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Application of nucleic acid - lipid conjugates for the programmable organisation of liposomal modules

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Abstract

We present a critical review of recent work related to the assembly of multicompartment liposomes clusters using nucleic acids as a specific recognition unit to link liposomal modules. The asymmetry in nucleic acid binding to its non-self complementary strand allows the controlled association of different compartmental modules into composite systems. These biomimetic multicompartment architectures could have future applications in chemical process control, drug delivery and synthetic biology. We assess the different methods of anchoring DNA to lipid membrane surfaces and discuss how lipid and DNA properties can be tuned to control the morphology and properties of liposome superstructures. We consider different methods for chemical communication between the contents of liposomal compartments within these clusters and assess the progress towards making this chemical mixing efficient, switchable and chemically specific. Finally, given the current state of the art, we assess the outlook for future developments towards functional modular networks of liposomes.

1. Introduction

Modular compartmentalisation of chemical processes and function is central to the organisation of living systems. Multiscale assembly from macromolecular complexes to organelles, cell, tissues, organs and organisms gives rise to sophisticated function across length scales from parallel biochemical modules that are in communication with one another and can sense changes in their environment. On colloidal lengthscales this compartmental organisation is predominately derived from the use of lipid bilayer membranes as functional interfacial barriers. These two-dimensional fluid interfaces host functional protein channels and receptors that regulate the passage of specific chemicals and biochemical signalling between compartments.

Mimicry of biology's compartmentalisation of its chemistry on micrometer and sub-micrometer length scales holds promise for technology development in several fields. One example is the design of multi-step micro-/nano-reactors for chemical process control; this would allow the maintenance of rare compounds, only available in small quantities, at high concentrations in self-assembled compartments while also allowing multi-step reactions in chemically incompatible environments (e.g. acidic and alkaline pH, or oxidising and reducing environments) as the reaction steps through each compartment (Fig. 1). While rational design of such sophisticated self-assembled multistep microreactors is some way from becoming a reality, the principle of single compartment, self-assembled catalytic capsules has already been demonstrated [1-3]. For example, enzymes encapsulated within polymersomes have recently been shown to be able to generate and release

antibiotics in bacterial cultures [4]. The next step towards design of multicompartment nanoreactors is the controlled assembly of modular capsules within close spatial proximity that might begin to allow communication between compartments.

A further application of compartmentalised nanostructures is within the field of nanomedicine [5]. Nanomedicine aims to use soft and nanoscale materials to control the temporal and spatial distribution of therapeutics within a patient by determining the biodistribution and drug release kinetics of a particular formulation in a predictable fashion. It is also desirable to deliver multiple therapeutic compounds simultaneously and preferably within the same particle such that they arrive at their target simultaneously [6]. Possible clinical applications include combination therapies to overcome multidrug resistant bacteria, codelivery of a prodrug with an activating agent and traceable delivery of therapeutics by combining the drug with an image contrast agent. In many cases it would be desirable that these compounds are kept physically isolated from one another within the particle structure to prevent unfavourable drug-drug interactions or store each compound in different favourable environments (e.g. pH). Therefore it would not always be favourable to encapsulate multiple active agents within a single compartment: multicompartment approaches will be required.

Synthetic biology is an emerging field broadly defined as the engineering of biological parts and devices as well as the redesign of natural biological systems [7, 8]. A bottom-up approach to synthetic biology refers to self-assembly approaches for engineering new systems created from biological components [9]. Within this context lies the ambitious challenge of building a functional cell from its fundamental molecular constituents. While many properties combine to define a living cell, including a metabolism, responsiveness to its environment, the ability to reproduce and to ultimately evolve [10], being able to replicate a small number of life-like properties within a synthetic system is currently considered to be a favourable outcome. For example, *in vitro* synthetic gene expression has been achieved in liposomes by encapsulation of a DNA plasmid with *E-coli* extract and incubating these liposomes within a "feeding solution" [10-12]. In terms of engineering cell-like materials within synthetic biology for new functional devices, reproduction and evolution of the "cell" may not be necessary for first generation technologies. However the ability to encapsulate metabolic processes that are responsive to their external environment will have many applications including environmental sensing, novel medicines and catalysis. Different functional elements could be combined in a modular fashion that is familiar to both engineering design and biological organisation, where each module is a synthetic gene network (e.g. BioBricks [13, 14]) expressed within a liposomal compartment in close communication with similar such modules in a

multicompartment architecture. This concept of modular compartmentalisation of function has already been demonstrated in protocell design, where light-activated release of lactose from a lipid organelle is coupled to the *in vitro* gene expression of green fluorescent protein within an emulsion droplet [15]. A similar photo-responsive synthetic organelle has previously been described where light-driven trans-membrane proton gradients are generated using the bacteriorhodopsin protein, which then drives F_0-F_1 ATP synthase to generate ATP, which could provide chemical potential energy to drive further downstream bioenergetic processes [16]. The advantage of designing bottom-up synthetic cells over reengineering existing organisms lies in the ability to eliminate potentially unwanted cross-talk between the host and synthetic biochemistries as well as providing a system of minimal biochemical complexity that is easier to understand, redesign and control.

The aforementioned applications in nanoreactors, drug delivery and synthetic biology provide ample motivation for the design and engineering of multicompartmental structures on the micro- and nano- scales. Several approaches towards this goal exist within the published literature. These include careful assembly protocols for encapsulation of smaller vesicles within larger ones to create multicompartmental "vesosomes" [17-19], "capsosomes" created by embedding liposomes within layer-by-layer polymer shells [20-22], encapsulation of aqueous two phase systems within single liposomes [23-25] and association of lipid vesicles via site-specific ligand-receptor interactions such as biotin-avidin bonds [26-28]. Many of these approaches were discussed in a recent review [29]. Here, the central focus will be on the use of nucleic acid functionalities on the surface of liposomes to mediate their associations. The two key advantages of nucleic acids for this purpose are [30]: (i) DNA (usually) forms an asymmetric interaction with its complement, allowing the controlled assembly of different liposomal compartments, compared with symmetric binding interactions, e.g. biotin-avidin-biotin, which cross-link liposomes from the same population; (ii) the high fidelity, sequence specific and directional (5′ → 3′) hybridisation of DNA duplexes mean that, in principle, an expansive array of specific interactions can be encoded within large multicomponent systems utilising the same chemistry.

DNA has long been used within materials science since the pioneering work of Nadrian Seeman on DNA nanostructures [31, 32]. The sequence specificity, binding fidelity and mechanical rigidity of the DNA molecule have made it a promising building block for the construction of new materials [33-35] and devices [36]. DNA has also been conjugated to other particles to direct their assembly into higher-order superstructures [37, 38], including encoding the delicate balance between attractive and repulsive interactions required to drive crystallisation in low density colloidal systems [39-41]. Hydrophobic modifications to DNA strands have recently been used to for the

higher-order assembly of DNA-cages and the fabrication of DNA cages with hydrophobic cores that can host poorly water soluble guest compounds [42]. Here we review the literature on functionalisation of soft, self-assembled liposomes with DNA functionalities related to their higher order assembly and applications within functional multicompartment materials (Fig. 2). Firstly we will assess the choice of hydrophobic modifications and other strategies that have been used to attach nucleic acids to the surface of membranes. We will then look at the properties of DNA when bound to a lipid bilayer, particularly those dependent upon the lipid composition of the membrane, that can influence the higher order assembly of liposome superstructures. Finally we will look at the prospects for communication between the contents of individual compartments either through irreversible fusion or specific channels embedded across the membranes.

2. Functionalisation of liposomes with DNA

In this section we look at the different strategies for encoding membranes with information by anchoring nucleic acid strands to their surface. The majority of these approaches involve the synthesis of nucleic acids with hydrophobic modification(s) resulting in surfactant molecules that spontaneously insert into amphiphile aggregate structures. Here we will primarily consider their structures and physical properties; details of their synthesis have been reviewed elsewhere [43, 44]. Cationic lipid lipoplexes designed for therapeutic delivery of nucleic acids will not be considered as part of this review [45-47].

a. Single hydrocarbon chains

Single hydrocarbon chains varying in length between C_{12} and C_{18} have been used to functionalise peptide nucleic acids (PNA) (Fig. 3a) [48, 49]. The modified PNAs can bind complementary DNA sequences without any significant perturbation to the hybridization free energy by the presence of the alkyl chain. These PNA amphiphiles bind transiently with micelles and vesicles, particularly when bound to a complementary DNA sequence. This allows micelles to be used as “drag tags” in conjunction with the PNA amphiphiles in electrokinetic chromatography, allowing the rapid separation of unlabelled DNA based upon their length and sequence [48, 50-52]. While useful in biotechnology applications for biomolecular separation, the temporary nature of single alkyl chain nucleic acid amphiphiles’ insertion into micelles and liposomes make them unsuitable for long-term tagging of liposome populations for high fidelity superstructure assembly. DNA block copolymers with single 1 kDa polypropyleneoxide (PPO) modifications have been used to stably label liposomes for triggered release studies (Fig. 3a) [53]. Another polymer-based anchoring unit, poly(2-vinyl-8-hydroxyquinoline-r-8-vinyl-1-naphthoic acid), that is photo-responsive such that

it becomes hydrophilic upon irradiation with ultra-violet light has been used for light-triggered release of liposomes anchored to surface substrates [54].

b. Cholesterol anchors

By far the most commonly used modifications within the literature are cholesterol-based anchors (Fig. 3b). The primary reason for their popularity is that they are commercially available (including strategies for creating double cholesterol anchoring), removing the need for specialist synthesis of modified nucleic acids and opening up their accessibility to a wider range of laboratories in biophysics, chemical engineering, bionanotechnology and soft matter. It was found that a single cholesterol anchor appeared to be insufficient to reliably label liposomes with 30 base DNA sequences for robust, addressable surface arrays of liposomes; a double anchoring protocol was devised where one short 5′ modified strand hybridizes to the lower (3′ proximal) end of a longer 3′ cholesterol modified strand (Fig. 3b) [55]. This leaves an overhanging single-stranded region of DNA to bind anti-sense strands in solution, while having two cholesterol moieties to reinforce the hydrophobic anchoring into a lipid bilayer. DNA strands with two parallel cholesterol modifications attached through a Y-shaped modification on one end of the DNA have also been reported for enhanced hydrophobic association with membrane surfaces [56, 57].

Single cholesterol anchors have been reported to quantitatively functionalise liposome surfaces using size exclusion chromatography [58]. The stability of the hydrophobic anchor within the membrane will, of course, be dependent on the length of the hydrophilic DNA sequence it is attached to; a thorough systematic study of this is yet to be reported. However single cholesterol anchors have successfully been used to functionalise liposomes with 38 base DNA-aptamers for reversible cell targeting [59] and to anchor 272 base hexagonal DNA nanostructures to liposomes, where surface anchoring permitted isothermal nanostructure assembly that isn't possible in bulk solution [60]. Multiple cholesterol anchors attached as modified bases along a section of the DNA strand have also been explored [58, 61]. While this yields a lower critical micelle concentration and stronger anchoring to the membrane, not all the cholesterol anchors insert into the membrane, which can cause aggregation of the modifications at the membrane interface. Therefore end-functionalised cholesterol-DNA amphiphiles (chol-DNA), in general, appear to be more compliant for bionanotechnology and soft materials applications. Very recently, double-cholesterol modified DNA has been used to functionalise lipid bilayer supported on microscale silica colloids, which brings the advantageous property of DNA lateral surface mobility to the assembly of hard, inorganic colloidal materials [62].

c. Double hydrocarbon anchors

Oligonucleotides with two hydrophobic tails as their hydrophobic modification, similar to the structure of natural lipids, generally appear to form stable anchors within liposome membranes. Thiol-derivatized DNA strands can be covalently attached to some commercially available, synthetic functional lipids (e.g. with maleimide moieties) [63, 64]. DNA-lipid conjugates can also be synthesized by first making a dialkyl lipid phosphoramidite, which can be added as the last base on a standard solid-phase DNA synthesizer (Fig. 3c) [65]. These DNA-lipid conjugates can then be mixed with preformed liposomes into which they spontaneously insert. This protocol removes the need to rely on efficient covalent conjugation of DNA strands with reactive groups at the membrane surface. Dipalmitoyl PNA conjugates and DNA containing two tocopherol modified deoxyuridine bases (Fig. 3c) have also been used to stably anchor nucleic acids to membranes *via* two hydrocarbon chains [66].

d. Other modifications

While hydrophobic nucleic acid modifications are the most commonly chosen method to label lipid bilayers, receptor mediated interactions have also been reported. Biotinylated DNA has been attached to biotinylated lipids within liposomes and lipid-coated emulsions using streptavidin cross-linkers (Fig. 3d) [67-69]. The challenge here is that streptavidin can directly cross-link biotinylated particles [26]. Therefore much care is required when setting up the functionalisation protocol for this technique to ensure that no direct streptavidin-driven cross-linking between liposomes of the same population occurs. The chemical components required for DNA-functionalisation via biotin-avidin bonds are also commercially available.

3. Properties & Assembly of DNA-functionalised liposomes

This section covers the properties of DNA-functionalized liposomes, their assembly into higher order superstructures and how DNA localization and binding is influenced by the lipid composition of the membrane.

a. Basic features and reversible liposome clustering in bulk solution

Detailed studies have been conducted for the functionalisation of planar bilayers [61] and liposomes [58] with single and multiple cholesterol anchors. Eighteen base single cholesterol-anchored DNA inserts into planar bilayers up to a saturation level of approximately 80:1 phospholipids:DNA, an average separation between strands of 5.3 nm, within the “brush” regime of polymer-functionalised interfaces [61]. The DNA moiety sits near-perpendicular to the bilayer,

moving within the volume of an inverted cone with a 2.6 nm radius; the dissociation constant with the bilayer is measured to be 2.0 ± 0.2 nM using Quartz Crystal Microbalance with Impedance (QCM-Z). DNA with multiple cholesteryl anchors along a poly-Thymine backbone behaves quite differently, with three adsorption regimes: a dilute regime where the DNA strand lies near-flat to the bilayer, a second regime where a 2nd layer of chol-DNA interdigitates and a more comb-like distribution of DNA forms, and a third "tetris-like" regime of complex multilayered multi-cholesterol DNAs. The aggregation between multi-cholesterol DNAs in the bilayer is thought to be driven by some of the cholesterol anchors which do not insert into the bilayer but instead stick out into the aqueous phase. However both single cholesterol DNAs and multi-cholesterol DNAs can reversibly bind their complementary strand without significant perturbation by the bilayer surface.

Little difference is found between planar bilayer and vesicle modification by cholesterol-DNAs [58]. The critical micelle concentration (c.m.c.) of an 18-mer single cholesterol DNA was measured to be 10 µM (about an order of magnitude higher than the multi-cholesterol DNA) using a combination of pyrene fluorescence and static light scattering. Therefore the free energy of transfer of one chol-DNA from an aggregate to the aqueous phase is calculated to be -52 kJ mol^{-1} (22 k_BT per molecule at 298 K), leading to an estimate of the contribution per base counter-acting the micellization of pure cholesterol of 0.8 kJ mol^{-1} (0.33 k_BT per molecule at 298 K). This is one of very few measurements of the c.m.c. of a DNA amphiphile; more such measurements are required for accurate comparison of the stability of membrane functionalisation by nucleic acid amphiphiles, and particularly how this changes with the increasing length of nucleic acid strands.

It should be noted that c.m.c. measurements of DNA amphiphiles need to be interpreted with care: when used for the functionalization of lipid membranes, these molecules are forming mixed aggregates with the lipids rather than single component micelles. When diluted within lipid membranes, the electrostatic and steric repulsion between the bulky nucleic acid headgroups that do not favour self-aggregation are significantly reduced and so c.m.c. measurements might provide misleadingly pessimistic predictions for their stability of incorporation within lipid membranes. Therefore the development of assays that are highly sensitive to the presence of free nucleic acid amphiphiles in the bulk solution that coexist with a population of liposomes would be more pertinent for determining their efficiency of membrane functionalisation. For example, partitioning of chol-DNA (18-mers) into preformed liposomes has been shown to be quantitative (within experimental error) using size exclusion chromatography [58].

The nucleic acid moieties of cholesteryl-conjugates are free to bind their complement from the bulk aqueous environment. DNA hybridization kinetics at the liposome surface can be analyzed

by dynamic light scattering through changes in the hydrodynamic radius once double-stranded (duplexed) DNA is formed [70]. DNA-decorated liposomes were also found to be stable for at least one week (the maximum duration tested).

Cholesterol-DNA conjugates have been shown to be able to mediate direct assembly interactions between two populations of liposomes functionalised by complementary strands [30]. This can be achieved for liposomes across a broad range of sizes from nanoscale (100 nm; LUVs) to giant (> 5μm; GUVs) liposomes. Clusters formed between liposomes were reversible by heating above the duplex melting temperature or reducing the salt concentration such the repulsion between charged sugar phosphate backbones dominates inter-strand interactions; however thermal melting of GUVs proved challenging, likely due to limitations of the temperature stage on the microscope. Three regimes of LUV assembly were observed by dynamic light scattering. Below 2.5 DNA/vesicle no noticeable aggregation was observed. Kinetically stable, small clusters were observed up to approximately 20 DNA/vesicle. At 39 DNA/vesicle and above, continuous agglomeration was observed until large flocculates, visible to the naked eye, dropped out of solution. Since chol-DNA is mobile within the bilayer, this is interpreted as the DNA saturating within adhesion plaques between pairs of liposomes until these regions saturate at around 20 DNA per vesicle. Above this DNA loading there is always excess DNA on the outside of clusters that can bind additional liposomes through further collisions. This work examined the interactions between two populations of vesicles. Other work has demonstrated that by using more complementary pairs of DNA strands, it is possible to form clusters of three different liposomes [68], moving towards the possibility of controlling the associations between an arbitrary number of liposome populations to create complex interaction networks. By fluorescent tagging of the DNA linkers, this also clearly showed the concentration of DNA-linkers within the adhesion plaques permitted by the lateral mobility of the DNA within the fluid bilayer membrane.

Multivalent interactions between particles and surfaces via surface-anchored “receptor” molecules can give rise to qualitatively different interaction behaviour when compared with direct interparticle interactions due to various entropic factors relating to the tethered “receptor” molecules [71]. For inter-vesicle interactions, in particular, the fluidity of the membrane, which allows receptor lateral mobility across the surface, and the deformability of the membrane during inter-liposome adhesion processes are important physical differences when comparing liposome assembly with similar studies for “hard” colloidal particles [30]. Changes in the conformational entropy of flexible tethers upon binding, combinatorial entropy arising from the multiplicity of states in a multivalent interaction and changes in the translational entropy of laterally mobile linkers can all

have significant contributions to the overall binding free energy [71]. Indeed the mobility of DNA strands within a fluid bilayer result in maximising the combinatorial entropy favouring adhesion as all possible DNA-DNA binding combinations can be explored, which counter-acts the unfavourable loss in translational entropy of the tethers which disfavours binding.

Enthalpy – entropy competition can arise in interesting ways during receptor binding interactions; for example flexible linkers or interfaces can permit optimal configurations for binding between complementary pairs that do not strain the bonds and thereby has the most favourable enthalpy, however flexible interfaces have a higher entropy cost than rigid interfaces during binding interactions [71]. Weak individual receptor interactions between surfaces can also be useful in controlling higher order assembly, where multivalent interactions that are not just sensitive to the presence of the complementary strands but also on their local surface density can be engineered into the system [72]. By applying a deeper understanding of the thermodynamics of multivalent interactions between the flexible, fluid interfaces of liposomes, a greater degree of control and complexity in the liposome assembly process should be possible, over and above what has already been demonstrated within experimental studies of such systems.

The significant physical differences between the DNA-mediated assembly of soft particles, such as liposomes, and hard particles has also been demonstrated using emulsion droplets, where the deformability of the individual particles and the fluidity of their interface also come into play [69].This study shows that the enrichment of DNA functionalities in adhesion contact sites gives the emulsion droplets an effective valency that can be controlled through the DNA surface density on these particles, similar to how the aggregation state of liposomes can also be controlled through tuning the DNA loading within the membranes [30]. Entropic effects of ligand binding are also found to be important in the growth of adhesion plaques between neighbouring droplets: DNA linkers with rigid double-stranded DNA spacers formed contact sites that were 60% larger than DNA linkers that were spaced from the droplet interface by flexible, single-stranded DNA [69]. A thermodynamic model for the size of contact sites as a function of emusion droplet radius; this model would in future be amenable to translation to liposome systems, where the deformation energy of the particles would be described by the curvature-elastic energy of the lipid membranes instead of the surface tension at the droplet interface. For the case of lipsomes, a further modification could be made to this model that accounts for the role of thermally excited membrane undulations in the thermodynamics of ligand binding, which has recently been addressed by theoretical and computational modelling [73]. Particle valency will also be addressed later in this review when

considering how liposome surface anisotropy generated through lipid phase separation influences their higher order assembly (section 3c).

In contrast to most of the examples discussed in this review, DNA strands have also been modified to have a hydrophobic anchoring group at both the 3´ and 5´ ends [74]. When mixed with liposomes these modified single stranded DNAs insert both hydrophobic ends into the membrane of the same liposome with the DNA lying across the membrane surface. Upon hybridization to the unmodified anti-sense strand, the DNA becomes near perpendicular to the membrane, exposing one of the hydrophobic groups. This hydrophobic group can then insert into the membrane of a different liposome, resulting in the assembly of higher-order liposome clusters. This clustering is reversible; when the DNA duplex melts the hydrophobic groups flip back to anchoring into the same liposome, maximising the entropy of the liposomes in the system. This approach results in reversible cross-linking between liposomes of the same population rather than bringing together liposomes with different contents. However this system could prove useful for DNA sensing assays: a 10 K suppression in melting temperature is observed between a 17 base, fully complementary anti-sense strand and strands containing a single base mismatch.

DNA functionalities have been used to form multicompartment assemblies between other soft nanostructures, these include attaching liposomes to gas microbubbles for medical theranostics [75], linking liposomes to layer-by-layer capsules [76] and assembly of lipid-coated oil-in-water microemulsions [67], which would allow extension to compartmentalisation of hydrophobic chemistries.

In the next subsections we will look beyond simply the DNA-mediated interactions between liposomes alone and consider ways in which the lipid compositions and membrane properties might couple with the specific DNA-binding interaction to add greater degrees of control to the assembly of multicompartment liposome architectures. We will also explore reducing the dimensions within which the liposomes are assembled by examining surface templating as a tool for mediating liposome interactions.

b. Influence of Membrane Interactions upon DNA thermodynamic stability

While we have already noted that DNA-mediated assembly using DNA amphiphiles is thermally reversible, it is of interest to explore this in more detail and in particular to investigate the dependence of the DNA hybridization thermodynamics upon the composition of membranes within which they are hosted. This is relevant for predicting the temperatures required for thermal annealing of DNA-directed liposomal superstructures as well as having biological relevance by

probing the coupling between local membrane composition and receptor binding strength within a simple model system with controllable parameters [77].

DNA strands anchored to lipid vesicles form thermodynamically more stable duplexes when mediating vesicle-vesicle interactions than the unmodified strands would in bulk solution [77]. This is not simply a local concentration effect from the localisation of the DNA at the vesicle surface; other thermodynamic effects make significant contributions to this change in hybridization free energy. These factors include the entropy loss from tethering the DNA to the vesicle surface, changes in vesicle entropy brought about by tethering them into clusters and the conformational steric restrictions imposed on the membrane-anchored DNA strands. Double-anchored chol-DNA formed from the hybridization of a long and short cholesterol-functionalised strand also show an enhanced thermodynamic stability of the duplex [78]; this could arise from enhanced inter-strand interactions through the hydrophobic moieties.

Intermembrane interactions can have a significant impact on the thermodynamic stability of DNA duplexes that act as tethers between liposomes [77]. When relatively short 10 base chol-DNA linkers were used, the melting temperature (T_m) of vesicle agglomerates was on average 11.6 °C lower for anionic POPG membranes than for neutral POPC membranes. While incorporating 10 mol% of cationic DOTAP lipid into POPC membranes raised T_m by an average of 8.6 °C. This is because electrostatic repulsion between anionic membranes weakens the DNA duplex, while attractive interactions caused by polyanionic DNA between cationic membranes stabilizes the DNA double helix. However when longer double-anchored chol-DNA conjugates were used, no difference in T_m was observed between POPC and POPG liposomes as the tethered membranes were now spaced far enough apart that their interaction energies were negligible.

Traditional thermodynamic models for sequence-dependent DNA melting [79-81] were modified to take into account inter-membrane interactions by applying a Bell-type model [82] to account for the tilting of the free energy landscape by an applied force [77]:

$$T_m = (\Delta H^0 - U^F)/(\Delta S^0 + k_B \ln(C_T/4)) \quad (3.1)$$

$$U^F = A_{DNA} \int_{D0}^{D0+\Delta x} P_{tot}(x)\, dx \quad (3.2)$$

Where ΔH^0 and ΔS^0 are the enthalpy and entropy changes per molecule, respectively, C_T is the concentration of single-stranded DNA, k_B is Boltzmann's constant, U^F is the total work done by intermembrane forces on the DNA duplex, A_{DNA} is the area per DNA in the adhesion plaques between vesicles, $P_{tot}(x)$ is the intermembrane pressure as a function of intermembrane distance (x), D_0 is the equilibrium intermembrane distance upon stable duplex formation and Δx is the distance

along the reaction coordinate (x) to the transition state where the DNA duplex melts. Theoretical models for intermembrane pressures (van der Waals, electrostatic double layer (edl), steric undulation and hydration interactions) show that edl interactions are dominant when comparing POPC and POPG liposomes. This allowed a calculated estimation of A_{DNA} of 41 nm^2; taken together with the estimate that adhesion plaques between 100 nm liposomes saturate at approximately 20 DNA duplexes (see section 3a), this predicts an average adhesion plaque diameter between 100 nm liposomes of 32 nm. This model also predicts an attractive energy of -1.4 k_BT per DNA duplex between 10% DOTAP membranes tethered by the 10 base pair DNA strands.

Lipid composition has also been shown to influence the T_m of DNA linkers between bilayer nanodiscs (Fig. 4) [83]. Anionic lipids are again found to destabilize the duplex, lowering the T_m of the DNA-linked structures. Lowering the ionic strength of the aqueous phase also significantly reduces T_m. Hysteresis in T_m was observed between heating and cooling directions, with the apparent T_m being higher during the heating cycle. This is interpreted as a result of needing to melt several DNA strands between each pair of nanodiscs in a cooperative unbinding interaction for disassembly, whereas only one DNA bond is required to form to tether two nanodiscs together, i.e. no cooperativity between strands is necessarily required. The cooperativity of the melting transition (determined by its full width, half maximum, FWHM) is relatively high at 100 mM NaCl (2 – 4 °C), compared with unmodified DNA strands (~10 – 15 °C). However the cooperativity of the melting transition is found to decrease with decreasing ionic strength. While the solvent environment is also known to be critical in determining the thermodynamics of DNA hybridization, e.g. nature and concentration of salts, osmolytes and cosolvents, there has been no systematic study of the effect of these parameters on DNA hybridization at a lipid bilayer surface. However, there are extensive examples in the literature of the study of solution effects on the hybridization of DNA oligomers in the bulk [84-88].

c. Breaking symmetry: phase separation and aspherical structures

To enhance the structural complexity and architectural control of liposomal assemblies it is desirable to break the spherical symmetry of the homogeneous surface distribution of DNA around the liposomes by clustering the nucleic acids within functional surface domains. Janus particles [89, 90], which consist of two faces of differing surface chemistry, or (more generally) particles with patchy surface morphologies [91] increase the complexity of materials that can be formed from assembly of the constituent building blocks. This is because by breaking the symmetry of the particle surface chemistry, inter-particle interactions are now not just dependent upon their relative

separations but also have an angular dependence from the relative orientations of the particles, thereby increasing the number of degrees of freedom within the system.

The membranes of lipid vesicles can be patterned by phase separation within multicomponent lipid mixtures. Lipid membranes can exist in several different phase states, including liquid disordered (L_α), liquid ordered (L_o) and several solid-like gel phases (e.g. L_β, L_β', P_β') [92]; lipid mixtures can therefore be developed that phase separate into two or more of these coexisting phase textures [93-96]. When impurities (e.g. fluorescent lipids) are incorporated into phase separated membranes in trace compositions, they thermodynamically partition into domains according to the relative free energy cost of insertion into each phase [97]. Therefore it is most common for impurities to partition into the most disordered phase available so that they do not incur a free energy penalty by disrupting the packing structure within the more ordered domains. Similarly, DNA amphiphiles will partition thermodynamically between available phases, opening the possibility for engineering specific functional domains within vesicle membranes.

The first demonstration of partitioning of DNA-amphiphiles within phase separated vesicles was using DNA strands modified by two hydrophobic α-tocopherol nucleotides [98, 99]. The partitioning behaviour of these molecules was investigated in liquid-liquid (L_α – L_o) phase separated GUVs composed of 1:1:1 POPC:Sphingomyelin:Cholesterol. Within this system, the DNA functionalities were observed to localize within the liquid disordered domains.

The partitioning behaviour of cholesteryl modified DNAs in phase separated GUVs has also been characterized [56]. Single and double anchored chol-DNAs predictably partition into the L_α phase of the various liquid-solid phase separated mixtures that were tested. However the behaviour in liquid-liquid phase separated DOPC/DPPC/cholesterol GUVs was found to be more complex. Single anchored chol-DNA partitioned fairly evenly between coexisting phases with only a slight enhancement in the L_o phase. The proportion of DNA in the liquid ordered domains could be enhanced by up to a factor of two when doubly-anchored chol-DNA was used instead. While cholesterol is known to be enriched in the L_o domains of GUVs, chemical modification of the cholesterol group means that these molecules cannot be assumed to have similar partitioning behaviour [56]. In fact, the cholesteryl-TEG anchors have been shown not to have the same lipid condensing behaviour as cholesterol in POPC membranes [78]. However it is suggested that the rigid fused ring structure of the cholesteryl anchor imposes an unfavourable entropic penalty on the conformational degrees of freedom available to “kinked” unsaturated lipids, which drives the moderate enhancement within the L_o phase [56].

It is desirable to move beyond a moderate enhancement towards a strong partitioning into L_o domains so that these phases can host distinct functionalities to their surrounding membrane. Two routes have been demonstrated to achieve this goal. Firstly, a physical route aimed at modifying the physical properties of the coexisting liquid phases to further enhance the partitioning of chol-DNAs into L_o domains has proven to be successful [100]. Incorporating highly unsaturated lipids into the lipid mixtures was predicted to enhance the entropic free energy cost for chol-DNA to partition into L_α domains; this indeed proved to be the case. The most potent lipid for this purpose proved to be 10 mol.% bovine heart cardiolipin (CL), which resulted in at least an order of magnitude enrichment of double cholesterol anchored DNA into liquid ordered domains (Fig. 5a-c). The small head group of the CL lipid combined with its four unsaturated acyl tails form an inverted-cone shaped lipid that exerts a lateral packing stress within the hydrophobic region of the L_α phase, into which this lipid partitions [101]. The enhanced lateral packing stress in the liquid disordered phase is thought to contribute to the strong enhancement of chol-DNA into the liquid ordered phase [100]; similarly a saturated dialkyl lipid-DNA was shown to strongly partition into the liquid ordered domains of liquid-liquid phase separated GUVs containing CL. When mixed population of Janus-textured GUVs with complementary DNA isolated to their L_o domains were studied, these liposomes formed size-limited clusters (Fig. 5d). Liquid-ordered domains localised within the adhesion plaques between liposomes leaving the DNA-depleted liquid disordered phase exposed on the exterior of the composite structures that do not favour the binding of further vesicles. Thus by breaking the symmetry of the DNA distribution on the vesicles, we have gained some control over the superstructure morphologies that can be formed.

A second approach to functionalising liquid ordered domains, we will refer to as the chemical route in that this solution is arrived at through chemical synthesis of novel DNA anchors designed to prefer the liquid-ordered phase. Loew *et al.* demonstrated that palmitoylated peptide nucleic acids partition almost exclusively to liquid-ordered domains [66]. When combined with the L_α-partitioning tocopherol anchors discussed earlier, this allowed the construction of GUVs where each phase was encoded with a different DNA functionality. These structures were shown to be reversible between well mixed DNA-functionalities within single phase GUVs and phase separated DNA-functionalities in liquid-liquid coexistence by heating above and cooling below the liquidus curve within the lipid phase diagram. Furthermore, by using strands that cross-link palmitoyl-PNA and tocopherol-DNA conjugates through hybridization into a composite molecule, the combined complex partitioned into liquid disordered domains [102]. However enzymatic cleavage of the linking DNA strand switched the palmitoylated PNA back into the liquid ordered domains (Fig. 6).

This proved an elegant demonstration of switchable domain partitioning of membrane-anchored nucleic acid molecules that is responsive to external stimuli in the form of enzymatic catalysis.

A second, but quite different, form of domain formation has also been demonstrated within DNA-functionalized membranes [103]. By using different length DNA tethers (24mers combined with either 48mers or 72mers) between a surface-supported lipid bilayer and a second tethered membrane, the laterally mobile DNA strands self-sort into domains of equal DNA length in order to minimise the total curvature energy of the tethered bilayer (Fig. 7). However when 48mer and 72mer tethers were combined, domain formation did not occur, likely due to flexibility or tilting of these longer DNA strands allowing for greater accommodation of DNA length difference that results in a much lower curvature elastic energy cost with the tethered membrane. This work has biological implications for the spontaneous organisation of cell surface receptors in the contact sites between interacting cells, such as the tight junctions of epithelial layers. Similar topographical domain formation has previously been observed in model systems that combine short-range ligand-receptor attractions with long-range steric repulsions within intermembrane contact sites [104].

Besides breaking symmetry by phase separation of different membrane textures within lipid bilayers, symmetry can also be broken by using constituent building blocks that are non-spherical in shape. This has been demonstrated by the DNA-mediated assembly of nanoscale bilayer discs (BioNanoStacks) [83]. Lipid nanodiscs are formed using amphipathic α-helical scaffold proteins derived from natural lipoproteins that form a belt around the lipid tails of the bilayer, minimising the hydrophobic line tension and stabilising the nanodisc morphology [105]. DNA-functionalities insert into the bilayer of the nanodisc oriented in opposite directions within each leaflet of the bilayer [83]. The directionality of the DNA functionalities within the disc shaped bilayer micelles results in the quasi-one-dimensional self-assembly of stacked nanodiscs when populations expressing complementary strands are mixed. The periodicity of stacking within these supramolecular polymer-like architectures can be tuned by selecting the length of the DNA tethers. Superstructures can be assembled that reach sizes visible by optical microscopy and these can be reversibly disassembled by thermal melting of the DNA duplex. While these nanodiscs do not contain an aqueous lumen, the hydrophobic interior of the membrane can incorporate membrane proteins and other hydrophobes [105]. These BioNanoStacks can also be further functionalised by attaching further molecules or particles to the poly-histidine (His) tags on the membrane scaffold proteins, for example Nickel-mediated assembly of gold nanoparticles containing nitrilotriacetic acid (NTA) surface functionalities onto the protein His-tags of bionanostacks has been reported [106].

d. Reduced dimensions: assembly on surfaces

One useful strategy for controlling the high-order assembly of materials is to confine the system to a lower dimensional space, for example template assembly upon a two dimensional surface. This approach has been used to assemble crystalline monolayers of λ-phage DNA-coated colloids above weakly attractive surfaces [107, 108]. There are numerous examples in the literature of DNA-mediated assembly of liposomes on surfaces, with numerous motivations besides controlled structure formation.

Liposomes had been assembled on solid interfaces, where the anchoring points remain fixed and hence the liposomes are laterally immobile [109-111], as well as fluid interfaces such as surface supported lipid bilayers, where the liposomes are then free to diffuse in two dimensions [112-116]. Surface immobilization of liposomes allows the use of sensitive surface analytical techniques to probe the binding and properties of liposomes for biosensor applications [109, 117-119]. These techniques can also be applied to develop new biophysical techniques for membrane biophysics, for example in investigating intermembrane interactions of unsupported bilayer membranes [113, 120]. Surface analytical techniques that have been applied to DNA-mediated surface-anchored liposomes include fluorescence interference contrast microscopy [63], Quartz Crystal Microbalance with Dissipation (QCM-D) [110], Total Internal Reflection Fluorescence Microscopy (TIRF-M) for label free detection of single base mismatches in DNA strands [117, 118], DNA detection by imaging mass spectrometry [119] and surface plasmon resonance (SPR) [109].

Micropatterned surfaces can be used to control the surface localisation of DNA-tethered vesicles and to create spatial domains of different vesicle populations upon the surface [55, 110, 111, 116]. The lateral mobility of planar bilayer-tethered liposomes has been characterised by FRAP [112] and single particle tracking [115]. FRAP studies with cholesterol-tagged DNAs found that liposome mobility was independent of tether length (in the range 15 – 30 bases) and liposome diameter (in the range 30 – 100 nm) [112]. However a 3 fold reduction in mobility is observed for double cholesterol anchored liposomes compared with their singly anchored analogues. If this is to be simply explained by the lateral mobility of the liposomes being dominated by the viscous drag of the hydrophobic anchors in the planar bilayer, then multiple cholesterol-DNA tethers must be anchoring each liposome to the surface (Fig. 8). This is because it was observed that individual cholesterol DNAs without their liposome cargo showed a six-fold and eleven-fold increase in lateral mobility for single chol-DNA and double chol-DNA respectively.

However it cannot be ruled out that more complex interfacial hydrodynamics are at play in determining the lateral mobility of liposomes in these systems; no difference in liposome lateral diffusion was observed by single particle tracking for increasing DNA surface loadings, which lead to

the apparently contradictory interpretation that only a single DNA tether binds the liposomes to the surface (albeit for a different lipid-derived hydrophobic DNA modification) [115]. The single particle tracking studies also observed an insensitivity of lateral mobility on liposome size (in the range 30 – 200 nm). Furthermore, liposome mobility was found to be insensitive to a 3-fold increase in bulk medium viscosity and the individual lipid-DNA anchors (without liposome cargo) diffused 3-5 times faster than the tethered liposomes. It would appear that further investigation is required to understand the full complexities of the hydrodynamics of tethered vesicle diffusion at planar bilayer interfaces.

Surface tethered liposomes can be manipulated by external fields, e.g. electric fields [114]. Under the application of an electric field in the bilayer plane, liposomes were found to move in the direction of electro-osmotic flow, the rate of which could be enhanced by incorporating anionic lipids in the supported bilayer. This allows liposomes to be concentrated at the boundaries of membrane corrals created by surface microfabrication. Adding anionic lipids into the liposomes slowed the electro-osmotic motion, eventually reversing it to the direction of electrophoresis at high anionic lipid content. Gradients of anionic lipids within the planar bilayer created zones within membrane corrals where electro-osmotic and electrophoretic mobility of liposomes were balanced; this allowed spatial separation of anchored liposomes within a planar bilayer based upon the electrostatic potential of their confining membranes (Fig. 9). Therefore electric fields offer a useful tool for sophisticated external control of the surface distribution of liposomes anchored to fluid bilayer interfaces.

Higher-order assembly of liposomes anchored to planar bilayer surfaces can be mediated by liposome functionalisation with further DNA-lipid molecules, where complementary interactions can be induced between different liposome populations [120]. Once two liposomes form a dimer by hybridisation of complementrary strands, the liposomes continue to diffuse in the bilayer plane as a colocalised unit. The docking probability between liposomes, which increases with DNA copy number, is higher for repeating DNA sequences than non-repeating sequences and increases for DNA binding domains that are located further from the liposome surfaces by non-binding spacer segments. A model has been developed for docking probability that depends on the product of three quantities: the collision rate between liposomes, the duration time of a collision where the liposomes are close enough for DNA to hybridize and the overlap volume between complementary DNA strands during this collision time.

Click chemistry has been used to covalently attach DNA-anchored liposomes to planar bilayers [113]. This results in the liposomes being irreversibly anchored to the planar bilayer even if

the DNA duplex melts. Interestingly dropping the salt concentration to low ionic strength rendered the liposomes laterally immobile. This is possibly due to strong attractive interactions with the planar bilayer, as polymer-induced depletion interactions between liposomes and the planar bilayer surface have previously been shown to arrest liposome mobility [115]. However incorporation of charged lipids in the liposomes and planar membrane that would be expected to negate moderate attractive interactions by electrostatic repulsion failed to prevent the arrest of liposomes at low salt [113].

Several intricate tools have so far been developed to control interactions and assembly of liposomes on supported membrane interfaces. Therefore surface-mediated assembly protocols would be one promising route to assembly of complex multicompartment liposome architectures. In the next section we go beyond the assembly of the multicompartment architectures themselves and consider methods for communication and transport between the encapsulated aqueous compartments of individual liposomes.

4. Communication between aqueous compartments

While DNA can be used to direct the assembly of compartmentalised liposome architectures, where morphology and interactions can be controlled through the delicate interplay between DNA and lipid interactions, methods for communication and transport of materials between the compartments needs to be realised for these materials to become useful as nanoreactors or synthetic cells or tissues. This section will primarily explore two bioinspired modes of chemical mixing: (i) irreversible fusion between liposomes, and (ii) functional membrane channels embedded within the membranes.

A third possibility for chemical mixing is triggered release of contents by targeted liposome rupture. One example of this approach from the literature uses DNA block copolymers to functionalise liposomes [53]. Complementary DNA containing a photo-sensitizer group hybridizes to the membrane-anchored strand. Photo-irradiation of the composite liposomes results in singlet oxygen generation at the bilayer surface, which locally oxidises the lipids and results in loss of membrane integrity. This approach could be used to sequentially release contents from multiple populations of differentially DNA-labelled liposomes within the same system. Targeted triggering of reversible vesicle to micelle transitions in high density DNA-lipid systems may also be an attractive route to targeted contents release [121].

a. Irreversible liposome fusion

Simply by changing the relative membrane-anchoring geometry of one of the DNA strands such that one is anchored at the 5′ proximal end and the other at the 3′ proximal end, membrane fusion can be achieved in contrast to just the relatively straight forward adhesion (docking) mode of action we have discussed in section 3 [65, 122]. This change in orientation of one of the interacting DNA strands means that the DNA now hybridizes in a zippering action that starts at the two membrane distal ends and proceeds towards the membrane proximal ends, thereby pulling the membranes into close apposition (Fig. 10). This mimics the action of natural SNARE fusion proteins in initiating vesicle fusion [123].

Traditional fluorescence assays for membrane fusion have been employed to study total lipid mixing, inner monolayer lipid mixing and contents mixing between interacting liposomes [57, 65, 122, 124]. Significant (up to ~80%) lipid mixing can be initiated between liposomes by the DNA-zippering mechanism [65]. Lipid mixing efficiency is not solely determined by the DNA properties, the lipid compositions of the liposomes is also an important factor, with “inverted-cone” shaped lipids such as DOPE and cholesterol amplifying the rate and extent of lipid mixing between liposomes [122]. These lipids increase the stored curvature elastic stress within lipid membranes, lowering the free energy barrier to the topological changes involved in development of highly curved hemi-fusion stalks and full fusion pores [125, 126].

Most studies of DNA-mediated liposome fusion have been conducted using a highly fusogenic liposome formulation of 2:1:1 DOPC:DOPE:cholesterol [57, 65, 122, 124]; inner monolayer mixing is found to be considerably lower than total lipid mixing within these systems, suggesting that a significant proportion of zippering interactions stall at, or reverse after, formation of the hemifusion state. More critical for the application of these systems for controlled chemical mixing between compartments is the prohibitively low contents mixing observed between the liposomes [57, 65, 124]. Contents mixing values as high as ~15% have been achieved [57, 65], however efficiencies of less than 2% are more common [65, 124], which may, in part, be explained by leakage of contents during the fusion process [57].

These DNA mimics of the SNARE fusion machinery are amenable to systematic variation of system parameters to investigate their effects on the rate and efficiency of membrane fusion events. Repeating poly-A – poly-T DNA zippers are found to be more efficient at initiating membrane fusion than non-repeating DNA zipper sequences [65]. Non-hybridizing spacer groups between the membranes and the DNA zipper sequences fairly predictably enhance the docking rate between liposomes but systematically reduce fusion efficiency due to the liposome membranes not being brought into as close proximity [124]. Perhaps more surprisingly, there appears to only be a slight

dependence of fusion efficiency on the length (and hence binding strength) of the DNA strands; while 27 base sequences are more efficient than short 12 base sequences, an increase to 42 base strands provides no significant enhancement [57]. This may be due to a strand length independence of the unzipping force between pairs of DNA bases.

Across the current reports on DNA-mediated liposome fusion, the effect of DNA-loading per vesicle is less clear cut and may be dependent on the chosen membrane-anchoring groups. Lipid-DNA conjugates showed systematically increasing fusion efficiencies with increasing DNA loading from <10> to <100> DNA/vesicle [65]. However, while chol-DNA conjugates showed some increase in efficiency with DNA-loading in ensemble lipid mixing assays, little increased benefit was found between loadings of 13 and 100 DNA/vesicle [57]. Furthermore, single vesicle lipid mixing experiments on planar supported bilayers, which we will discuss in more detail below, found that 10 – 16 bivalent cholesterol-DNAs/vesicle were optimal for fusion with higher DNA loadings hampering fusion [127]. This could be a result of steric restrictions at higher loading during the zippering process where more complicated toe-hold strand displacement mechanisms need to take place within the bivalent cholesterol anchoring system [57, 122, 127]. Single cholesterol anchors were found not to be as efficient in instigating membrane fusion as these anchors were prone to flipping between membranes due to the high repulsive pressures that arise between membranes during fusion, resulting in undocking of the liposome complexes [57].

DNA-mediated vesicle fusion has also been investigated at the single vesicle level using image techniques on surface supporting bilayer membranes [127, 128]. TIRF microscopy was employed to directly visualize lipid mixing when liposomes fused with lipid bilayers supported directly on a glass cover slip [127]. Upon liposome docking, a few DNA tethers formed, with the mean liposome lateral diffusion decreasing with increasing DNA-loading and, by implication, tether number. Fusion could only proceed through the zippering mechanism once 10 – 16 tethers had formed, suggesting that multiple zippers are required to overcome the repulsive membrane interactions and trigger fusion. Fusion was also found to be Ca^{2+}-dependent. The calcium ions could have two roles: firstly in screening electrostatic repulsions between the phosphates of the DNA backbones, and secondly by creating direct bridging interactions between phospholipid head groups, since calcium alone is known to be able to instigate fusion between phospholipid membranes [129].

TIRF microscopy has also been used to directly observe contents release across a surface-tethered membrane [128]. Surface tethered membranes were positioned away from the glass cover slip upon DNA “stilts”, i.e. DNA tethered in an adhesion geometry such that they provide rigid spacers preventing direct interaction with the substrate. By spacing the membrane away from the

glass cover slip, this allowed full fusion of liposomes with, and contents release across, the membrane without restrictions incurred by strong supported bilayer – glass substrate interactions. This experimental geometry allowed simultaneous, time-resolved observation of lipid mixing and contents release during liposome docking and fusion events at the single vesicle level. Further investigation of fusion at these surface-tethered membranes has revealed that arrested hemi-fusion is the dominant state of these systems with full fusion occurring in less than 5% of cases [130].

DNA-mediated fusion interactions have been used to demonstrate an artificial secretory cell [131]. Liposomes loaded with catechol were directly inserted into GUVs by micropipette injection. The liposomes and GUV membranes were functionalised with complementary "zipper" DNAs to stimulate excretory fusion events at the GUV surface. Fusion was triggered following the addition of Ca^{2+} ions and release of catechol across the membrane was recorded by an amperometric electrode. Qualitatively similar exocytosis events were observed in this model system when compared with PC12 excretory cells.

The release of compounds across membrane compartments by the stochastic fusion of many liposomes may be one route to reliable and predictive chemical mixing in multicompartmental systems. However DNA-mediated content mixing by direct fusion is not currently a viable technology for general applications where reliable one-to-one fusion events between compartments are desired. Further work will be required to find the ideal conditions (lipid composition, DNA properties and solution environment) that allow high efficiency, non-leaky fusion to proceed. Despite the challenges to be faced in applying DNA-mediated membrane fusion to new chemical technologies, these systems are already proving valuable in testing biophysical theories as models for SNARE fusion proteins. One possible exciting advance in this area would be the demonstration of reversible kiss-and-run fusion between DNA-mediated liposomes, where reversible fusion events allow efficient recycling of liposomes, as is observed for natural synaptic vesicles in neuronal signalling [132]. This would allow fundamental investigation of the biophysical factors that differentiate between full fusion and kiss-and-run fusion within a model system.

Beyond direct fusion of compartments, nature controls chemical mixing between isolated membrane-bound environments by material transport through membrane-embedded channels. The prospects for incorporating this second scenario within multicompartment liposome architectures will be explored in the next section.

b. Trans-membrane channels

Integrated membrane channels that allow direct passage of chemicals between the aqueous compartments of DNA-linked liposomes are yet to be achieved. This will require transport of matter across two bilayers that are separated by rigid double-stranded DNA spacers. Many double membranes exist in biology, including the membranes of the nucleus, mitochrondria and gram-negative bacteria. Looking to nature for inspiration for natural proteins that span a double membrane is one possible solution, where numerous such examples exist, including the nuclear pore complex [133], connexins within gap junctions between cells [134] and bacterial drug efflux transporters [135]. However these larger trans-bi-membrane proteins may be challenging to readily functionally reconstitute as a component within functional liposome networks, in particular the gargantuan nuclear pore complex with its tens of constituent proteins and >100 MDa molecular weight. Synthetic membrane channels, whose features can be designed specifically for a desired functional role within a liposome network, might be a better route toward this goal, where DNA nanotechnology could again provide the solution.

Recent innovative work has demonstrated the ability of DNA origami to create transmembrane channels that have electrophysiological properties similar to some integral membrane proteins (Fig. 11) [136, 137]. Langecker and co-workers designed a DNA origami membrane plug with a central channel that penetrates through the membrane [137]. The channel, which was inspired by the bacterial toxin α-hemolysin, is anchored to the lipid bilayer by 26 cholesteryl-DNA anchors which provide a strong hydrophobic association with the bilayer that forces the inner channel to penetrate through the membrane. The penetrating column does not contain any hydrophobic groups that would interact favourably with the hydrophobic tail groups of the lipids, therefore it is anticipated that the lipid bilayer itself rearranges to form a torroidal pore around the DNA nanochannel such that the lipid head groups protect the hydrophobic chains from the highly charged sugar-phosphate DNA backbones. The pore had an internal diameter of 2 nm and a length of 47 nm (Fig. 11 A-D).

A second example of a synthetic transmembrane channel was formed from six interconnected DNA duplexes approximately 15 nm long, again with a central pore of around 2 nm and is therefore considerably smaller than the previous example while having a similar internal diameter (Fig. 11 E,F) [136]. On this occasion, targeted chemical modification of the DNA backbones is used to insert a ring of hydrophobic ethyl groups that match the hydrophobic thickness of the lipid bilayer. This allows the DNA nanochannel to insert stably into the membrane forming a tight seal with the surrounding lipid matrix in a similar manner to how transmembrane proteins fold with exposed hydrophobic amino acids along the intra-bilayer-contacting face of its structure.

These first generation DNA nanostructure membrane channels are currently fairly non-specific in their transport properties except for the size-exclusion effects of the pore's finite diameter. Further engineering of these structures might yield additional biomimetic properties of transmembrane proteins such as chemical specificity and controlled gating. The nanochannel designed by Langecker et al. did demonstrate some stochastic gating within the channel recordings that was assumed to be derived from thermal fluctuations in the form of temporal, labile strand melting within the central pore; this assumption was supported by an increase in gating phenomena when a single stranded loop was engineered within the channel structure [137]. However this stochastic gating is unlikely to be amenable to external control; strategies analogous to the stimuli-responsive lids of open-close DNA origami boxes may offer an elegant route to smart gating phenomena [138]. While these DNA nanochannels currently only span a single bilayer, it is straightforward to envisage how these structures could be modified to span a second membrane opening up new opportunities in chemical transport between liposomal modules.

5. Outlook and future prospects

Significant technical advances have been made towards using nucleic acid amphiphiles to template the self-assembly of multicompartment liposome architectures. Current research has demonstrated several degrees of assembly control by specifying the DNA copy number per liposome (or DNA surface density), lipid composition relating to surface potential and lateral structural heterogeneity within the membrane, and using surface substrates as templates to control inter-liposome distributions and interactions. Further control is likely achievable through an understanding of the roles of interaction strength per DNA bond and entropic factors relating to the lateral mobility of the ligands and the roles of the flexibility of spacer groups that position the DNA strands away from the liposome surface.

Several major challenges lie ahead, including development of a general framework for programming the interconnections and superstructures formed from an arbitrary number of liposome populations, going beyond the binary systems most commonly studied. Theoretical developments for the assembly of complex, multicomponent structures from hard colloidal particles will likely be a promising starting point toward this goal [139]. A second challenge is to efficiently control the transport of chemicals between compartments with chemical specificity and the possibilities of control of transport through responsive gating mechanisms. This would be a significant step change from current techniques for trans-membrane transport in such synthetic systems, where non-specific pores or complete contents mixing or release are more common. With respect to the potential drug delivery applications of size-limited liposome clusters, studies need to

be done to understand the interactions of these structures with cells and whole organisms to test the viability of this concept. Future studies may go beyond use of lipid-based confining layers and extend the concept of DNA-mediated assembly to structurally more robust polymersomes [3, 140, 141], or hybrid vesicles, “lipopolymersomes”, composed of both lipids and block copolymers [142-144].

The advances over the past decade in controlling the functionalisation and interactions of liposomes using nucleic acids means it is now possible to start exploring combining structure formation and chemical transport to develop materials of increased complexity and emergent functionality. We anticipate that in the coming years examples will start to appear in the literature of proof of concept demonstrations of chemical process control within modular liposome networks.

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Figure Captions

Figure 1. Schematic cartoon depicting the concept of biomimetic chemical process control within a network of liposomal modules. Each compartment has a different chemical environment and catalytic function but is in communication with its environment and the other modules allowing transport of chemical species and a cascade of sequential reactions within consecutive compartments.

Figure 2. Schematic cartoon depicting the functionalization of different populations of liposomal modules with DNA-amphiphiles and their subsequent assembly into higher-order composite architectures [100] - Reproduced by permission of The Royal Society of Chemistry.

Figure 3. Structures of amphiphilic nucleic acids with example literature references. (a) Single hydrocarbon chains: monoacyl functionalised PNA (left) and polypropylene oxide modifications (right). (b) cholesterol anchors: single cholesterol modification (left), double cholesterol modification (centre) and a schematic cartoon of double-anchored chol-DNA using two single cholesterol modified DNAs (right; reprinted with permission from Pfeiffer I., Hook F., *J. Am. Chem. Soc.* 2004;126:10224-5 [55]. Copyright 2004 American Chemical Society). (c) Double hydrocarbon anchors: structure of a dialkyl lipid-DNA conjugate (left) and an α-tocopherol modified base which can be inserted within the DNA chain to create two (or more) hydrophobic anchors (right). (d) Biotin-avidin linkers for functionalisation of lipid bilayers with DNA: avidin binds to biotinylated lipids within the membrane; subsequent addition of biotinylated DNA attaches the DNA to the avidin on the membranes. Inset cartoon of biotin-avidin conjugation to lipid bilayers and subsequent membrane adhesion reprinted with permission from reference [68].

Figure 4. Reversible assembly of BioNanoStacks formed from the DNA-mediated assembly of lipid nanodiscs. (a) representative melting curves from UV-vis spectroscopy. (b) Melting temperatures as a function of salt concentration for different lipid compositions separated by heating and cooling directions. (c) Melting cooperativities represented by the full width half maximum (FWHM) of the melting curves. (d,e) Negative staining TEM images of BioNanoStack morphologies. Reprinted with permission from Beales P.A. *et al.*, *J. Am. Chem. Soc.* 2013;135:3335-8 [83]. Copyright 2013 American Chemical Society.

Figure 5. Assembly of DNA-functionalized “Janus” vesicles. (A) confocal fluorescence microscopy sections of DNA-functionalized Janus vesicles with the liquid disordered domain labelled in red and the DNA labelled in blue. (B) Fluorescence intensity line profiles of the liquid disordered dye (Rh-DOPE) and DNA (A647-DNA) from the image in part A. (C) 3D-reconstructed z-stack of the

morphology of DNA-functionalized Janus vesicles. (D) Assembly of Janus vesicles into size limited clusters with the liquid disordered phase labelled red and the liquid ordered phase labelled green; the fluorescence intensity line profile demonstrates the presence of an unfused double membrane in the adhesion plaques between liposomes [100] - Reproduced by permission of The Royal Society of Chemistry.

Figure 6. Enzymatic-switching of DNA domain partitioning. (A) Hybridization of a palmitoylated PNA and DNA tocopherol causes both molecules to partition into liquid disordered domains. (B) Cleavage of the linking DNA strand by EcoR1-HF switches the palmitoylated PNA into the liquid ordered domain. Reprinted with permission from Schade M. *et al*., *J. Am. Chem. Soc.* 2012;134:20490-7 [102]. Copyright 2012 American Chemical Society.**Figure 7**. Linker self-sorting by length between DNA-tethered membranes. (A) cartoon showing a mixture of 24mer and 72mer DNAs separating a silica supported bilayer from a second tethered bilayer. (B) Fluorescence interference contrast (FLIC) image showing height differences of a tethered lipid bilayer labelled with a Texas Red lipid; brigher areas represent higher membrane domains. (C) The same patch showing fluorescence from Alexa-488 labelled 24mer DNA. The scale bars represent 10 μm [103] - Reproduced by permission of The Royal Society of Chemistry.

Figure 8. Cartoon showing liposomes tethered to a planar bilayer by multiple DNA tethers deduced from lateral mobility measurements. The DNA could form a concentrated patch of DNA at the contact site (left) or move out in a crown-like ring that would increase the effective contact area between the liposome and planar membrane. Reprinted with permission from Benkoski J.J., Hook F., *J. Phys. Chem. B* 2005;109:9773-9 [112]. Copyright 2005 American Chemical Society.**Figure 9.** Competition between electroosmotic flow and electrophoretic flow of charged liposomes tethered to (A) a neutral supported membrane and (B) a supported membrane containing charged lipids in the presence of an applied electric field. (C) Separation of vesicles by charge due to their different electrophoretic mobilities: egg PC + 1% Texas Red DHPE + 4% DPPS liposomes (red) and egg PC + 2% Oregon Green DHPE liposomes (green) on a supported membrane of egg PC + 2% DPPS. Reprinted with permission from Yoshina-Ishii C., Boxer S.G., *Langmuir* 2006;22:2384-91 [114]. Copyright 2006 American Chemical Society.**Figure 10**. Schematic cartoon of the steps of liposome fusion using chol-DNAs that zip the liposome membranes into close apposition, stimulating lipid mixing and fusion in an analogous mechanism to the natural SNARE fusion complex. Reprinted with permission from Stengel G. *et al*., *J. Phys. Chem. B* 2008;112:8264-74 [57]. Copyright 2008 American Chemical Society.**Figure 11**. DNA nanostructures as trans-membrane pores. (A-C) Cartoons illustrating the DNA nanostructure designed by Langecker *et al.* along with (D) TEM images of the structure. From

Langecker M., *Science* 2012;338:932-6 [137]. Reprinted with permission from AAAS. (E, F) Illustrated structure of the transmembrane pore designed by Burns *et al.* Reprinted with permission from Burns J.R. *et al.*, *Nano Lett.* 2013;13:2351-6 [136]. Copyright 2013 American Chemical Society.

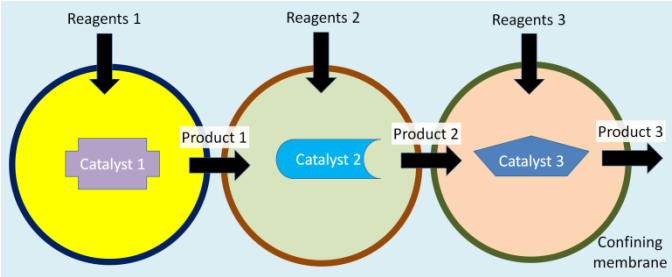
Reagents 1
Reagents 2
Reagents 3
Catalyst 1
Product 1
Catalyst 2
Product 2
Catalyst 3
Product 3
Confining membrane

Figure 1.

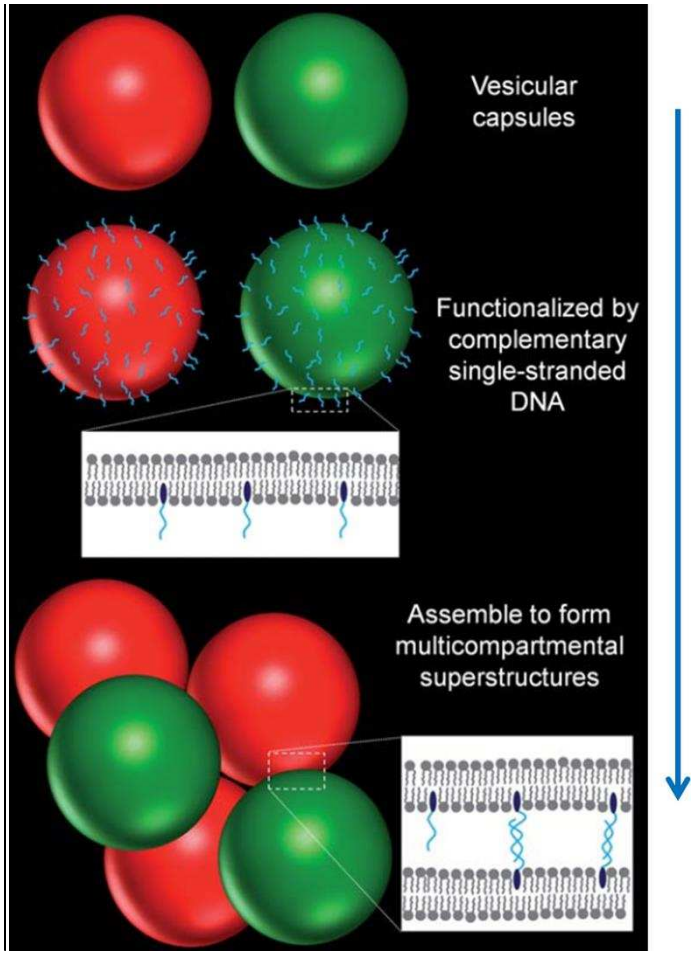
Vesicular capsules
Functionalized by complementary single-stranded DNA
Assemble to form multicompartmental superstructures

Figure 2.

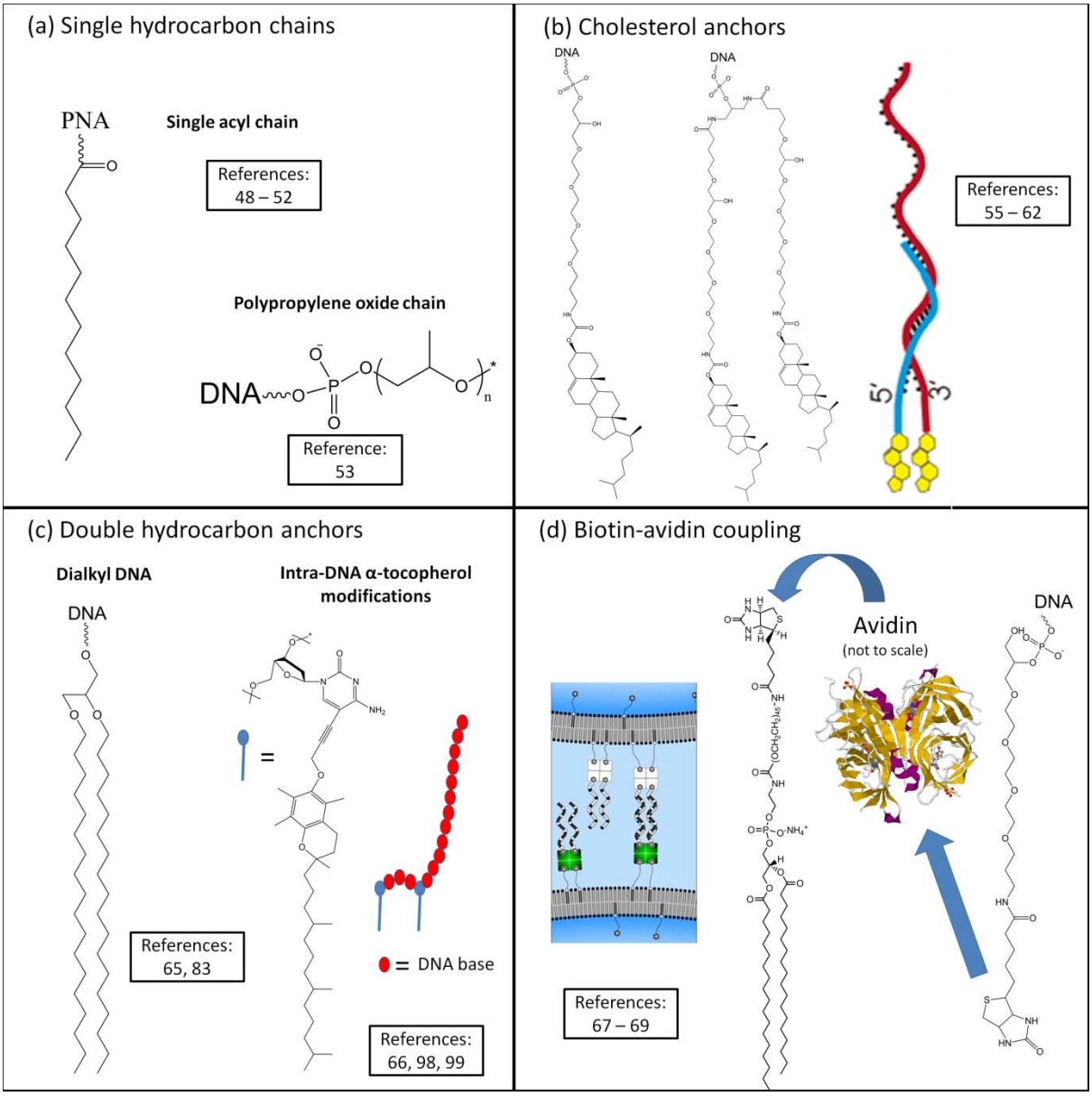
(a) Single hydrocarbon chains
PNA
Single acyl chain
References: 48 – 52
Polypropylene oxide chain
DNA
Reference: 53
(b) Cholesterol anchors
DNA
DNA
References: 55 – 62
5'
3'
(c) Double hydrocarbon anchors
Dialkyl DNA
DNA
References: 65, 83
Intra-DNA α-tocopherol modifications
= DNA base
References: 66, 98, 99
(d) Biotin-avidin coupling
Avidin
(not to scale)
DNA
References: 67 – 69

Figure 3

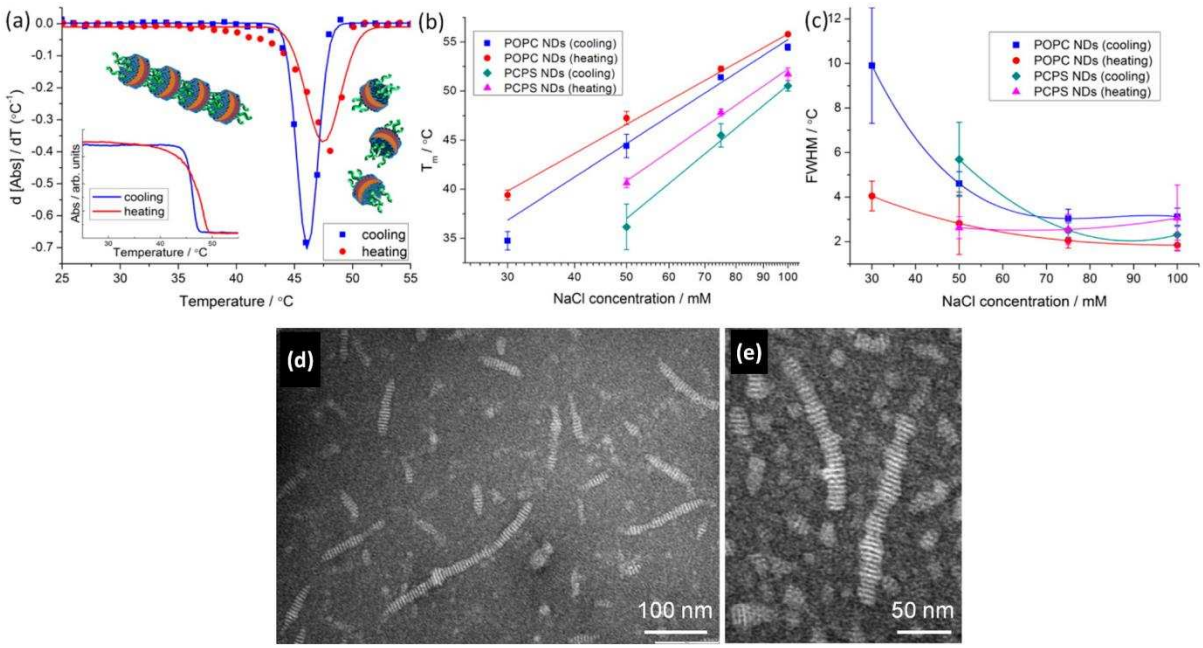
(a)
d [Abs] / dT (°C⁻¹)
Temperature / °C
cooling
heating
Abs / arb. units
(b)
POPC NDs (cooling)
POPC NDs (heating)
PCPS NDs (cooling)
PCPS NDs (heating)
T_m / °C
NaCl concentration / mM
(c)
FWHM / °C
NaCl concentration / mM
(d)
100 nm
(e)
50 nm

Figure 4.

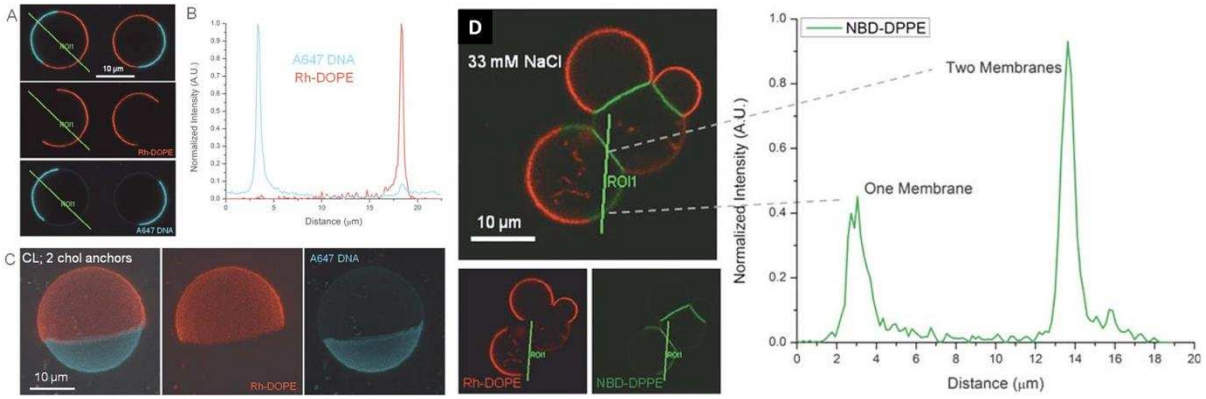
A
ROI1
10 µm
Rh-DOPE
A647 DNA
B
A647 DNA
Rh-DOPE
Normalized Intensity (A.U.)
Distance (µm)
C
CL; 2 chol anchors
A647 DNA
10 µm
Rh-DOPE
D
33 mM NaCl
ROI1
10 µm
Rh-DOPE
NBD-DPPE
NBD-DPPE
Two Membranes
One Membrane
Normalized Intensity (A.U.)
Distance (µm)

Figure 5.

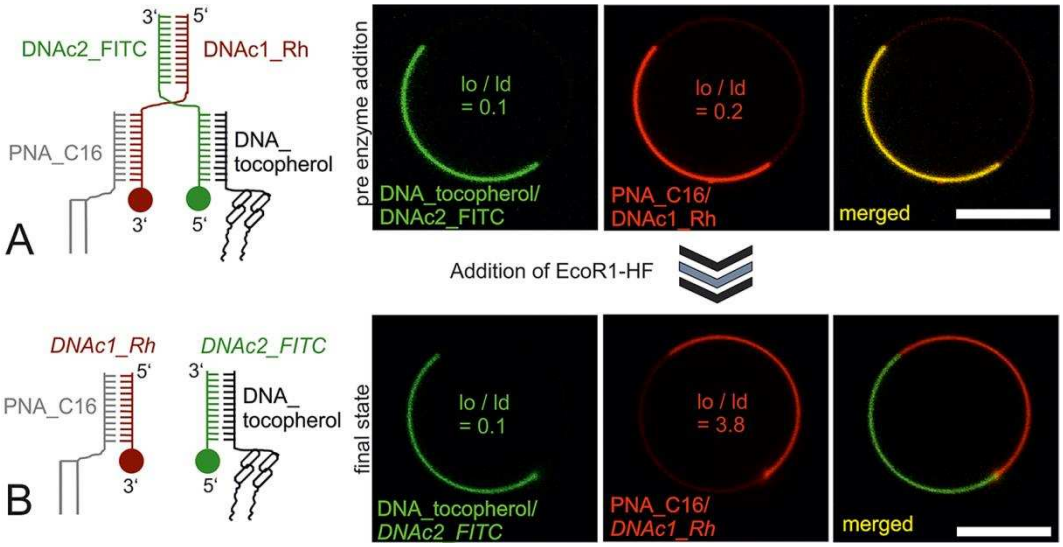
3'
5'
DNAc2_FITC
DNAc1_Rh
PNA_C16
DNA_
tocopherol
3'
5'
A
pre enzyme additon
Io / Id
= 0.1
DNA_tocopherol/
DNAc2_FITC
Io / Id
= 0.2
PNA_C16/
DNAc1_Rh
merged
Addition of EcoR1-HF
DNAc1_Rh
DNAc2_FITC
5'
3'
PNA_C16
3'
DNA_
tocopherol
5'
B
final state
Io / Id
= 0.1
DNA_tocopherol/
DNAc2_FITC
Io / Id
= 3.8
PNA_C16/
DNAc1_Rh
merged

Figure 6.

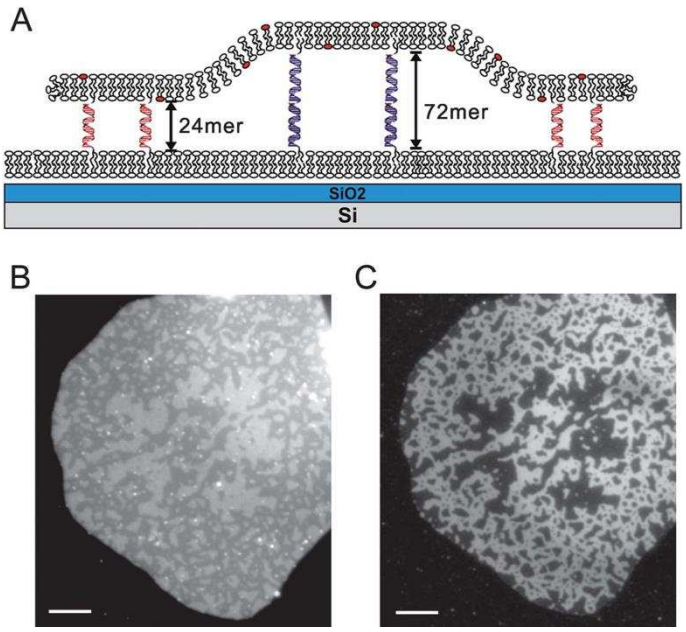

Figure 7.

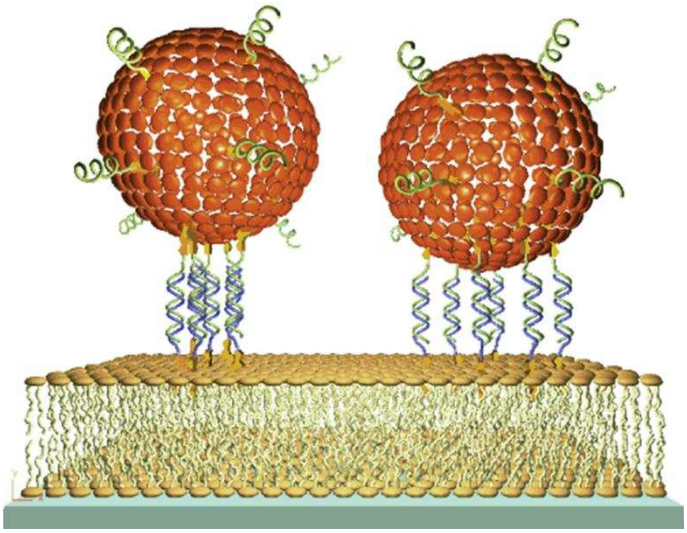

Figure 8.

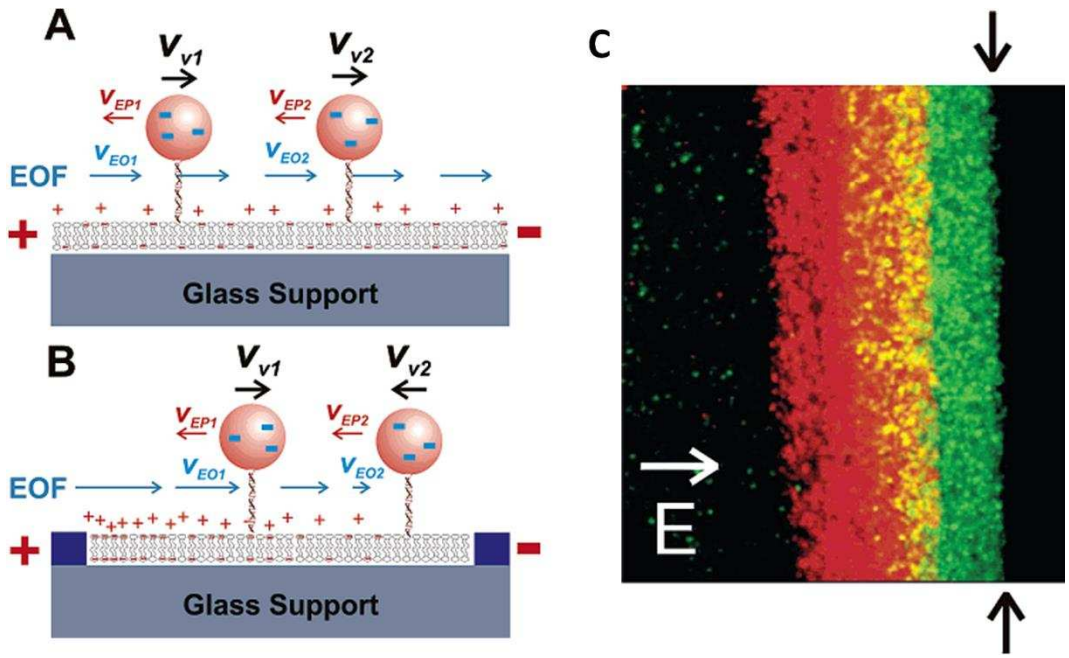
A
V_{v1}
V_{v2}
V_{EP1}
V_{EP2}
V_{EO1}
V_{EO2}
EOF
+
-
Glass Support
B
V_{v1}
V_{v2}
V_{EP1}
V_{EP2}
V_{EO1}
V_{EO2}
EOF
+
-
Glass Support
C
E

Figure 9.

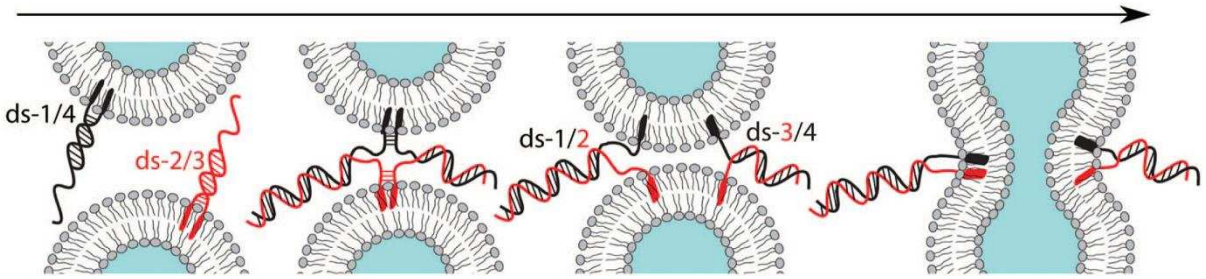

Figure 10.

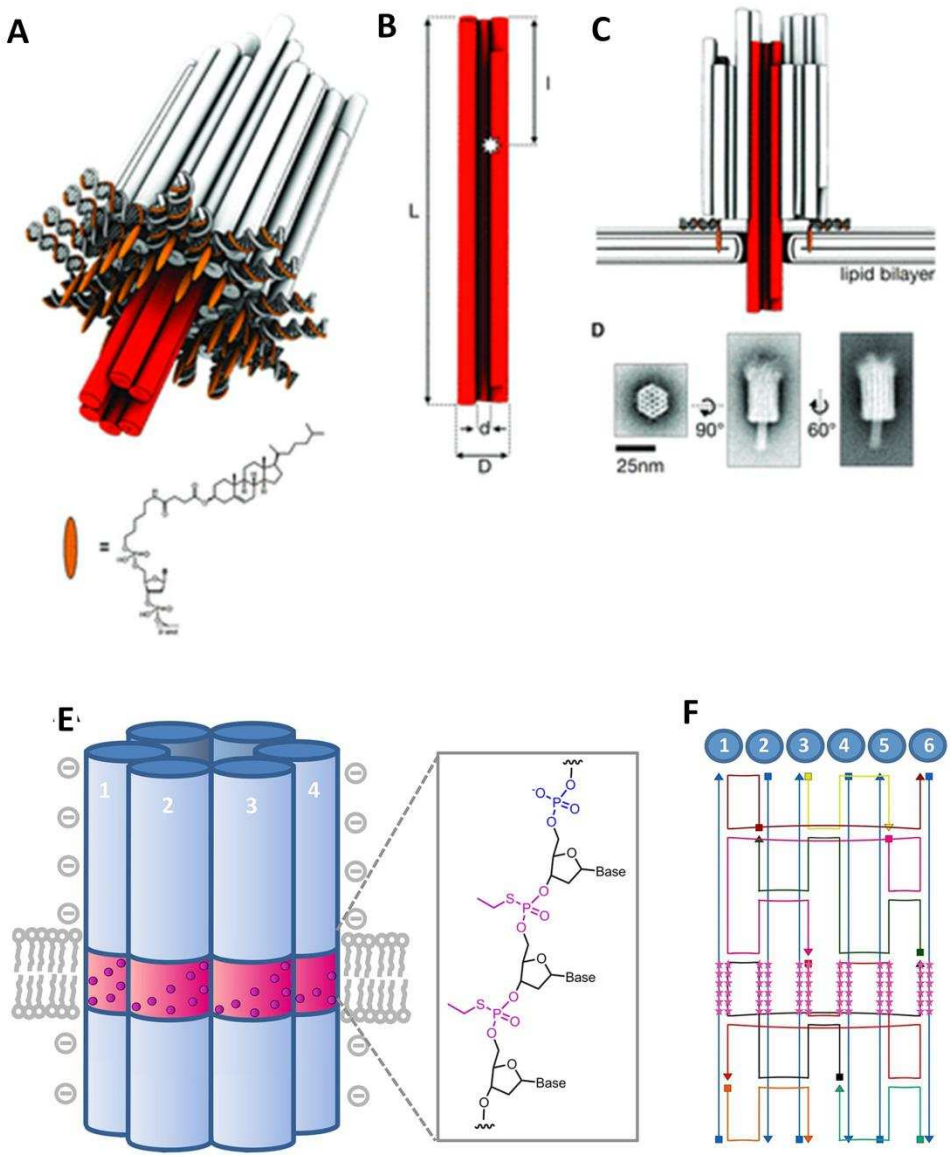
A
B
C
l
L
d
D
lipid bilayer
D
90°
60°
25nm
E
1
2
3
4
Base
Base
Base
F
1
2
3
4
5
6

Figure 11.

Highlights

- DNA with hydrophobic modifications can functionalise liposome membranes.
- Membrane-anchored DNA can act as adhesion receptors between liposome compartments.
- Tuning lipid composition modulates properties of DNA-mediated liposome assemblies.
- DNA nanotechnology allows transport of chemical information between compartments.
- This biomimetic toolbox will enable applications in medicine and synthetic biology.

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