DNA nanotechnology as a tool to manipulate lipid bilayer membranes
Meng, Zhuojun

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Chapter 5

Performing DNA Nanotechnology Operations on a Zebrafish Surface

This chapter has been submitted for publication.
5.1 Introduction

Zebrafish is an ideal model vertebrate system with robust, external, and transparent development allowing sophisticated imaging and experimental techniques. In just a few days, embryos develop from single cells to remarkably complex body structures and organ systems (Fig. 5.1). Zebrafish embryos exhibit the great feature that they develop as "see through" embryos, meaning that, all internal development can be clearly observed from the outside in the living system.

Fluorescent probes, such as synthetic organic dyes, fluorescent proteins, and quantum dots have been used extensively to monitor biomolecules and biologically relevant species in vitro and in vivo. Due to their transparent properties, zebrafish embryos have received great attention for live vertebrate imaging due to the possibility to conduct high resolution in vivo imaging. Compared to mammals, many structures and processes are similar in zebrafish such as the brain and spinal cord. Because of its outstanding suitability for imaging and transgenesis approaches, the developing zebrafish has become a leading vertebrate model for studies in drug discovery and a variety of human diseases. In many cases, molecular imaging using fluorescent probes has been carried out within cells. However, live cell imaging is not completely acceptable for obtaining detailed information about the biological effects of analytes in a tissue context. In contrast, images of the insides of live animals provide a more informative view of these effects. Moreover, fluorescent imaging
studies using organisms that are genetically close to humans have become highly attractive (Fig. 5.2).\textsuperscript{11}

![Diagram showing live cell and animal imaging using fluorescent probes.](image)

\textit{Fig. 5.2} Live cell and animal imaging using fluorescent probes. The trend for detection of analytes has changed from live cell imaging to whole animal imaging.\textsuperscript{11}

Owing to its small size, optical transparency, external fertilization and easy manipulation, zebrafish are perhaps the most suitable vertebrate model animals for \textit{in vivo} imaging using fluorescent probes.

In this chapter, we implemented a membrane engineering related DNA nanotechnology on the surface of a living animal. We investigated whether it was possible to insert the lipid-modified DNA sequences into the membrane of live zebrafish to function as an artificial receptor. Firstly, the immobilization of membrane-anchor-functionalized oligonucleotides on a zebrafish was demonstrated. Then, functionalization of protruding single-stranded DNA atop the fish was realized by Watson-Crick base pairing employing complementary DNA sequences. In this way, small molecules and liposomes were guided and attached to the fish surface. The anchoring process can be designed to be reversible allowing exchange of surface functionalities by simple addition of DNA sequences. Finally, a DNA based amplification process was performed atop of the zebrafish enabling the multiplication of surface functionalities from a single DNA anchoring unit.
5.2 Results and Discussion

5.2.1 DNA hybridization on a zebrafish surface and strand replacement

A DNA-based receptor can be used to modify the cell membrane with new functions, such as for immobilization of surface probes or other payloads for targeted delivery onto or through the lipid bilayer. For that purpose, 1 µM lipid-DNA U4T-18 was utilized and incubated with zebrafish embryos of 1 day post-fertilization (dpf) for 1 h. Previously the group of Irvine\textsuperscript{12} performed cell membrane insertion of oligonucleotides carrying diacyl-lipid (C18) or cholesterol anchors using an incubating time of 30 min. Because electrostatic repulsion might delay incorporation into the membrane, we prolonged the time for incubation of lipid-DNA with zebrafish membrane to 1 h.

Subsequently, the 20mer oligonucleotide C594, which is partially complementary to U4T-18 and contains the red emitting fluorophore ATTO594, was added to hybridize to the lipid-modified surface anchor. Fluorescence microscopy showed staining of the fish surface as evidenced by the red emission (Fig. 5.3A) indicating that U4T-18 was successfully incorporated into the fish skin and that the protruding single-stranded DNA chain can selectively undergo sequence specific hybridization.

Next, we investigated if it is possible to dynamically exchange the red label by a removal strand. This strategy of strand replacement was previously introduced in the context of a DNA fueled molecular machine.\textsuperscript{13} Here, we employed this strategy for the reversible and gentle labeling of the skin of a living animal. Therefore, 2 µM 20-mer DNA oligonucleotide that is fully complementary to C594 was introduced to peel off C594 from U4T-18. The removal of C594 was proven by disappearance of red fluorescence on the fish surface (Fig. 5.3B). After removal of C594, U4T-18 remained on the skin and kept the ability to hybridize with other complementary DNA sequence such as C488, a 14mer DNA oligonucleotide complementary to U4T-18 and labeled with the green emitting fluorophore ATTO488. Clear evidence for the strand replacement was the green fluorescence observed on the exterior of the fish (Fig. 5.3C). Control experiments in which U4T-18
lipid-DNA or 20-mer oligonucleotides were omitted (Fig. 5.4) showed no (changes in) fluorescence.

Fig. 5.3 Fluorescent labelling and DNA replacement on the surface of zebrafish embryos. (A) Lipid DNA (U4T-18) is anchored on the skin membrane of 1 dpf zebrafish embryos and hybridizes to 1 μM ATTO594 fluorescently labeled complementary DNA (C594), resulting in red fluorescence on the zebrafish surface. (B) A 20-mer oligonucleotide replaces C594 by means of strand displacement, resulting in loss of fluorescence of the fish. (C). Addition of 1 μM ATTO488 fluorescently labeled complementary DNA (C488) hybridizes with U4T-18, results in hybridization with U4T-18 and the appearance of a green fluorescence at the zebrafish surface. Red channel = ATTO594; Green channel = ATTO488.
Fig. 5.4 Fluorescence images of two control experiments: (A) untreated 2dpf zebrafish without DNA anchoring unit was incubated with 1 µM ATTO594 labeled complementary DNA (C594) for 1h. No fluorescence labelling was detected. (B) 2dpf zebrafish was decorated with U4T-18, incubated with 1 µM C594 for 1h, washed three times with egg water, and subsequently exposed to solution containing 1 µM ATTO488 labeled complementary DNA (C488). Although the green labelled DNA was added to the fish, red fluorescence was detected on its surface. This is due to lack of the removal strand. After all treatments of the fish with DNA, washing three times with egg water was performed. Red channel = ATTO594; Green channel = ATTO488.

5.2.2 Loading larger containers to the zebrafish surface by Watson-Crick base pairing

After demonstrating that base pairing is a very efficient tool to attach small oligonucleotides to the live animal surface, we attempted to load larger cargo to the zebrafish membrane. Therefore, phospholipid bilayer of liposomes of 120 nm diameter was loaded with rhodamine-functionalized phospholipid (Rh-DHPE), which is characterized by a red emission. Likewise, the surface of the vesicles was decorated with lipid-modified DNA that is complementary to that on the zebrafish (Fig. 5.5A). Proof of successful loading of the fish surface by supramolecular bonds was provided by fluorescence microscopy showing characteristic red fluorescence originating from the liposomes, which are bound to the fish surface (Fig. 5.5B). This result opens the way for potential DNA-mediated delivery of liposomal cargo. These experiments demonstrate that the DNA hybridization overcomes the repulsive hydration forces between the lipid
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head groups and brings the two lipid bilayers with complementary DNA in close proximity to achieve surface docking (aggregation).

Fig. 5.5 DNA duplex formation between U4T-18 and CrU4T-18 decorated liposomes on the surface of zebrafish. (A) Schematic representation of liposomes docking on the surface of zebrafish embryos by lipid-DNA hybridization. Confocal images of 2dpf zebrafish treated with (B) U4T-18 for 1 h, followed by incubation with CrU4T-18 decorated liposomes or (C) treated with 1 µM CrU4T-18 decorated liposomes in absence of U4T-18. The concentration of total lipids (DOPC:DOPE:CHO = 2:1:1mol%) was 0.5 mM. Red Channel: Rh-DHPE.
5.2.3 Nucleic acid mediated amplification process on live fish surface

To demonstrate the broad versatility of zebrafish surface engineering enabled by lipid-DNA, we performed a DNA-based amplification process on the animal, i.e. hybridization chain reaction (HCR). Previously, this method was employed for augmenting the signal during nucleic acid detection.\textsuperscript{14} Later, this technique was utilized for surface modification with DNA hydrogels.\textsuperscript{15} Here, we demonstrate that this supramolecular polymerization can be performed on the exterior of the living animal.

Since initiation of HCR from a lipid membrane was not demonstrated before we first established a HCR protocol for decorating the rim of liposomes with a DNA layer (see chapter 4). Then, the optimized DNA anchors and sequences were employed for modification of the fish surface (Fig. 5.6). Compared to the previous experiments, for membrane anchoring U4T-28, a 28mer lipid-DNA with 4 modified lipid bases, was bound to zebrafish skin. Next, hairpin strands M1 (partially complementary to U4T-28) and M2 (partially complementary to M1) were added (sequences see chapter 4).\textsuperscript{16} Hybridization of M1 to U4T-28 results in liberation of its loop that subsequently can hybridize with M2. Opening of the M2 hairpin exposes a sequence that binds to a new M1 monomer from the solution. In turn, opening of the M1 hairpin exposes a sequence that can bind new M2. This effectively triggers the “supramolecular polymerization” of M1 and M2 with surface anchor U4T-28 as initiator, leading to extended DNA on the zebrafish membrane (Fig. 5.6A). The realization of HCR was investigated on the membrane of 1 dpf zebrafish embryo. As shown schematically in Fig. 5.6B, U4T-28 at the concentration of 1 μM was exposed to zebrafish embryos for 1 h, followed by the incubation with a mixture of 2 μM M1-FAM and 2 μM M2-Cy3 for 2 h. Both monomers were labeled with two different fluorophores (FAM and Cy3). Green and red fluorescence could be clearly observed due to progression of polymerization of M1-FAM and M2-Cy3 from the initiator (Fig. 5.6C).
Fig. 5.6 DNA hybridization chain reaction (HCR) on the surface of zebrafish embryos. (A) Schematic representation of DNA HCR on the surface of zebrafish embryos. $a'$, $b'$, and $c'$ are regions that are complementary to regions $a$, $b$, and $c$, respectively. Hairpin M1 can be unfolded by hybridization with initiator U4T-28, resulting in growing DNA strands. (B) Addition of M1-FAM and M2-Cy3 to the U4T-28 pre-treated zebrafish resulted in DNA HCR and concomitant increase in fluorescence. (C) Fluorescence images of 1 dpf zebrafish embryos after incubation with U4T-28 for 1 h, and subsequent exposure to 1 µM M1-FAM and M2-Cy3 for 1 h. Green channel: 6-FAM; Red channel: Cy3. (D) Normalized fluorescence intensity of attached DNA on the surface of zebrafish embryos. Fluorescence intensities of images (C) and Fig.5.7A were calculated by Image J and plotted as a percentage relative to the fluorescence of M1-FAM or M2-Cy3 of Fig. 5.6C. The intensities of Fig. 5.6C were set to 100%.
Two control experiments were performed to prove the HCR was initiated on the membrane of zebrafish (Fig. 5.7). As shown in Fig. 5.7A, free M1-FAM was washed away before the addition of M2-Cy3. In this case, the fluorescent signals of M1-FAM and M2-Cy3 were 10 and 20 times lower, respectively, than those obtained in the presence of HCR (Fig. 5.6D). Also, when the monomer M1-FAM was omitted the HCR could not proceed and consequently no fluorescence could be detected on the fish surface (Fig 5.7B).

Fig. 5.7 HCR on zebrafish skin depends on DNA hybridization between U4T-28, M1 and M2. Fluorescence images of 1 dpf zebrafish (A) that were first incubated with U4T-28 and M1-FAM for 2 hours, subsequently washed 3 times with egg water before the addition of M2-Cy3, or (B) similar to (A) but without M1-FAM. Green channel: 6-FAM; Red channel: Cy3.

The signal increase by HCR was also clearly demonstrated by an experiment involving non-fluorescent M2 and M1-FAM. In case of HCR approximately 10-fold stronger green fluorescence was observed on the surface of 2 dpf zebrafish embryos (Fig. 5.8A) compared to labeling with a single fluorophore per anchor unit (Fig. 5.8B).
Fig. 5.8 In vivo DNA HCR enhances the fluorescence intensity of labeling. (A) Fluorescence images of 2dpf zebrafish embryos that were first decorated with U4T-28, followed by 3 times washing with egg water, incubation with M1-FAM for 1 h and M2 for another 1 h. (B) Only FAM fluorescent labeled M1 (M1-FAM) was added after anchoring of U4T28 on the fish and washing three times with egg water. Green channel: M1-FAM.

5.3 Conclusion

Previously, oligonucleotides were covalently attached to live cells by metabolic oligosaccharide engineering allowing the introduction of orthogonal chemical handles on the cell surface for DNA anchoring without dependence on endogenous receptors. Besides oligosaccharides, cell-surface proteins were exploited for the chemical modification of cells with DNA. Similarly, cell-surface proteins were decorated with DNA by non-covalent interactions. An alternative strategy for introducing artificial DNA receptors on live cell surfaces represents the utilization of oligonucleotides carrying hydrophobic membrane anchors, as described in this study. Based on such an anchoring strategy, Watson-Crick base pairing was exploited for the programmed synthesis of three-dimensional tissues. The examples above demonstrate that anchored DNA in a lipid bilayer developed into a powerful tool for realizing exciting functionalities in the context of synthetic and natural membranes, even including live cells. On the other hand, DNA nanostructures were employed in higher organisms in the context of functional in-vivo imaging and for the
targeted delivery of siRNA. To the best of our knowledge, there is no experiment about DNA-based membrane engineering in a living animal involving a wide variety of functions yet.

In this chapter, we demonstrated that lipid-DNA sequences with four anchoring units could be readily incorporated in the surface layer of zebrafish embryos. The single-stranded DNA present on the surface can be functionalized by Watson-Crick base pairing enabling the sequence specific functionalization of the live animal with small molecules or larger cargos, for example, liposomal systems. The payloads connected by the supramolecular tether DNA can be reversibly removed employing a removal strand, which represents a very mild stimulus just requiring the addition of a DNA sequence not affecting the life of the fish. Finally, we successfully demonstrated the performance of a DNA mediated amplification process on the fish skin. The hybridization chain reaction allows attachment of multiple moieties on a single anchored DNA strand allowing multiplication of cargoes or signals on the surface. Moreover, it was shown that surface modification of model membranes in form of liposomes by various DNA nanotechnology procedures could be easily transferred to the live animal. This allows establishment of DNA based surface functionalization procedures and their facile and fast implementation in zebrafish. However, challenges in the application of lipid-DNA in the transition of zebrafish to higher mammals remain. For instance, the DNA part in lipid-DNA is susceptible to the deoxyribonuclease in the circulation system of mammals. On the other hand, unlike zebrafish, mammals are not clear and transparent, which makes the direct visualization of fluorescently labeled tissues impossible. Nevertheless, due to the broad application of zebrafish as animal model in drug development, toxicology and nanoparticles characterization, we believe that the platform presented here allows amalgamation of DNA nanotechnology tools with live animals and enables efficient bio-barcoding as well as \textit{in vivo} tracking.
5.4 Experiment Section

5.4.1 Materials

Cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, USA) (purity >99%) and used without further purification. Headgroup-labeled phospholipid, lissamine rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (triethylammonium salt) (Rh-DHPE) and N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (triethylammonium salt) (NBD-DHPE) were purchased from Invitrogen (Amsterdam, Netherlands), and used as received. The DNA-dye conjugates C488, M1-FAM and M2-Cy3 were purchased from Biomers.net GmbH (Ulm, Germany). Anhydrous CHCl₃ was purchased from Acros Organics (Geel, Belgium) and stored over molecular sieves.

5.4.2 Zebrafish strain, husbandry, and egg collection

Wildtype zebrafish were used in this study, and were maintained and handled according to the guidelines from http://zfin.org. Fertilization was performed by natural spawning at the beginning of the light period, and eggs were raised at 28 °C. All experimental procedures were conducted in compliance with the directives of the animal welfare committee of the Leiden University.

5.4.3 Microscopy images

Zebrafishes were seeded in a glass bottom flask with egg water. After incubation for 1 h with lipid-DNA, they were washed three times and then incubated with other DNA oligonucleotides for 1 h. For live imaging, zebrafish embryos were anaesthetized with 0.003% tricaine and mounted on 0.6% low-melting agarose. Fluorescent images were acquired using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) and merged with Leica application suite advanced fluorescence software (Leica Microsystems) or ImageJ software (National Institutes of Health, Bethesda, MD, USA). A Leica MZ16FA stereo microscope was used for stereo images. Images were adjusted for
brightness and contrast using ImageJ. The wavelength settings for C488 were Ex/Em: 495/520nm (Ex laser: 488 nm), for C594 Ex/Em: 601/627 nm (Ex laser: 532 nm), for M1-FAM Ex/Em: 494/518 nm (Ex laser: 488 nm), and M2-Cy3 Ex/Em: 550/570 nm (Ex laser: 532 nm).

Author contributions

Yang J conducted the experiments and performed data analysis. Yang J and Meng Z designed the experiments and prepared the manuscript. Liu Q synthesized lipid-DNA. Yasuhito S produced the zebrafish embryo. Herman S and René CLO interpreted the data. Kros A and Herrmann A supervised the project. All authors edited the manuscript.
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