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Co-Delivery of Hydrophobic and Hydrophilic Drugs from Nanoparticle–Aptamer Bioconjugates

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Numerous nanoparticle (NP) drug delivery vehicles have emerged during the past two decades including, for example, polymeric NPs, block copolymer micelles, liposomes, dendrimers, and hydrogels, many of which have been engineered for improved serum stability, biocompatibility, and in vivo circulation time.[1–4] A promising application of nanoparticle (NP) drug delivery systems is the targeted delivery of therapeutic agents in a cell-, tissue-, or disease-specific manner. This goal may be achieved by the surface-modification of NPs with antibodies, nucleic acid ligands (aptamers; Apt), peptides, or small molecules that bind to antigens present on the target cells or tissues.[5,6] Another promising application of NPs may be the co-delivery of different drugs using the same NP delivery device. Compared to delivering a single drug, co-delivery of multiple drugs has several potential advantages, including: 1) synergistic effects,[7] 2) suppressed drug resistance,[8] and 3) the ability to tune the relative dosage of various drugs to the level of a single NP carrier.

Liposomes and cationic core-shell NP have been developed to simultaneously deliver drugs and DNA plasmids to cells.[9,10] More recently, a novel multifunctional system comprising of polymeric NPs entrapped within liposomes has also been described that can deliver two hydrophilic drugs in a temporal manner.[11] One major challenge in engineering multifunctional nanoparticles is maintaining the simplicity in design to assure future scale-up, while incorporating the desired functionalities such as targeted delivery, temporally regulated drug release, and the ability to simultaneously deliver distinct drugs. A logical approach would be to develop systems where a minimum number of components can achieve the cumulative desired functionalities.

We had previously reported the development of targeted polymeric NPs and demonstrated their efficacy against prostate cancer (PCa) cells in vitro and in vivo.[12,13] More recently we reported the development of targeted physical conjugates of Doxorubicin and the A10 RNA Apt that binds to the prostate specific membrane antigen (PSMA) on the surface of PCa cells.[14] Herein, we report a novel NP drug delivery system that bridges these two simple concepts to co-deliver both hydrophobic and hydrophilic drugs in a temporally distinct and targeted manner. The components of the NPs include 1) aptamers that target the delivery and uptake of nanoparticles to a subset of cells while simultaneously acting as fast releasing carriers for hydrophilic intercalating drugs, and 2) polymeric NPs that act as relatively slow releasing carriers for hydrophobic drugs. As illustrated in Figure 1, using docetaxel (Dtxl) as a model small molecule hydrophobic drug; doxorubicin (Dox) as a model intercalating hydrophilic drug; the A10 RNA Apt which binds to the PSMA on the surface of PCa cells as a model aptamer targeting ligand;[15] and poly(D,L-lactic-co-glycolic acid)-block-poly(ethylene glycol) (PLGA -b-PEG) block copolymer as a model controlled release polymer system, we developed targeted NPs that can co-deliver Dox and Dtxl to PSMA expressing PCa cells intracellularly. The A10 PSMA Apt is a 57 base pair nuclease-stabilized 2′-fluoropyrimidine RNA molecule with a single 5′-CG-3′ sequence in its predicted double stranded stem region that is the preferred binding site of Dox.[16] Incubation of Dox with the A10 PSMA aptamer results in formation of a reversible physical conjugate, with a final Dox/Apt stoichiometry of 1:1.1, consistent with the intercalation of the Dox into a single CG sequence present in this aptamer, as previously described.[14] In this study, we hypothesized that the conjugation of a Dox-loaded aptamer with Dtxl-encapsulated polymeric NPs may result in vehicles for targeted delivery of Dox and Dtxl—two widely used chemotherapeutics with distinct water solubility properties.

We used the biocompatible and biodegradable PLGA-b-PEG copolymer to formulate Dtxl encapsulated NPs (~1 wt% Dtxl) with a diameter of 62 ± 1.5 nm using the nanoprecipitation method.[17] NP surfaces were next functionalized with the A10 PSMA Apt that was preloaded with Dox. The resulting targeted NP-Apt bioconjugate carries and releases both Dtxl and Dox. The drug loading efficiency and release rate of Dtxl and Dox from the NP–Apt system was determined in PBS at 37 °C under gentle stirring. The drug content in solution was assayed over time and quantified using high performance liquid chromatography (HPLC) (experimental section). Our data suggests that the relative carrying capacity of Dtxl to Dox in each NP–Apt bioconjugate is approximately 9:1 (molar ratio), respectively, a property that can be tuned by controlling the total amount of each drug in the formulation process. The release profiles of Dtxl and Dox from the NP–Apt bioconjugates is shown in Figure 2. Approximately 50% and 80% of the initial dose of Dtxl was released from the polymeric core of the NP–Apt during the first 6 and 25 h, respectively. Conversely, Dox re-

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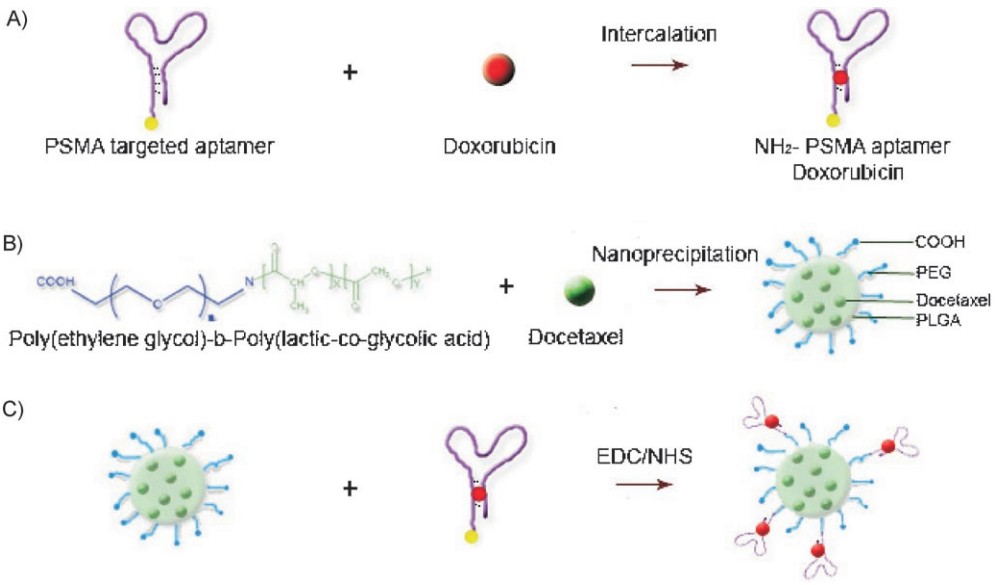

Figure 1. Schematic illustration of A) the intercalation of a hydrophilic anthracycline drug, such as Dox within the A10 PSMA aptamer; B) the encapsulation of a hydrophobic drug, such as Dtxl, within the PLGA-b-PEG nanoparticles using the nanoprecipitation method; and C) nanoparticle–aptamer (NP–Apt) bioconjugates comprised of PLGA-b-PEG nanoparticles surface functionalized with the A10 PSMA aptamer for co-delivery of Dtxl and Dox. Both drugs can be released from the bioconjugates over time.

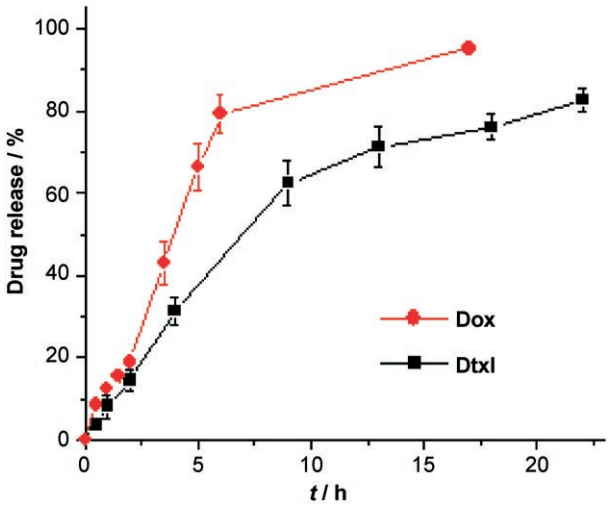

Figure 2. Drug release of docetaxel (black squares) and doxorubicin (red circles) from NP–Apt bioconjugates at 37 °C in PBS measured by HPLC. The average molar ratio of docetaxel to doxorubicin carried by each bioconjugate is 9:1.

lease from the aptamer component of the NP–Apt was relatively fast, such that 50% and 80% of intercalated Dox was released within four and six hours, respectively. The difference in release rates between Dtxl and Dox is attributed to their relative hydrophobicity and their distinct loading mechanisms. The former has low water solubility (0.025 mg L^{-1}) and is readily encapsulated within the hydrophobic core of NPs. The release of Dtxl depends on diffusion through the polymer matrix and on the hydrolysis of the PLGA polymer. Conversely, Dox is more soluble in water (10 g L^{-1}) and is more exposed to the aqueous solution, with release requiring only dissociation from the surface-bound aptamer.

Next we demonstrated that NP–Apt bioconjugates are capable of co-delivering Dtxl and Dox selectively to target cells. LNCaP prostate adenocarcinomas, which express the PSMA antigen on their plasma membrane, were chosen as the target cell line for in vitro testing; PC3 prostate adenocarcinomas, which do not express the PSMA antigen, were employed as a negative control.[13] To visualize cell uptake of drugs using fluorescence microscopy, a hydrophobic fluorescent probe, NBD-cholesterol (22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol) (Excitation/Emission = 460 nm/534 nm), was encapsulated inside the PLGA-b-PEG NPs as an analogue of a hydrophobic drug, while taking advantage of the fluorescence emission spectrum of Dox, which is in the red region to track the delivery of Dox (experimental section). Figure 3 shows that both NBD and Dox were effectively delivered into LNCaP cells by NP–Apt bioconjugates. Consistent with our expectations, no NBD was delivered into PC3 cells, and the relatively small amount of Dox signal appearing in the PC3 nucleus may represent a portion of free Dox released from the NP–Apt bioconjugates during incubation with cells, consistent with the observation that Dox release from the conjugates is relatively fast.

After having confirmed the feasibility of using NP–Apt bioconjugates to co-deliver model hydrophobic and intercalating hydrophilic drugs to target cells, we further examined the in vitro cellular cytotoxicity of NP–Apt bioconjugates carrying both Dtxl and Dox [NP(Dtxl)–Apt(Dox)], Dtxl alone [NP(Dtxl)–Apt], Dox alone [NP–Apt(Dox)], or no drug [NP–Apt] to LNCaP and PC3 cell lines. The MTT cell proliferation assay results (Figure 4) show that for LNCaP cells treated with the same dose of drugs, NP(Dtxl)–Apt(Dox) are more cytotoxic than all controls. Relative

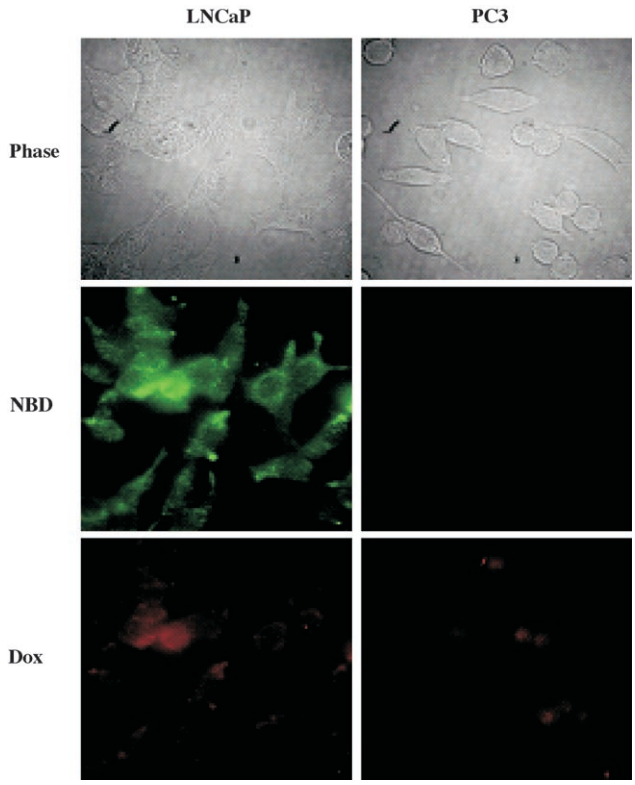

Figure 3. Binding of PSMA targeted nanoparticle–aptamer bioconjugates to LNCaP (+PSMA) and PC3 (-PSMA) prostate epithelial cells. The data demonstrates that NBD (green), serving as an analogue of a hydrophobic drug encapsulated within the nanoparticles, and doxorubicin (red) serving as a model hydrophilic anthracycline drug intercalated within the aptamers were both selectively delivered to LNCaP cells which express the PSMA protein (left panel), but not PC3 cells (right panel). The dim red fluorescence in PC3 cells is likely due to small amount of doxorubicin released from the bioconjugates during incubation with the cells; note that Dox can diffuse through cell membranes.

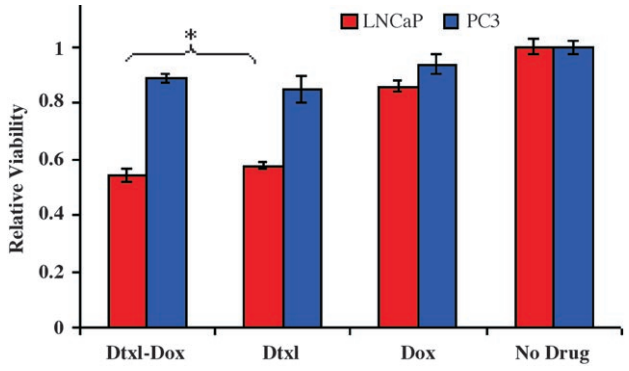

Figure 4. MTT assay to measure the cytotoxicity of NP–Apt bioconjugates carrying both Dtxl and Dox [NP(Dtxl)–Apt(Dox)]; Dtxl alone [NP(Dtxl)–Apt]; Dox alone [NP–Apt(Dox)]; or no drug (NP–Apt) to LNCaP and PC3 cell lines. All NP–Apt bioconjugates were incubated with cells for 6 h, and the cells were subsequently washed and incubated in media for a total of 72 h before assessing cell viability in each group ($n=4$); *denotes statistical significance by one-sided two-sample *t*-test with equal variances, $p=0.029$.

cell viability of NP(Dtxl)–Apt(Dox) is 54% in contrast to 58%, 86%, and 100% using NP(Dtxl)–Apt, NP–Apt(Dox), and NP–Apt respectively. A one-sided, two sample t-test with equal variances was used to confirm that the observed differences between NP(Dtxl)–Apt(Dox) and NP(Dtxl)–Apt were statistically meaningful ($p=0.029$, $n=4$), and the differences between NP-(Dtxl)–Apt(Dox), NP–Apt(Dox), and NP–Apt are all statistically significant ($p<0.005$, $n=4$). Equality of variances was confirmed by F-test. These results suggest that co-delivery of Dtxl and Dox is more efficient than treating cells with the same amount of single drugs. The synergistic effects between the two chemotherapeutic drugs were obtained but did not reach a statistically significant level in this study. One possible reason is that the molar ratio of Dtxl to Dox carried by each NP–Apt bioconjugate on average is 9:1. Speculatively, an alternative aptamer with rich CG bases would enhance Dox carrying capacity, thereby, enhancing synergistic effects between drugs. The relative lack of toxicity on PC3 cells confirm the specificity of the NP(Dtxl)–Apt(Dox) bioconjugate system although some prereleased drugs during the period of sample preparation induced cell apoptosis. This is consistent with what was found in Figure 2.

In summary, herein we report a novel targeted drug delivery system consisting of NP–Apt bioconjugates that can simultaneously deliver both hydrophobic taxane and hydrophilic nucleic acid intercalating drugs to cancer cells. The loading capacity and relative ratio of the two drugs are amenable to fine tuning by varying the amount of each drug during formulations. Other strategies, such as the incorporation of multiple CG sequences within the stem of the aptamer used for targeting, may further titrate the relative loading of the Dox to Dtxl on the nanoparticle surface for each unique clinical application. The future optimization of these simple targeted nanoparticles may allow co-delivery of two distinct classes of drugs for cancer therapy, a disease which is often treated using combination chemotherapy. This targeted co-delivery system may also allow for temporally distinct release of drugs, which may have implications for delivery to distinct anatomical locations.[11]

Experimental Section

HPLC measurements: The release of docetaxel (Dtxl) and doxorubicin (Dox) from targeted nanoparticle–aptamer bioconjugates [NP(Dtxl)–Apt(Dox)] was performed in PBS buffer at 37 °C using a Slide-A-Lyzer MINI dialysis microtube with a molecular weight cutoff of 3500 (Pierce, Rockford, IL). To measure the drug release profile of Dtxl, NP(Dtxl)–Apt(Dox) PBS solutions (10 mg mL^{-1}, 3 mL) were split equally into 30 MINI dialysis microtubes (100 μL per microtube). These microtubes were dialyzed in PBS buffer (4 L) at 37 °C with gentle stirring. At each data point, NP(Dtxl)–Apt(Dox) solutions from three microtubes were collected separately and mixed with an equal volume of acetonitrile to dissolve the nanoparticles. The resulting free Dtxl content in each microtube was assayed using an Agilent (Palo Alto, CA) 1100 HPLC equipped with a pentafluorophenyl column (Curosil-PFP, 250 × 4.6 mm, 5μ; Phenomenex, Torrance, CA). Dtxl absorbance was measured by a UV/Vis detector at 227 nm and a retention time of 12 min in 50/50 acetonitrile/water mobile phase (1 mL min^{-1}). To measure the release of Dox, the same NP(Dtxl)–Apt(Dox) solution (300 μL) was equally distributed into three MINI dialysis microtubes. These microtubes were each dialyzed in PBS buffer (1 mL) at 37 °C with gentle stir-

ring. At each data point, 100 μL samples from each dialysate were gathered, replaced by the same amount of fresh PBS buffer, and then assayed by HPLC with a UV/Vis detector at a wavelength of 490 nm and a retention time of 3 min in 40/60 acetonitrile/water mobile phase (1 mL min^{-1}).

Fluorescence microscopy measurements: To visualize cell uptake of drugs using fluorescence microscopy, a hydrophobic fluorescent dye, NBD-cholesterol (22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol, Invitrogen, Carlsbad, CA), was encapsulated inside PLGA-b-PEG nanoparticles as an analogue of a hydrophobic drug. The fluorescence emission spectrum (Excitation/Emission = 460 nm/534 nm) of NBD was detected mainly in the green channel (490 nm/528 nm) of a Delta Vision RT Deconvolution Microscope. The fluorescence emission spectrum of Dox (Excitation/Emission = 540 nm/600 nm, Pierce, Rockford, IL) allowed it to be visualized in the red channel (560 nm/617 nm) of a Delta Vision RT Deconvolution Microscope. In the study, the prostate LNCaP and PC3 cell lines were grown in 8-well microscope chamber slides in RPMI-1640 and Ham's F-12K medium, respectively, both supplemented with aqueous penicillin G (100 units ml^{-1}), streptomycin (100 μg mL^{-1}), and 10% FBS (Fetal Bovine Serum) at concentrations to allow 70% confluence in 24 h (that is, 40000 cells per cm^2). On the day of experiments, cells were washed with prewarmed PBS buffer and incubated with prewarmed fresh media for 30 min before adding NP(NBD)–Apt(Dox) bioconjugates with a final dye concentration of ~1 μg mL^{-1} ($n = 4$). Cells were incubated with the bioconjugates for 2 h at 37 °C, washed twice with PBS (300 μL per well), fixed with 4% formaldehyde, and mounted with nonfluorescent mounting medium DAPI (Cector Laboratory, Inc., Burlingame, CA). The cells were then imaged using a Delta Vision RT Deconvolution Microscope.

MTT cell viability assay: The prostate LNCaP and PC3 cell lines were grown in 24-well plates in RPMI-1640 and Ham's F-12 K medium, respectively, both supplemented with aqueous penicillin G (100 units mL^{-1}), streptomycin (100 μg mL^{-1}), and 10% FBS (Fetal Bovine Serum) at concentrations to allow 70% confluence in 24 h (that is, 40000 cells per cm^2). On the day of experiments, cells were washed with prewarmed PBS buffer and incubated with prewarmed fresh media for 30 min before adding NP(Dtxl)–Apt(Dox) bioconjugates with a final drug concentration of ~1 μg mL^{-1} ($n = 4$), approximately 0.93 μg mL^{-1} Dtxl and 0.07 μg mL^{-1} Dox bearing in mind that the molar ratio between Dtxl and Dox is about 9:1. Three other systems, NP–Apt bioconjugates carrying Dtxl (~0.93 μg mL^{-1}) alone [NP(Dtxl)–Apt], Dox (~0.07 μg mL^{-1}) alone [NP–Apt(Dox)], and no drug [NP–Apt], were chosen as controls for both cell lines. Cells were incubated with the bioconjugates for 6 h at 37 °C, washed twice with PBS (1 mL per well), and then incubated in fresh growth media for a total of 72 h. Cell viability was assessed colorimetrically with the MTT reagent (ATCC) following the standard protocol provided by the manufacturer.

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