Targeted Aptamer-Nanoparticles to Diminish Drug Resistance of Cancer Cells *in vitro* Study

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ABSTRACT

Using prostate cancer as a model, we report the drug release of Docetaxel and doxorubicin from aptamer-poly (D,L lactic co glycolic acid)-poly(ethylene glycol) (PLGA-PEG) nanoparticles used to target prostate-specific membrane antigen (PSMA), a well characterized antigen that is expressed on the surface of prostate cancer cells. In our study, the doxorubicin non-covalently intercalated in the aptamer on the surface of the nanoparticles and the Docetaxel is encapsulated within the bulk polymer unit for a powerful delivery method of both drugs at different release rates acting synergistically. *In vitro* toxicity assays have shown the targeting efficiency of these nanoparticles and the combinatorial effect of the two drugs are higher than a single drug delivery system. These targeted drug delivery nanoparticles could be used as a powerful therapeutic tool, in comparison to the single drug approach, to combat cancer cells displaying drug resistance.

Keywords: aptamer, prostate cancer, targeted delivery, poly (D,L-lactic-co-glycolic acid) (PLGA), doxorubicin

1 INTRODUCTION

Chemotherapeutic drug resistance significantly hinders the effective treatment and cure of the majority of cancers. A chemotherapeutic strategy currently being used is the implementation of a combination of drugs that will interfere with multiple cellular processes, which decreases the chance of recurrence. Prostate cancer is known to exhibit drug resistance where continuation of single drug treatment has no effect. Multiple drugs have shown positive effects in eliminating cancer cells when locally administered such as docetaxel and doxorubicin. Docetaxel leads to the accumulation of microtubules, thereby stopping the formation of mitotic spindles which interrupts cell division of the cancer cells. Due its unique structure, doxorubicin functions as an inhibitor of topoisomoerase II progression which kills the cells by stopping replication. It is also used for its fluorescent properties and its notable quenching when bound to nucleic acids. This drug has shown to increase cardiotoxic side effects due a decline in mitochondrial oxidative phosphorylation leading to an increase in reactive oxygen species and damaging myocytes; therefore should be used in targeted delivery in clinical practice [1].

Targeted polymeric nanoparticles with therapeutic uptake represent a powerful technology on the frontier of drug delivery for widespread applications in medicine such as cancer treatment. Recently, our group and others have used aptamers for therapeutic and diagnostic targeted delivery [2]. In prior experiments, we have shown successful targeted deliverance of docetaxel-encapsulated nanoparticles (NPs) *in vivo* against prostate cancer cells [2]. Additionally, we have shown specific intercalation of doxorubicin into aptamers to allow release within a time frame of a few hours [1]. Using prostate cancer as a model, we examined the effect of using novel multi-drug targeted nanoparticles in decreasing the thereby providing potential to overcome the effect of drug resistance in cancer cells.

2 MATERIALS & METHODS

2.1 Materials

All chemicals were acquired from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Poly($_{D,L}$-lactic co glycolic acid) (PLGA; inherent viscosity of 0.67 dL/g) was purchased from Absorbable Polymers International (Pelham, AL, USA). Polyethylene glycol (PEG) polymer with both carboxylic acid and terminal amine functional groups (NH_2-PEG_{3400}-COOH) was synthesized by Nektar Therapeutics (San Carlos, CA). The A10 2'-fluoropyrimidine RNA aptamer (selected sequence: 50-NH2-spacer-GGG/AGG/ACG/AUG/CGG/AUC/AGC/ CAU/GUU/UAC/GUC/ACU/CCU/UGU/CAA/UCC/UCA/ UCG/GCiT-30 with a 50-amino group attached by a hexaethyleneglycol spacer, 20-fluoro pyrimidines, and a 30-inverted T cap) was purchased from RNA-TEC (Leuven, Belgium). Buffers for molecular biology were purchased from Boston BioProducts (Worcester, MA,

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USA). Tissue culture materials and both LNCaP and PC3 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA).

2.2 Aptamer-Drug Intercalation

Doxorubicin was intercalated with the PSMA aptamer using the same method as in prior studies [1]. The ratio of doxorubicin to aptamer used was 1:1.2, with non conjugates rinsed out prior to the nanoparticle-aptamer conjugation step as seen in Figure 1A.

2.3 Nanoparticle Synthesis

The carboxylate- functionalized copolymer (PLGA-b-PEG) was synthesized using EDC/NHS chemistry as done previously [6]. Docetaxel encapsulated nanoparticles were generated using nanoprecipitation using a 5% drug load [3]. The nanoparticles were formed by dissolving both drug and polymer in organic solvents and then adding this mixture dropwise to water with a volume ratio of 1:3 to mix for 4 hours at room temperature. The mixture was purified by three rounds of ultrafiltration (15 min, 3000g in Amicon Ultra, Ultracel membrane with 100,000 NMWL, Millipore, Billerica, MA, USA) [6]. Quasi-elastic laser light scattering using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, NY; 15 mW laser with an incident beam of 676 nm) was used to determine he size, 60 nm, and the surface zeta potential, -20 (mV, of the nanoparticles.

2.4 Nanoparticle-Aptamer Conjugation

The conjugation of the Docetaxel encapsulated PLGA-PEG nanoparticles with the doxorubicin conjugated PSMA RNA aptamer was performed using EDC/NHS chemistry as done in prior research, with the aptamer being first dissolved in DNAse/RNAse free water and then heated to an activated conformation at 90°C for 5 minutes [5]. The

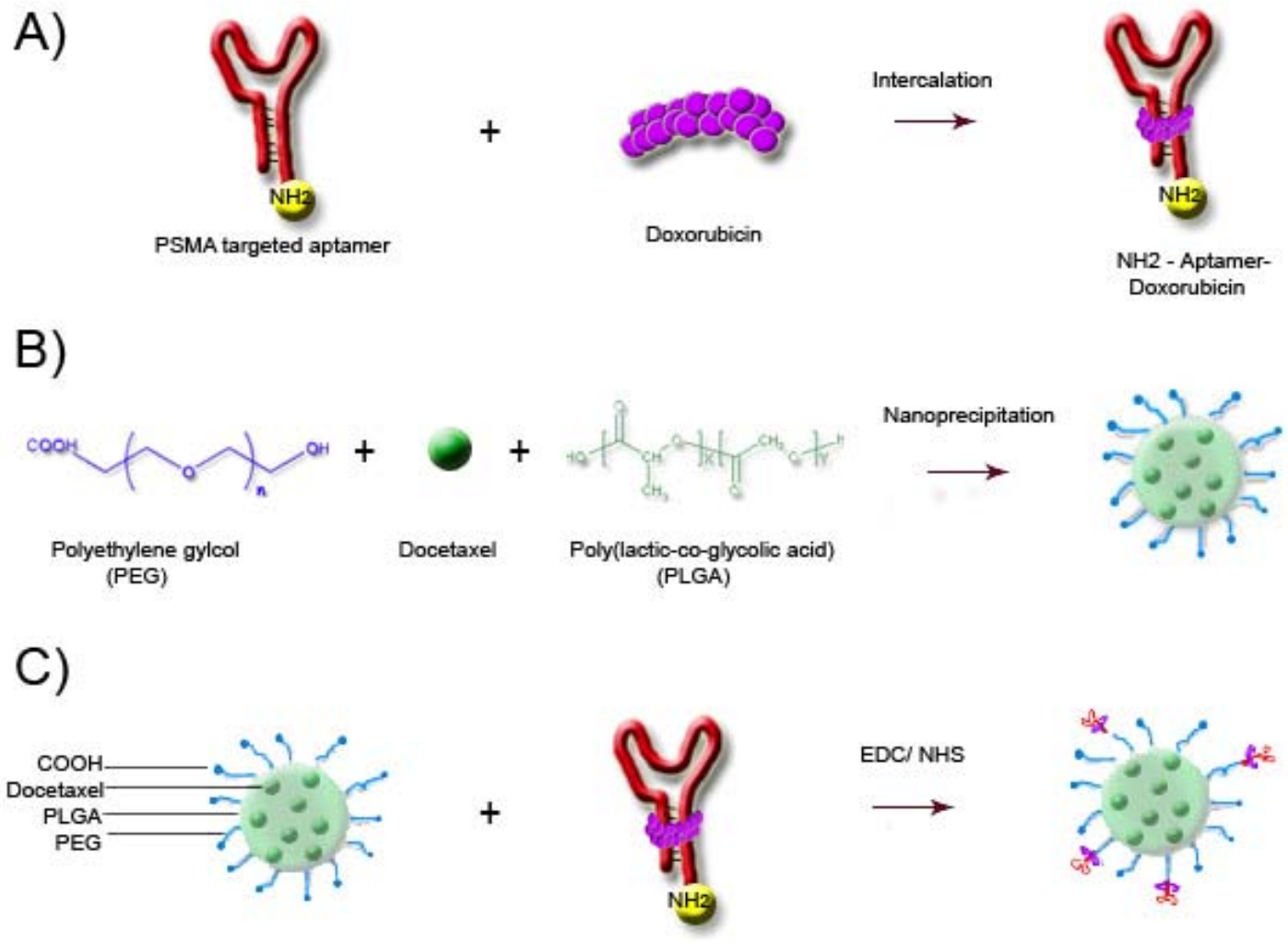

Figure 1: Schematic illustrating the steps required to create a duo-delivery nanoparticle system. A) Intercalation of the doxorubicin and PSMA aptamer B) Docetaxel encapsulated nanoparticles are generated providing functional groups on the PEGylated surface C) The conjugation of the PSMA aptamer using EDC/NHS chemistry yields the final drug delivery vehicle.

resulting NP-Aptamer bioconjugates were washed with ultra pure water by ultra filtration to remove aptamer that did not attach.

2.5 Drug Content Determination

The conjugation of the docetaxel encapsulated PLGA-PEG nanoparticles with the aptamer-doxorubicin conjugates was performed using EDC/NHS chemistry. To determine the docetaxel content, the nanoparticles were dissolved in acetonitrile and measured by HPLC in triplicates. The equipment used was the Agilent 1100 HPLC (Palo Alto, CA) equipped with a UV detector and a reverse-phase pentafluorophenyl column (Curosil-PFP, 250_4.6 mm, 5 m, Phenomenex, Torrance, CA, USA) using a non-gradient mobile phase of water and acetonitrile (v/v 50/ 50) at a constant flow rate of 1 mL/min. The docetaxel and doxorubicin peaks were measured at wavelength of 227 nm and 490 nm respectively, and then determined quantitatively by comparing with standard curves.

2.6 Cell Uptake of Multi-Drug System

The prostate cancer PC3 and LNCaP cell lines were grown in chamber slides in Ham's F12K medium and RPMI 1640, respectively, both supplemented with 100 units/mL aqueous penicillin G, 100 g/mL streptomycin, and 10% fetal bovine serum at concentrations to allow 70% confluence in 24 h (*i.e.*, LNCaP: 40,000 cells/cm^2). The day of the experiment, the cells were washed with prewarmed PBS and then incubated with prewarmed phenol-red–reduced OptiMEM media for 30 minutes before the addition of nanoparticle-aptamer bioconjugates with a concentration of 2 ug/ml. Cells were incubated for two hours at 37°C, washed with PBS three times, fixed with 4% paraformaldehyde, mounted with non-fluorescent 4,6-diamidino-2-phenylindole. To visualize cell uptake of nanoparticle-aptamer bioconjugates using fluorescence microscope, hydrophobic fluorescent probe NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yl) (Ex/Em=460nm / 534 nm) was encapsulated inside PLGA-PEG nanoparticles, while fluorescence emission from doxorubicin was employed to illuminate itself and its conjugates with aptamer. In study, fluorescent images were carried out using a computerized Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging, Thornwood, NY) with an x40 air objective (Numerical aperture 0.7).

3 RESULTS & DISCUSSION

In development of our steps to conjugate the nanoparticles, we had to examine the role of surface charge of positively charged doxorubicin to the negatively charged polymer surface. In examination of fluorescence spectra of triple washed doxorubicin and PLGA-PEG nanoparticles without aptamer, less than 7% of doxorubicin bound to the nanoparticles (Figure 2). This indicates that the nanoparticle itself is not a significant carrier of free doxorubicin. With the conjugation of aptamer on the surface, even less doxorubicin is able to reach the surface. Nonetheless, the conjugation protocol was adapted to ensure that only intercalated doxorubicin is able to reach the cell, thereby to better the control of the drug release system (Figure 1).

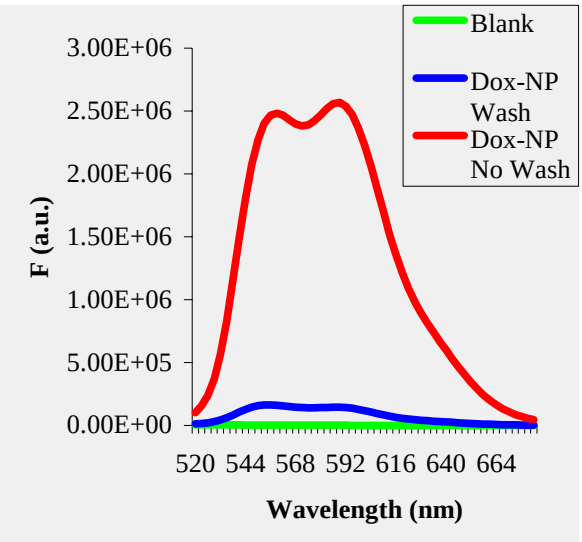

Figure 2: Fluorescence spectra of doxorubicin solution and 1 mg/mL nanoparticles used as a control to show the affinity of doxorubicin to the surface of the polymer nanoparticles.

In cellular uptake studies, the multi-drug delivery system showed successful targeted delivery to LNCaP cells which is known to display PMSA antigens on the surface in comparison to a negative control, the PC3 cell line which is known to not have a detectable amount of PSMA protein on the surface. Since both doxorubicin and docetaxel were fluorescently tagged, the nanoparticle and drugs uptake by the cell is able to be qualitatively visualized (Figure 3). These images suggest that both the ligand used is effective at localized targeting as well as that multiple drugs can be taken up simultaneously, thereby providing a new tool to effectively administer therapeutics.

4 CONCLUSION

As a key strategy to fighting cancer, multiple drug therapy is crucial in combating the effects of single drug resistance. We have studied the effects of docetaxel encapsulated targeted nanoparticles and doxorubicin intercalated aptamer targeting separately and have combined our techniques to create a novel platform for multi-drug delivery that promises to be therapeutically more effective than current treatment.

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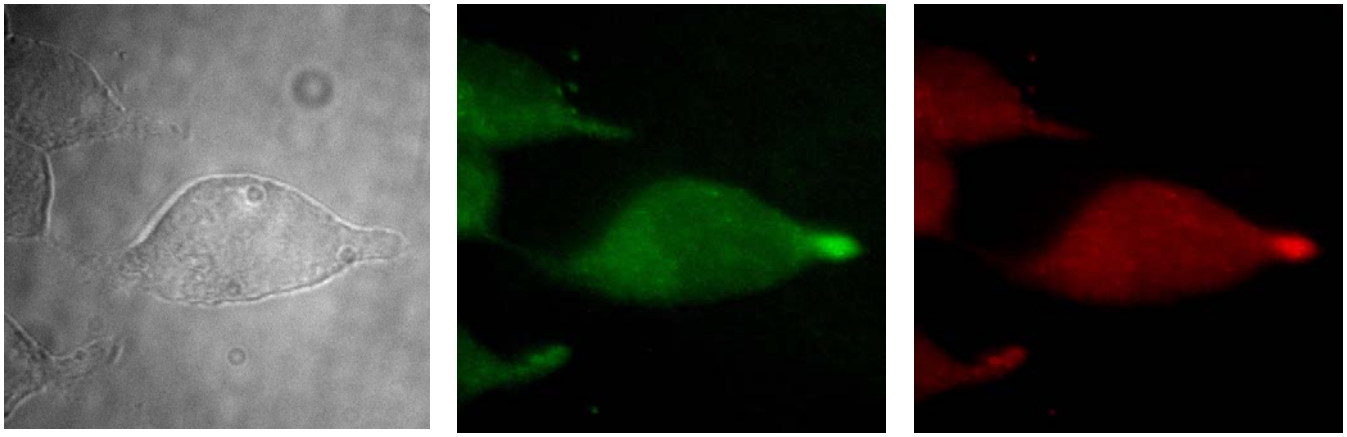

Figure 3: DeltaVision captured fluorescence spectra of doxorubicin solution and nanoparticles used as a control against doxorubicin NP binding. [Left] Phase image of individual cells; [Center] Fluorescence image of NBD shows the uptake of nanoparticles by cells;[Right] Fluorescence image of doxorubicin shows the uptake of uptake of doxorubicin only or the bioconjugates. However, the highly matched fluorescent images indicated that the nanoparticle-aptamer bioconjugates were kept intact after taken by cells.

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