscopy. Purified triskelia bearing cholesterol anchors (9 cholesterol per triskelion) and labelled with Alexa-647 fluorescent dye (6 dye per triskelion) are then added to the system. Triskelia could be polymerised by adding a 10-fold excess of 4 bp 2 $\leftrightarrow$ 3 polymerisation staples to the 6T dimer triskelia.

Co-localisation of the DNA origami and lipids in the GUV was observed using a Nikon Eclipse Ti microscope (epifluorescence), or a Nikon Eclipse Ti-E inverted microscope (confocal). The wavelengths of the lasers used for the detection of fluorescence was 474 nm with a 525/39 nm Band Pass filter (lipids, ATTO488-DOPE), and a laser at 644 nm with a 680/42 nm Band Pass filter (DNA-conjugate, Alexa-647).

A.12 ELECTRON MICROSCOPY

TEM micrographs were acquired using a JEOL JEM100CX with a tension of 120 eV at LMU, Germany, and a FEI Tecnai 12 with a tension of 100 eV at University of Oxford, UK.

Protocol and setup used for grid preparation (formvar film and carbon coating) can be found in Chapter 6, section 6.1; details on the negative stains and staining method is described in section 6.2. A general sample preparation for the DNA star (Chapter 2), the triskelion (Chapter 3 and 4), and the tetrahedron (Chapter 5) for imaging is described:

A.12.1 TEM grid preparation

A 5-10 μ L droplet of DNA sample (1-50 nM) was placed on a glow-discharged grid for 3 minutes with the grid held by a reverse action tweezer. Excess liquid was removed by carefully touching the edge of the grid with a piece of filter paper. The sample was then quickly washed with 7 μ L of stain solution (UFO in LMU, UAc in Oxford University) and then blotted once more with filter paper. A second droplet of stain was then applied to the adsorbed sample and left on the grid for 10 seconds before being blotted away. For DNA sample of concentration between 50 and 300 nM, the deposition time was reduced to 1-2 minutes before being blotted with filter paper and stained as described above.

A.12.2 Cryo-EM

Cryo-EM micrographs of the DNA tetrahedron (Chapter 5) were acquired on a Tecnai TF30 (FEI), operating at 300 kV, equipped with a GIF Quantum LS energy filter (Gatan) and a K2 direct electron detector (Gatan), using a Gatan 626 side entry holder. Images were constructed by Lindsay Baker at the Division of Structural Biology (Strubi), Wellcome Trust Centre for Human Genetics, University of Oxford. The tetrahedron samples for imaging were prepared by Emma Silvester at the Clarendon Laboratory, University of Oxford.

After PEG purification of the DNA origami tetrahedron and removal of residual PEG using the QIAquick PCR purification kit by Qiagen in TE buffer with 5 mM MgCl₂, a final concentration of 2 μ M was obtained. The purified origami solution (2 μ L) was mixed with mammalian cell lysate (6 μ L, from freeze-thaw technique^[334]). The sample was then prepared and imaged as described in reference [302]. In brief, a EM grid (type

Quantifoil 2/1) was glow discharged and washed with acetone before applying 4 μL of the tetrahedron-lysate mixture. Excess liquid was removed after 10 seconds with a filter paper, as for negative staining, and the guillotine (holding the grid) immediately released into the liquid ethane recipient (located in a pool of liquid nitrogen).

A.13 ATOMIC FORCE MICROSCOPY (AFM)

AFM images were acquired in LMU, Germany, using a Veeco Dimension and AFM probes Olympus TR400-PSA variety, using the smaller cantilever. A volume of a few μL of annealed DNA strands was applied to a freshly cleaved mica surface, allowed several minutes to deposit the DNA three-way junction on the mica disc, and then imaged. Images were recorded in tapping mode, where the imaging buffer was 1 x TAE with 12.5 mM MgCl_2 and 4 mM nickel chloride (NiCl_2). Micrographs were then analysed using the software NanoScope Analysis 1.5.

A.14 MELTING CURVE

Melting curves were recorded in LMU, Germany, using a Mx3005P QPCR System, Agilent Technology. The melting curve was recorded as follows: increase of temperature from 25°C to 95°C at a rate of 1°/min, hold at 95°C for 15 min, decrease the temperature from 95°C to 25°C at a rate of 1°/min, and hold at 25°C for 15 min. This cycle was repeated 3 times, and fluorescence intensity was recorded 3 times at every change of temperature (i.e: 3 measurements per degree).

A.15 ADDITIONAL TEM IMAGES FOR SECTION 3.2.6

The following figure shows selected electron micrographs of triskelia with 90 bp long bridges and either the initial bridge (one DNA helix), or the reinforced, stiff bridge (three DNA helices). This illustrates the irregular arm positions observed on the EM grid when the weaker bridge was used. Triskelia with stiff bridges showed improved dihedral angle distribution, as a large majority of the structure ($>95\%$) exposed the expected $120^\circ \pm 5^\circ$ angle between them (Figure A.6B). Stiff bridges were visible on the micrograph, confirming the successful integration of the strands composing the stiff bridges. Single dsDNA bridges were not observable, which is why we have to consider the possibility of a ssDNA bridge (Figure A.6D).

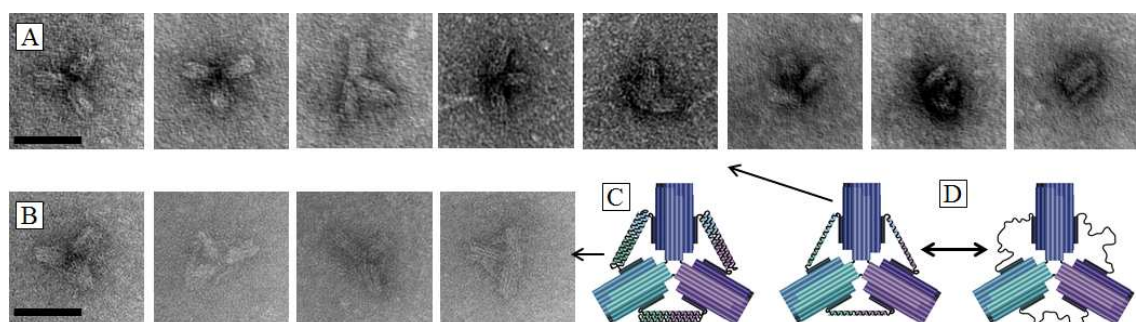


Figure A.6: Electron micrographs showing the range of arm positioning for triskelion (A) with one double helix bridge and, (B) with three double helices bridge. Scale bars 50 nm. Illustration of (C) triskelion with stiff bridges of three double helices, and (D) triskelion with bridge of one double helix or ssDNA.

It is important to stress that the triskelia in Figure A.6 are structures with bad conformations and are presented only for the purpose of illustration of the improvement made by the stiffer bridge. Analysis of the TEM images showed that, out of 400 counts, 60% (243/400) of the triskelia folded and imaged by EM with the single double DNA helix were in the expected configuration with their arms spaced by $120 \pm 5^\circ$, against 95% (382/400) when folded with the reinforced bridge.

A.16 DNA SEQUENCES

A.16.1 Chapter 2

	SEQUENCE STAR
strand name	sequence
S1	TCA ACT ACT AGC ATC
S2	CCG GAG ACT CCA GCT
S3	TAT TGC TGG AGA AGT GAC GGT CAG ACA CCT GCT GGC AAG CCT ACG ATT GGA CGC GGT ATG GAA CGT ACA CGT GCC GA
S4'	CCG TCA CTT CGC TTC TGT ATT CGA TCC GGT TCG TCT CGC CAA TCT AGT ATC GCG CAG TCGGCA CGT GTA GTT GTT TA
S5	AGC ACC AAT CGT AGG TTT TTC TTG CCA GCA CCA ATC GTA GGT TTT TCT TGC CAG CAC CAA TCG TAG GTT TTC TTG CC
S6	TGG CTT TGA GAC GAA CCA CTA GAT TGG CTT TGA GAC GAA CCA CTA GAT TGG CTT TGA GAC GAA CCA CTA GAT
S7	AAT AGA TGC TAG TAC GTT CCA TAC CGC GTG GTG TCT GA
S8	CTG CGC GAT GGA TCG AAT ACA GAA GCG TCT CCG GTA AA
S8'	CTG CGC GAT GGA TCG AAT CAT TCT CCT ATT ACT ACC
S9	ACA GAA GCG TCT CCG GTA AA
S1	TTG TCA ACT AGC ATC
S1' for 3bp	A CA ACT ACT AGC ATC A
S1' for 2bp	AA CA ACT ACT AGC ATC TA
S2 for 4bp	CCG GAG ACT CCA GCT
S2 for 3bp	ACCG GAG ACT CCA GCA
S2 for 2bp	TA CCG GAG ACT CCA GC AA
S4	CCG TCA CTT CGC TTC TGT ATT CGA TCC GGT TCG TCT CGC CAA TCT AGT ATC GCG CAG TCG GCA CGT GTT GAC ATT TA
chol-DNA	GGT AGT AAT AGG AGA ATG /3CholTEG/

red: sequence of sticky ends, or T-loops or T-cap,

light green: improved sequence on S1' and S4'

dark green: extra nucleotide in compensation of a shorter sticky end

orange: cholesterol handle sequence

A.16.2 Chapter 3 and 4

SEQUENCE TRISKELION

black staples on CaDNAno file: common to all the triskelia in the thesis

CORE and VERTEX STAPLES (black in caDNAno: see A.15)

core_staple_45[95]33[108]	GAG CAA CAG TTT ACT AGA AAA AGC CTG TTT AAT AAA GCT AAA GTA TTC AGC TAT TC
core_staple_53[102]45[94]	GGA AGA GGT CAT TTT TGT TGA CCA GAA GCA AAA ACA GTA ATG CAG GTA A
core_staple_21[109]25[101]	AGG CGG GGT TGA GGG TTC GTA CTC AGG AGG TAG AA
core_staple_69[70] 71[90]	GTA ACG TAG ATG ATT ACC TTT TAC ATC GCA GAA ATA GCT CAT TTC GAT C
core_staple_21[74] 13[69]	CGG CAT ATC GGC CTT GCC ATG GAA TGG ATT AGG CCA AC
core_staple_67[70] 69[83]	GGC GCA TAA ACA TAT TTG AAT TAC CTT TCA AAA GAG CCG GAA
core_staple_71[91] 73[112]	GGT GCG GAT TTG CAT ATA ATC CTG ATT GTA AAA GTG GTT TTC CCA GTT TT
core_staple_16[112] 21[108]	TTT CGC AGT CTC TGA TTT GAT GAT ACA GGT GCC TTG TAA G
core_staple_68[112] 83[112]	TTT AGG CTG CGC AAC GGG AAA CTG CGC TCA CTA TTA AAG AAT TT
core_staple_19[77] 27[80]	AGA GTA GCG TCA TAT ATA AAC TAT TCT GGC GCC GCT ACA GGG TTA G
core_staple_63[98] 69[112]	TAC ATA ATA TTC ATC ATT CGC CAT TCT TT
core_staple_70[112] 74[98]	TTT TTA CGC CAG CTG AAA GTG TCA TAG CT
core_staple_81[102] 58[95]	GTG TGG CCC TGA GAG AGA GAG TCC ACT GCC CGA AGC ATG CGA AAG ACG CCA GTT G
core_staple_30[58] 46[63]	CGC GCC TGA TAT TAT ACA GAG GCA AAA GAA TTC TTT CC
core_staple_25[91] 10[87]	CAC CCT CTT AGT ACA TAA GTG CCA CCA CCA GAG GCC ACC CTC TCA G
core_staple_27[81] 24[66]	TAA GTG AGA ATA GAA AGG AAC AAC CGT CTT TAC TG
core_staple_52[45] 40[52]	CAT GCG GTT TAT CAG CTT TGA CAA CTC GCT GAT AGG
core_staple_15[60] 22[63]	GCT TGG TAA TAA ACA GGA GGC CCT TAT GGT TAC CAC CAG GAA GGG
core_staple_32[72] 44[70]	TAA CCA GTA ATA AGA GAA ATT GAG AAT TAC GAA TCA TCT TTG ACC
core_staple_33[109] 38[102]	CAA GAG AAC AGA CTT GCT GCT ATT TTG CAC CAC TTT AAC TG
core_staple_42[112] 46[98]	TTT CCA CAT TCA ACT TCA GAA AGA TAG CG
core_staple_43[91] 45[112]	CAG TTG AAA CGC GCG TAC CGA CAA AAG GCA ACG CTC AAC ACT ATC ATT TT
core_staple_83[81] 80[67]	GTT AAA CCG TCT ATC AGG GCG ATG GGG TTG AGG T
core_staple_79[84] 60[73]	ATG CAT TGG GCG CCA GTC GGT TGT ACC ACT CAT ATT AGG AAC TTA AAT CAA AG
core_staple_23[84] 4[73]	AAT GCG GCG CCA CCC TCG TGG CGA GAA ACA CCC GCT CTG AAA ATA CCT AAA TT

A.16 DNA sequences

core_staple_35[56] 48[59]	AAG GCT TCC AAT AGA ACC GGA AGG CGC AGG CTT GCG CAT
core_staple_33[67] 38[56]	TGT ACC GCG CAT CCG GTA ACG CTA ACG AGC GCC AGA ACC AAA GCT
core_staple_57[84] 64[84]	GAA GTA TAC ATT ATT CAT CAA CGT AAA AGG AGA AAA CCT GAG TTT AAT GCT TAG AA
core_staple_41[70] 43[90]	TAC CCA AAA TCA TTA GAA ACC AAT CAA TTC AAC AAT TGA AAG AGT TCA T
core_staple_29[77] 33[66]	CAC CGG ATT CTG ACC TAA ATT TAA TGG TTA AAT AAA TAT TTA TTT CGA GGT CCT GAA GCA
core_staple_74[97] 70[84]	GTT TCC TAT GCC TGA AAC GAC GGC CAG TTT GGG TAG GGG ATG
core_staple_5[98] 15[112]	TTA TTC ATA AGT TTT TGA CAG GAG GTT TT
core_staple_4[72] 16[70]	CAT ATT ACG CAG TAT GTA ACC GAG CAA ATA ATA CTG TCC ATC ACG
core_staple_54[112] 53[90]	TTT CGC AAA TGG TCA ATA ACC TGT TTA GCA TTT AGT CGG ATG GGC AAA CT
core_staple_49[109] 53[101]	GAA GAC TAT TGA TTA AGT AAT TCG AGC TTC AGT CA
core_staple_77[109] 81[101]	CCT ATT GCG TCT GTC GTG GGA GAG GCG GTT TAC CA
core_staple_78[62] 77[52]	TAT GAC CAT AAA GCC CCA GCA GGC GAA AAA TAG CCG GGT GAG TGA T
core_staple_5[109] 7[97]	AGG GGG AAT TCG TCA GAT AGC CCC CTT ATT AGC GTT AAA AGT TTG
core_staple_79[46] 80[35]	GCA ACT TTT GCG GGA GAA AGA ATT TTG ATG G
core_staple_14[83] 19[76]	ATA TTC AAA CCT ATA GTT AAT GCC CCC TGA GTA AA
core_staple_39[81] 39[69]	GAG GAC GTT GGG AAA TCA ACG TAA GAG TAG T
core_staple_24[65] 21[73]	AGT GAC GGG GAA AGC CGG CGA ACA CCA TGT ACC GTA ACC CAG ACG CGC GTA CAT TT
core_staple_24[45] 12[52]	CTA CGG AGC CCC CGA TTT CGA AAG GTC ACG CTT GGC
core_staple_1[77] 5[66]	AAT AAG ACA AAG TCA GAG GGT AAT TGA GGA GTT AAA ATA ATA ACT CCT TAT GGT TTC CGA
core_staple_57[77] 61[66]	GGA TTT ATA TCT AAA ATA TCT TTA GGA GGA TAA TAA AGA AAC TGA TTA TTT GCG TAG TAA
core_staple_77[74] 69[69]	ACA AGA GGA AGA TTG TAA TTT TTG GCC ATC ATG AGC GA
core_staple_12[112] 27[112]	TTT CCG CCA CCA GAA CCG TCG ATT GCT CAT GGG ATT TTG CTT TT
core_staple_39[70] 41[83]	AAA TTG GTC TTA CCA TTC TAA GAA CGC GTC GTA GGG AAA AAT
core_staple_70[83] 75[76]	TGC TGC ACA ACA TAA TTG TTA TCC GCT CGT CTG GA
core_staple_80[45] 68[52]	CTG TAG CAA AAT TAA GCA CTG TAA TAG GAT AAT TCG
core_staple_11[81] 11[69]	TGA CGC CAC CCT TAG AAC CCT TCT AGT CTT T
core_staple_82[112] 81[90]	TTT CGT GGA CTC CAA CGT CAA AGG GCG AAT GGA ACA TTG CAG CTG GTT TT
core_staple_26[112] 25[90]	TTT AAA CAA CTT TCA ACA GTT TCA GCG GAA TGA ATT TAG GAA CGA ACC GC
core_staple_13[70] 15[90]	AGA GAC ATT ACC ATA GGG AGG GAA GGT AAA TAG AAC ATT TTG ACC CGC C
core_staple_23[45] 24[35]	GCG GAG CGG GCG CTA GGG CCT AAA GAA CGC CT
core_staple_42[83] 47[76]	ACG CCA ACC CTC AAA TCG TCA TAA ATA TAC ACT AA

A.16 DNA sequences

core_staple_11[70] 23[83]	AAT GCG CAT ACC GAG TAA TCA GTA GCG AAG CAC CAG AAC CGC TCA ATC GCG CGC TT
core_staple_75[77] 83[80]	GCA AAC AAG CTT GCG TGT GAA CGA GCC GGC ATG CCT GAG TAA TCC A
core_staple_80[66] 77[73]	TTG CCT CAG AGC ATA AAG CTA AAG GAA GCG GTC CAC GCT GTG TTG TTG TGT AGT TCC
core_staple_81[91] 79[83]	TCT TTT CGC GTA TTT CCA GTC TGT TGG GAA GGG TTT AAC CAA ATT TTA A
core_staple_25[102] 17[94]	CCG GAT AGC AAG CCC AAT TCT GTA GTA CCA GAA ACA TGG ACG ATT TTA A
core_staple_40[112] 55[112]	TTT ACA ACA TTA TTA TCA AAA AAT AGT CAT TAG ATA CAT TTT TT
core_staple_46[97] 42[84]	TCC AAT AGC GAG AGC CAG ACG ACG ATA AAG GCA TAA TAC ATA
core_staple_61[98] 71[112]	GGA TTC GCA AAA TTG CCT CTT CGC TAT TT
core_staple_1[84] 8[84]	GCA AGA AAG AAG GAT AGC AAA CAC AAT CAA TAT TGC ACC AGT CAG AAT CCA TCG CC
core_staple_43[60] 50[63]	AAC GCT CCA TTT CAT GAG GAA GAT AAA GAC AAG GGA GTA GTT GCG
core_staple_5[67] 10[56]	TTG TAG CAA GCA GCA CCA CGA ACC ACC AGC AGG CTA TTG ACC TGA
core_staple_63[56] 76[59]	ATT TAA CAC ATC AAT TAA ATG AAA ATA AAA ATT TTT TCA
core_staple_15[91] 17[112]	GCC AGC AAT TTT GTC GTA GAA AAT ACA TCG AAC AAC AGA ATG GAA AGT TT
core_staple_67[81] 67[69]	ACC CCG CTT CTG AAC CCG TCG GAT GTA GAT G
core_staple_60[72] 72[70]	AAA CAG ATG ATG GCA ATC ATT TTG GAT TAA GGC CAA GAG AAT CGA
core_staple_41[84] 35[97]	CTA CGT TCA GTC AGA TGG TTT AAT TTC ACA GCT ACT TAG CGA
core_staple_52[65] 49[73]	AAT AGG TGA ATT TCT TAA ACA GAA CTT AGA GCT TAA TTC TGC GAA AGC AGC GTG AA
core_staple_22[62] 21[52]	AAG AAA GAG AGC TTT TCG TCA CCA GTA CAT CTA AAC GAG CAC GGG A
core_staple_14[112] 18[98]	TTT TGA GGC AGG TCA AAA GTA TAG TAA CA
core_staple_76[58] 80[46]	AAA CGA GAT AGC CCA CTA CGC CCA AAT CAA GTC AAA AGA TC
core_staple_49[74] 41[69]	TCC AAA ATC CGC GAC CTC GAA CTG ACA GAC CTA TTC AT
core_staple_18[97] 14[84]	GTG CCC GCA TGG CTA TTT ACC GTT CCA GAT CCT CAG GCC TTG
core_staple_20[58] 24[46]	TGA GTT TTG TTA AAG GAA TTG TTT TTT CAC GCG TAA CGA AA
core_staple_51[84] 32[73]	GCG GGC GCC AGA CCG GCT TGA TAC CGA TTA AAG GCA CGG TGT ACC AAC TTA GA
core_staple_33[98] 43[112]	CCT TAT CAA TGC AGG ATT TAG GAA TAT TT
core_staple_44[112] 49[108]	TTT AAC CCT CGT TTA GCT TTT GCA AAA GAT AGA CTG ACG A
core_staple_2[58] 18[63]	AAT TGA GTA GAC CGT TGT CAG TGA GGC CAC CGC GAT TA
core_staple_17[95] 5[108]	AGC AGT TAC CAC AAT GAA ATA GCA ATA GCT AAG ATA GCA CAT AAA CAC GGA ATT AA
core_staple_7[56] 20[59]	TCG ATA GGC CGG AAA CAT TCT TTT ACA TGC GCG TAG CTT

A.16 DNA sequences

core_staple_53[91] 51[83]	CCA ACA GAA GCG AAG ATT GCA CAG GTA GAA AGA GAC AGA TGA CGC TTT T
core_staple_47[77] 55[80]	AAC ACA CCA AAA TAC TGC GGA ATG CTT TAG ATC GTC ACC CTC CGA G
core_staple_69[84] 63[97]	ACC AGG CTC CGG CAG TGC ATC TGC CAG TAT TTT CCG AAA CAG
core_staple_29[84] 36[84]	ATC ATA AAG GGC TTA TAT AAA CTG TTT AAA TCG GCA TTT TCA AGG CGT TAA TTT TA
core_staple_51[45] 52[35]	TCG GAA CCA TCG CCC ACG ATT GTA TTT TTA AA
core_staple_58[58] 74[63]	AGA TCA GAA ATA ATC GTG TCA TTG CCT GAG AAC CTG AT
core_staple_58[94] 61[108]	AGT ATA GAC TTT ACA AAC AAT TCG ATT AAT TTT TTG GAT ACC ATA TCC TG
core_staple_7[98] 13[112]	CCT TTA GAG AGC CAC CAC CCT CAG AGT TT
core_staple_72[112] 77[108]	TTT CAC GAC GTT GTA CAG GTC GAC TCT AGT CAT GGT AAA G
core_staple_71[60] 78[63]	TAA TAA GCA ACA ACC GTT CTA GAA GTA AAG ATA GAA CCA AAA CAT
core_staple_50[62] 49[52]	CCG ACA AGC TTT CGA TAA TGC TGT AGC TTA TAA CAA ACG AGG AGA C
core_staple_55[81] 52[66]	TAG TAT ATT TTC ATT TGG GGC GCG CCC AAT TGC TG
core_staple_48[58] 52[46]	CGG GTT GAT TAG CTG AAA AGT TCT ACT AAT ACA TTC CAC AA
core_staple_35[98] 41[112]	ACC TCC CAG CAA GCC GAA CTA ACG GAT TT
core_staple_61[109] 67[101]	ATT GGC GAA TAT CAA TAG TCG CTA TTA ATT ATT GA
core_staple_61[67] 66[56]	CAG GAA ACA AAA TTT CAG CGA TAG CTT AGA TCG TTG GTT CTC CGT
10[123]11[123] -not in contact	TTT GAA CCA GAG CCA CCA CAT AAT CAA AAT CAC CGT TT
38[123] 39[123] -not in contact	TTT TAT GCG ATT TTA AGA ATC ATT GTG AAT TAC CTT TT
66[123] 67[123] -not in contact	TTT GCC TCA GGA AGA TCG CGA CGA CGA CAG TAT CGT TT
triskelion_vertex_6T_21[53] 2[42]	GCT ATC CAG AAC TTG CCA CCC AAA AGT TTT TT
triskelion_vertex_6T_68[51] 58[59]	CGT AAA ATT CAG GTT TAA CTT TTT TTT TAT TAT CAT CAT ATT CCC ACC
triskelion_vertex_6T_79[27] 82[21]	TTT TTT CCT TTA TTT GGA GAC ATT ATA AAT TTT TTG GGG TCG AGG TGC CG
triskelion_vertex_6T_12[51] 2[59]	AGA ACA GGA ACC AAA GAC ATT TTT TTT TTT TAA CTG GCA TGA TTA AGA CGG
triskelion_vertex_6T_46[62] 44[31]	ATT AAA CGG GTA AAA AAC GAA CAA GCG CGA AAC AAA GTA CTT TTT T
triskelion_vertex_6T_83[28] 74[27]	AAA TCC CGT TTT TTT TTT TTT CAA ATC ACC GCT ATT TTT TTT TTT
triskelion_vertex_6T_73[31] 70[31]	TTT TTT TAT GTA CCC CGG AAT TGT AAA CGT TTT TTT
triskelion_vertex_6T_9[42] 11[80]	TTT TTT ACA GAG GTG AGG CGG TCA GTA TTA ACA CCG CCT GCA AAT TAA AAG AAC
triskelion_vertex_6T_23[27] 26[21]	TTT TTT CTG GCA AGT GTG CTT TTA GTT AGT TGA AAA TCT CCA AAA AAA AG
triskelion_vertex_6T_74[62] 72[31]	AAA TTA ATG CCG GAC TAT CAG AAA ACT AGC ATG TCA ATC ATT TTT T

A.16 DNA sequences

triskelion_vertex_6T_18[62] 16[31]	AAG GGA TTT TAG ACT TTA TAA TAG CAA TAC TTC TTT GAT TTT TTT T
triskelion_vertex_6T_19[27] 23[44]	TTT TTT AAT CCT GAG AAG TGT TAG GAA CGT CAG AGC GTA TAA CGT A
triskelion_vertex_6T_41[31] 38[31]	TTT TTT CAA GAG TAA TCT CAG TGA ATA AGG TTT TTT
triskelion_vertex_6T_51[27] 54[21]	TTT TTT TAA CCG ATA CGG CTA CGA AGT TTG TAG TAG CAT TAA CAT CCA AT
triskelion_vertex_6T_65[41] 67[80]	TTT TTT GAG AAG AGT CAA TAG TGA ATT TAT CAA AAT CAT AGG TTC CTT GAC GTA
triskelion_vertex_6T_55[28] 46[27]	GTG TCT GAG TTT TTT TTT TTT AGG CTT TGA ATG CCA CTA TTT TTT
triskelion_vertex_6T_69[31] 66[31]	TTT TTT TAG CCA GCT TTC AAC GGC GGA TTG TTT TTT
triskelion_vertex_6T_77[53] 58[41]	ATT ATA TTT ATT GAT AAA GGA GCG GAT TTT TT
triskelion_vertex_6T_27[28] 18[27]	GCC CTC ACC TTT TTT TTT TTT TCG TTA GAA GTA CGC CAG TTT TTT
triskelion_vertex_6T_47[27] 51[44]	TTT TTT CGA AGG CAC CAA CCT AAT ACG TAG GAC TAA GTA GCA ATA T
triskelion_vertex_6T_17[31] 14[31]	TTT TTT AGT AAT AAC ATC ACA ATA TTA CCG TTT TTT
triskelion_vertex_6T_37[41] 39[80]	TTT TTT AGC CTA ATT TGC CAG TTA CAA AAT AAA CAG CCA TAT TTC CTG AAG CTT
triskelion_vertex_6T_40[51] 30[59]	CTG AAT CAT AAA ATA ATA TTT TTT TTT TTT TAT TTA GGC AGA GGC ATA CAA
triskelion_vertex_6T_49[53] 30[41]	TTT GTT ACT TTA TCA TCC AAC ATG TAT TTT TT
triskelion_vertex_6T_29[41] 28[41]	TTT TTT GTG ATA AAT AAG GCG TTT GAA ATA CCG ACC GTT TTT TT
triskelion_vertex_6T_15[31] 12[31]	TTT TTT CCA GCC ATT GCA TTC ACC AGT CAC TTT TTT
triskelion_vertex_6T_43[31] 40[31]	TTT TTT GCG CAG ACG GTC GCT GAC CTT CAT TTT TTT
triskelion_vertex_6T_75[27] 79[45]	TTT TTT GAG AGA TCT ACA AAG GGA GGG TAA TCA ATA AAA GGC CCA AC
triskelion_vertex_6T_1[41] 0[41]	TTT TTT ATA ACC CAC AAG AAT TCG CTA ATA TCA GAG AGT TTT TT
triskelion_vertex_6T_13[31] 10[31]	TTT TTT ACG ACC AGT AAT AGA ATA CGT GGC TTT TTT
triskelion_vertex_6T_57[41] 56[41]	TTT TTT ATT AGA GCC GTC AAT ACA CTA ACA ACT AAT AGT TTT TT
triskelion_vertex_6T_45[31] 42[31]	TTT TTT AAC GGA GAT TTG AGC CGG AAC GAG TTT TTT
triskelion_vertex_6T_71[31] 68[31]	TTT TTT TAA TAT TTT GTT CTG GCC TTC CTG TTT TTT
triskelion_vertex_6T_66[55] 71[59]	GGG AAC AAT CAA CAG AAA ACA AAT TTT TTT TTT TTG TCA GAT GAA TAT ACA GAT TTT CGC AT
triskelion_vertex_6T_10[55] 15[59]	AAG CGT AAA AAG GGA CGT CAC CAT TTT TTT TTT TTA AAG GGC GAC ATT CAA ACC AGC GAA AC
triskelion_vertex_6T_38[55] 43[59]	GCT CAT TTG ACA AGC AAG CAA ATT TTT TTT TTT TTC CCA TCC TAA TTT ACG ACA AGA AAG GG
extra_support_25[21] 22[27]	CAC TAA ATC GGA ACC GTT TTT T

A.16 DNA sequences

extra_support_53[21] 50[27]	AAA AGG AGC CTT TAC ATT TTT T
extra_support_80[34] 55[27]	TGG TTC CGA AAT TTT TTT TTT TTT TGT ACG
extra_support_52[34] 27[27]	TAT GCA ACT AAA TTT TTT TTT TTT TTA GAC A
extra_support_81[21] 78[27]	ATA CAG GCA AGG CAA GTT TTT T
extra_support_24[34] 83[27]	GTA GCA TTC CAC TTT TTT TTT TTT TTC GGC A

STIFF BRIDGES three DNA helices (curved 60 bp and flat 90 bp)	
bp60_2_85	CGTGCATTCTCGCCATGTTCCGTCAGTACAAGTTGAAGAGTCG GTCACGTTAGAACAACG
Duplex_62_87	CGCCTCACTAACATGTAAGTGTAATTGAATGTGAGATTCAGCT CGAGAGACTAACACACAGC
bp60_1_87	GCGCTGTCGTGCCGACGTGTAAGTAAGGTTAGCGTACTGACA GGTCGAACCGGCGCTGC
bp60_2_87	GCGGTCCAGTGACCGCCATTCCGACCGCTCTCACACACACGA ATAACGAAGTCAAGGTGC
1*-flat_84	CCGGCGCGACCAGTTAAGTTATGATGGCAAATATATTTTAGTT AATTTTAAAGTGTCTGGTTT
2*-flat_84	CAACAGTTTGGGTTATATAACTATATGTAAAAACGCGAGAAA ACTTTTAAACACGT
3*-flat_84	TGGCGGGACTTCCGCGCGGCCCTGCGTTGGTCGGTAGTTCTC TCTGTCT
4*-flat_84	AACCTCCGGCTTAGGAGTTATAGCCGCACTCGACCTCATGTGT ATTGTAC
5*-flat_84	GAGACTACGAGATAGGAAGAAGCGCGCACAACAACTGTCGGC GTGCGACACTTTTT
1*-curved_84	CCGGCGCGACCAGTTAAGTTATGATGGATATATTTTAGTTAAT TT
2*-curved_84	CAACACTAGGTTGGGTTATATAACTATATGTGCGAGAAAACCT TTTCAAAACACGT
3*-curved_84	TGGCGGGACTTCCGCGCGGCCCTGCGTTGGTCGGTAGTTCTC TCTGTCT
4*-curved_84	TTTAACCTCCGGCTTAGTTATAGCCGCACTCGACCTCATGTGT ATTGTAC
5*-curved_84	GAGACGAGATAGGAAGAAGCGCGCACAACAACTGTCGGCGTG CGACATACCTT
1*-flat_85	GAACCGCGGTAGGAGAGGCACCTGAGAACAGTTGAAAGGAAT TGAGGATTGTCATACTGCTTT
2*-flat_85	GTGACCGAATCTAAAGCATCACCTTGCTGAATGGTCAGTTGGC AAATCACTCTCGG
3*-flat_85	ATCTCCGCTCCGAGCGCGTTGCTCCGGAGTTGCATTGTACTTA GGTCTAG
4*-flat_85	CAGCAGCAAATGAAAACCTTCCGACGACAGGATCCACAGTT CTTCTAAC
5*-flat_85	GCCACGCTGAACATGGAATGCACGCGCAGTCCGGTCAACTTG TACTGACGGAGAGC
1*-curved_85	GAACCGCGGTAGGAGAGGCACCTGAGAGTTGAAAGGAATTGA GGA
2*-curved_85	GTGACCGAAAAATCTAAAGCATCACCTTGCTTCAGTTGGCAAA TCAACACTCTCGG
3*-interm_85	ATCTCCGCTCCGAGCGCGTTGGTCCGGAGTTGTATTGTACTTA GGTCTAG

A.16 DNA sequences

4*-interm_85	AGCCAGCAGCAAATGACTCTTCCGACGACAGGATCCACAGTT GTTCTAAC
5*-interm_85	GCCACGAACATGGAATGCACGCGCAGTCCGGTCAACTTGTAC TGACGGCTGAG
1*-flat_87	GAGGCGGCTGTGTCGAGCTGAATCTCAGGGAGAATTAAGTGA ACACCCTTTCTCTGTTACTTT
2*-flat_87	CGTTATTGTTTAACGTGAAAAATCAAAATAGCAGGGAAGCGC ATTAGACCATTCAT
3*-flat_87	TTACACACGACAGCGCGCACCTTACTTACACGTGCGGCTTACA TGTTAGT
4*-flat_87	AAGAAACGATTTTTTTCGTGTGTCGACCTGTCAGTACGCTAACC TTGACTT
5*-flat_87	ATCCCAATAATGGCGCTGGACCGCGCAGCGCCGGTTGTGAGA GCGGTCCGCCAAAT
1*-interm_87	GAGGCGGCTGTGTCGAGCTGAATCTCAAGAATTAAGTGAACA CCC
2*-interm_87	CGTTATTGTTTAACGTCAAAAATCAAAATAGCAGGGAAGCGC ATTAGACCGTTCAA
3*-interm_87	TGATTGTACATTCGGCGTGACATTCATTCCACGCGCGACAGC ACACATT
4*-interm_87	AATAAGAAAGCATTTTCGTGTGTCGACCTGTCAGTACGCTAACC TTGACTT
5*-interm_87	ATCCCAATGGCGCTGGACCGCGCAGCGCCGGTTGTGAGAGCG GTCCGAAT

POLYMERISATION STAPLES (4 bp sticky ends arm 2-3 only)

30[131]29[131] 2<->3, 4bp	GAC GAC AAA TTC TTA CCA GTG TAT CAT ATG CGT TAT AGT C
32[131]31[131] 2<->3, 4bp	CTG AGA CAA TAA ACA ACA TGA TTC TGT CCA GAC GAC TGC A
34[131]33[131] 2<->3, 4bp	GCA CAA GTA CCG CAC TCA TCG AAC GGG TAT TAA ACC CAG T
36[127] 35[127] 2<->3, 4bp	TTT TTA AAT CAA GAT TAG TGG GAG GTT TTG AAG CCT CAG
48[131]49[131] 2<->3, 4bp	ACG TAT CAG GTC TTT ACC CTT GAC CAT AAA TCA AAA CGT G
50[131]51[131] 2<->3, 4bp	TTT TTC AAA TAT CGC GTT TAG GAA GCC CGA AAG ACG TCA
58[131]57[131] 3<->2, 4bp	GTG CCT TTG CCC GAA CGT TAC AAC TCG TAT TAA ATC CTG A
60[131]59[131] 3<->2, 4bp	TCA GGG AAG GGT TAG AAC CTT ATA CTT CTG AAT AAT ACT G
62[131]61[131] 3<->2, 4bp	CGT CTA CAA AAT CGC GCA GAG CTT TGA ATA CCA AGT TGC T
64[127] 63[127] 3<->2, 4bp	TTT CTT GCT TCT GTA AAT CTA TGT GAG TGA ATA ACG ACT
76[131]77[131] 3<->2, 4bp	ACG TGA GCT AAC TCA CAT TAG GGG TGC CTA ATG AGT TGA C
78[131]79[131] 3<->2, 4bp	TTT AAT CGG CCA ACG CGC GGC CAG CTG CAT TAA TGC ACG
INVADING STRANDS (6T arm 1-1)	
2[121]1 [133] 1<->1, 6bp TTT	AAA AGT AAG CTC TTA CCG AAG CCC TTT TTT CGG CA

A.16 DNA sequences

4[121]3 [133] 1<->1, 6bp TTT	GCA AAG ACA CGG TGG CAA CAT ATA AAT TTC CGA CT
6[121]5[133] 1<->1, 6bp TTT	TGA GCC ATT TTG AAT TAT CAC CGT CAT TTA GAA AC
8[121] 7[133] 1<->1, 6bp TTT	TTT TCG GTC ACT GTA GCG CGT TTT CAT TTT TTA AG
20[121]21[133] 1<->1, 6bp TTT	TTA GGA TTA GCT GAG ACT CCT CAA GAT TTG GAA TA
22[121]23[133] 1<->1, 6bp TTT	GGT GTA TCA CGA TAT AAG TAT AGC CCT TTG AAG GA

A.16.3 Chapter 5

	SEQUENCE TETRAHEDRON	
	each colour correspond to one edge	
strand Name	Sequence	
0[55]1[69]	GGGGTCTAGTGAGGCACCTATCTCAGCGTTGATCTAACTCAC	
0[94]4[70]	TTTTTTATCTCAAGAAGATCCTATCTGTCTCTGACA	
1[28]5[41]	AAACGTTAATACCGCTCTTGCCCGGCGTAAGTCATTTCTGTG	
1[42]4[56]	GCGAACGCTCAGTGTTACCTAAATTAATAAATGAAATGCTTA	
1[70]3[78]	GTTAAGGGATTTTTTTTTTTTTTTTGGTCATGAGATATCAATCTA	
2[17]0[1]	CAGAACTTTTTTTTTTTTTTTTAAAAGTGCTCATAGATCCAGTTTTTTTTT	
3[79]5[94]	AAGTATATATTTTTTTTTTTTTTGGAGTAACTTGGTATTTTCGTTTTTTTTT	
4[25]2[18]	AGTGTATGCGTTTTTTTTTTTTTGGCACCAGTTGCGCCACATAG	
4[55]1[41]	ATCAGTACTCAACCCACTTTTAGATCATAACGGGATCTTCGGG	
4[69]0[56]	GTTACCAGTTTTTAATATCAAAAAGGATCGAACGAATTTCTAC	
5[2]1[27]	TTTTTTTGTCATGCCATCCGTAAGCTGTTGCATTGGA	
5[42]4[26]	ACTGGTGGAACCTCTCAAGGATCTTACCGATGCTTTCTGAGAAT	
6[55]7[69]	ACAGGCAGCTAAGCGGATGCCGGGAGCATTGTTGCCGTTTGG	
6[94]10[70]	TTTTTTAATAGTTTGCGCAACGGACAAGCTCCCGGA	
7[28]11[41]	GAAGTAATGTGCAAAATAGGCGTATCACTCTAAGATTTCCCC	
7[42]10[56]	GCAGTGTGGTGTGAGAGTTACTTTCGTCTCGCGCGACAGCTT	
7[70]9[78]	TATGGCTTCATTTTTTTTTTTTTTTCAGCTCCGGTTCGATGACGGT	
8[17]6[2]	AGCTCCTTCTTTTTTTTTTTTTTGGTCCTCCGATCTGCATAATTCTTTTTT	
9[79]11[94]	GAAAACCTCTTTTTTTTTTTTTTGACACATGCAGCCCGTCAGGGTTTTTTT	
10[25]8[18]	TATCATGACATTTTTTTTTTTTTTTAACCTATAAAAAAAGCGGTT	
10[55]7[41]	GTCTGCACCTGACGGAGGCCCATGATCCCCCATGTGTTGGCC	

A.16 DNA sequences

10[69]6[56]	GACGGTCTTTCGGTCCAACGATCAAGGCCGCTCGTCATTGCT	
11[1]7[27]	TTTTTTAAACAAATAGGGGTTCGCAGCACGTTGTCA	
11[42]10[26]	GAAAAGTTTCGTTATCACTCATGGTTATGGCGCACAAACCATTAT	
12[34]15[22]	TATCGCCGTCGTGTGGCCCCAGTGCTGCAATGATTTTTTTTTTTTACCGC G	
12[55]13[69]	TTTCTCACGTTAATTGTTGCCGGGAAGCGGGAAGCTCTCAGT	
12[94]16[70]	TTTTTTCGCCTTTCTCCCTTCTAGAGTAACTTTA	
13[42]16[56]	CCCGGGCTCACGCTCGAACCCATATCAGCAATAAACCCATCCA	
13[70]15[78]	TCGGTGTAAGTCTTTTTTTTTTTTTGTTTCGCTCCAAGCCGGAAGGG	
15[23]15[34]	AGACCGCCTTATCCTTTTTTTTTTTTTGGTAACTATCGTCTTGCCGCTGCCA CGCTC	
15[35]12[35]	ACCGGCTACCATCTAGATAACTACGATATATAAGACACGACT	
15[79]17[94]	CCGAGCGCAGTTTTTTTTTTTTTAAGTGGTCCTGCAGTAGTTCGTTTTTTT	
16[55]13[41]	GTCTAGGAGGGCTTCCAGATTCCTCGTTCAGCCCGAAGTCCAA	
16[69]12[56]	TCCGCCTCAGCCAGCTGGGCTGTGTGCAGTAGGTAGTGGCGC	
17[2]12[1]	TTTTTTTAGTTGCCTGACTCCCCACTGGCAGCAGCCACTGTTTTTTT	
18[34]21[22]	ACGGTTAGCACCACAACAACCGCTTCCAGTCGTTTTTTTTTTTTTGGAAAC C	
18[55]19[69]	TACAGAGGTTTTTTTTGTTTGCAAGCAGCTATGTAGAACTACG	
18[94]21[78]	TTTTTTGATTAGCAGAGCGAGGAGATTACCAGTTACCTTCGGAGAGAGGC GGTTTATTT	
19[42]22[56]	CACTCCTTGAAGTGGCTTCTCGGCCAACGCGCGGGAAAAGAG	
19[70]18[56]	GCTACACTAGAAATTTTTTTTTTTTTGAACAGTGCGTATTGGGCGCTCTTCCG TGGCCTGCGGTGC	
21[23]21[34]	TGTCGCGGTCTGTTCTTTTTTTTTTTTTGGCTGCGGCGAGCGGTCTGCGCTTG CCAGC	
21[35]18[35]	TGCATTATCCGGCACGCTGGTAGCGGTGTTAAAGGCGGTAAT	
21[79]23[94]	GGTATCTGCGTTTTTTTTTTTTTCTCTGCTGAAGCGCGCAGAAATTTTTTT	
22[55]19[41]	TTGGTAGCTCTTGAATGAATCCGCTCACTGACTCGATCAGCT	
23[1]18[2]	TTTTTTATTGCGTTGCGCTCACTTCCACAGAATCAGGGGATTTTTTT	
24[55]25[69]	TAATATGGGCGCTCTCCTGTTCCGACCTGTCGGGGTACTGA	
24[94]28[70]	TTTTTTAGCGGGTGTGCGGGGCTGCCGCGCGTTTC	
25[28]29[41]	TCGGTGCAGGCGCCCCCTGACGAGCATCAAGGCCGCCAGCAA	
25[42]28[56]	TTCGCGGCATCAGAGTAAGGATCGACGCTCAAGTCAAGCTCC	
25[70]27[78]	GAGTGCACCATATTTTTTTTTTTTTTTCGGTGTGAAAGCGAAACCC	
26[17]24[1]	CAGGCTGCGTTTTTTTTTTTTTCAACTGTTGGGATGTGCTGCAATTTTTTT	
27[79]29[94]	GACAGGACTATTTTTTTTTTTTTTAAAGATACCAGTTACCGGATTTTTTTT	
28[25]26[18]	CGTTTTTCCATTTTTTTTTTTTTTAGGCTCCGCCCATTTCGCCATT	
28[55]25[41]	CTCGTAACCGTAAAAACAAAAGAAAATACCGCATCGGGCCTC	
28[69]24[56]	CCCCTGGAGAGGTGTACCGCACAGATGCGCAGATTGCTGGCT	
29[2]25[27]	TTTTTTAGGAAAGAACATGTGAGAGGGGGAAGGGCGA	
29[42]28[26]	AAGGCCAGCTATTACGCCAGCTGGCGAACAAAAGGCGTTGCTGG	

A.16 DNA sequences

30[55]31[69]	ATCTTTTGTCTCATGAGCGGATACATATAACTGATGGGTGAG
30[94]34[70]	TTTTTTACCCACTCGTGCACCCTTGAATGCACGGAA
31[28]35[41]	TACGAGCTGTTTCCGACGGCCAGTGAATCAATATTTTATCAG
31[42]34[56]	ATAAATTTACACCAGTGGCGTACGGTACCCGGGGATATACTCA
31[70]33[77]	CAAAAACAGGAATTTTTTTTTTTTGGCAAAAACCTGAGTCGTGC
32[17]30[2]	TGTTATCCGTTTTTTTTTTTTCTCACAATTCCATGAGCTAACTTTTTTT
33[78]35[94]	CGCAAAAAGGTTTTTTTTTTTTGAATAAGGGCGATATTTAGAATTTTTT
34[25]32[18]	CCCAGTCACGTTTTTTTTTTTTACGTTGTAAACTGTGTGAAAT
34[55]31[41]	TACTCTTCCTTTTTTCGAGCTATCATGGTCATAGCCGGAAGC
34[69]30[56]	ATGTTGACCTCTAGCAGGCATGCAAGCTCGTTTCTCTTCAGC
35[2]31[27]	TTTTTTAAGTTGGGTAACGCCAGTAATGAGCACAACA
35[42]34[26]	GGTTATTACGTGTAAAGCCTGGGGTGCCGGAGCATATTGATTTT

Cholesterol-DNA conjugates sequence is the same as the one for the triskelion and for the star.

DNA staples for cholesterol functionalisation of the tetrahedron		
arm1-lipid-hdle	CATTCTCCTATTACTACC GCGACCGAGTTGCGCCACATAGCA	instead of 4[25]2[18]
arm1-new str.	ACTGGTGGAACCTCTCAAGGATCTTACCGATGCTTTCTGAGAATAGTG TATGCGTTTTTT	instead of 5[42]4[26]
arm2-lipid-hdle	CATTCTCCTATTACTACC CAGCTCCGGTTCGATGACGGT	instead of 7[70]9[78]
arm2-new str.	ACAGGCAGCTAAGCGGATGCCGGGAGCATTGTTGCCGTTTGGTATGG CTTCATTTTTTTT	instead of 6[55]7[69]
arm3-lipid-hdle	CATTCTCCTATTACTACC TACCGCGAGACCGCCTTATCCTTTTTTTTTT TTGGTAACTAT	instead of 12[34]15[22]
arm3-new str. (1)	CGTCTTGCCGCTGCCACGCTCACCGGCTACCATCTAGATAACTACGAT ATATAAGACACG	instead of 15[23]15[34]
arm3-new str. (2)	ACTTATCGCCGTCGTGTGGCCCCAGTGCTGCAATGATTTTTT	instead of 15[35]12[35]
arm4-lipid-hdle	CATTCTCCTATTACTACC GGAAACCTGTCGCGGTCGTTCTTTTTTTTTT TTGGCTGCGGC	instead of 18[34]21[22]
arm4-new str.	GGAAACCTGTCGCGGTCGTTCTTTTTTTTTTTTTGGCTGCGGC	instead of 21[23]21[34]

A.17 CaDNAno ROUTING FOR DNA ORIGAMI

A.17.1 Triskelion

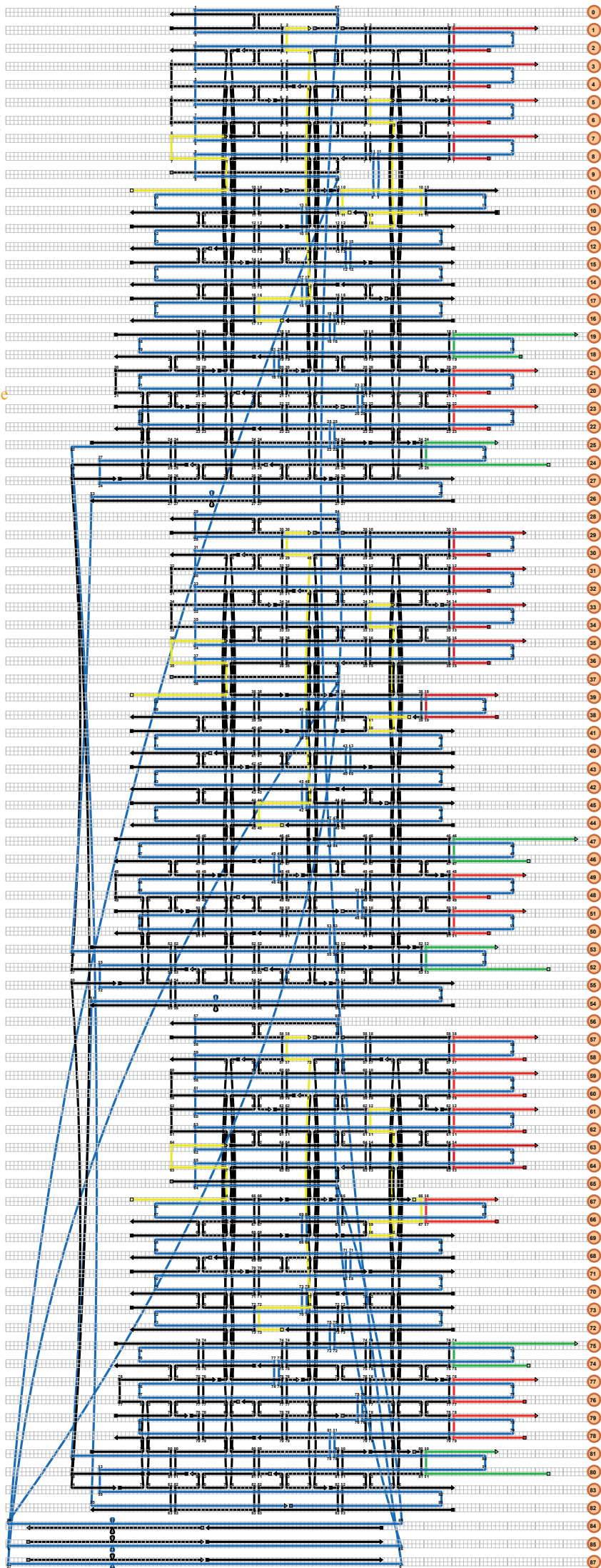
blue: DNA scaffold

black: main staples, common to all
the triskelia (except the one bridges
(HB84, HB85 and HB87 at the bottom)

red: staples involved in the sticky end
and forming the invading strands

green: strands bearing dye handle

yellow: strands bearing cholesterol handle

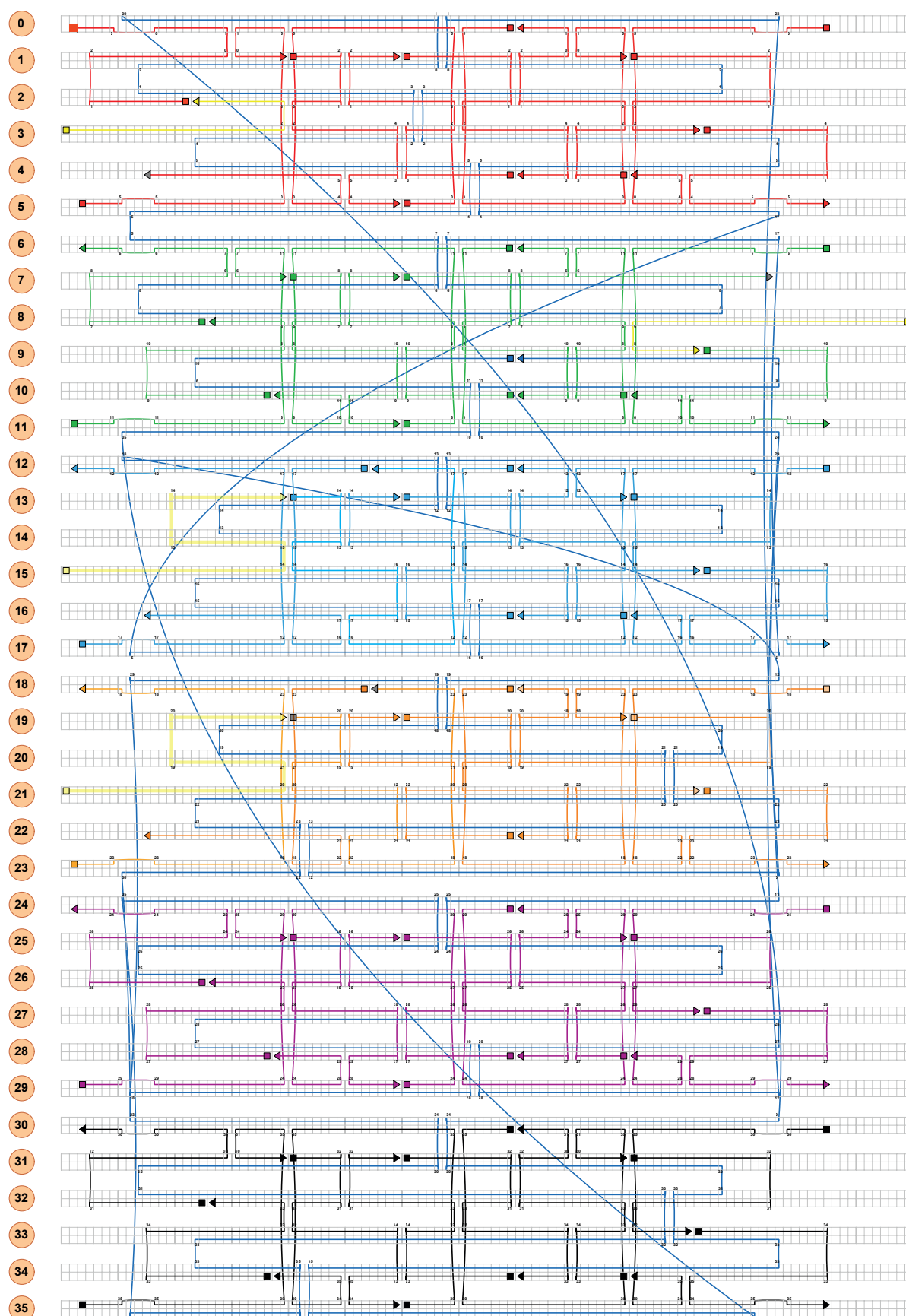


A.17.2 Tetrahedron

blue: DNA scaffold

The staples for each edge are marked in colour code (red, green, blue, orange, purple and black)

yellow: strands bearing cholesterol handle (4 strands)



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