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Aptamer-based liposomes improve specific drug loading and release

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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 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.

1. 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-

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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.

2. 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 min at 95 °C, vortexed for 1 min 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 min at 37 °C in the same cuvette, producing DNA concentrations ranging from 0.001 to 25 μM with incrementing volumes <20% of final volume. Emission scans were taken at a high resolution of 100 nm/min and data were smoothed with a 10-neighbors Savitzky-Golay factor. The area under curve of the full scan was considered for the analysis. The dissociation constant (K_D, nM) was calculated using GraphPad 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 magnitude and M_3 is the K_D. In the definition of Poly-Doxapt concentrations, one strand A complexed with one strand A′ was considered as one aptamer molecule (two binding sites).

2.3. Preparation and characterization of liposomes

All liposome formulations, except Doxil-like, were prepared using the hydration method. Briefly, stock solutions of lipids in chloroform (20–40 mg/mL) were stored under argon at −80 °C before use. For the preparation of cationic liposomes, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(polyethylene glycol)-2000 (DSPE-PEG_{2000}) solutions were combined in a 10-mL round bottom flask in a 50/48/2 molar ratio to get 30 μmol total lipid amount. The solvent was evaporated under reduced pressure at 50 °C. The dried lipid film was hydrated 30 min at 60 rpm with 1 mL of 5% dextrose and 5 mM NaCl. "No cationic lipid" formulation consisted in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), cholesterol and DSPE-PEG_{2000} in a 55/40/5 molar ratio. All liposomes were extruded through 400 and 200 nm polycarbonate membranes using a LiposoFast manual extruder (Avestin Inc., Ottawa, ON, Canada) at room temperature. Doxil-like liposomes were prepared following the same procedure with minor modifications. Lipid composition was 55% 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 40% cholesterol and 5% DSPE-PEG_{2000} (10 μmol total lipid amount). The dried lipid film was hydrated 30 min at 65 °C (60 rpm) with 1 mL of a 120 mM ammonium sulphate salt solution. Extrusion was performed at controlled temperature (65 °C) to ensure the fluidity of the lipids, using a homemade heating block for the manual extruder. Finally, Doxil-like liposomes were purified on a 1 × 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 MilliQ 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 min at 95 °C, vortexed for 1 min 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 (0.5–15). 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 min, 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 >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 (1–150 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 min at 18,500*g* at room temperature. The supernatant was diluted to fit in the linear range (1–150 nM) and SYBRGreen 100× (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