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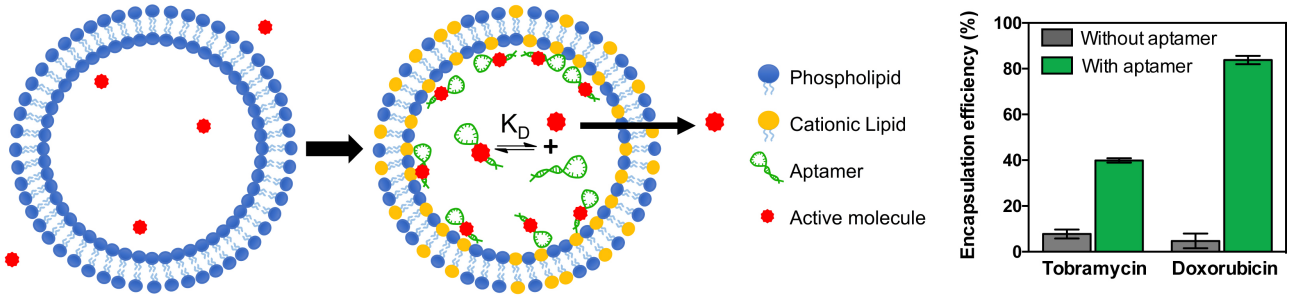
Abstract

Aptamer technology has shown much promise in cancer therapeutics for its targeting abilities. However, its potential to improve drug loading and release from nanocarriers has not been thoroughly explored. In this study, we employed drug-binding aptamers to actively load drugs into liposomes. We designed a series of DNA aptamer sequences specific to doxorubicin, displaying multiple binding sites and various binding affinities. The binding ability of aptamers was preserved when incorporated into cationic liposomes, binding up to 15 equivalents of doxorubicin per aptamer, therefore drawing the drug into liposomes. Optimization of the charge and drug/aptamer ratios resulted in ≥ 80% encapsulation efficiency of doxorubicin, ten times higher than classical passively-encapsulating liposomal formulations and similar to a pH-gradient active loading strategy. In addition, kinetic release profiles and cytotoxicity assay on HeLa cells demonstrated that the release and therapeutic efficacy of liposomal doxorubicin could be controlled by the aptamer's structure. Our results suggest that the aptamer exhibiting a specific intermediate affinity is the best suited to achieve high drug loading while maintaining efficient drug release and therapeutic activity. This strategy was then successfully applied to tobramycin, a hydrophilic drug

suffering from low encapsulation into liposomes, where its loading was improved six-fold using aptamers. Overall, we demonstrate that aptamers could act, in addition to their targeting properties, as multifunctional excipients for liposomal formulations.

Keywords: liposome, aptamer, doxorubicin, encapsulation efficiency, controlled release, active loading

Graphical abstract



Introduction

Aptamer technology, although discovered for 25 years, is still evolving to fulfill the requirements of more precise diagnosis and personalized therapy [1, 2]. Aptamers are RNA or DNA sequences generated to exhibit high affinity and specificity against a broad range of targets, ranging from small molecules to whole cells or tissues [2-4]. Like antibodies, they recognize their specific targets due to the unique three-dimensional structure they adopt. Nucleotides aptamers, however, exhibit several improved properties when compared to antibodies, such as lower immunogenicity, higher thermal stability, rapid and large-scale synthesis and lower production costs [5, 6]. To date, they have shown a high potential for clinical translation, especially in the field of drug and biomarker discovery [7, 8], biosensor design [9, 10], vaccines [11] and molecular imaging [12, 13].

The pioneering work of Farokhzad *et al.* first demonstrated the potential of conjugating an aptamer to the surface of polymeric nanoparticles for targeting prostate cancers *in vivo* [14]. Since then, aptamers have been conjugated to multiple nanocarriers to provide specific recognition of biological targets, showing much promise in targeted cancer therapeutics [15, 16]. However, loading sufficient therapeutics into nanocarriers, while controlling its release rate in order to reach therapeutic concentrations at the target site, still remains a major limitation of nanocarriers [17-19]. Liposomes, for instance, offer unique benefits for clinical applications, such as large internal volume for high drug loading, prolonged circulation times and controlled biodistribution, as well as excellent biocompatibility and biodegradability [20]. To improve drug loading capacity, current strategies exploit a trans-membrane gradient, such as pH- or ion- gradient, to actively load and retain the drug into the liposomal core [21]. The most successful example is Doxil, commercialized

liposomes of doxorubicin, able to reach up to 10 000 molecules of doxorubicin per liposome, most of which existing in the crystalline phase [21]. The liposome formulation significantly reduced the cardiotoxicity of doxorubicin, but the strong entrapment of the drug within the core significantly reduced its release, and, by extension, its therapeutic efficacy [22]. In extreme cases, such as liposomal cisplatin, the therapeutic efficacy has even been abolished [23]. Alternative methods are therefore pursued to provide a better control over the loading and release of the encapsulated drug [24]. Recent studies have used ATP-binding aptamers to selectively release doxorubicin in an ATP-rich environment from nanogels [25, 26], graphene nanosheets [27] or cross-linked microcapsules [28]. Aptamer-functionalized hydrogels have also been programmed to release various and multiple therapeutics when needed through specific nucleic acid recognition and complementary hybridization process [29, 30].

In this study, we propose to use drug-specific aptamers to improve drug loading into liposomes. Indeed, specific aptamers have been designed to show a tunable affinity for a variety of drugs such as doxorubicin [31] [32], cocaine [33] or neomycin [34]. Interestingly, the loading and release rate of the drug from aptamer-drug complexes is a function of the sequence [32, 34] and the number of binding sites [31]. However, these complexes suffer from a low stability in the blood, limited drug loading capacity and some inherent immunogenicity of the aptamers [15, 35]. We hypothesize that incorporating the drug-aptamer complex into liposomes will improve specific drug loading and offer a better control over the release rate to improve the therapeutic efficiency. We have designed specific aptamer sequences to tune the binding affinity of doxorubicin and incorporated them into liposomal formulations. The impact on drug loading, drug release and therapeutic efficacy was investigated. This proof-of-concept was first demonstrated with doxorubicin and applied to tobramycin, a hydrophilic drug suffering from low encapsulation into liposomes.

Material and methods

2.1 Chemicals and material

All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). DNA aptamers and control nucleotide sequences were purchased from Sigma-Aldrich custom oligonucleotide synthesis service (Oakville, ON). The sequences are detailed in Table S1. Doxorubicin hydrochloride was purchased from Sigma-Aldrich and tobramycin sulphate was purchased from AK Scientific (CA, USA). EMEM (ATCC® 30-2003™), PBS and TrypLE Express was purchased from GE Healthcare (Baie-d'Urfé, QC). All sterile consumables were purchased from Sarstedt (Montreal, QC). All reagents, solvents and salts were either purchased from Sigma-Aldrich (Oakville, ON) or Fisher Scientific (Whitby, ON). HeLa cells (ATCC® CCL-2™) were kindly provided by Prof. Marc Servant (University of Montreal).

2.2 Dissociation constants of aptamer-doxorubicin complexes

Dissociation constants for the different aptamer-doxorubicin complexes were obtained by monitoring the quenching of doxorubicin fluorescence at various aptamer concentrations. DNA aptamer solution (0.1 mM in a solution of 5% dextrose and 5 mM NaCl) was denatured 5 minutes at 95 °C, vortexed for 1 minute and annealed at room temperature. Doxorubicin sample concentration was kept constant in all samples (100 nM in 5% dextrose and 5 mM NaCl). Fluorescence emission spectrum (λ_{ex} 485 nm, λ_{em} 520-700 nm) was recorded at 37 °C on a Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, Mississauga, ON). Increasing amounts of DNA were added and equilibrated 1 minute at 37 °C in the same cuvette, producing DNA concentrations ranging from 0.001 to 25 μM with incrementing volumes less than 20% of final volume. Emission scans were taken at a high resolution of 100 nm/min and data were

97 smoothed with a 10-neighbors Savitzky-Golay factor. The area under curve of the full scan was
98 considered for the analysis. The dissociation constant ($K_{D,}$ nM) was calculated using GraphPad
99 Prism 6 with the equation $Y=M_1 + (M_2*X)/(X+M_3)$, where M_1 is the initial value of Y, M_2 is its
100 magnitude and M_3 is the K_D. In the definition of Poly-Doxapt concentrations, one strand A
101 complexed with one strand A' was considered as one aptamer molecule (two binding sites).

102 2.3 Preparation and characterisation of liposomes

103 All liposome formulations, except Doxil-Like, were prepared using the hydration method. Briefly,
104 stock solutions of lipids in chloroform (20-40 mg/mL) were stored under argon at -80 °C before
105 use. For the preparation of cationic liposomes, 1,2-dioleoyl-3-trimethylammonium-propane
106 (DOTAP), cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-(polyethylene
107 glycol)-2000 (DSPE-PEG_{2000}) solutions were combined in a 10-mL round bottom flask in a
108 50/48/2 molar ratio to get 30 µmol total lipid amount. The solvent was evaporated under reduced
109 pressure at 50 °C. The dried lipid film was hydrated 30 minutes at 60 rpm with 1 mL of 5%
110 dextrose and 5 mM NaCl. "No cationic lipid" formulation consisted in 1-palmitoyl-2-oleoyl-*sn*-
111 glycero-3-phosphocholine (POPC), cholesterol and DSPE-PEG_{2000} in a 55/40/5 molar ratio. All
112 liposomes were extruded through 400 and 200 nm polycarbonate membranes using a LiposoFast
113 manual extruder (Avestin Inc., Ottawa, ON, Canada) at room temperature. Doxil-Like liposomes
114 were prepared following the same procedure with minor modifications. Lipid composition was
115 55% 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 40% cholesterol and 5% DSPE-
116 PEG_{2000} (10 µmol total lipid amount). The dried lipid film was hydrated 30 minutes at 65 °C (60
117 rpm) with 1 mL of a 120 mM ammonium sulfate salt solution. Extrusion was performed at
118 controlled temperature (65 °C) to ensure the fluidity of the lipids, using a homemade heating block
119 for the manual extruder. Finally, Doxil-Like liposomes were purified on a 1 x 20 cm Sephadex G-

50 (Medium) column equilibrated in a pH 7.4 buffer (5 mM Tris and 145 mM NaCl) to exchange external medium. All liposome preparations (total final volume ~ 2 mL) were stored in darkness at 4°C in 4 mL glass vials.

Liposome hydrodynamic diameter and ζ-potential were measured at 25°C using a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK) using the automatic algorithm mode at a scattering angle of 173°. Size measurements, reported in intensity, were performed in a pH 7.4 buffer (5 mM Tris and 145 mM NaCl). ζ-potential measurements were obtained using the Smoluchowski model by diluting the liposome sample in MiliQ purified water, using 0.1 mM total lipid concentration in the cuvette. Experiments were run in triplicates or more.

2.4 Preparation of aptamer-loaded lipoplexes

Aptamer spiking solution (5% dextrose and 5 mM NaCl) was denatured 5 minutes at 95 °C, vortexed for 1 minute and annealed at room temperature. Aptamer solution was then added dropwise under stirring into 2.5 mM liposomal solution (1:1 v/v) at predefined N/P ratios (0.5 to 15). N is the number of amines (molar quantity of DOTAP) and P is the number of phosphorous groups of aptamers (corresponding to the number of nucleotides). Lipoplexes were incubated in a VorTemp 56 (Labnet, Edison, NJ) for 25 minutes, at 1000 rpm and 30 °C. Lipoplexes (total final volume ~ 1 mL) were immediately used after incubation to determine the encapsulation efficiency of aptamers. N/P ratio was optimized for each formulation to encapsulate more than 90% of aptamers so that no further removal of unencapsulated aptamer was required.

2.5 Encapsulation efficiency of aptamers

Two methods were used to determine the encapsulation efficiency of aptamers into cationic liposomes. Through the indirect method, the residual aptamer concentration in solution (1 to 150 nM) was quantified using fluorescent intercalating probes SYBRGold or SYBRGreen (Thermo

Scientific) for Apt-Ctrl-2 or all other aptamers, respectively. An aliquot of lipoplexes was diluted to 300 µL in 5% dextrose and 5 mM NaCl and centrifuged 60 minutes at 18500 g at room temperature. The supernatant was diluted to fit in the linear range (1 to 150 nM) and SYBRGreen 100 X (or SYBRGold for Apt-Ctrl-2) was added (5% total volume). 150 µL of each sample was added to a 96-well plate and analyzed with a Safire microplate reader (Tecan, Männedorf, Switzerland) (λ_{exc} 496 nm, λ_{em} 523 nm for both SYBRGreen and SYBRGold). A calibration curve was determined for each DNA sequence. The amount of free DNA was determined and the encapsulation efficiency was calculated using equation 1.

Equation 1: $EE\ (\%) = \frac{Total\ DNA - Free\ DNA}{Total\ DNA} * 100\%$

In addition, the amount of encapsulated aptamers within the lipoplexes was quantified directly according to a second fluorescent assay. 2 mL of a solution of 1X SYBRGreen (or SYBRGold) in 5% dextrose and 5 mM NaCl was placed in a 3 mL cuvette. The fluorescence of this solution was monitored over time on a F-2710 Spectrophotometer (Hitachi High Technologies America Inc., Schaumburg, IL, USA) at wavelengths of 496/523 nm ($\lambda_{exc}/\lambda_{em}$), under weak agitation. At t = 30 seconds, lipoplexes (corresponding to 100 nM DNA final concentration) were added in the cuvette, and the accessible DNA increased the fluorescence of the SYBR probe. At t = 100 seconds, 10 µL of Triton X100 10% (v/v) was added to disrupt the liposomes, allowing the total amount of DNA to be complexed by the SYBR probe. Final fluorescence was recorded at t = 200 seconds. The percentage of aptamer encapsulation was calculated using the equation 2, where the initial intensity is the average fluorescence intensity between time points 30 and 100 seconds and the final intensity is the average fluorescence intensity between time points 100 and 200 seconds.

Equation 2: $EE\ (\%) = 100 - (\frac{Initial\ intensity}{Final\ intensity}) * 100\%$

2.6 Stability of lipoplexes

Lipoplexes, containing Doxapt-30 at N/P = 3, were prepared in 5% dextrose and 5 mM NaCl and kept at 37 °C. Encapsulation efficiency of aptamers was measured by direct and indirect methods at 0, 4 and 24 h to quantify the release of the Doxapt-30 aptamer. A similar study was conducted on lipoplexes containing Apt-Ctrl-1 at N/P = 3. Encapsulation efficiency of aptamers was measured at 0, 1, 2, 6, 8, 12 and 24 h in PBS at 37 °C. Colloidal stability studies were conducted on another batch of lipolexes containing Doxapt-30 at N/P = 3 diluted 3:1 in phosphate buffered saline (PBS) at pH 7.4. The hydrodynamic diameter and polydispersity index were measured at days 0, 3, 5 and 7.

2.7 Doxorubicin-loaded lipoplexes

Doxorubicin stock solutions were prepared in Tris/NaCl buffer (5 mM/145 mM, pH 7.4). Equal volumes of lipoplexes and doxorubicin solutions were combined at various doxorubicin/aptamer molar ratios (1:1 to 25:1). Noteworthy, Poly-Doxapt was composed of two pre-complexed strands and considered as one molecule in the calculations (124 nucleic acid for one DNA molecule). The mixture was incubated for 25 minutes, at 1000 rpm and 30 °C, and stored in the refrigerator in the dark before use. Samples were used immediately to determine their encapsulation efficiency. N/P ratio and drug/aptamer ratio were optimized for each formulation (Table 3). Optimized formulations of doxorubicin-loaded lipoplexes were used without purification for *in vitro* release and cytotoxicity studies, to ensure that doxorubicin total amount was identical in all samples.

As for Doxil-Like liposomes, doxorubicin stock solution was added to pH-gradient liposomes (1:1 v/v) to obtain drug/lipid ratio of 0.1 and the mixture was incubated at 50 °C, for 25 minutes and 1000 rpm. No further purification method was applied for subsequent testing.

Doxorubicin encapsulation was determined indirectly by fluorescence assay. Doxorubicin-loaded lipoplexes were centrifuged 60 minutes at 18,500 g at room temperature and supernatant was

collected. Free doxorubicin was quantified by fluorescence using a Safire microplate reader (λ_{ex} 485 nm; λ_{em} 585 nm) against a calibration curve. Doxorubicin encapsulation efficiency was determined using Eq 3. The final Drug/Lipid (D/L) ratio was determined using Eq 4.

Equation 3: $$EE\ (\%) = \frac{(Feeding\ doxorubicine\ ) - (Free\ doxorubicin)}{(Feeding\ \ doxorubicin)} * 100\%$$

Equation 4: $$D/L\ (w/w) = \frac{(Feeding\ doxorubicin - Free\ doxorubicin)}{Total\ lipid + Feeding\ doxorubicin}$$

2.8 Release kinetics of doxorubicin-loaded lipoplexes

Each formulation of doxorubicin-loaded lipoplexes was prepared in quadruplicate and used without purification to ensure identical initial doxorubicin amounts in each condition. 1 mL of doxorubicin-loaded lipoplexes (13 µM of doxorubicin) was added to dialysis bags (6-8 kDa MWCO, Spectra/Por, Spectrum Laboratories, Inc) and immerged into 100 mL of PBS pH 7.4 (n = 3) or acetate buffer pH 5 (n = 1). The whole set-up was moderately stirred at 37 °C and protected from light. 0.8 mL samples were withdrawn from the external medium at predetermined time points over 48 hours and replaced by fresh medium. Samples were stored at 4°C before analysis with a UPLC-Fluorescence against a calibration curve prepared from the initial doxorubicin solution. Briefly, the UPLC system (Shimadzu-Prominence UFLC, Shimadzu USA Manufacturing inc. Mandel) consisted of a LC-20AD binary pump, a DGU-20A5 solvent degasser, a SIL-20AC HT refrigerated, a CT0-20AC column oven and a RF-20AXS fluorescence detector. Mobile phase A: water/0.1% formic acid. Mobile phase B: acetonitrile. Flow rate: 0.7 mL/min. Gradient A decreased linearly from 90 to 60% between 0.25 to 5.5 min; followed by recovery of 90% A in 0.2 min, then equilibration for 4.5 minutes at 90% A. Total run time: 9 minutes. Injection volume: 50 µL. The column (Poroshell 120 EC-C18, 3.0 x 30 mm, 2.7 micron equipped with a pre-column Agilent EC-C18, 3.0 x 5 mm, 2.7 micron) was kept at 30 °C and retention time of doxorubicin was of 5.4 min. Detection of doxorubicin was measured at λ_{exc} = 485 nm/ λ_{em} = 585 nm. The kinetics

curves were also fitted using a double exponential function and the amplitude and rate constants for drug release and drug degradation were obtained from the best fit.

2.9 Cell viability assay

HeLa cells were initially cultured in Eagle's Minimum Essential Medium (EMEM, ATCC® 30-2003™) supplemented with 10 % fetal bovine serum (FBS) and 100 units/mL Penicillin-streptomycin (Life Technologies, Burlington, ON). Cells were incubated at 37 °C with 5 % CO_2. Cells were seeded in a 96-well plate at a density of 5 x 10^3 cells per well. After 24 h, fresh culture medium and optimized formulation were added to each well, with final concentrations of doxorubicin ranging from 0.01 to 25 µM per well. In each plate, the blank formulation (without doxorubicin) was tested for its cytotoxicity at its highest concentration used. After 48 h incubation, the cells were rinsed with DPBS 1X. Resazurin in EMEM (final concentration of 44 µM in each well) was added. The cells were incubated an additional 3 hours. Absorbance was measured at 570 nm and 600 nm to determine the metabolic reduction of resazurin. IC_{50} was determined using GraphPad Prism 6 and a normalized dose-response inhibition curve fitting. Each curve was made in triplicate or more.

2.10 Tobramycin-loaded lipoplexes

Tobramycin sulphate was fluorescently labeled by Cy5. Synthesis details and characterization are described in supplementary information. The binding affinity of Apt-Ctrl-1 for tobramycin-Cy5 was measured by fluorescence assay as described in supplementary information.

Before liposome preparation, Ctrl-Apt-1 was denatured 5 minutes at 95 °C, vortexed for 1 minute and annealed at room temperature. Aptamer and Tobramycin-Cy5 were first mixed in equal volumes at increasing molar ratios (0.5, 1, 2, 3, 5 tobramycin/aptamer) in 5% dextrose and 5 mM NaCl. Liposomes (DOTAP/Chol/DSPE-PEG_{2000} 50/48/2 molar ratio) were previously prepared in

5% dextrose and 5 mM NaCl as described in section 2.3. They were mixed at equal volume (N/P = 3) with the tobramycin/aptamer solutions or tobramycin solutions without Apt-Ctrl-1 (negative control). The lipoplexes were incubated for 25 min at 30 °C at 1000 rpm before being centrifuged 60 minutes at 18,500 g at room temperature. Free tobramycin-Cy5 was quantified in the supernatant by fluorescence using a Safire microplate reader (λ_{exc} 649 nm; λ_{em} 670 nm) against a calibration curve. Tobramycin encapsulation efficiency and the final Drug/Lipid (D/L) ratio were determined using Eq.3 and Eq.4 adapted to tobramycin. Experiment was run in triplicate.

2.11 Statistical analysis

Statistical analysis was carried out using PRISM 6.01 software (Graphpad, CA, USA). For zeta potential measurements, statistical analysis was performed with two-tailed Student's t-test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. To determine statistically significant encapsulation efficiency differences, a two-way ANOVA analysis was performed. To determine statistically significant IC_{50} differences, a one-way ANOVA analysis was performed. In both cases, P values for multiple comparisons were adjusted using the Bonferroni correction. A P value of ≤ 0.05 was considered significant.

Results

3.1 Aptamer design and affinity for doxorubicin

In this study, we used aptamers as the driving force to actively load a drug into the liposomes. Doxorubicin was selected as a model drug for several reasons. First, it represents a good example of successful active drug loading via ammonium sulphate gradient into liposomes [22]. Second, a doxorubicin-binding DNA aptamer has already been reported and validated in the literature [33,

36]. Third, binding of doxorubicin to aptamers can be easily monitored by fluorescence measurements, which makes it a suitable model drug candidate for formulation optimization [37]. We hypothesized that tuning the affinity of the aptamers should impact the loading and the release rate of the drug from liposomes, therefore we designed a series of aptamer sequences with various binding properties, (Figure 1A, Table S1). All the aptamers include the doxorubicin specific sequence, designed as Doxapt-28 [36]. Doxapt-30 possesses an extra base pair that should stabilize the double strand section and increase the affinity of the aptamer for its ligand [33]. Coop-Doxapt, a two-binding-site aptamer reported by Simon *et al.,* contains two binding sites and displays a cooperative binding behaviour [33]. Inspired by polymer-like aptamers [38], we also created Poly-Doxapt to improve aptamer packing into the liposome. Finally, two sequences were used as negative controls: Apta-Ctrl-1 is a 21-nucleotide aptamer designed for tobramycin, which presents a hairpin structure, but no specificity for doxorubicin [39] and Apta-Ctrl-2 is a 30mer of poly-thymine without any secondary structure. Following the proof-of-concept demonstration, the strategy was applied to another drug, tobramycin, using the reported specific aptamer sequence Apt-Ctrl-1 (Figure 1A) [39].

Once intercalated into a DNA, doxorubicin fluorescence is quenched, which allows monitoring of its interaction with different aptamer structures (Figure 1B) [37]. Dissociation constants (K_D) of the various aptamers in the medium used for liposomal formulation (dextrose 5%, 5 mM NaCl) can thus be obtained by fitting the fluorescence intensity of doxorubicin to increasing concentrations of aptamers. A representative example of such curves is displayed in Figure 1C (see also Figures S1-S5) and the summary of all K_D is provided in Table 1. Doxapt-28 (380 nM) and Doxapt-30 (334 nM) exhibit K_D values in agreement with previous reports, despite variation of the medium (Table 1) [33]. Both Coop-Doxapt and Poly-Doxapt exhibited a higher affinity for

doxorubicin (160 ± 36 nM and 68 ± 6 nM respectively), confirming the relevance of multivalent aptamers. However, under the liposomal formulation conditions, however, Coop-Doxapt did not display a higher cooperativity for doxorubicin binding as this has been previously observed at higher temperature (30°C) and ionic strength (50 mM) (Figure S3) [33]. This is likely due to the fact that the cooperativity level of DNA recognition elements are highly dependent on temperature and ionic strength variations [40]. Finally, Apt-Ctrl-1 displayed reduced affinity for doxorubicin (K_D > 1000 nM) while Apt-Ctrl-2 did not quench the fluorescence at an appreciable amount to determine a dissociation constant. Overall, this collection of aptamers exhibits dissociation constants for doxorubicin ranging from 68 nM to > 1000 nM.

3.2 Lipoplex preparation and characterization

Aptamers were incorporated into cationic liposomes via electrostatic interactions. The liposome composition (DOTAP/Chol/DSPE-PEG_{2000} 50/48/2) was inspired by the widely reported lipid formulations used in gene delivery [41]. Similarly to these latter systems, the physico-chemical properties of lipoplexes rely on the charge ratio N/P (amino group of DOTAP *vs.* phosphate group of nucleotide). As illustrated by Doxapt-30 lipoplexes, the liposome increased in size upon incubation with aptamers and exhibited aggregation at the charge ratio N/P = 2, as witnessed by the higher diameter and polydispersity index (Figure 2A). Colloidal stability is recovered with an excess of cationic charges (N/P ≥ 3), resulting in lipoplexes smaller than 200 nm diameter, with low polydispersity and positive ζ potential (Figure 2A&B). Similar behaviour was confirmed for all aptamers (Figure S6). Aptamer complexation efficiency, quantified by the remaining DNA in solution, was also improved with increasing charge ratios. Aptamers presenting one binding site (Doxapt-28 and Doxapt-30) required N/P = 3 to be fully encapsulated into liposomes, whereas N/P

= 2 was sufficient for Coop-Doxapt and Poly-Doxapt to achieve complete DNA complexation (Figure 2C). These results were confirmed by directly measuring the concentration of aptamers within lipoplexes (Table S2). Except for Coop-Doxapt and Poly-Doxapt, the values obtained (≥ 94% for all aptamers) demonstrated that DNA was mainly encapsulated within lipoplexes; only a minor quantity was adsorbed on the surface. Noteworthy, ζ potential of Poly-Doxapt and Coop-Doxapt were still negative at N/P = 2, suggesting the presence of DNA strains adsorbed on the surface of the lipoplexes (Table 3). This could be due to the longer sequence of nucleotides of Coop-Doxapt and Poly-Doxapt (86 and 124 nucleotides, respectively) as compared to other aptamers (28-30 nucleotides). Adsorption of Coop-Doxapt was confirmed since only 46% of the aptamer was actually encapsulated within the lipoplex structure, as determined by direct measurement (Table S2). As for Poly-Doxapt, direct and indirect methods corroborate that more than 83% of aptamers were encapsulated within liposomes (Table S2). This result suggests that, despite the negative zeta potential, aptamers were tightly bound to the lipoplex structure and were not accessible to the fluorescent dye.

In addition, the aptamers remained encapsulated for at least 24h at 37°C, for Doxapt-30 (Table 2) as well as for the control sequence Apt-Ctrl-1 (Table S3). For each aptamer, optimal N/P ratio was selected as the lowest ratio leading to stable lipoplexes and maximal encapsulation of aptamer (Table 3). Final lipoplexes exhibited diameters ranging from 170 to 290 nm with low polydispersity indexes, and almost complete complexation of aptamers. Lipoplexes exhibited good colloidal stability for 7 days in PBS at 37°C (Figure S7).

3.3 Encapsulation of doxorubicin

Aptamer-loaded lipoplexes were further incubated with doxorubicin at increasing doxorubicin/aptamer molar ratios, and doxorubicin encapsulation was quantified by fluorescence of free drug after centrifugation. A representative behaviour is reported on Figure 3A with Doxapt-30. Encapsulation efficiency (EE) reached ≥ 85 % doxorubicin for up to 2 drug molecules per aptamer, close to the efficiency of the sulphate gradient method of Doxil-Like liposomes (Figure 3A). The active role of aptamers in the loading capacity was confirmed by a series of controls. Passive encapsulation was estimated to range from 5 to 25% using cationic or plain liposomes, respectively (Figure 3A). Moreover, Apt-Ctrl-2 (Poly-thymine) was not able to drag doxorubicin into the liposomes whereas Apt-Ctrl-1, presenting a hairpin structure, displayed 45% reduction compared to the highest doxorubicin encapsulation value, which confirmed the higher specificity of Doxapt-30 for doxorubicin. Interestingly, the lipoplexes of Doxapt-30 enabled the loading of more than 2 doxorubicin molecules per aptamer, although this sequence is designed to have only one binding site. Above this doxorubicin/aptamer ratio, additional drug loading was reduced, leading to a decrease of EE %. Nonetheless, the final drug/lipid ratio (D/L) still increased up to 20 doxorubicin/aptamer (Figure 3A). Similar trends were also observed for Coop-Doxapt and Poly-Doxapt, which were found to load up to 8 and 15 equivalents of doxorubicin with more than 80% EE for Coop-Doxapt and Poly-Doxapt, respectively (Figure 3B). Therefore, the adsorption of Coop-Doxapt on the surface of lipoplexes did not seem to impact its loading capacity. In contrast, Doxapt-28 loaded lipoplexes were not able to encapsulate more than 45 % doxorubicin. This could be due to the deletion of one base pair in the Doxapt-28 sequence (Figure 1A), which leads to a less stable stem-loop (1 GC less, around 2 kCal/mol) [42]. The Doxapt-28 stem-loop may be destabilized in the lipoplex structure, which may impair doxorubicin binding. Therefore, Doxapt-28 appears as a negative control of Doxapt-30, exhibiting the closest sequence as possible with

346 significantly different loading properties. A summary of the optimized formulations is presented
347 on Table 3. For each aptamer, we selected the formulation exhibiting high encapsulation of both
348 aptamer and doxorubicin, with a minimum amount of excipients. Furthermore, the addition of
349 doxorubicin to lipoplexes did not significantly affect neither the size nor the polydispersity of the
350 formulations (Table 3). Further studies were conducted using twice the doxorubicin amount as
351 compared to the number of binding sites, to maximize drug loading for both single and double-
352 binding sites aptamers.

353 3.4 Release kinetics of doxorubicin-loaded lipoplexes

354 Aptamers improve the loading capacity of liposomes but do their presence impact drug release
355 rate? To test this question, we measured the release kinetics of doxorubicin from the aptamer-
356 loaded lipoplexes by employing a dialysis method (Figure 4). Lipoplexes were prepared at optimal
357 ratios and were not purified before dialysis, to ensure similar initial doxorubicin concentration.
358 Kinetics display a bi-exponential profile with the fastest transition representing the release of
359 doxorubicin from the lipoplexes and the slowest transition the elimination of doxorubicin over
360 time (Table 4 and Figure S8). The elimination of doxorubicin concentration after several hours
361 (Figure 4) might be due to the degradation of doxorubicin at pH 7.4 at 37°C and its adsorption to
362 surfaces (dialysis bags as well as sampling vials), as previously described [43, 44]. In agreement
363 with literature, Doxil-Like liposomes exhibited a lower release efficiency (less than 15% after 48
364 hours, Figure 4) [22, 45] but the observed release rate was found similar to the rate obtained for
365 free doxorubicin control (Table 4). This could be due to some doxorubicin adsorbed on the
366 liposome surface, which was washed out in the first hour. Interestingly, quite different profiles
367 were obtained for the series of lipoplexes. The high-loading Coop-Doxapt lipoplexes, for example,

368 released 5-fold more doxorubicin than the Doxil-like liposomes at a 5-fold slower rate (0.20 ± 0.05
369 hr^{-1}) reaching 80% of drug release after 10 hours (Figure 4). This suggests that doxorubicin release
370 was slowed down by the binding to the aptamer but the adsorption of Coop-Doxapt on the lipoplex
371 surface favored doxorubicin extensive release. Poly-Doxapt, which possesses the highest affinity
372 for doxorubicin, released doxorubicin 5-fold slower than the Coop-Doxapt (0.04 ± 0.01 h^{-1}), which
373 resulted in only 20% of doxorubicin being released after 48 h, similar to the Doxil-Like
374 formulation (Figure 4). Doxapt-30, which has an intermediate affinity (K_D = 334 nM), displayed a
375 sustained release, achieving 30% of release after 12 hours with a release rate similar to Coop-
376 Doxapt (0.23 ± 0.06 h^{-1}). Doxapt-28 also displayed a similar release rate (Table 4), despite its low
377 drug loading efficiency, while Apta-Ctrl 1 displayed a significantly faster release rate of 0.38 ±
378 0.07 h^{-1}, in agreement with its lower affinity for doxorubicin. Both latter formulations released
379 more doxorubicin than Doxapt-30 (75% and 45% of initial doxorubicin after 12 hours,
380 respectively), probably due to the residual unencapsulated doxorubicin. As a control, we
381 demonstrated that no leakage of Doxapt-30 nor the Apta-Ctrl-1 was detected over a 24h period in
382 the same conditions, showing no sequence specific effect on aptamer release (Table 2 & Table
383 S3). We also investigated the behaviour of doxorubicin loaded lipoplexes at acidic pH, to mimic
384 the endosomal conditions. Interestingly, the relative behaviour of aptamers was maintained albeit
385 the acidic conditions decreased doxorubicin elimination, in agreement with literature [44], which
386 slightly increased the extent of drug release (Figure S9 and S10). Coop-Doxapt and Doxapt-30
387 still exhibited similar release rates (0.30 and 0.28 h^{-1}, respectively, Table 4). Here again, Coop-
388 Doxapt released over 90% of its drug in 12 hours (Figure S9) whereas Doxapt-30 sustained its
389 release (53% doxorubicin released in 12 hours). The higher affinity of Poly-Doxapt severely
390 reduced doxorubicin release (22% in 12 hours, Figure S9) as well as the release rate (0.078 h^{-1},

391 Table 4). The negative controls, Doxapt-28 and Apta-Ctrl-1, still quickly released 82 and 72% of
392 their content after 12 hours, respectively (Figure S9). Overall, these results suggest that aptamers
393 slowed down the release of drug according to their specific affinity, which allows to control drug
394 diffusion and release from lipoplexes.

395 3.5 Cell viability assay

396 Modifying the release kinetics of a drug has a direct impact on its therapeutic efficacy. To explore
397 this impact, we completed our study by assessing the cytotoxic activity of doxorubicin in the
398 optimized formulations on HeLa cells (Figure 5A). Free doxorubicin presented an IC_{50} of 1.5 ±
399 0.6 µM, in agreement with the literature [46]. Doxil-Like formulation exhibited significantly
400 higher values of IC_{50}, up to 38 µM, reflecting of the low immediate bioavailability of doxorubicin
401 from these liposomes. Doxapt-30 improved 4 times the therapeutic efficacy of Doxil-Like
402 formulation, exhibiting similar cytotoxicity as free doxorubicin, notwithstanding its sustained
403 release. Coop-Doxapt and Apta-Ctrl 1 also exhibited similar cytotoxicity to free doxorubicin,
404 probably due to their extensive drug release over 48 hours. Conversely, Poly-Doxapt exhibited
405 similar cytotoxicity to Doxil-Like formulations, in agreement with its low doxorubicin release, in
406 addition to its negative zeta potential and low N/P ratio, which may reduce cellular uptake [47].
407 As a control of lipoplex influence, the cell viability was determined after incubation with the
408 highest concentration of aptamer-loaded lipoplexes without doxorubicin (Figure 5B). The four
409 blank lipoplexes formulations exhibited no toxicity on HeLa cells at their highest concentration,
410 and were better tolerated than Doxil-Like formulation. Overall, these results demonstrate that
411 aptamer binding to doxorubicin did not prevent therapeutic efficacy of the drug, and an adequate

sequence design could even improve its availability as compared to ammonium sulphate gradient liposomes.

3.6 Application to tobramycin

Encouraged by this proof-of-concept, we further applied the strategy of aptamer-loaded lipoplexes to another drug, tobramycin. In the treatment of lung infection, liposomal tobramycin demonstrated drug retention in the lung and improved *in vivo* efficacy against bacterial infection as compared to the free drug [48]. Liposomes have also allowed a better accumulation close to the bacterial biofilm but the antibiotic activity was limited due to the low encapsulation efficiency of liposomes [49]. Indeed, tobramycin aminoglycoside structure is highly hydrophilic, which prevents its efficient encapsulation within liposomes. Therefore, this drug would significantly benefit from an active loading strategy using drug specific aptamers. Apt-Ctrl-1 was selected for its reported affinity for tobramycin [39]. To facilitate detection, tobramycin sulphate was labeled by fluorescent Cy5 (see synthesis and characterization in supporting information). Binding affinity of Apt-Ctrl-1 for tobramycin was determined to be 1.15 ± 0.24 μM, slightly higher than doxorubicin-binding aptamers (Figure 6A). Encapsulation of tobramycin in cationic liposomes was compared with or without aptamers (Figure 6B). Interestingly, aptamers do also improve the encapsulation of tobramycin, reaching 5.8 times greater EE at high concentrations of tobramycin (45% against 8%, with and without aptamer, respectively) as well as drug/lipid ratio. These results demonstrate that an aptamer-loading strategy could successfully be used to improve the loading efficiency of various drugs into liposomes, especially for drugs with low encapsulation properties.

Discussion

433 Loading of drugs into liposomes is a critical step of the liposomal formulation, since it determines
434 the amount of excipient required, as well as the factors governing the release rate of the drug [21].
435 In the passive loading method, the drug dissolved in the aqueous phase equilibrates with the
436 liposome's internal medium, which limits its encapsulation efficiency. Higher scores can be
437 achieved using a gradient method, such as pH or ions (ammonium sulphate, hydrogenophosphate,
438 or metallic ion such as copper or manganese salts) [19]. In this strategy, the drug, when present
439 inside the vesicle core, is converted to an ionic/salt form, which is unable to diffuse back through
440 the lipid membrane, thus is retained into the liposome core. Unfortunately, this strategy also
441 strongly impairs the release rate of the drug, and often requires an additional trigger [24]. This is
442 confirmed by our results of Doxil-Like formulation, which exhibited limited release (Figure 4),
443 high IC_{50} on HeLa cells, and even a non-negligible toxicity of the liposomes itself (Figure 5).

444 We prepared liposomes with drug-specific aptamers to improve the loading capacity of liposomes.
445 We selected aptamer technology because (i) their affinity can be tuned by controlling their
446 nucleotide sequence or length [42], allowing to optimize the aptamer sequence according to the
447 desired release properties; (ii) the binding does not change the protonation of the drug, therefore
448 maintaining its diffusion and release ability [34] and (iii) aptamers could be designed for almost
449 any target, suggesting this strategy could be applied to a large range of molecules [3].

450 The series of aptamers was designed to study the relationship between the sequence and the
451 properties of aptamer-loaded lipoplexes. As expected, the affinity of aptamers varied according to
452 their sequences and binding sites (Table 1). The results confirmed the specificity of the aptamer
453 sequences for its drug since the binding is higher than the natural affinity of doxorubicin for
454 random single or double-stranded DNA (Table 1) as well as aptamer/doxorubicin complexes

455 reported in literature ($K_D \approx 600$ nM) [37]. However, Doxapt-28, presenting a similar affinity to
456 Doxapt-30, was not able to encapsulate more than 50% of doxorubicin. Adding a base pair to the
457 hairpin probably allowed a higher stabilization of the complex, which has already been reported
458 [42]. The three other structures, although exhibiting different binding constants, demonstrated
459 similar drug loading capacity (Table 3). This might be explained by the high concentration of the
460 drug in the liposomal formulation (more than 10 times K_D) resulting in the saturation of aptamers.
461 These conditions might also reveal secondary binding sites on aptamers, which results in the
462 binding of more than 2 doxorubicin molecules per aptamer for Doxapt-30 and more than 8 and 15
463 molecules for Coop-Doxapt and Poly-Doxapt, respectively (Figure 3). For the latter cases, the
464 higher loading capacity may be linked to their longer sequence of nucleotides (86 and 124
465 nucleotides, respectively), revealing probably additional binding sites for doxorubicin.
466 Interestingly, this behaviour was also observed for tobramycin (Figure 6B), which was able to
467 encapsulate up to 2.5 tobramycin equivalents, although designed with only one binding site.
468 Although this non-specific binding was not detected in the binding affinity measurements (Figure
469 1), it can be explained by the natural affinity of doxorubicin for nucleic acids or the electrostatic
470 interactions of both cationic drugs with anionic nucleobases [37, 50]. In addition, incorporation of
471 DNA sequences into liposomes has been reported to strongly impact the structural organization of
472 the liposome, resulting in densely packed lipoplexes, which might favor the retention of the drug
473 [50, 51]. Overall, aptamer incorporation into liposomes significantly improved their loading
474 capacity, through specific as well as non-specific interactions with the drug.

475 Interestingly, our results show that release kinetics and therapeutic efficacy could be tuned
476 according to the aptamer structure. We showed that drug binding to the aptamer slowed down the
477 release rate of the drug in comparison to the free drug, according to its affinity constant. Doxapt-

30 exhibited a slower release than Apt-Ctrl-1, which allowed a sustained release of the drug over 48 hours. Nevertheless, a too high affinity might compromise the drug release from the lipoplex, as exemplified by Poly-Doxapt (K_D = 68 nM, less than 15% release in 48 hours). Therefore, this latter system did not improve commercial formulation, as confirmed by the same therapeutic efficiency as Doxil-Like (Figure 5). Apart from the affinity, our results suggest that other sequence parameters impact the overall behaviour of the system. Coop-Doxapt, maybe due to its longer stem-loop, resulted in low encapsulation of aptamer within the lipoplex. The adsorption of ~ 50% Coop-Doxapt on the surface of liposomes still allowed doxorubicin complexation but favored its release in physiological conditions. Therefore, this system was not improved compared to free doxorubicin. In the other hand, the short Doxapt-28 sequence demonstrated that a minimal stability of the stem-loop is required to ensure drug binding ability within the lipoplex structure. In our study, Doxapt-30 exhibited the advantages of Doxil-Like formulation without its limitations. Indeed, this sequence exhibits a specific affinity for doxorubicin and a high encapsulation efficiency within cationic lipoplexes, which resulted in a sustained release profile and an excellent therapeutic efficiency. In addition, this nucleotide sequence length limited the excipient mass required for doxorubicin loading, resulting in the highest drug/lipid ratio (Table 3).

Conclusion

In conclusion, we developed a new strategy of drug loading, by designing drug-specific aptamers to drive drugs into liposomes. Combining the advantages of aptamer specific properties and liposomal controlled release enable to achieve tunable properties according to the structure of the aptamer. We demonstrated the proof-of-concept using doxorubicin, but this strategy may be applied to a large variety of drugs, since aptamers can be synthetically prepared against about any

target, from small molecules to whole cells. In particular, we showed that aptamers significantly improved the loading of hydrophilic tobramycin into liposomes. These results demonstrate the potential of aptamer technology for multifunctional drug delivery systems. In particular, adding drug specific sequences to a cancer-targeted aptamer could lead to better controlled drug delivery systems. Further improvements are currently focused on the drug/lipid ratio and the application to larger biomacromolecules.

Acknowledgements

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514 Table 1. Characteristics of aptamers used in this study and their affinity constants (K_D) for
515 doxorubicin.

Formulation	Doxapt-28	Doxapt-30	Coop-Doxapt	Poly-Doxapt	Apt-Ctrl-1	Apt-Ctrl-2
Nucleotides	28	30	86	62 +62	21	30
Binding sites	1	1	2	2	0	0
K_D (nM)[1]	380 ± 45	334 ± 29	160 ± 36	68 ± 6	> 1000	ND[2]

516 [1] *Determined by fluorescence assay, as shown in Fig 1, S1-S5.*
517 [2]*Fluorescence of doxorubicin was not quenched sufficiently to determine a dissociation constant (see Figure S5).*
518

519 Table 2. Stability of encapsulated aptamers over 24 h at 37 °C in dextrose 5% NaCl 5 mM.
520 Liposomes DOTAP/Chol/DSPE-PEG_{2000} 50/48/2 encapsulating Doxapt-30 (ratio N/P = 3) were
521 assayed for their content in aptamers using the direct and indirect method. Results are reported as
522 the mean value of 3 experiments, ± standard deviation of the mean.

Time (h)	0	4	24
Direct method	98.3 ± 0.1 %	98.1 ± 0.1 %	98.2 ± 0.1%
Indirect method	98.6 ± 0.6 %	99.1 ± 0.3 %	97.0 ± 1.2 %

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524

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Table 3: Summary of the characteristics for the optimized formulations of liposomes, lipoplexes containing aptamers and doxorubicin-loaded lipoplexes. All liposomes were cationic liposomes (DOTAP/Chol/DSPE-PEG_{2000} 50/48/2 molar ratio) except for Doxil-Like (DSPC/Cholesterol/DSPE-PEG_{2000} 55/40/5 molar ratio). Results are reported as the mean value of 3 measurements, ± standard deviation of the mean.

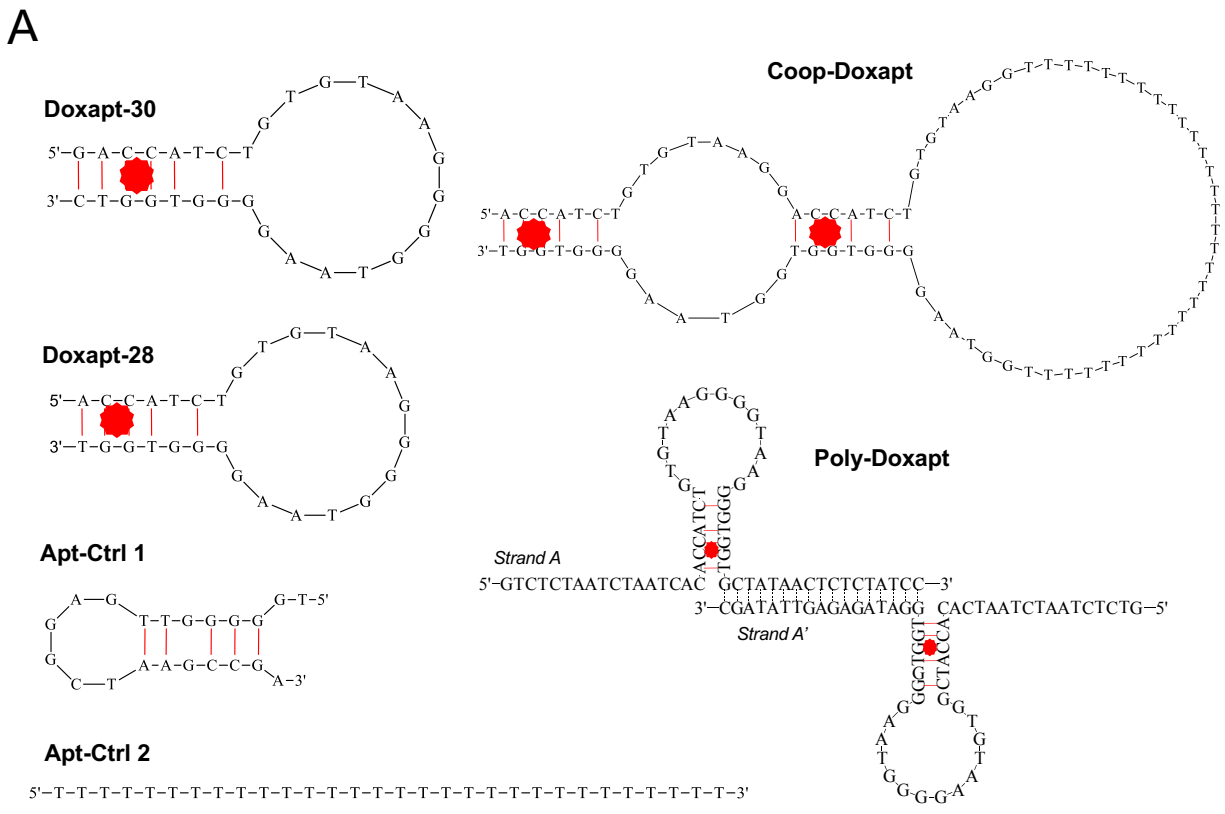
Formulation		Doxapt-28	Doxapt-30	Coop-Doxapt	Poly-Doxapt	No Apta	Doxil-Like	Apt-Ctrl-1	Apt-Ctrl-2
Liposomes	Z-Average Diameter (nm)	139 ± 3	154 ± 2	139 ± 3	139 ± 3	139 ± 3	187 ± 6	139 ± 3	139 ± 3
	PdI	0.082 ± 0.001	0.049 ± 0.012	0.082 ± 0.001	0.082 ± 0.001	0.082 ± 0.001	0.038 ± 0.036	0.082 ± 0.001	0.082 ± 0.001
Aptamer-loaded Lipoplexes	Z-Average Diameter (nm)	171 ± 3	286 ± 2	230 ± 3	283 ± 7	139 ± 3	187 ± 6	242 ± 5	206 ± 4
	PdI	0.058 ± 0.018	0.241 ± 0.018	0.058 ± 0.019	0.222 ± 0.023	0.082 ± 0.001	0.038 ± 0.036	0.069 ± 0.013	0.048 ± 0.033
	Zeta potential (mV)	22. 4 ± 1.2	28.2 ± 0.6	-19.4 ± 0.6	-24.7 ± 0.6	24.9 ± 0.6	-4.8 ± 1.1	4.53 ± 0.6	17.6 ± 1.6
	Ratio N/P	3	3	2	2	N/A	N/A	3	3
	EE% Aptamers[a]	94 ± 2%	100 ± 0%	99 ± 2%	98 ± 1%	N/A	N/A	94 ± 1%	99 ± 0%
Doxorubicin and aptamer-loaded lipoplexes	Z-Average Diameter (nm)	197 ± 2	236 ± 6	254 ± 2	312 ± 4	154 ± 2	181 ± 3	285 ± 5	204 ± 2
	PdI	0.058 ± 0.018	0.047 ± 0.048	0.062 ± 0.002	0.172 ± 0.007	0.074 ± 0.008	0.063 ± 0.010	0.075 ± 0.011	0.068 ± 0.029
	Zeta potential (mV)	16.3 ± 0.8	15.9 ± 0.7	-28.1± 1.7	-28.8± 1.2	20.9 ± 0.9	-11.5± 0.1	8.81 ± 0.7	5.7 ± 0.5
	Ratio Dox/Apta	2	2	4	4	N/A	N/A	2	2
	EE% Dox[b]	28 ± 7%	84 ± 2%	73 ± 3%	86 ± 1%	5 ± 3%	98 ± 1%	39 ± 0%	7 ± 5%
	Final D/L ratio[c]	0.0034	0.0092	0.0088	0.0069	0.0006	0.098	0.0062	0.0008

[a] *Determined by indirect method using Eq 1.*
[b] *Determined by indirect method using Eq 3.*
[c] *D/L: Drug / Lipid ratio (w/w) determined using Eq 4.*

Table 4. Kinetic parameters of doxorubicin release from the aptamer-loaded lipoplexes at pH 7.4 (see Figure 4) and pH 5 (See Figure S9). All kinetics were fitted using a bi-exponential fit. The fastest transition (described by Amplitude 1 and Rate 1) represents the release of doxorubicin from the lipoplexes and the slowest transition (described by Amplitude 2 and Rate 2) the degradation of doxorubicin over time. No degradation was evidenced at pH 5.

		Free Dox	Doxil-Like	Coop-Doxapt	Poly-Doxapt	Doxapt-30	Doxapt-28	Apta-Ctr-1
pH 7.4	Amp 1[1]	-102 ±8	-16 ± 2	-136 ± 23	-21 ± 4	-52 ± 9	-130 ± 20	48 ± 2
	Rate 1[2]	0.8 ± 0.1	1.2 ± 0.3	0.20 ± 0.05	0.04 ± 0.01	0.23 ± 0.06	0.20 ± 0.04	0.38 ± 0.07
	Amp 2[1]	99 ± 8	16 ± 1	128 ± 25	-	49 ± 10	123 ± 21	-
	Rate 2[2]	0.039±0.006	0.016±0.005	0.025±0.007	-	0.028±0.007	0.027±0.007	-
pH 5	Amp 1[1]	-	-	-93 ± 2	-34 ± 1	-55 ± 2	-80 ± 5	73 ± 3
	Rate 1[2]	-	-	0.30 ± 0.01	0.078 ± 0.007	0.28 ± 0.03	0.52 ± 0.07	0.30 ± 0.06

[1] *In %*
[2] h^{-1}



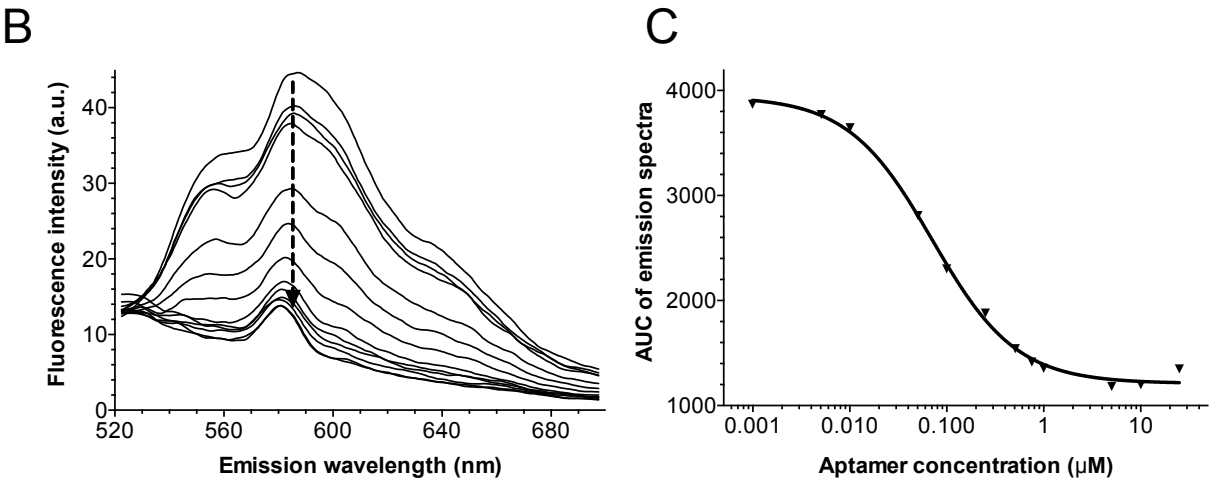
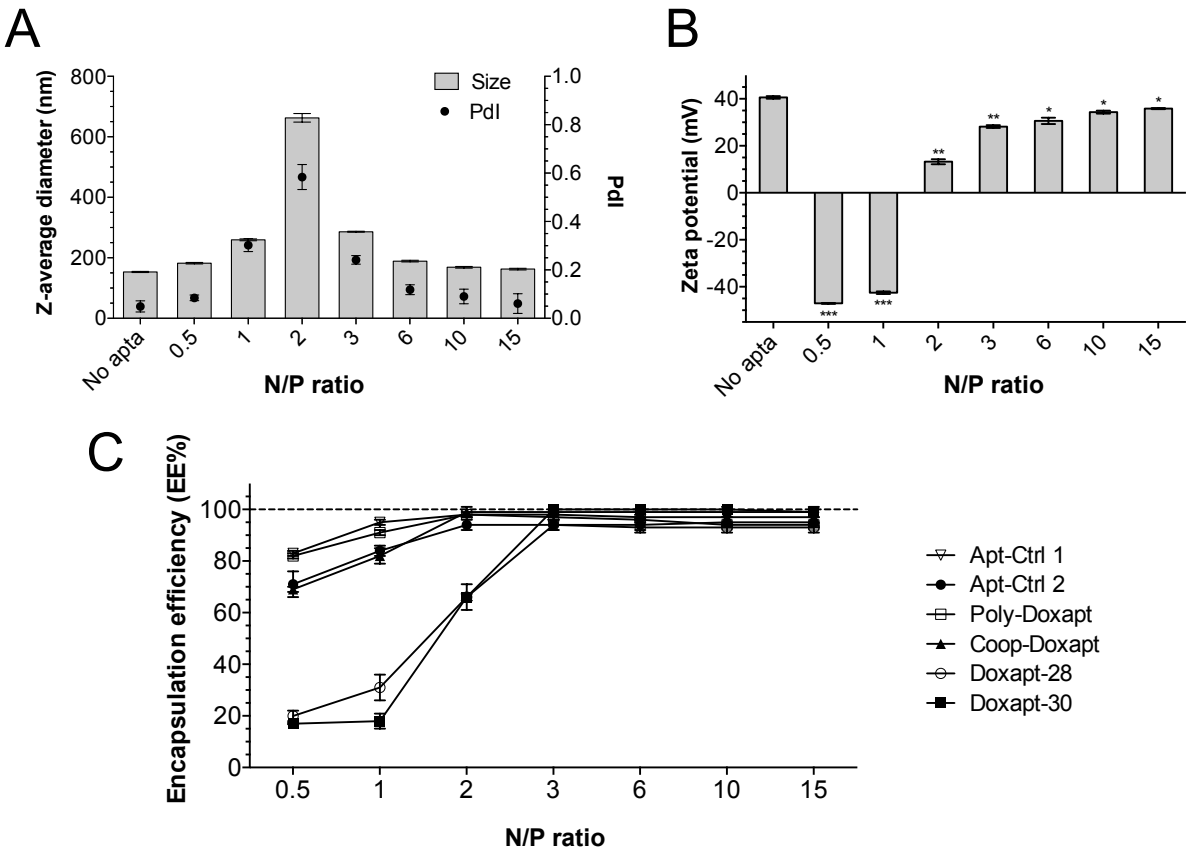


Figure 1. Design of doxorubicin-binding aptamers with various affinities. A) Structure of DNA sequences designed for doxorubicin. The binding site for doxorubicin is suggested by a dot. B) Smoothed emission spectra of doxorubicin (100 nM in 5% dextrose and 5 mM NaCl) with increasing concentration of Poly-Doxapt (from top to bottom, 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.25, 0.75, 1, 5, 10, 25 μM). C) Representative determination of affinity binding constant K_D for Poly-Doxapt. Each point represents the area under the curve of the fluorescence emission spectra showed in B).



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567 Figure 2. Optimization of aptamer-loaded liposomes (DOTAP/Chol/DSPE-PEG$_{2000}$ 50/48/2 molar
568 ratio) according to the N/P ratio (amine of DOTAP/phosphate of nucleobases). A) Hydrodynamic
569 diameter (Size) and polydispersity index (PdI) and B) Zeta potential measurements of lipoplexes
570 encapsulating Doxapt-30. Statistical analysis performed with two-tailed Student's t-test: *$p < 0.05$;
571 **$p < 0.01$; ***$p < 0.001$. C) Encapsulation efficiency of all aptamers within cationic lipoplexes.
572 Results are reported as the mean value of 3 measurements, ± standard deviation of the mean.

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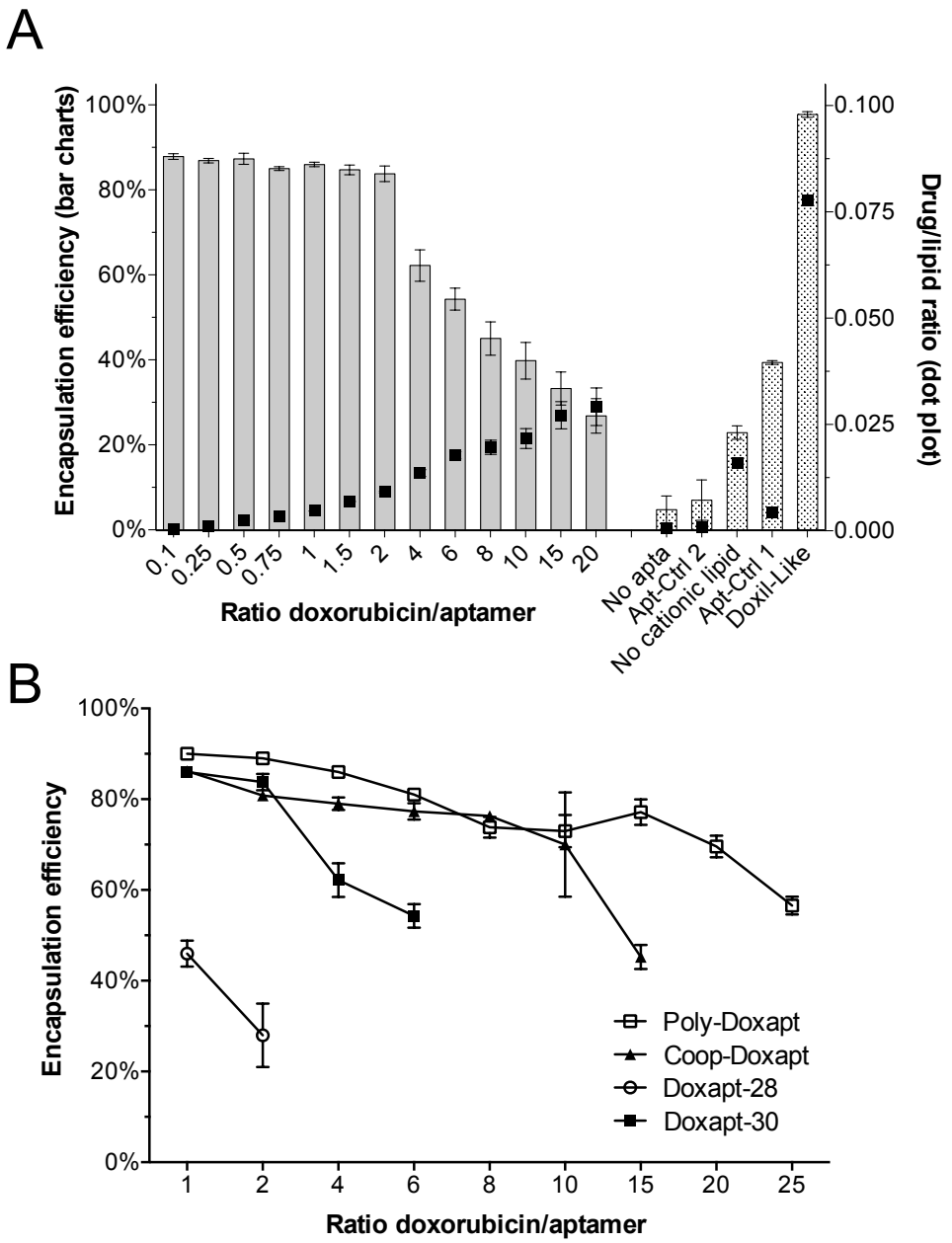

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575 Figure 3. Optimization of doxorubicin loading into aptamer-loaded lipoplexes. A) Doxorubicin
576 encapsulation efficiency (%, bar charts, left) and final Drug/Lipid (D/L) ratio (w/w, dot plot, right)
577 of Doxapt-30 lipoplexes (N/P = 3) at increasing molar ratios of doxorubicin/aptamer. 'No apta'
578 control consisted of the cationic liposome (DOTAP/Chol/DSPE-PEG_{2000} 50/48/2 molar ratio)
579 without any DNA. Apt-Ctrl-1 and Apt-Ctrl-2 were assessed at N/P = 3 and Doxorubicin/aptamer
580 = 2. 'No cationic lipid' is a liposome of POPC/Cholesterol/DSPE-PEG_{2000} 55/40/5 representative
581 of a neutral formulation for passive encapsulation, tested at D/L = 0.086. Doxil-Like liposome was
582 assessed at a D/L = 0.1. B) Encapsulation efficiency of doxorubicin within all aptamer-loaded
583 lipoplexes. For each aptamer, N/P ratio was selected according to the optimized formulation (Table
584 3): N/P = 2 for Poly-Doxapt and Coop-Doxapt. N/P = 3 for Doxapt-28 and Doxapt-30. Results are
585 reported as the mean value of 3 experiments, ± standard deviation of the mean.

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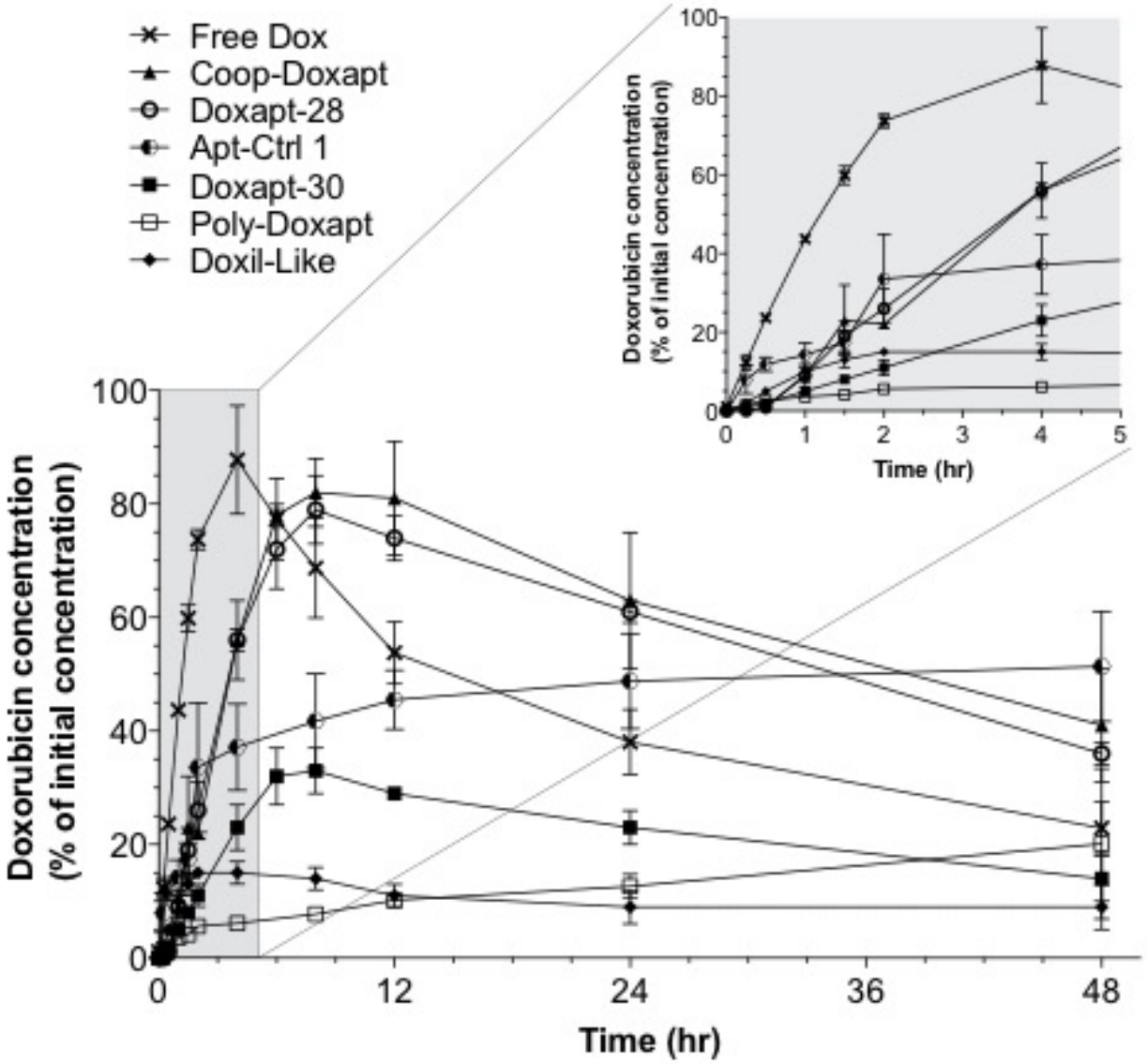

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588 Figure 4. Release of doxorubicin at 37 °C in PBS (pH 7.4) from optimized formulations of
589 doxorubicin-loaded lipoplexes (see Table 3). 'Free Dox' corresponds to the initial amount of
590 doxorubicin (1 mL of 15 μM doxorubicin) placed in the dialysis bag. Results are reported as the
591 mean value of 3 experiments, ± standard deviation of the mean.

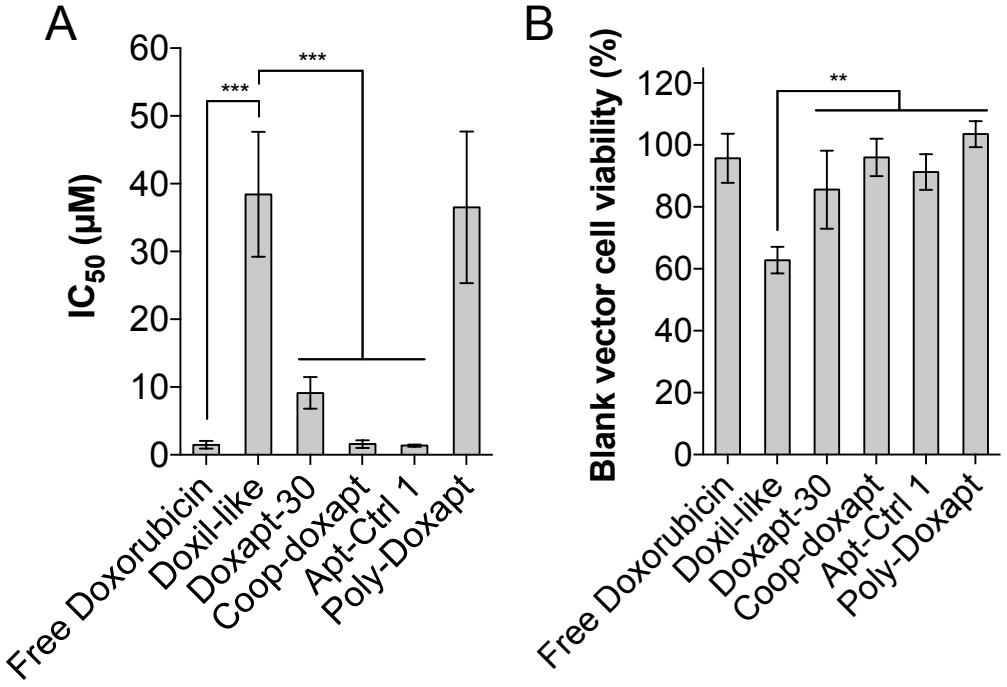

Figure 5. Cytotoxic activity of doxorubicin in optimized formulations (see Table 3) on HeLa cells. A) IC_{50} values of doxorubicin in optimized formulations after 48h. B) Cell viability of aptamer-loaded lipoplexes without doxorubicin at their highest concentration used for IC_{50} assay (corresponding to 25 µM doxorubicin). Results are reported as the mean value of 3 experiments, ± standard deviation of the mean. *** $P \leq 0.05$.

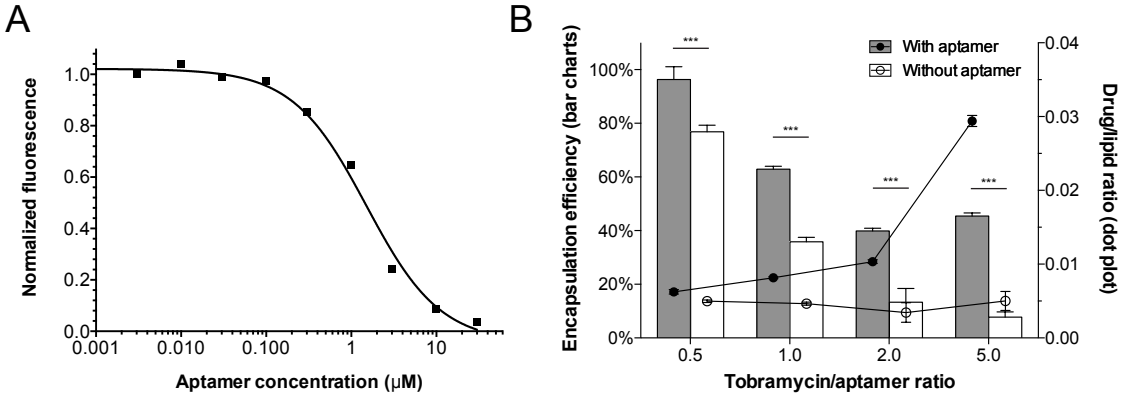

Figure 6. Tobramycin encapsulation using aptamers. A) Binding affinity of Apt-Ctrl-1 for Tobramycin-Cy5. $K_D = 1.15 \pm 24$ µM. B) Tobramycin encapsulation efficiency (%, bar charts, left) and final Drug/Lipid ratio (w/w, dot plot, right) of aptamer-loaded lipoplexes (DOTAP/Chol/DSPE-PEG_{2000} 50/48/2 molar ratio, aptamer Ctrl-1, N/P = 3). Results are reported as the mean value of 3 experiments, ± standard deviation of the mean. *** $P \leq 0.05$.

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