Project Title
DNA-mediated fusion of spheroplasts with synthetic liposomes

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DNA-mediated fusion of spheroplasts with synthetic liposomes

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Summary
The project aims at controlling the fusion of spheroplasts or mammalian cells with artificial liposomes using amphiphilic DNA nanostructures. These complexes mimic the function of SNARE proteins (soluble N-ethylmaleimide sensitive factor (NSF) attachment protein receptors), inducing membrane fusion by bridging across two lipid bilayers and driving them to close proximity. Liposomes can be used to deliver efficiently large amounts of cargoes into the live cells, needed for instance for genetic editing or otherwise interfering with the cell's metabolism, mechanical properties etc.
Various candidate DNA nanostructures were designed, assembled and characterised. Then their ability of inducing fusion of liposomes of different size with other liposomes was successfully demonstrated.
In parallel, a protocol for the preparation of giant spheroplasts from filamentous *E. coli* was implemented and the possibility of functionalising the spheroplasts with the DNA nanostructures demonstrated.
We are currently attempting the fusion of spheroplasts with liposomes, but preliminary results are inconclusive.
In parallel, we started exploring the possibility of functionalising mouse stem cells with the same DNA complexes, with the objective of delivering CRISPR machinery for genetic editing. This part of the project is done in collaboration with Dr A. Russel (CRUK).

Report and outcomes

DNA nanostructures for induced membrane fusion were designed using the online tool NUPACK. The final designs are shown in Figure 1. The nanostructures are formed from 4 cholesterol functionalised single-stranded DNA (ssDNA) molecules labelled as A, A', B and B', binding in pairs. DNA was purchased from Integrated DNA Technologies or Eurogentec. Initially, 2 nanostructures (see Figure 1, left) are prepared by mixing strands AB and A'B' in stoichiometric ratio, heating up to above the ssDNA melting temperature and slowly cooling down by means of a PCR machine. The so-formed nanostructures are then incubated with liposome/cell solutions for a few hours (or overnight) until partitioning on the membrane occurs (Figure 1 left).
When two samples of liposomes/cells featuring AB and A'B' constructs are mixed, the nanostructures bind to each other as shown in Figure 1 (centre) until AB and A'B' complexes are broken to form AA' and BB' nanostructures (Figure 1, right). The latter complexes are thermodynamically more stable than AB and A'B', ensuring the irreversibility of the described DNA reaction. AA' and BB' nanostructures force the two membranes in very close proximity, inducing fusion.
Liposomes were prepared from synthetic DOPC lipids using either extrusion or electroformation. The former procedure produces Large Unilamellar Vesicles (LUVs) with an approximate diameter of 100 nm, the latter produces Giant Unilamellar Vesicles (GUVs)

with diameter ranging from ~5 to ~50 μm. A small fraction of fluorescently labelled DHPE lipids was mixed with the DOC to monitor membrane fusion by means of confocal microscopy or fluorimetry.
Giant spheroplasts, with diameters of 3-5 μm were prepared by removing the cell wall of filamentous *E. coli* following conventional protocols. Once prepared, the spheroplasts were purified via repeated centrifugation and resuspension in clean medium.

Figure 2 shows the outcome of a fluorimetry experiment aimed at inducing the fusion of LUVs. The two types of liposomes (functionalised with complementary AB and A'B' nanostructures respectively) were mixed at time t=0, and the percentage of lipid mixing was monitored over time exploiting Forster Resonant Energy Transfer (FRET) between fluorescently labelled lipids. The fraction of lipid mixing exceeded 50% after 2 hours. In contrast, when the two liposome species were functionalised with the same (non complementary) DNA nanostructure (either AB or A'B'), no mixing was observed over the same time period.

Figure 3 demonstrates the liposome fusion in GUVs. Two species of GUVs (red and cyan in confocal images) are initially labelled with different fluorescent lipids and functionalised with different (AA and A'B') nanostructures. Initially adhering GUVs (t=0) show no membrane mixing, while complete fusion is observed after 120 minutes.

Figure 4 demonstrates the possibility of functionalising spheroplasts similarly to what can be done with the liposomes. Here the DNA nanostructures have been made fluorescent by adding a short, fluorescently labelled, ssDNA binding to one of the unpaired overhangs. The fluorescent interface of the spheroplasts demonstrates the partitioning of the nanostructures on the plasma membranes.

Initial experiments on the fusion of liposomes with spheroplasts are still inconclusive. Bulk fluorimetry assays aimed at inducing LUV-spheroplast fusion did not evidence lipid mixing.

Possible reasons for the lack of fusion are impurities (residual of the wall removal process) present at the surface of the spheroplasts as well as in the solution of the spheroplast samples, impairing the contact with the liposomes. Also the presence of a thick protein layer on the plasma membrane of the spheroplasts could block fusion by burying the DNA linkers. We plan to explore some protocols for cleaving these membrane tethers. The work we could do with Martin Howard is on hold until we succeed in this fusion step to the cell.

In parallel we started exploring the possibility of using the nanostructures to mediate the fusion of liposomes with mouse stem cells. To this end, we shared some DNA nanostuructures with Dr A. Russel, CRUK (Cambridge). Preliminary flow cytometry experiments indicate that the nanosturctures can indeed partition on the stem cells, and that these survive being functionalized. Further experiments are necessary to understand if the nanostructures are present on the plasm membrane or uptaken by the cell.

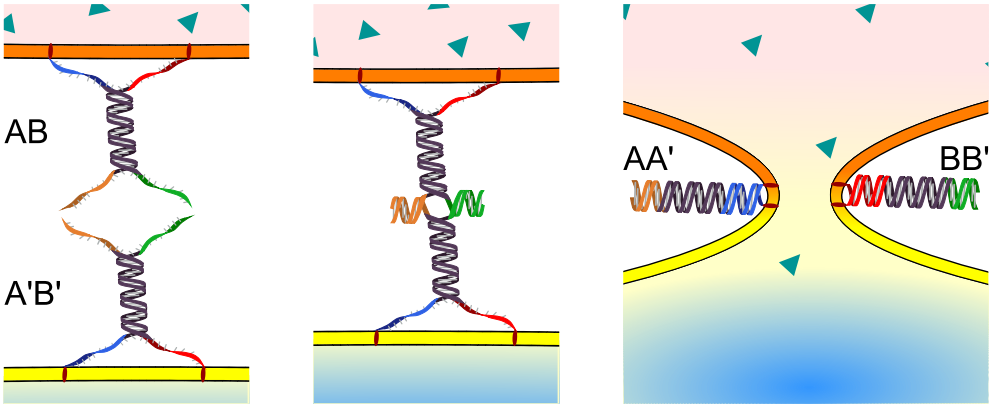

Figure 1. Design and function of membrane-fusing DNA nanostructures. Two samples of liposomes/cells are functionalised with complexes AB and A'B' respectively (left). When the two samples are mixed the complexes bind, bridging together the membranes (centre). Binding progresses all the way to the anchoring point of the nanostructures and complexes rearrange to form AA' and BB' duplexes. At this stage the membranes are taken in close proximity and fusion is induced (right).

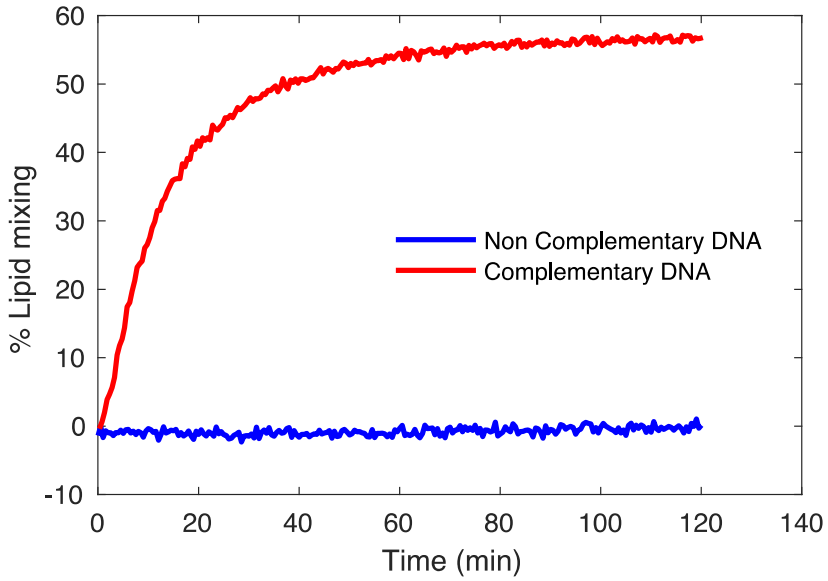

Figure 2. Bulk fusion (fluorimetry) assay between functionalised LUVs. In samples were LUVs carry complementary DNA fusion is observed. If non-complementary DNA is used the show no fusion.

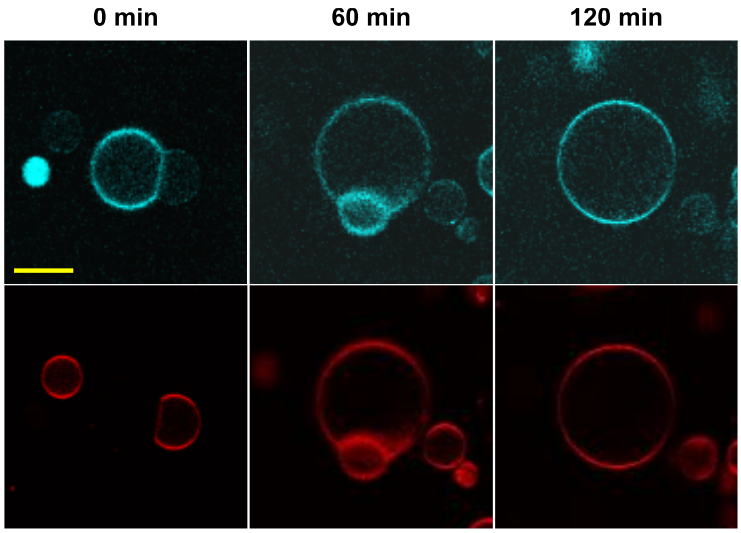

Figure 3. Confocal GUV fusion experiment. Two families of GUVs are labelled in with different fluorophores visualised in the two fluorescent channels (red and cyan). Initially 2 GUVs are adhering thanks to the formation of DNA bonds, but their lipid membranes remain distinct (no mixing of the fluorophores). Over time the membranes fuse and after 120 minutes a single (larger) GUV with both dyes in the membrane is present. Scale bar: 10 μm.

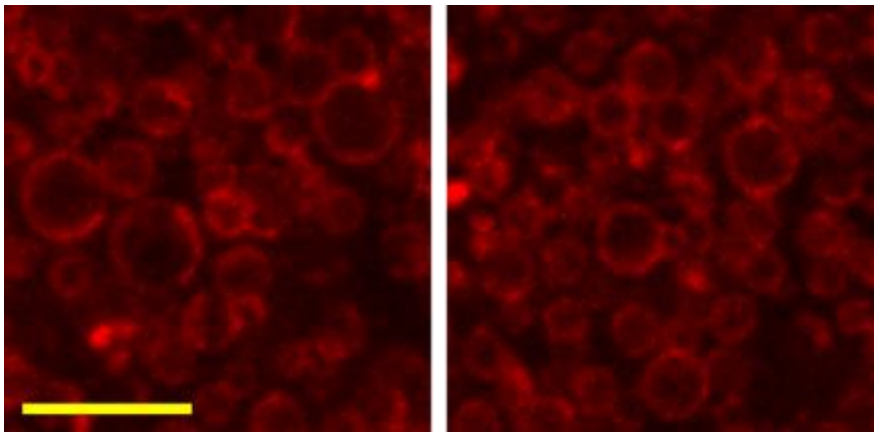

Figure 4. *E. coli* giant spheroplasts functionalised with fluorescent DNA nanostructures. Scale bar: 10 μm.

Expenditure

- Fluorescent lipids: £196
- Functionalised DNA: £1974
- Cuvettes for fluorimetry: £753
- Various (consumables, access to lab space etc.): £882

Total expenditure: £3805

Follow on Plans

We plan to continue experiments on the fusion of spherolasts with liposomes. We will try confocal experiments involving GUVs rather than LUVs, as the former have shown greater tendency to fusing.
We plant to optimise the purification protocol of the spheroplasts to remove more impurities from the samples.
Besides try with the current nanostructures, will try designing and producing longer DNA linkers that can be accessed also in the presence of a protein brush covering the plasma membrane.

We plan to continue the optimisation of the protocol to functionalised stem cells with our DNA nanostructures combining flow cytometry with confocal microscopy.

We plan to use the remaining funds (£195) and the additional £1000 to purchase a new DNA stock for preparing the nanostructures (both the initial and "longer" designs).

As well as enabling high-risk research, this exploratory project is extremely useful to us in testing ideas and generating preliminary results that we are hoping to develop into both a grant on lipid systems, and a grant on new technology for gene editing in cells.