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COMMUNICATIONS

Published on Web 03/20/2008

Reductively Labile PRINT Particles for the Delivery of Doxorubicin to HeLa Cells

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Herein we report a Trojan horse PRINT (Particle Replication in Nonwetting Templates) particle composition based on a disulfide cross-linker that releases doxorubicin in response to a reducing environment and that is capable of killing HeLa cells in vitro without the addition of an external reducing agent. The application of nanotechnology to biological systems is fundamentally altering the landscape of medicine. One such example is the translation of precision fabrication technologies developed for use in the microelectronics industry to the realm of bioengineering. PRINT has now made it possible to fabricate discrete organic nanoparticles with complete control over particle size, shape, and composition.[1,2] With PRINT, these parameters can be tuned independently providing the flexibility critical to the field of engineered drug therapies where strategies for targeting, stealthing, cargo loading, and release must be pre-engineered into the delivery vector.[3] Nanoparticles synthesized using the PRINT technology offer distinct advantages over other delivery vectors currently in use, such as liposomes,[4,5] dendrimers,[6,7] conjugates,[8,9] viruses,[10] and micelles.[11] One of these advantages lies in the ability to alter the matrix chemistry of the nanoparticle, which allows one to design in a cargo release strategy without affecting other characteristics of the particle such as shape or size.

The goal in designing an activated release delivery system is to engineer a delivery vector that is stable during circulation, but degrades rapidly in response to a predefined stimulus at the desired location to release its cargo.[12] Activated release of therapeutics can reduce systemic exposure to the drug and, when coupled with an effective targeting strategy, offers a powerful tool to reduce adverse side effects to traditional chemotherapeutics. Fréchet and co-workers[13,14] have prepared particles containing a pH-sensitive acetal cross-linker that were shown to breakdown rapidly in response to a drop in pH like the one known to occur in endosomes. More recently, Matyjaszewski and co-workers have reported[15] the synthesis of nanogels containing a disulfide cross-linker that were destabilized upon exposure to glutathione, which aims to capitalize on the fact that the cytosol is known to be reducing.[16–18] A number of groups have taken advantage of the reductive intracellular environment by conjugating drugs or biomolecules directly to carriers through disulfide bonds, which cleave intracellularly facilitating release.[19]

Trojan horse PRINT particles (cube side length = 2 μm) with or without a reductively labile disulfide cross-linker were prepared using the monomers shown in Figure 1 in the proportions shown in Table 1. Doxorubicin (Dox) encapsulation was achieved by dissolution in the prepolymer matrix solution. Molds having the desired feature shape and size were filled with these prepolymer solutions (65:35, solids/solvent in DMSO), and the contents were solidified using standard free radical

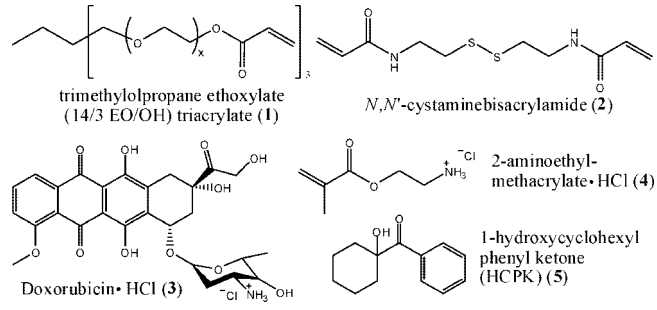

Figure 1. Prepolymer matrix components of Trojan horse PRINT particles.

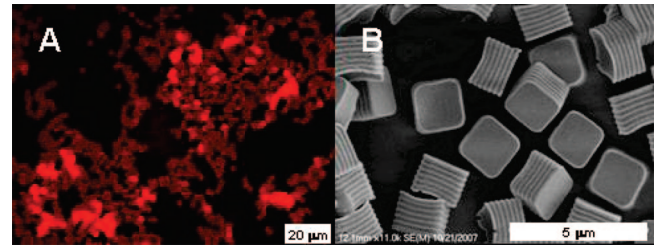

Figure 2. Fluorescence (A), and SEM (B) images of cube side length = 2 μm, Dox-loaded Trojan horse PRINT particles (composition A). Ridges observed in panel B are due to Bosch etch lines, which originate in the silicon master template and are an artifact of the etching process.

Table 1. PRINT Particle Compositions

composition	1	2	3	4	5
A	57	30	2	10	1
B	87	0	2	10	1
C	57	30	0	10	1

chemistry.[1,20] Because Dox is fluorescent, encapsulation could be confirmed using fluorescence microscopy (Figure 2). A burst release of Dox (84% of Dox encapsulated) occurred upon stirring the particles in aqueous solution overnight. HPLC analysis confirmed that the major compound being released was unmodified Dox.

Cell studies were conducted using unmodified, positively charged particles (primary-amine containing). Positive charge was used to facilitate particle uptake by HeLa cells. Particles used for cargo release studies were further modified to have avidin on their surface and have a negative ζ-potential. These particles more closely reflect those that will be used in future targeting studies; however, in the absence of targeting ligands they were not expected to be internalized by HeLa cells. The negative charge attenuates nonselective cellular uptake while avidination allows for the attachment of biotinylated targeting ligands. Details of avidination will be reported elsewhere.

10.1021/ja801436j CCC: $40.75

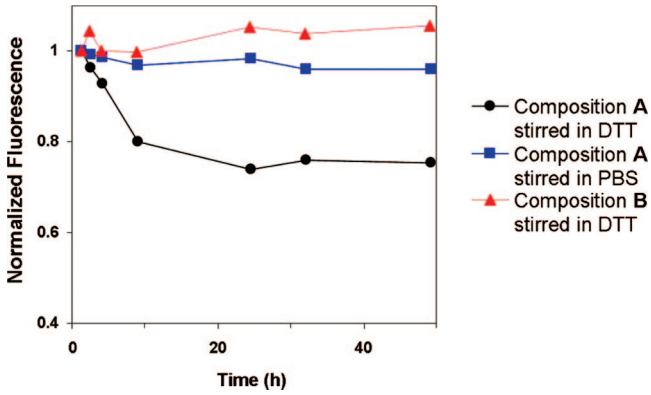

Figure 3. Normalized mean fluorescence intensity as a function of time for avidinated composition A stirred in PBS alone or PBS + 100 mM DTT, and avidinated composition B stirred in PBS + 100 mM DTT.

Particles used in the studies described here had cube side length = 2 μm (Figure 2).

Flow cytometry was used to monitor release of Dox from the particles. Briefly, individual particles were identified by their characteristic forward and side scatter properties, gated, and their mean fluorescence determined. Avidinated PRINT composition A stirred in PBS solution for 48 h showed little change (~4%) in their mean fluorescence indicating that Dox was not being released from the particles during that time (Figure 3). A second set of identical particles stirred in a PBS solution containing 100 mM dithiothreitol (DTT) for the same period of time exhibited markedly different behavior (Figure 3). With reductant present, the mean fluorescence of the particle population decreased by roughly 25%, indicating significant release of Dox.[21] These two pieces of data clearly demonstrated that Dox release from Trojan horse PRINT particles can be triggered by a reducing environment.

Further evidence was obtained using a second set of particles where the disulfide cross-linker was replaced with a triacrylate one. These particles were also stirred in 100 mM DTT in PBS; however, little change in their mean fluorescence was observed over the same 48 h period (Figure 3). These experiments confirmed that release of Dox from Trojan horse PRINT particles was triggered by reductant and was not the result of passive diffusion. We believe that reduction of the disulfide bonds leads to a decrease in the mesh density of the particle making it more porous, which allows Dox to diffuse out.

Next, we examined the ability of these Trojan horse PRINT particles to kill HeLa cells in vitro. Unmodified PRINT composition A, B, C, or free Dox were dosed on HeLa cells for 72 h followed by assessment of cell viability using a cyQUANT assay (Figure 4).

Cell viability at the highest dosing of composition C showed no toxicity to the cells and indicated a general level of biocompatibility of the PRINT particle matrix materials. PRINT composition A was extremely efficient at killing HeLa cells with more than 50% of the cells killed at a dosing of 160 μg/mL PRINT particles while PRINT composition B showed no toxicity. At the highest particle dosing, PRINT composition A was almost as efficient at killing HeLa cells as free Dox with only 10% of the cells viable (at the highest dosing a small reduction in viability for PRINT composition B was also observed, presumably due to passive release).

In summary, a Trojan horse PRINT particle composition was developed that incorporates a reductively labile cross-linker to

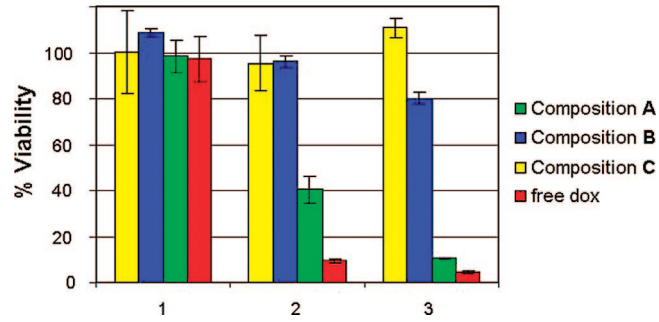

Figure 4. Cell viability data for HeLa cells dosed with unmodified PRINT composition A, B, C, or free Dox. Dosing of particles and Dox for 1–3 was 2.5, 160, 640 μg/mL, and 0.05, 3.2, and 12.8 μg/mL, respectively. (Dox concentrations are based on total dox encapsulated.)

achieve activated release of Dox. Particles of discrete size and shape (cube side length = 2 μm) containing 30 wt % of a disulfide-based cross-linker and 2 wt % Dox were synthesized and could be surface-functionalized with avidin allowing for the incorporation of targeting ligands for future studies. This PRINT composition was shown to release Dox in response to a reducing environment as measured by flow cytometry and was found to be proficient at killing HeLa cells in vitro.

Acknowledgment. We thank Chris Luft for many useful discussions. This work was funded in part by Liquidia Technologies, Inc., DARPA (W911NF-06-1-0343), Juvenile Diabetes Research Fund (A08-0273-001), STC Program of the NSF (CHE-9876674), and NIH U54-CA119343 (the Carolina Center of Cancer Nanotechnology Excellence).

Supporting Information Available: Complete details of particle fabrication, flow cytometry experiments, cellular uptake, and cell viability assays.

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(21) Elution time of released product was identical to that of an authentic Dox solution prepared as a standard.

JA801436J