DNA nanoparticles as ocular drug delivery platform

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3. Ocular adhesion of lipid-DNA nanoparticles

3.1 Introduction
Currently, the vast majority of chronic and acute ophthalmic diseases that are not managed surgically are treated using eye drops - a non-invasive mode of treatment that can be self-administered without medical supervision. However, only a small percentage of the active compound present in eye drops reaches its target tissue as it is rapidly cleared from the eye by tear fluid and eye lid movement\cite{1-2}. As a consequence, frequent administration of highly concentrated eye drops is necessary. Aside from the inefficiency of this dosage form, the requirement of frequent administration leads to poor compliance\cite{3-5}. On the other hand, high concentrations of bioactive compounds produce side-effects that can range from simple irritations to, in extreme cases, a life threatening anaphylactic shock\cite{6-7}. Thus, increasing the half-life of the drug on the eye is an important goal for more efficient treatment of eye diseases with fewer side effects.

To address these challenges nanotechnological approaches have been pursued in the field of ophthalmology\cite{8-9}. Of special interest are polymeric nanoparticles that adhere to the cornea and increase the bioavailability of the released drug. Although satisfactory effectiveness has been demonstrated in -vitro and in-vivo, these delivery vehicles still face several shortcomings\cite{10-11}.
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The nanoparticles are characterized by a broad size distribution, easily exceed sizes of 100 nm, and their composition needs to be greatly varied to accommodate drugs with different physicochemical properties.

Here we overcome these limitations with the help of a novel carrier system based on DNA nanotechnology. This field has progressed rapidly in the past decades and many strategies have been presented to fabricate nucleic acid nanoarchitectures with well-defined sizes, periodicities and shapes in one-, two- and three dimensions[13-16]. Therefore, it is not surprising that this type of nanostructures has attracted great interest from researchers working in the field of nanomedicine where well-defined multifunctional nanostructures are urgently needed. DNA nanoobjects can be exclusively self-assembled from nucleic acids by Watson-Crick base pairing and functionalization is achieved by hybridization with oligonucleotides. Examples are a DNA icosahedron functionalized with a target cell-recognizing aptamer or a 140 nm-long DNA tube both loaded with anticancer drugs[17-18]. On the other hand, DNA strands can be chemically attached to a nanoscopic inorganic template and easily equipped with targeting units and drug molecules by hybridization with complementary oligonucleotide conjugates[19-20]. A very similar type of nanoparticles for drug delivery was introduced by our group where the inorganic material is replaced by a soft matter core consisting of hydrophobic polymer units that is covalently attached to an oligonucleotide to form a DNA block copolymer[21].

So far, all DNA nanocarriers have been only applied for cancer therapy and proven functionality in-vivo is very rare[22]. In this chapter the successful selection of a lipid-modified DNA NPs suitable as ophthalmic drug delivery vehicle is presented. After synthesis and characterization of several different constructs, their in-vitro and in-vivo adherence properties to the corneal tissue are investigated. For the amphiphile showing the highest affinity the residence time on the eye is elucidated.
3.2 Results and discussion

3.2.1 Nanoparticle design and characteristics
To obtain the desired nanoparticles (NPs), we replaced the hydrophobic polymer unit of our earlier presented DNA block copolymer delivery system by several alkyl-modified 2’-deoxyuridine nucleotides (U) (See Fig. 3.1a). When introduced into an aqueous environment, these DNA amphiphiles self-assemble into micellar nanoparticles (NPs) through microphase separation and thus exhibit a corona of single stranded DNA surrounding a lipid core\(^{[23]}\). For the purpose of imaging we hybridized an oligonucleotide functionalized with a fluorophore (See Fig 3.1b). Previously it was shown that these micelles have a small hydrodynamic diameter of approximately 7 nm, depending on the number of hydrophobically-modified nucleotides and the aggregation number of similar lipid-DNA constructs was around 25\(^{[24]}\).

Figure 3.1. Structure of lipid modified nucleotide and functionalization strategy of DNA NPs. (a) Chemical structure of dodecyne-modified deoxyuracil, represented as U in the text. This nucleoside is incorporated into the oligonucleotide chain during DNA synthesis to impart hydrophobic properties. (b) In an aqueous environment, U-modified DNA strands self-assemble to form DNA nanoparticles due to their amphiphilic nature. The nanoparticles can then be functionalized through hybridization with a complementary DNA strand bearing a covalently attached fluorescent dye (green).
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Micellar systems are highly dynamic; therefore, it was hypothesized that they can interact with the outermost hydrophobic layer of the cornea. To confirm this assumption and get more insights about the structural requirements for adhesion, several DNA lipid nanoparticles were exposed to corneal epithelium of porcine eyes. The particles were composed of different DNA amphiphiles: U2-12, U4-12, U4-18, U6-12, U6-20 (See Table 3.1). Here, the name of the lipid modified oligonucleotide is annotated as UX-Y, wherein X and Y represent the number of hydrophobic deoxyuridine units and the total number of nucleotides, respectively. For example, U2-12 contains 2 Us at the 5' terminus and in total is composed of 12 nucleotides. The NPs are characterized by CMC values which are ranging from 4 to 27 µM, confirming the micellar nature of the amphiphile aggregates (See Fig. 3.8).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’→3’)</th>
<th>Lipid modified bases (# (%))</th>
<th>CMC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2T-12</td>
<td>UUTGCGGATTC</td>
<td>2 (17)</td>
<td>27.4</td>
</tr>
<tr>
<td>U4T-12</td>
<td>UUUUGCGGATTC</td>
<td>4 (33)</td>
<td>5.2</td>
</tr>
<tr>
<td>U4T-18</td>
<td>UUUUGCGGATTCGTCGTGC</td>
<td>4 (22)</td>
<td>4.6</td>
</tr>
<tr>
<td>U6T-12</td>
<td>UUUUUUGGATTC</td>
<td>6 (50)</td>
<td>24.3</td>
</tr>
<tr>
<td>U6T-20</td>
<td>UUUUUUGCGGATTCGTCGTGC</td>
<td>6 (30)</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 3.1. Sequences and characteristics of lipid modified oligonucleotides used to formulate NP-containing eye drops.

3.2.2 Determination of best NP binding in-vitro and in-vivo

To determine the adherence capabilities of the designed NPs to the cornea first in-vitro experiments on porcine eyes were performed. Therefore, the eye was incubated with the fluorescently labeled NPs at a concentration of 20 µM for 15 minutes followed by washing in PBS buffer. Cryo sections of the treated eyes were prepared and imaged using a fluorescence microscope (See Fig. 3.2). As control a double stranded (ds) DNA with the same sequence as one of the DNA amphiphiles, but lacking the lipid-modified nucleotide, was also exposed to the cornea in a similar fashion (NoU4-12).
In the figure above the cornea is visualized through nuclear staining using 4’,6-diamidino-2-phenylindol (DAPI) and therefore is visible in blue. In contrast, the NPs are labeled using a green fluorescent dye. As can be seen, for all the tested NPs good adherence to the porcine cornea is observed while the unmodified control sequence shows no affinity. Among the amphiphiles no clear difference in affinity was notable.

In the next step, the binding capabilities of the different NPs were investigated under realistic conditions by performing in-vivo experiments on living rats. The drops containing the DNA amphiphiles at a concentration of 20 µM were administered to conscious rats using a single drop of approximately 30 µL and 30 min, 2 or 24 h after application the animals were sacrificed. Similar as before, cryo sections of the treated eyes were prepared and imaged using fluorescence microscopy. As controls both the single stranded (ss) and ds fluorescently labeled unmodified DNA sequences were also administered. The best delivery system was determined as the NP carrier with the highest percentage of eyes exhibiting the DNA amphiphile and, in the case of similar results, by visual comparison of the cryosections (See Fig. 3.3 and Table 3.2).
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Figure 3.3. Representative fluorescent images of all NPs (green) screened for adherence to the rat cornea (blue).
### 3.2 Results and discussion

#### 3.2.1 Overview of number of rat eyes tested positive for the presence of NPs out of total number of eyes to which the NPs were administered at various time points.

![Table 3.2](image)

Among the tested amphiphiles, U4-12 shows the best adherence to the corneal epithelium while both ss and ds 12mer control sequences (NoU4-12 and cU4-12) without any lipid modification do not show any affinity at all. From the results, we can deduce that the optimal ratio between standard and hydrophobic bases that promotes efficient adhesion is around 2:1. NPs composed of ds oligonucleotides with lower (U2-12) or higher (U6-12) ratios exhibit significantly lower affinity. When comparing amphiphiles with a similar percentages of U content (U4-12 and U6-20), strands with a smaller number of nucleotides show better adherence. Therefore, both the number of lipid modifications and the total length of the amphiphile are important parameters determining adhesion to the cornea.

### 3.2.3 Time dependent adherence of U4-12

Upon identifying U4-12 as the best carrier amphiphile, the adherence time on the cornea was evaluated. To this end, eye drops of the labeled NPs were administrated and the rats were sacrificed after 5, 15, 30 minutes, 1, 2, 4 or 6 hours (See Table 3.3 and Fig. 3.4). Also here, the unmodified ss and ds controls were included at the first time point. As can be observed, the NPs of U4-12 are already visible 5 min after application of the eye drops, thus showing a fast adherence to the cornea. Importantly, neither one of the controls were found at this time period, indicating no interaction of the pristine DNA with the tissue. In contrast, even after a period of four hours the NPs were still present as the distinct green fluorescence of the carrier was clearly visible. This adherence time is significantly longer than of any currently applied ocular medication, thus making these DNA NPs a promising vehicle for ocular drug delivery if equipped with a drug that can be released.
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Figure 3.4. Evaluation of the time-dependent NP adhesion for the U4-12 system. Adhesion of U4-12 (green) to the rat cornea (blue) followed by fluorescence microscopy.
3.3 Conclusion

DNA based nanoparticles have many advantages over NPs composed of other materials. Due to the unique self-recognition properties of DNA the formed architectures are uniform in size and functionality can easily be introduced at any desired place with complete control over the spatial orientation (inside the NP or at the surface of the NP).

Here, we demonstrated for the first time the successful application of DNA nanoparticles in the field of ophthalmology. These nanoparticles are composed of lipid-modified DNA strands which allows for facile modification by simple hybridization. For imaging purposes, fluorescent dye-modified oligonucleotides were attached to the DNA NPs by Watson-Crick base pairing. It was found that through alteration of the lipid content and the total length of the amphiphile the adherence properties of the NPs can be tailored. Furthermore, they exhibit a dramatically increased affinity to the cornea of living animals compared to pristine DNA. The best adhering system was found on the cornea even 4 hours after application of a single eye drop to conscious rats, which is significantly longer than medication in pristine eye drop-based dosage forms.

<table>
<thead>
<tr>
<th>Time</th>
<th>U4-12 adherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min</td>
<td>4/4</td>
</tr>
<tr>
<td>15 min</td>
<td>4/4</td>
</tr>
<tr>
<td>30 min</td>
<td>6/6</td>
</tr>
<tr>
<td>1 hour</td>
<td>4/4</td>
</tr>
<tr>
<td>2 hours</td>
<td>4/6</td>
</tr>
<tr>
<td>4 hours</td>
<td>3/4</td>
</tr>
<tr>
<td>6 hours</td>
<td>0/4</td>
</tr>
</tbody>
</table>

Table 3.4. Summary of number of NP positive eyes out of total number of eyes to which the NPs were administered at several time points.
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3.4 Experimental

3.4.1 Materials
All chemicals and reagents were purchased from commercial suppliers and were used without further purification, unless otherwise noted. The 1-dodecynyl, copper(I)iodide, tetrakis(triphenylphosphine)palladium(0) and diisopropylamine were purchased from Sigma-Aldrich and used as received. Other special chemicals acquired from different chemical sources were 5’-DMT-5-ido deoxy uridine (Chemgenes). All lipid modified oligonucleotides (ODNs) were synthesized using standard automated solid-phase phosphoramidite coupling methods on an ÄKTA oligopilot plus (GE Healthcare) DNA synthesizer. All solvents and reagents for oligonucleotide synthesis were purchased from Novabiochem (Merck, UK) and SAFC (Sigma-Aldrich, Netherlands). Solid supports (Primer Support™, 200 μmol/g) from GE Healthcare were used for the synthesis of DNA. Oligonucleotides were purified by reverse-phase high performance liquid chromatography (HPLC) using a C15 RESOURCE RPC™ 1 ml reverse phase column (GE Healthcare) through custom gradients using elution buffers (A: 100 mM triethylammonium acetate (TEAAC) and 2.5% acetonitrile, B: 100 mM TEAAc and 65% acetonitrile). Fractions were desalted using centrifugal dialysis membranes (MWCO 3000, Sartorius Stedim) or a HiTrap Desalting column (GE Healthcare). Afterwards the oligonucleotides were characterized by MALDI-TOF mass spectrometry using a 3-hydroxypicolinic acid matrix. Spectra were recorded on an ABI Voyager DE-PRO MALDI-TOF (delayed extraction reflector) Biospectrometry Workstation mass spectrometer. The concentrations of the DNA were measured on a SpectraMax M2 spectrophotometer (Molecular Devices, USA) using 1 cm light-path quartz cuvette. Fluorescently labeled oligonucleotides were purchased from Biomers.net at HPLC purification grade. H¹-NMR and P³¹-NMR spectra were recorded on a Varian Mercury (400 MHz) NMR spectrometer at 25 °C. Column chromatography was performed using silica gel 60 Å (200-400 Mesh).
3.4.2 Synthesis and characterization of amphiphilic oligonucleotides

The modified 5-(dodec-1-ynyl)uracil phosphoramidite 3 was synthesized in two steps as previously reported in our group starting from 1 (See Fig. 3.5)[24]. The modified uracil phosphoramidite was dissolved in CH$_3$CN to adjust the concentration to 0.15 M, in the presence of 3 Å molecular sieves. The prepared solution was directly connected to the DNA synthesizer. All oligonucleotides were synthesized on a 10 μmol scale on an ÄKTA oligopilot plus (GE Healthcare) DNA synthesizer using standard β-cyanoethylphosphoramidite coupling chemistry. Deprotection and cleavage from the PS support was carried out by incubation in concentrated aqueous ammonium hydroxide solution for 5 h at 55 °C. Following deprotection, the oligonucleotides were purified by using reverse-phase chromatography, using a C15 RESOURCE RPC™ 1 ml reverse phase column (GE Healthcare) through custom gradient elution (A: 100 mM triethylammonium acetate (TEAAc) and 2.5% acetonitrile, B: 100 mM TEAAc and 65% acetonitrile). Fractions were desalted using centrifugal dialysis membranes (MWCO 3000, Sartorius Stedim). Oligonucleotide concentrations were determined by UV absorbance using extinction coefficients. Finally, the identity and purity of the oligonucleotides were confirmed by MALDI-TOF mass spectrometry and analytical anion exchange chromatography using a linear gradient elution, respectively (See Fig. 3.6 and Fig. 3.7).
3. Ocular adhesion of lipid-DNA nanoparticles

Figure 3.6. Characterization of amphiphilic oligonucleotides by MALDI-TOF mass spectrometry. (a) Spectrum of U2-12 (calc. 3968 g/mol, found 3967 g/mol), (b) U4-12 (calc. 4243 g/mol, found 4245 g/mol), (c) U4-18 (calc. 6089 g/mol, found 6087 g/mol), (d) U6-12 (calc. 4534 g/mol, found 4527 g/mol), (e) U6-20 (calc. 6998 g/mol, found 6994 g/mol).
Figure 3.7. Characterization of the amphiphilic oligonucleotides by analytical anion exchange chromatography. A linear gradient to 100 %B in 62.5 ml was used. Chromatograms show (a) U2-12, (b) U4-12, (c) U4-18, (d) U6-12 and (e) U6-20.
3.4.3 Preparation of functionalized NPs
Micelles were prepared in low bind tubes (Eppendorf) in 1x TAE buffer (40 mM Tris-Acetate, 1 mM EDTA, 20 mM NaCl, 12 mM MgCl2, pH 8.0) at a concentration of 20 µM. The lipid modified oligonucleotide of interest was prepared at the desired concentration and one equivalent of the complementary DNA was added and hybridized using a thermal gradient (90 °C, 30 min; -1 °C/2 min until room temperature (RT)). When nanoparticles were used for fluorescent imaging a 5’ Atto488 functionalized complementary DNA was used.

3.4.5 Critical micelle concentration determination
For CMC determination, firstly 10 pmol of 1,6-diphenyl-1,3,5-hexatriene (DPH) was loaded in Eppendorf DNA low-bind tubes using a 1 µM solution in acetone. The solvent was allowed to evaporate at room temperature (RT) for 5 h after which 100 µl of DNA amphiphile solution was added. The oligonucleotides were prepared at concentrations ranging from 0.0025 to 1 g/L in 1x TAE buffer (10 mM Tris-Acetate, 0.2 mM EDTA, 20 mM NaCl, 12 mM MgCl2, pH 8.0) and thermally cycled (90 °C, 30 min; -1 °C/2 min until RT) before use. After addition to the DPH containing tubes, the solutions were incubated overnight at 37 °C. Subsequently, fluorescence spectra (375 – 500 nm) were recorded on a Varian Cary Eclipse fluorimeter (Varian Nederland B.V.) at RT using an excitation wavelength of 350 nm (See Fig. 3.8).
Figure 3.8. Determination of critical micelle concentrations of the amphiphilic oligonucleotides. Fluorescence spectra of micelle-incorporated 1,6-diphenyl-1,3,5-hexatriene (DPH) at different concentrations (g/L) (Left) and intensity at 425 nm (maximum) plotted against the logarithm of the concentration (right) for (a) U2-12, (b) U4-12, (c) U4-18, (d) U6-12 and (e) U6-20.
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3.4.4 Determination of adherence to porcine eye

Pig eyes were obtained from a local slaughterhouse and kept humidified at 4°C until further use. Before applying the NPs the eyes were washed and allowed to adjust to room temperature. For NP application, the eyes were placed on 6-well plates with the cornea facing up and 2 rubber rings (a smaller one in the center of the cornea and a larger one around the cornea) were used to prevent spillage of the solution. To every eye 50 µl of NP solution (20 µM) was applied and the eyes were incubated for the designated time (15 minutes). Afterwards the rings were removed and the eyes were washed in excess PBS buffer. Then the eyes were frozen in Tissue-Tek O.C.T. (Sakura Finetek) in liquid nitrogen. Frozen sections were longitudinally cut (12 µm) on a cryostat (Leica CM 1900), thaw-mounted onto glass slides (Superfrost plus, R. Langenbrinck Labor- & Medizintechnik) and stored at -30 °C until further use. For visualisation sections were fixed with methanol and to stain nuclei the sections were further incubated in a solution containing 0.2 µg/ml DAPI for 1 min. Stained sections were embedded in FluorSave (Calbiochem) and imaged using a fluorescent microscope (Axioplan2, Zeiss with Openlab software, Improvision)[25].

3.4.5 Selection of best adhering NP and evaluation of adherence time on the cornea

Adult Lister Hooded Rats were obtained from Harlan Winkelmann (Germany). Nanoparticles, prepared as described above, were administered to conscious rats at a concentration of 20 µM using a single drop of approximately 30 µl. For the eye drop applications the conscious rats were very shortly fixated and a drop was administered to the eye using a single drop device as in medical applications. Blinking of the eyes was not hindered during drop application or afterwards. After the designated incubation time, the rat was sacrificed with carbon dioxide inhalation. After sacrificing the animal, the eyes were enucleated and frozen in Tissue-Tek O.C.T. (Sakura Finetek, Germany) using liquid nitrogen. The samples were stored at -30 °C until further use and processed as described in section 3.4.4. Animals were treated according to the Principles of laboratory animal care
(NIH publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Human Care and Use of Laboratory Animals (revised 1986) and the German animal protection law (Research permission AK3/11 to Sven Schnichels).

3.5 References

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