Investigating the acoustic release of doxorubicin from targeted micelles

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Abstract

The main problem associated with the administration of anti-cancer medication is that the drug is delivered throughout the body causing undesirable side effects. Therefore, it is important to synthesize drug carriers capable of minimizing the adverse side effects of chemotherapy by preferentially targeting tumor cells both actively (e.g. a folate receptor) and using external stimulus (e.g. ultrasound). In this paper, we report the synthesis of Pluronic P105 micelles with a folate targeting moiety (with a yield of 48%) containing doxorubicin (Dox). We applied low frequency ultrasound as an external stimulus and measured the amount of release of Dox from these folated micelles. The results showed that the percent drug release increases as the power intensity of ultrasound increases. The maximum amount of release (14%) was measured at 5.4W/cm^2. A power density threshold at approximately 0.55W/cm^2 exists below which no statistically significant release was observed. This lower threshold suggests that cavitation plays an important role in triggering drug release from targeted micelles.

1. Introduction

While small polymeric drug carriers reduce the harmful side effects of chemotherapeutic agents on the body and demonstrate enhanced permeation and retention (EPR) at the tumor site, they have several drawbacks. These include a short blood half-life and low specificity. Nano-sized carrier assemblies are rapidly eliminated from the blood due to the recognition by the macrophages of the mononuclear phagocyte system (MPS) as a consequence of the adsorption of blood proteins onto the surface of the carriers. This causes the accumulation of the carriers in MPS organs, such as the liver and the spleen. For this purpose polyethylene glycol (PEG) is used in the modification of the carrier surface to increase its circulation time in the blood [1]. However, to enhance intracellular uptake of drug delivery vehicles, an active targeting moiety is helpful. Selective targeting to tumor cells is provided by non-antigenic ligands such as folic acid, which is reported to solve the problem of site-specific targeting and to induce endocytotic internalization [2].

Site-specific targeting is possible because a wide variety of human tumors have shown a significant overexpression of a folate binding protein, a glycosylphosphatidylinositol-anchored cell surface receptor for folic acid (FA) [3]. The approach is to use targeting of drug carriers with phospholipid- anchored folate conjugates. Carriers containing the chemotherapeutic drug must be long circulating and possess the ability to bind to the folic acid receptors on surface of tumor cells [4].

Experiments with folate-targeted doxorubicin (Dox) have shown that cells that overexpress the folate receptor, including ovarian and breast carcinomas, demonstrate significant uptake of the drug [5]. Flow cytometry and confocal image analysis showed that micelles with conjugated folate were taken up by cervical cancer cells (KB cells) overexpressing folate receptors at a significantly higher rate when compared to non-targeted micelles [6].

Control cells (e.g. W1-38 fibroblasts) take up a lesser amount of the drug since they do not express the folate receptor when compared to Hela and MCF 7 cells (folate-positive cell lines). Studies have also shown that the number of folic acid targeting ligands needs to be optimized in order to optimize Dox uptake [7]. Furthermore, the folic acid/polymer ratio has been found to influence selective cytotoxicity, cellular uptake and intracellular localization of the drug delivery vehicle [8,9].

Our ideal carrier is envisioned to be capable of securely retaining the drug and releasing it upon the application of an external stimulus. While these new micelles will enter the tumor using the EPR effect similar to other drug delivery vehicles, they will bind to the tumor cells instead of flowing on through and being collected by the lymphatic system. Thus we expect their accumulation in the tumor will be higher than other micelles that depend solely on the EPR effect. Ultrasound has been found to be a viable drug delivery trigger mechanism due to its ability to propagate into deep tissue and the fact that it can be specifically focused on the target tumor [10]. Ultrasound releases the drug from the carrier and enhances intracellular uptake of both released and encapsulated drug [11–18]. The advantages of ultrasound are that it is non-invasive, penetrates deep into the body, has a synergistic effect on the activity of drugs, and enhances transport of drugs through various membranes and tissues [15]. Furthermore, ultrasonic waves can be carefully localized through focusing, and are capable of inducing hyperthermia.

The purpose of this study is (1) to report the synthesis of Pluronic P105 micelles labeled with a folic acid moiety and (2) to quantify the acoustic release of Dox from these newly synthesized micelles.

2. Materials and methods

P105-FA was synthesized using 1,1-carbonyldiimidazole (CDI, Sigma–Aldrich) [19]. Approximately 1.026 g of folic acid (FA, Sigma–Aldrich) was dissolved in 100 ml of dried DMSO. Then 0.414 g CDI was added and allowed to react for 4 h under dark conditions at room temperature. After this activation of the FA, 30.2 g of Pluronic P105 (BASF), which was dried overnight under vacuum, was added to the above solution. The activated FA and Pluronic P105 were allowed to react for 20 h at room temperature in darkness. At the conclusion of the reaction, the product was dialyzed (Spectra Millipore MWCO 3500) against DMSO for 2 days and then against DD-water for 2 days. The purified product was then lyophilized and stored at −20◦C. The formation of P105-FA was confirmed using NMR which showed a broad peak at 3.7 ppm (attributed to the PEG backbone) and characteristic peaks attributed to folic acid at 2.3, 6.6, 7.6 and 8.6 ppm. These NMR peaks were also used to calculate a yield of 48%.

For release experiments, P105-FA was dissolved in PBS to make a final concentration of 5 wt.% (5 wt.% P105-FA, 95 wt.% phosphate buffered saline (PBS)). Dox (Sigma–Aldrich) was introduced into the micelles by mixing at room temperature to a final concentration of 10 g/ml. The drug-loaded micellar solution was degassed overnight. The average hydrodynamic diameter was measured by dynamic light scattering measurements (DLS) using a Brookhaven instrument (model BI-DSI) equipped with a Lexel 95 laser source (514 nm at room temperature) at a fixed angle of 90◦. The average diameter of these micelles was measured to be 10.2 ± 2.2 nm.

A custom chamber was built to measure the change in fluorescence [20] and hence the Dox release in the presence of ultrasound. The beam of an argon ion laser (Ion Laser Technology, Model 5500A) was directed to a metal-film neutral density beam splitter. The power of the split portion of the beam was measured by a photodetector (used to monitor the laser power) and the other portion of the beam was directed into a fiber optic bundle.

The drug concentration was quantified by measuring fluorescence emissions produced by an excitation wavelength of 488 nm. A fiber optic probe (100 bundled multimode fibers, approximately 40 cm in length) was used to deliver the excitation light to the sample and to collect fluorescence emissions. The emitted light was passed through a dielectric bandpass filter (Omega Optical Model 535DF35) to a silicon photodetector (EG & G 450-1). The filter was used to cut off any emissions below 517 nm, including any Rayleighscattered laser light. Fluorescence measurements were digitized for computer storage and processing. To mimic physiological conditions, the temperature of the ultrasonic exposure chamber was maintained at 37◦C using a thermostated bath.

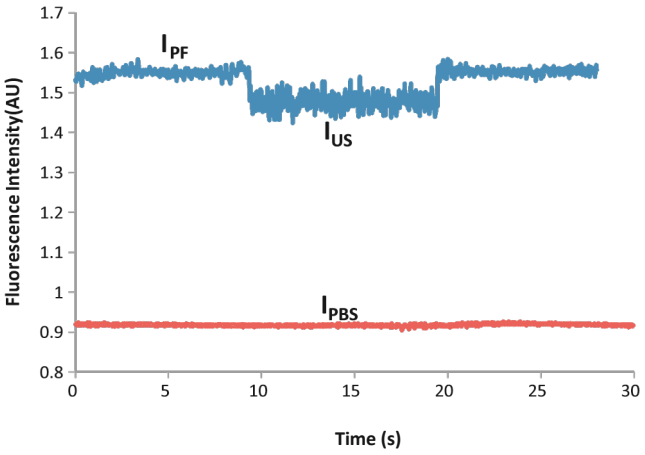

Fig. 1. An example showing one release pulse for folated micelles at 5.01 W/cm^2.

The chamber described above was used to measure the kinetics of acoustically activated drug release from micelles. Dox exhibits a large decrease in fluorescence when transferred from the hydrophobic core of the micelle to the surrounding aqueous solution (Fig. 1). Therefore, the release can be determined by measuring the decrease in fluorescence intensity upon the application of ultrasound. Fig. 1 is a representative example of one release pulse at 5.01W/cm^2 for acoustic release of Dox from targeted micelles.

The three distinct regions that make up the pulse correspond to the fluorescence intensity of Dox prior to insonation (I_{PF}), the intensity when the ultrasound is switched on (I_{US}), and the intensity when the ultrasound is switched off (where re-encapsulation of drug occurs). The flat profile below the pulse corresponds to the fluorescence intensity of Dox in phosphate-buffered saline (PBS) and represents 100% release where all the Dox molecules interact with water molecules. Additionally, Fig. 1 reveals fast reencapsulation of the released drug when the ultrasound is turned off. This is important because it suggests that non-internalized drug would go back inside the drug delivery vehicle and circulate in the encapsulated form upon leaving the sonicated volume, which in a clinical setting, will reduce unwanted drug interactions with normal tissues. In these release experiments, we used a 15 ml micellar solution at a Dox concentration of 10 g/ml. When calculating the percent release, fluorescence data were corrected to account for Dox quenching by folic acid. Ultrasound was applied using a 70kHz ultrasonicating bath (SC-40, Sonicor, Copiaque, NY) equipped with a single piezoceramic transducer. The best description of the acoustic waveform is that of a 70-kHz wave amplitude modulated sinusoidally at about 0.12 kHz. The bath was powered by 60-Hz AC voltage from a variable AC transformer (variac). The voltage from the variac to the sonicating bath was adjusted to produce differing intensities of continuous ultrasound, and these intensities were measured at the point of optical fluorescence excitation using a calibrated hydrophone (Bruel & Kjaer model 8103, Decatur, GA). The ultrasound was manually turned on and off every 10 s.

3. Results and discussion

After successfully synthesizing the folate-labeled Pluronic P105 micelles, we measured the amount of Dox release as a function of

ultrasound intensity at 70 kHz. In evaluating the results, we relied on the fact that Dox fluorescence changes according to its microenvironment as mentioned above.

Fig. 2 summarizes the percent of drug release from folated micelles as a function of acoustic power density at 70 kHz. There are three distinctive regions in this plot. At low powers, the measured drug release is very small and not significantly different

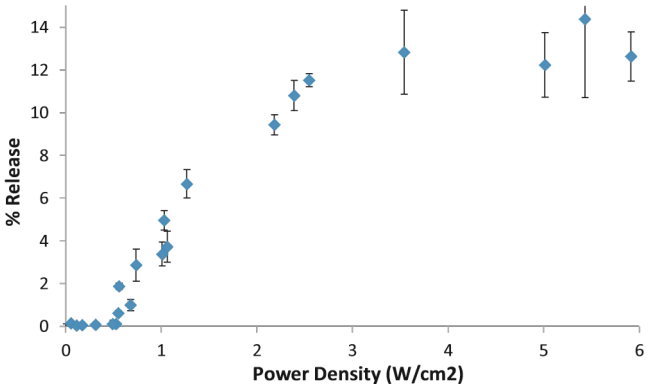

Fig. 2. Percent release of Dox from targeted micelles as a function of intensity at 70-kHz ultrasound. Error bars represent standard deviations (N=8).

($p>0.085$ observed at 0.53W/cm^2) from release in sham conditions (no ultrasound). The next region is bounded on the lower end by a threshold at about 0.55W/cm^2 ($p=0.028$—the first power density with statistically significant release compared to the sham experiments), above which release increases somewhat linearly with power density up to about 3W/cm^2. The third characteristic region is a constant amount of release at power densities above 3W/cm^2.The amount released is approximately 12%, which is slightly higher than previously reported release of about 9% from non-folated micelles [2]. The existence of thresholds in this data implicates a role of inertial cavitation in the drug release. Assuming planar wave models of ultrasound, the threshold of 0.55W/cm^2 at 70kHz correspond to a mechanical index (MI) of 0.46 in water [21]. Above this level of MI, inertial cavitation is possible in water, depending upon the presence of seed microbubbles in the fluid. The beginning of the plateau at 3W/cm^2 corresponds to a MI of 1.13. While there is no theoretical explanation for an upper plateau, it has been observed in other experiments [22,23]. It may not be a true plateau in disruption of micelles, but rather the achievement of a net steady state in which the rate of re-encapsulation of Dox equals the release rate [24].

We posit that shock waves produced by transient cavitation events disrupt micelles and allow their contents to be released transiently into the aqueous environment. In vivo work is currently being conducted to test the feasibility of using these newly synthesized folated micelles in combination with ultrasound as a drug delivery system.

Although degassed solutions were used in these in vitro experiments, and we did not add contrast agents (bubbles), there apparently were enough gas cavitation nuclei to generate cavitation at a mechanical index of 0.46. This is slightly higher than similar experiments using non-folated micelles [22], but we cannot determine whether the difference is due to folate labeling or to better degassing. When this technology is translated to an in vivo application, there may be even fewer gas cavitation nuclei in blood or interstitial tissues. However our previous experiments with nonfolated micelles show that there are sufficient cavitation nuclei in vivo to produce a therapeutic effect without resort to extremely high values of mechanical index [14]. We anticipate the forthcoming in vivo experiments will address these issues.

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