Chapter 3

Lipid modified aptamers as vehicles for ophthalmic drug delivery
3.1 Introduction

Aptamers are DNA or RNA oligonucleotides, which bind to specific ligands with selectivity and affinity comparable to antibodies.\textsuperscript{1} The aptamer-ligand binding is facilitated by electrostatic interactions, hydrogen bonding, π-π stacking, van der Waals interactions and geometric complementarity between the stable three-dimensional aptamer structure and the target molecule.\textsuperscript{2, 3} Due to their broad range of affinities, aptamers have been widely employed in nanomedicine as metabolite quenchers and targeting probes delivering active pharmaceutical ingredients (APIs) or contrast agents to the site of action.\textsuperscript{4-6} Up to now, oncology has been the main area of application for such aptamer-derived therapeutics, however, the treatment of other pathologies like ocular, infectious, and cardiovascular diseases is also being performed.\textsuperscript{7-9} Surprisingly, aptamers have hardly been employed as drug-loading moiety for medical applications, despite a large number of aptameric sequences available for medically relevant molecules.\textsuperscript{10} Recently, Herrmann et al. introduced a novel, cornea-adhering DNA-based NP architecture for ophthalmic indications and presented the concept of using aptamers as a drug-loading moiety.\textsuperscript{11}

In fact, ophthalmic drug delivery faces many challenges, such as poor bioavailability and solubility of active compounds and their rapid clearance from the eye.\textsuperscript{12} Additionally, an increasing failure rate of drugs under development is not being compensated by an adequate number of potential drug candidates for either known or emerging indications. Hence, significant effort is directed into appropriately formulating available drugs to boost their efficacy.\textsuperscript{9, 13} Topically applied formulations, i.e. eye drops, are convenient and the most accepted form of drug administration that is available to treat anterior chamber diseases. However, research in eye drop development still faces a lot of challenges and therefore they remain inefficient.\textsuperscript{14} The effectiveness of topical therapy suffers from limited permeability of the corneal epithelium and insufficient residence time of the drug at the side of action due to lacrimal fluid production.\textsuperscript{15} To overcome these difficulties, several ophthalmic formulations based on natural and synthetic polymeric nanoparticles (NPs), hydrogels, emulsions and liposomes were proposed.\textsuperscript{9, 14, 16} Such formulations can significantly improve the drug bioavailability, pharmacokinetic profile, and reduce potential immunogenicity and cytotoxicity due to minimized drug exposure.\textsuperscript{14} Yet, they seldom reach the market, which indicates the necessity for novel solutions.

In this work, we present a strategy to turn virtually any aptamer into a drug-tailored nanocarrier with intrinsic corneal adhesive properties and we introduce lipid-
modified aptamers (lApts) as a class of engineered vehicles for topical ocular therapies.

3.2 Result and Discussion

3.2.1 Synthesis and characterization of lApts

![Synthesis of 5-(dodec-1-ynyl) deoxyuracil, 5-(dodec-1-ynyl) deoxyuracil phosphoramidite and lApts.](image)

The lApts were synthesized using a DNA synthesizer (Figure 3.1). First, the commercially available 5'-DMT-5-iodo deoxyuridine was converted into 5'-DMT-5-(dodec-1-ynyl) deoxyuridine through Cul mediated Sonogashira coupling. Then, the obtained product was activated into 5-(dodec-1-ynyl) deoxyuracil phosphoramidite which was further incorporated into DNA sequences using a DNA synthesizer. The
obtained lApts were purified employing reverse-phase chromatography (RPC-HPLC) and the purity was confirmed by Matrix-Assisted Laser Desorption Time of Flight Mass Spectrometry (MALDI-TOF) and RPC-HPLC (Figure 3.2).

### 3.2.2 Characterization of lApt micelles

The lApt design is based on an aptamer molecule which is extended with a domain containing four alkyl-modified 2'-deoxyuridine nucleotides (U) as hydrophobic modification (Figure 3.2A). In such a molecule, the aptamer sequence is selected to promote specific interactions with a chosen drug and therefore to facilitate the direct loading into the micelle corona. At the same time the lipid tails govern the lApt self-assembly into nanosized particles in an aqueous solution. It has to be noted that pristine oligonucleotides do not exhibit corneal adhesive properties, however the introduction of the lipid tails in a geometrically defined manner results in significant affinity to ocular tissue.\(^{11}\) In the previously reported system a drug-binding aptamer moiety needed to be synthesized in a separate step and afterwards hybridized to the basic carrier molecule (Figure 3.3A).\(^{11}\) Although this approach allowed for versatile functionalization and rapid screening of new formulations, it requires more synthetic effort and, therefore, is less cost and time efficient. In fact, cost efficiency is a significant difficulty in pharmaceutical industry and hence it has to be considered in development projects. The direct synthesis of lipid modified aptamers allows avoiding several cumbersome purification steps and simplifies the formulation process, thereby significantly improving the earlier reported platform.

To demonstrate the generality of this approach, three medically relevant ligands (Figure 3.3C) were chosen. Kanamycin B, is an aminoglycoside antibiotic used to cure eye infections. A DNA binding aptamer (Kan) for this particular drug was developed earlier.\(^{17}\) Brimonidine and travoprost represent two classes of intraocular pressure lowering (IOP) APIs commonly used to control the progression of glaucoma. The brimonidine and travoprost binding DNA aptamer sequences (Bri and Tra, respectively) were developed for this study employing a competitive binding assay. Anti-glaucoma medication and anti-infectives currently cover the biggest part of ophthalmic market world-wide.\(^{18}\) The aptamers were elongated at the 5'-end with the hydrophobic domain consisting of four U lipid bases during automated synthesis (Figure 3.3D). As expected, the resulting lApt\textsuperscript{Kan}, lApt\textsuperscript{Bri} and lApt\textsuperscript{Tra} sequences exhibited an amphiphilic character and therefore self-assembled into NPs above their critical micelle concentrations (CMC). The formed NPs exhibited diameters around 10 nm for lApt\textsuperscript{Kan} and lApt\textsuperscript{Bri}, and 22 nm for the lApt\textsuperscript{Tra} (Figure 3.8), with CMC values ranging from 6.7 to 13.3 µM (Figure 3.7). Isothermal titration calorimetry (ITC) was performed to assess the binding capabilities of the
hydrophobically modified aptamers and see if the introduced alkyl chains influence the ligand binding affinity (Figure 3.9). It was revealed that lipid domain does not compromise the aptameric binding and the binding constants are comparable.

**Figure. 3.3.** (A) lApt nanoparticle assembly from the old generation of aptameric NPs; (B) The new design of a lApt molecule which self-assembles into a NP and the aptamer-assisted loading strategy; (C) Chemical structures of ophthalmic APIs included in the study; (D) Sequences of corresponding lApts (U indicates lipid modified uracil base).

### 3.2.3 Lipid aptamer NP evaluation *ex-vivo*

The cornea binding properties of the aptameric NPs were initially investigated using *ex vivo* porcine corneal tissue. The lApts were equipped with a fluorescent tag (FAM) at the 3’ end of the aptamer sequence to facilitate imaging (Figure. 3.A). Such approach allowed the aptamer to maintain its secondary conformation and
eliminated the necessity to introduce additional DNA probes, which could impair aptamer functionality upon hybridization. An eye drop of 50 µL of lApt solution (20 µM) was applied topically on the porcine eyes and, after a short incubation period, tearing was simulated by washing with PBS buffer for the indicated time (Figure 3.B). All investigated lApts exhibited remarkable affinity to the ex-vivo tissue and were detected even after 60 minutes of washing. In contrast, the control consisting of aptamers lacking hydrophobic modification, were not found on the corneal surface.11

![Figure 3.4](image)

**Figure 3.4.** A. Design of fluorescently labeled lApts employed in corneal adherence study ex-vivo and in-vivo; B. Representative images of adherence of the lApt^{Kan}, lApt^{Bri} and lApt^{Tra}, which were present on ex vivo porcine tissue even after 60 min of tear simulation. C. Representative images of the corneas of rodent eyes at 15, 30, 60 and 120 min after drop application. Scale bars correspond to 50 µm.

### 3.2.4 Lipid aptamer evaluation in vivo

The corneal binding was subsequently investigated in an in vivo rodent model. Therefore, a single eye drop (~30 uL) of the FAM labeled lApt at a concentration of 20 µM was administrated to a conscious rat using a medical dropping device. The animal was shortly fixated during eye drop application, however, lid movements were not hindered throughout the duration of the experiment. The animals were sacrificed 15, 30, 60 and 120 minutes after eye drop application and cryosections of
the eyes were visualized by fluorescence microscopy (Figure. 3.C). The \textit{I}Apt\textsuperscript{Tra} residence time reached 60 min, while the \textit{I}Apt\textsuperscript{Kan} and \textit{I}Apt\textsuperscript{Bri} were detected on rat corneas even after 120 min from drop application (Table 1). The observed difference in residence time is likely due to varying length of the employed aptameric sequences. The hydrophobic-hydrophilic balance within the amphiphilic molecule is an important parameter that governs the micellization process and morphology. As a consequence, it has an influence on the NP’s interactions with cellular components.\textsuperscript{19} The \textit{I}Apt\textsuperscript{Tra} sequence is the longest among employed aptamers (42 mer) and it forms the largest \textit{I}Apt particles as demonstrated with dynamic light scattering (DLS) (Figure 3.8). Hence, its aggregation behavior is presumably different than that of the shorter DNA equipped with the same UUUU functionality (23 mer and 34 mer for \textit{I}Apt\textsuperscript{Kan} and \textit{I}Apt\textsuperscript{Bri}, respectively). It is very likely that this alters the ocular affinity. This finding also indicates that intrinsically short or truncated aptamers are more suited for corneal-adhesive NP formation.

\textbf{Table 1.} Evaluation of \textit{I}Apts adherence to in vivo rodent eyes presented as a number of rat eyes tested positive for the presence of \textit{I}Apts out of total number of eyes to which the NPs were administered.

<table>
<thead>
<tr>
<th>\textbf{Time from eye drop application (min)}</th>
<th>5</th>
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<th>30</th>
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<th>120</th>
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<tr>
<td>\textit{I}Apt\textsuperscript{Kan}</td>
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<td>3/4</td>
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<td>\textit{I}Apt\textsuperscript{Bri}</td>
<td>1/4</td>
<td>3/4</td>
<td>2/4</td>
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<tr>
<td>\textit{I}Apt\textsuperscript{Tra}</td>
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\textbf{3.2.5 Toxicity testing}

For an ophthalmic drug delivery system, it is very important to address its toxicological profile in an early stage. Therefore, the \textit{I}Apts were evaluated on primary ocular cell lines regarding their influence on cell viability and apoptosis induction. The primary epithelial cells were isolated from freshly inoculated porcine eyes and incubated with \textit{I}Apt NPs at an amphiphile concentration of 20 µM for 24 h. No toxicity was found and the \textit{I}Apts treated cells were indistinguishable from the cells treated with the pristine buffer. This finding is of crucial importance as it indicates that the lipid modification introduced into the biodegradable aptamer does not result in a toxic effect at the investigated conditions.
In-vivo treated rodent eyes were subjected to a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to screen for apoptosis induction in vivo. In this fluorescence assay, almost no TUNEL-positive cells were found, which indicates that lApt do not induce apoptosis in in vivo rodent eyes (Figure 3.5).

![Figure 3.5. TUNEL stained cryo-sections of lApt treated rodent eyes showed almost no apoptosis positive cells when compared to the assay positive control.](image)

### 3.2.6 Efficacy

The aptamer corona of the NP presented in this study is more compact and sterically crowded than in the situation when aptamers are introduced onto the NP via a linker molecule. To investigate if the altered design influences the lApt functionality as drug delivery system, we tested the bactericidal efficacy of lApt Kan NPs. The aminoglycoside based system was selected as it presents an easy to measure endpoint, the bacterial growth. Additionally, no in vitro intra ocular pressure (IOP) based glaucoma models exist, which would be relevant for transcorneal drug delivery.20

First, we studied whether the API can be liberated from the lAptKan formulation. The antibiotic loaded lAptKan NPs were compared to the free antibiotic solution in a minimum inhibitory concentration test (MIC-test) using Escherichia coli (E. coli). The bacterial growth was monitored over 5 h at 37 °C by measuring the optical density of the bacterial culture at a wavelength of 600 nm (Figure 3.6A). The experiment was performed in the presence and absence of DNase to mimic situation in the relevant body fluid, i.e. tears.21 The MIC tests showed that under in vitro conditions, lAptKan nanocarriers exhibited comparable level of activity to the free drug. The API was liberated from the formulation also in the absence of externally added nuclease and the measured difference in activity was not significant. It was
concluded that the steric hindrance introduced by designing a more compact nanoparticle has no significant impact on the drug release profile.

**Figure. 3.6. In vitro efficacy of lApt\textsuperscript{Kan}.** A. E. coli growth inhibition by lApt\textsuperscript{Kan} formulated antibiotic in the presence (blue) and absence (red) of DNAse and by pristine kanamycin B (black) proves that the antibiotic is liberated from the NPs on antibiotic. B. *P. aeruginosa* growth inhibition on infected porcine corneas.

As a final point the efficacy of the lApt\textsuperscript{Kan} NPs at the site of action was addressed. Porcine *ex vivo* corneas were infected with *P. aeruginosa* and afterwards treated with free and lApt\textsuperscript{Kan} formulated kanamycin B solution. It was revealed that the antibiotic formulated within lApt\textsuperscript{Kan} is significantly more potent than when administrated as the free solution. The improved performance can be attributed to cornea-adhesive properties of the formulation, which contributes to prolonged presence at the infected tissue. Hereby, the 5min washing time to simulate tear fluid flow probably plays a very important role (see supplementary information).

### 3.3 Conclusion

The direct functionalization of aptamers with a lipid moiety represent a novel approach for the formation of NPs used for drug delivery intended to treat ophthalmic applications. The drug tailored NPs were synthesized for three ophthalmic APIs: kanamycin B, brimonidine and travoprost. It was demonstrated that the lipid modification resulted in the corneal adherence of the aptameric NPs both *in vitro* and *in vivo*, and that it did not contribute to toxicity of the amphiphiles. The drug release was also proven *in vitro* and the efficacy of the formulated drug was even demonstrated *ex vivo*. The created NPs offer an additional drug loading modality as the lipid core of the micelle can accommodate water insoluble medication.\textsuperscript{22} As such, their performance as drug delivery system can be further developed by introducing an additional load for co-therapy.
Utilizing aptamers as a building block makes the presented strategy readily adaptable to other pharmaceutical ingredients. Moreover, the affinity between aptamer and its ligand can be regulated and therefore the IAPts can be fine-tuned to obtain a controlled release profile. In conclusion, the developed NPs formulation is a promising candidate for development of multifunctional nanotechnology products for therapeutic purposes in the field of ophthalmology.

3.4 Experimental Section

3.4.1 Materials

All chemicals and reagents were purchased from commercial suppliers and were used without further purification. The 1-dodecyne, copper(I)iodide, tetrakis(triphenylphosphine)palladium(0), kanamycin B sulfate and brimonidine were purchased from Sigma-Aldrich and used as received. Travoprost was purchased from Tocris Bioscience. All solvents and reagents for oligonucleotide synthesis were purchased from Novabiochem (Merck) and SAFC (Sigma-Aldrich) and 5'-DMT-5-Iodo deoxy Uridine was obtained from Chemgenes. The concentrations of the DNA were measured on a SpectraMax M2 spectrophotometer (Molecular Devices) using 1 cm light-path quartz cuvette. 1H-NMR and 31P-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 lipid modified aptamers

The modified aptamers synthesis was performed in 10 μmol scale on an ÄKTA oligopilot plus DNA synthesizer (GE Healthcare) using standard β-cyanoethylphosphoramidite coupling chemistry on polystyrene solid support (Primer Support™, 200 μmol/g GE Healthcare). The lipid modifier 5-(dodec-1-ynyl)uracil phosphoramidite (U) was synthesized according to previously published procedure. All conventional phosphoramidites and U phosphoramidite were dissolved in anhydrous CH₃CN at the final concentration of 0.15 M under argon atmosphere and connected to the DNA synthesizer. For adhesion studies the aptamer sequences were functionalized with a 5-FAM fluorophore at the 3’ hydroxyl group. After synthesis, deprotection and cleavage from the solid support were carried out by incubation in concentrated aqueous ammonium hydroxide solution for 5 h at 55 °C. Crude mixture of modified oligonucleotides was purified by reverse-phase chromatography, on a C15 RESOURCE RPC™ 1 ml reverse phase column (GE Healthcare) through a custom gradient elution (A: 100 mM triethylammonium
acetate (TEAAc) and 2.5% acetonitrile, B: 100 mM TEAAc and 65% acetonitrile). Fractions were concentrated and 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 using a 3-hydroxypicolinic acid matrix (ABI Voyager DE-PRO MALDI TOF Biospectrometry Workstation) and analytical anion exchange chromatography using a linear gradient elution, respectively (Figure 3.2).

### 3.4.3 Determination of critical micelle concentration

The critical micelle concentration (CMC) was determined by hydrophobic dye solubilization method. The lApts were prepared at concentrations ranging from 0.25-100 µM in 1xTAE buffer (10 mM Tris-Acetate, 20 mM NaCl, 12 mM MgCl₂, 0.2 mM EDTA, pH 8.0), and annealed by heating up to 90 °C for 30 min and slowly cooling down to RT (-1 °C/2 min). Subsequently 100 µl of lApt solutions were added to tubes charged with 10 pmol of dry 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma Aldrich) and 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 (Figure 3.7).

![Figure 3.7. Determination of critical micellization concentration of lApts.](image-url)
3.4.4 Nanoparticle size determination by dynamic light scattering (DLS)

Figure 3.8. Dynamic Light Scattering of IApt nanoparticles.

The amphiphiles were prepared at a concentration of 50 µM in 1x TAE buffer and annealed as described above. The 1xTEA buffer was filtered through a 0.22 µm syringe filter prior to use. Measurements were performed in a temperature controlled set up at 25 °C and under a scattering angle of 90° on an ALV/CGS-3 goniometer system working in autocorrelation mode and using the JDSU 1145/P HeNe laser (λ=632.8 nm). The average hydrodynamic radius was obtained from 3 measurements of 30 seconds each (Figure 3.8).
3.4.4 Isothermal titration calorimetry (ITC)

Figure 3.9. Isothermal Titration Calorimetry results of lApt nanoparticles: A. lApt\textsuperscript{Kan} + kanamycin B; B. lApt\textsuperscript{Bri} + Brimonidine; C. lApt\textsuperscript{Tra} + travoprost.

Isothermal Titration Calorimetry was used to determine the binding constants between lApts and drugs using a MicroCal ITC 200 Microcalorimeter (Northampton, MA). A 1mM drug solution was injected from a 40 µL rotating syringe into an isothermal sample chamber containing 200 µL of 7 µM lApt solution. A TAE buffer (pH 8.0) was used to prepare both solutions (Absolute ethanol was used for travoprost), as well as to load the reference cell. The experiments were carried out
at 25 °C and with a stirring speed of 750 rpm. Typically, injections of 1.2 µL of titrant were added into the cell with a delay between injections of 100 seconds, up to a lApt/drug ratio of 1 : 5. The effective heat of the drug-lApt interaction upon each titration step was corrected for dilution effect by subtracting the values obtained in the titration of 1 into buffer solution. Each injection generated a point for point heat curve (microcalories per second vs time) which was conveniently integrated in order to obtain the heats of the bimolecular interactions associated with those injections. Then the normalized heat signals were analyzed by using the ITC non-linear curve fitting functions for 2 binding sites from MicroCal Origin 7.0 software (MicroCal, Inc.; Northampton, MA). The satisfactorily fitted cure was used to determine the molar enthalpy change for binding (ΔH °) and the corresponding binding constant (K_a). Fundamental thermodynamic equations were used to determine the molar free energy of binding, ΔG °, and the molar entropy change (ΔS °). Each drug-lApt titration was repeated at least 3 times in order to increase the accuracy of the thermodynamic parameters.

3.4.5 Corneal adherence ex vivo

The fluorescently labeled lApt solutions were prepared at a concentration of 20 µM. Porcine eyes were obtained from a local slaughterhouse and washed with PBS buffer before use. To every eye 50 µl of lApt solution was applied and, after short incubation, the eyes were immersed in PBS for the designated time (5, 15 or 30 min) to simulate tearing. Afterwards, the eyes were enucleated and snap frozen in Tissue-Tek O.C.T. (Sakura Finetek) using liquid nitrogen. Frozen sections were longitudinally cut (12 µm) on a cryostat (Leica CM 1900, Germany), thaw-mounted onto glass slides (Superfrost plus, R. Langenbrinck Labor- und Medizintechnik) and stored at -30 °C before use. For fluorescence imaging the sections were fixed with methanol and 4’,6-diamidino-2-phenylindol (DAPI) nuclei staining procedure was performed as described elsewhere. Stained sections were embedded in FluorSave (Calbiochem, Germany) and imaged using a fluorescent microscope (Axioplan2 imaging®, Zeiss, Germany) and imaged using the Openlab software, Improvision, Germany).

3.4.6 Corneal adherence in vivo

The lApt solutions were prepared at a concentration of 20 µM and transferred to a single drop medical device. During the eye drop application the conscious Lister Hooded rats (Charles River, Germany) were very shortly fixated without hindering blinking during drop application or afterwards. After the designated incubation time,
the rats were sacrificed with carbon dioxide inhalation. The eyes were enucleated and the sections were prepared and visualized as described in section 3.4.5.

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. The animal research was conducted under research permission AK3/11 granted by the Regierungspräsidium Tübingen to Sven Schnichels.

3.4.7 Preparation of drug loaded nanoparticles

The lAptKan NPs were prepared at the needed concentration in 1x TAE buffer and annealed as described above. One equivalent of drug was added and the solution was incubated at RT for a minimum of 30 min prior to use.

3.4.8 Minimum inhibitory concentration tests

Kanamycin B loaded lAptKan NPs and free antibiotic stock solutions were prepared at concentration of 250 µM. *Escherichia coli* (*E. coli*) was grown in 1X LB medium (0.5% yeast extract, 1% tryptone, 1% NaCl, BD) at 37 °C. The obtained culture was diluted to OD$_{600}$=0.1 with 1X LB medium and loaded in a 96 well plate to the final volume of 200 µl/well. Subsequently, the wells were supplemented with the antibiotic or antibiotic loaded lAptKan NPs to the final concentrations of 0-12 µM and the OD$_{600}$ was monitored every 5 minutes over 5 hours at 37 °C on a Synergy HT multi-mode microplate reader (BioTek). For studies including DNAse, 2 µl of 10 mg/ml DNAse solution was added to each well. Experiments were performed in triplicate.

3.4.9 Evaluation of antibiotic activity on porcine cornea

Kanamycin B loaded lAptKan NPs and unformulated antibiotic solution were prepared at a concentration of 500 µM. *Pseudomonas aeruginosa* (ATCC® 10145GFP™) was inoculated into LB medium (agar 3%, tryptone 1%, yeast extract 1%, Irgasan, Sigma aldrich 25 µg/ml, pH=7.0) and incubated overnight at 37°C. *Ex-vivo* cornea punches (diameter 7 mm) were prepared from porcine eyes obtained from the local slaughterhouse. The tissue was infected with *P. aeruginosa* by incubation with 40 µl 0.45-0.6 OD$_{600}$ bacterial solution for 15 min. Afterwards each corneal punch was washed with 0.5 ml PBS every 8 hours and finally supplemented with 0.3 ml DMEM medium. Kanamycin B loaded NPs were prepared at a
concentration of 500 µM as described above. Treatment was performed by incubating every cornea punch with 40 µl solution of unformulated kanamycin or antibiotic loaded lApt\textsuperscript{Kan} NPs. The punches were incubated for 5 min with the solution and subsequently washed for 5 min with PBS. For the placebo, the infected punches were incubated with buffer solution. Treatment was performed for 24 h. In the latter case the above described procedure was repeated a second time. Afterwards, the punches were shaken for 20 min at 24 °C at a thermomixer and serial dilutions (1:10) of the supernatants were incubated on PIA (Sigma Aldrich) plates. The number of colonies was determined by counting in duplicates.

**Statistical analysis of bactericidal effect on porcine cornea:** Data is represented as mean +/- SD. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.) ANOVA analysis with Tukey-Kramer post-hoc test was used for statistical evaluation of the individual time-points and the negative cornea samples. Differences were considered to be significant at *: p<0.05; **: p<0.01; ***: p<0.001

3.4.10 TUNEL assay

TUNEL assay was performed according to manufacturers procedure.

3.4.11 Toxicity studies

Cell culture studies were performed as previously described in detail.\textsuperscript{25, 26} A short description and changes are noted below. Statistical analysis of cell culture assays

Data are represented as mean +/- SD. With every assay five-six different experiments were conducted per cell line and U4-12 or buffer, respectively (n=5-6). Statistical analysis was performed using JMP® (version 11.1.1, SAS Institute Inc., Cary, NC, USA). ANOVA analysis with Dunnett’s post-hoc test was used for comparison between buffer and NP Differences were considered to be significant at p<0.05.

3.4.12 MTS viability assay

24 h after supplementation, 20 µL of the CellTiter 96® AQueous One Solution Reagent (Promega) was directly added to the culture wells and incubated for 90 minutes. Then the absorbance was recorded at 490 nm with a Microplate Reader (BioTek, Synergy HT) with the correction of interference at 690 nm.
3.4.13 Crystal violet staining

After the MTS assay, medium was removed and the cells fixed overnight with 4% paraformaldehyde. After washing three times, the cells were stained with crystal violet solution (Sigma Aldrich), washed again and incubated with 1% SDS for 1h. Absorbance was determined at 595 nm (BioTek, Synergy HT).

3.4.14 Caspase 3/7 activity assay

24 h after supplementation, caspase 3/7 activity was determined using CaspaseGlo 3/7 activity kit (Promega, Madison, USA) according to the manufacturer’s protocol. Luminescence was measured with a luminometer (BioTek, Synergy HT).

Author Contribution

In this chapter, Qing Liu performed the synthesis of the phosphoramidite building block of lipidated 2’-deoxyuridine and the synthesis, purification and characterization of lipidated drug-aptmer oligonucleotides. Moreover, he performed the characterization of the resulting micelles by dynamic light scattering and determined critical micelle concentrations. Moreover, he investigated the binding of the drugs to the aptamer and established a loading protocol for incorporation of the drugs into the soft matter DNA nanoparticles. The in-vitro and in-vivo tests of the resulting nucleic acid materials were carried out by personnel of our collaborator at the University Eye Hospital Tübingen.
Reference


