Degradation kinetics of stabilized Pluronic micelles under the action of ultrasound

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Abstract

The majority of research in the area of acoustically-activated drug delivery from stabilized micelles has been focused on the rapid release of chemotherapy drugs from the core of such nano-carriers. Previous publications have shown that low-frequency ultrasound is able to release approximately 2% of Doxorubicin (Dox) from the core of Pluronic P105 micelles stabilized using a cross-linked network of N,N-diethylacrylamide (NanoDeliv™) within 2 s of applying 70-kHz ultrasound. Here we use a custom-made ultrasound exposure chamber with fluorescence detection to measure the long-term fluorescence emissions of Dox from the NanoDeliv™ after 2 h of exposure to two ultrasound frequencies, 70 and 476 kHz, at a mechanical index of 0.9. Fluorescence measurements are then used to deduce the degradation kinetics of stabilized Pluronic micelles during 24 h following exposure to ultrasound. Results show that ultrasound does disrupt the covalent network of the stabilized micelles, but the time constant of network degradation is very long compared to the time constant pertaining to drug release from micelles. Experiments also show no significant difference in degradation rates when employing the two frequencies in question at the same mechanical index.

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1. Introduction

The kinetics of drug release from nanosized vehicles constitute an integral aspect of the carrier's design. Many research efforts have been focused on careful measurements of such kinetics since rates of drug release and carrier degradation dictate clinical drug concentrations and durations of administration in addition to the extent of side effects experienced by the patient. A plethora of reports are found in literature related to real-time measurements of drug release and carrier degradation kinetics. These reports include release from liposomes [1–3], polymeric micelles [4–16], solid particles [17–22] and polymersomes [23].

For the past decade, our group has directed considerable attention towards examining the rates of Doxorubicin (Dox) release by ultrasonic stimulation from stabilized and unstabilized polymeric micelles in an attempt to design a carrier capable of targeted delivery while minimizing the adverse side effects of systemic chemotherapy. Initially, we focused our efforts on studying the feasibility of using micelles formed by dissolving 10% Pluronic P105 in phosphate buffered saline (PBS) to retain hydrophobic agents and to cause their release by ultrasonic stimulus. Using the comet assay, we started by investigating the DNA damage induced by Dox delivered to human leukemia (HL-60) cells from Pluronic P105 micelles with and without exposure to ultrasound [6,15]. Results showed remarkable and beneficial synergism between ultrasound, Dox and Pluronic micelles.

Then we utilized a custom-made ultrasonic exposure chamber with real-time fluorescence detection to measure acoustically-triggered drug release from Pluronic P105 micelles under continuous wave (CW) or pulsed ultrasound. Drug release was greatest with 20-kHz ultrasound and diminished with increasing ultrasound frequency despite higher power densities at higher frequencies [7].

More recently, we published several articles on the extent and kinetics of Dox release from stabilized Pluronic micelles. Stabilized micelles were synthesized using Pluronic P105 with a cross-linked network of N,N-diethylacrylamide (NNDEA) capable of holding its core intact upon dilution [24]. Thus, its micellar integrity was conserved even following a 1000-fold dilution [24]. Fluorescence measurements using Dox encapsulated in stabilized micelles (NanoDeliv™) revealed a lower degree of drug release at the same frequencies and power intensities when compared to unstabilized micelles [10]. That less Dox is released from NanoDeliv™ is not surprising since the stronger integrity of the stabilized micelle cores renders them less susceptible to the shearing forces of cavitation microstreaming and shockwaves believed to cause the acousticallyactivated drug release. Further investigation of acoustically-activated micellar drug release correlated the onset of Dox release with the emergence of a subharmonic peak and a baseline shift in the acoustic spectra, suggesting a fundamental role of inertial cavitation in this release phenomenon [10,25].

The question remained as to whether cavitation events were releasing the drug by disrupting the interpenetrating network that stabilizes the micelle or if these events were shearing the micelle (and partially releasing the drug) without permanent structural damage to the micelle stability. In this work, we investigated the degradation kinetics of stabilized micelles for several hours following a two-hour application of ultrasound (US) at one of two frequencies, 70 kHz and 476 kHz. In order to properly compare the rate of degradation between the two frequencies under investigation, the same mechanical index (MI) was used throughout the experiments. The MI is calculated as the ratio of the peak negative pressure (in MPa) to the square root of the frequency (in MHz) [26]. This parameter indicates the likelihood and intensity of inertial cavitation events. The results presented herein show that ultrasound does disrupt the covalent network of the stabilized micelles, but the time constant of "post-insonation" release due to such degradation is very long compared to the very short time constant pertaining to rapid drug release from micelles during insonation.

2. Methods

2.1. Materials and methods

2.1.1. Materials

Pluronic® P-105 was provided by BASF Corp. (Mount Olive, NJ); N, N-diethylacrylamide (NNDEA) was obtained from Polyscience (Warrington, PA); N,N-bis(acryoyl)cystamine (BAC) was obtained from Fluka (Milwaukee, WI); and 2,2′-azobis(isobutyronitrile) (AIBN) was obtained from Aldrich (Milwaukee, WI).

Doxorubicin (Doxorubicin HCl, lyophilized, Bedford laboratories, Bedford, Ohio, USA) was obtained in dosage form (a 1:5 mixture with lactose). It was dissolved in PBS and sterilized by filtration through a 0.2 μm filter.

2.1.2. Drug encapsulation in stabilized Pluronic® micelles (NanoDeliv™)

To form the stabilized micelles (NanoDeliv™), a 40-mL aliquot of double distilled water containing 10 wt.% P105 was added to a roundbottom flask. Then NNDEA (monomer), BAC (cross-linker) and AIBN (initiator) were added. The resulting solution was connected to a water condenser and purged with nitrogen for 1 h. The system was allowed to polymerize for 24 h at 65 °C with magnetic stirring and a nitrogen purge for the first 4 h. Dox stock solution was added at room temperature to the resulting NanoDeliv™ to make a final concentration of 10 μg/ml. As a control formulation, the same drug concentration was also prepared in PBS.

2.1.3. Ultrasound

2.1.3.1. 70-kHz exposure conditions. Ultrasound was applied using a 70-kHz ultrasonicating bath (SC-40, Sonicor, Copiaque, NY) equipped with a single piezoceramic transducer driven at about 70 kHz. The best description of the waveform is that of a 70-kHz wave that is amplitude modulated sinusoidally at about 0.12 kHz. The bath was powered by 60-Hz AC voltage coming from a variable AC transformer (variac). The voltage from the variac to the sonicating bath was kept at a maximum (140 VAC) to attain the highest power density. To execute the experiments, the end of a coaxial fiber optic probe (which both introduces the excitation light and collects the fluorescence emission) was centered inside a polypropylene test tube placed at an acoustically intense position in the ultrasonicating bath. The bath was filled with degassed water and the tube containing the fiber optic probe was filled with the solution under investigation.

At 70 kHz, ultrasonic power density measurements were obtained using a calibrated hydrophone (Bruel and Kjaer model 8103, Decatur, GA) whose response was measured with an oscilloscope as follows. After measurements of Dox fluorescence, the fiber optic probe was replaced with the hydrophone in the same location, and the hydrophone response was recorded at the same settings as used for the fluorescence measurements. The average acoustic power density was calculated from $I=V^2_{rms}Q^2/Z$, where Q is a calibration factor obtained from the hydrophone manufacturer, Z is the acoustic impedance of water (1.5×10^6 kg/m^2 s), and V_{rms} is the root-mean squared voltage of the hydrophone signal. Using the hydrophone, the power density at 70 kHz was measured to be 1.89 W/cm^2, corresponding to an MI of 0.9. Dox-loaded micelles were sonicated continuously for 2 h before monitoring their fluorescence.

2.1.3.2. 476-kHz exposure conditions. The chamber used to expose stabilized micelles to 476-kHz ultrasound was an aluminum box of ~2 L volume lined with acoustically absorbing rubber on the bottom and sides. Placed on one wall was a 476-kHz ultrasonic transducer (Sonic Concepts, Woodinville, WA). An X–Y stage on top of the box positioned a tube containing the sample at the focal point of the transducer. A continuous sine wave at 476 kHz–produced by a signal generator (Hewlett Packard, model 33120A) – was sent to a power amplifier (ENI Model 240 L, Rochester, NY), which in turn sent the amplified signal through a matching network to the ultrasonic transducer. The signal was monitored on a digital oscilloscope. Power density (intensity) of the ultrasound at the focal point was measured in preliminary experiments using a calibrated hydrophone (HNR 1000, ONDA Corp., Sunnyvale, CA). To match the same MI of 0.9 used at 70 kHz (and thus be able to make proper comparison between the frequencies employed), continuous ultrasound was applied for 2 h at 13.5 W/cm^2 at the focal point of the 476-kHz transducer.

2.1.4. Measuring degradation kinetics of NanoDeliv™ with and without ultrasound

An ultrasonic exposure chamber with fluorescence detection was used to measure the long-term degradation kinetics of Dox from NanoDeliv™. Briefly, an argon-ion laser was used to excite Dox molecules at 488 nm. Emissions were then collected (at 580 nm) using a coaxial fiber optic collector. Dox fluoresces well in a hydrophobic environment (i.e., the micelle interior), but its fluorescence is partially quenched by water when it is released from the micelle. Thus by monitoring the fluorescence level for a period of 24 h after ultrasonication, we could deduce the kinetics of Dox release from the degradation of the micelles. Fluorescence measurements were digitized for computer storage and processing. Because of the long term nature of these experiments (24 h), a drift in the laser power was observed and recorded. In order to make proper comparison between our experiments, the fluorescence signal was normalized by dividing by the measured laser power, thus correcting for laser drift. For more details on our experimental apparatus, refer to our previous paper [10].

In addition to monitoring the fluorescence of insonated NanoDeliv™, we measured the decay in fluorescence of a Dox/PBS solution not exposed to ultrasound as a control. Furthermore, to make proper comparisons, we measured the decrease in fluorescence of a Dox/ NanoDeliv™ solution (of the same concentration) not previously exposed to ultrasound.

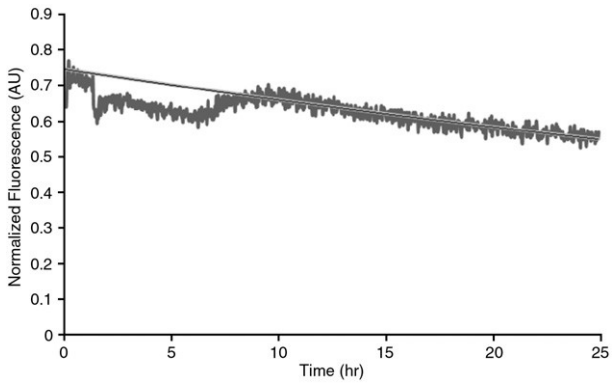

Fig. 1. A typical normalized fluorescence degradation curve. A NanoDeliv™ solution, loaded with 10 μg/ml Doxorubicin, was subjected to ultrasound for 2 h at 70 kHz immediately prior to this measurement. Note the inconsistent behavior during the first 10 h.

3. Results and discussion

Fig. 1 shows a typical normalized fluorescence degradation curve following 2 h of insonation at 70 kHz. As mentioned above, the raw fluorescence data were divided by the laser power to account for the slow drift in laser power. Throughout the course of all experiments, we noticed that fluorescence emission was unstable during the first 10 h, but decayed exponentially thereafter. For example, during some experiments we observed an initial increase in fluorescence followed by a decrease, while other experiments showed an initial decrease followed by a slow recovery in fluorescence value. In order to study the source of this inconsistency, we attached a CCD camera to a microscope and monitored the surface of the fiber optic probe, paying careful attention to the emergence and disappearance of macroscopic bubbles. The camera was mounted in such a way to enable the capture at 10-minute intervals of bubble events occurring at the tip of the fiber optic probe, where the Dox is excited and fluorescence is collected. Bubbles appeared to nucleate, migrate toward or away from the probe for the first several hours. However, images collected after 16 h revealed no macroscopic bubbles at the tip of the probe. We attribute the fluorescence behavior during the first 10 h (in Fig. 1) to the nucleation, movement and dissolution of macroscopic bubbles in the neighborhood of the fiber optic probe for the first several hours.

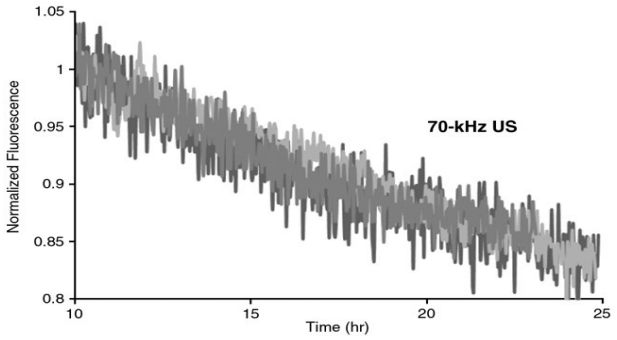

Fig. 2. Normalized fluorescence beginning 10 h after ultrasonicating NanoDeliv™ solutions, loaded with 10 μg/ml Doxorubicin, using a 70-kHz bath. The data display three superimposed experiments, each indicated by a different gray-scale and are scaled to start at 1 (or 100% encapsulation of Dox inside these micelles).

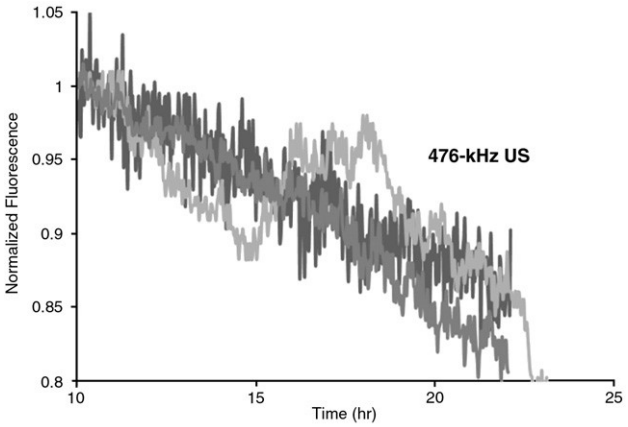

Fig. 3. Normalized fluorescence beginning 10 h after ultrasonicating NanoDeliv™ solutions, loaded with 10 μg/ml Doxorubicin, using a 476-kHz transducer. The data display three superimposed experiments, each indicated by a different gray-scale and are scaled to start at 1 (or 100% encapsulation of Dox inside these micelles).

Since these bubbles disappeared after about 10 h, we attributed the observed fluorescence decrease in the latter part of the experiment to the degradation of the NNDEA cross-linked network used to stabilize these micelles. The solid line shown in Fig. 1 is a first-order exponential decay that was fit to the final 14 h of the experimental data, which is expected to predict the fluorescence behavior in the absence of macroscopic bubbles. Since most bubbles appear to subside after 10 h of initiating our detection system, this paper analyzes only the last 14 h to calculate and compare the rate of micellar degradation with and without 2 h of insonation.

Figs. 2 and 3 show plots of normalized fluorescence beginning 10 h after ultrasonicating NanoDeliv™ solutions, loaded with 10 μg/ml Doxorubicin, using the 70-kHz bath and the 476-kHz transducer, respectively. Fig. 4 reports fluorescence behavior after incubating the Dox-micellar solution for 10 h as a control. All of these figures have the same scale for easy comparison. These plots demonstrate that a 2hour exposure to ultrasound increases the rate of Dox quenching (compared to the rate in the absence of prior insonation). In a micellar solution, the decrease in Dox fluorescence is attributed to the change in the drug's environment whereby it is transferred from the hydrophobic micelle core to the surrounding aqueous solution or to the penetration of water into the hydrophobic core where Dox molecules are concentrated. We attribute fluorescence quenching reported in Figs. 2, 3 and 4 to the degradation of the polymeric mesh that holds

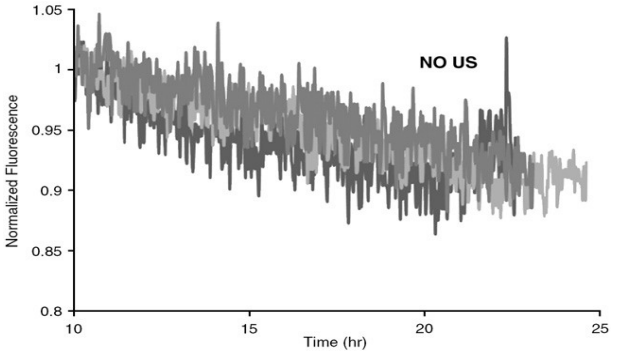

Fig. 4. Normalized fluorescence behavior after incubating the Dox-micellar solution (NanoDeliv™ solutions, loaded with 10 μg/ml Doxorubicin) for 10 h. No prior insonation was used in these experiments. The data display three superimposed experiments, each indicated by a different gray-scale

the micelle intact, and as a result, Dox is able to escape into the surrounding aqueous environment, thus partially quenching its fluorescence. We hypothesize that shockwaves and microstreaming events associated with inertial cavitation during 2 h of insonation are able to stress and damage the NNDEA network, thus adversely affecting the stability of the micellar structure, leading to Dox molecules being "released" into the surrounding solution.

An alternative hypothesis explaining these results is that the decrease in fluorescence is due to degradation of the interpenetrating network, which allows more water to penetrate into the micelle core.

Table 1
Time constants of micelle degradation with and without ultrasound.

Exposure	Time constant (τ)	p-value∗
No ultrasound	145.6±11.3[a]	N/A
70-kHz ultrasound	80.3±1.74[a]	0.000583
476-kHz US	78.1±18.6[a]	0.00574

∗p-value using a 1-sided t-test in comparison to the control without US.
[a] Mean and standard deviation of 3 experiments.

Such water can also partially quench the fluorescence of Dox molecules residing in the micelle core.

Table 1 shows the mean and standard deviation of the degradation time constants without ultrasound, and following 2 h of insonation. Time constants were obtained by fitting the normalized fluorescence (which decreases in time) to a decay function, $F=Ae^{-t/\tau}$. A longer time constant τ indicates slower rate of Dox quenching, implying a slower rate of NanoDeliv™ degradation.

We also compared the rate of micellar degradation at the two frequencies 70 kHz and 476 kHz at the same mechanical index. The data show that the degradation time constants are not statistically different from each other ($p=0.847$). At both frequencies, the degradation appears to take place at a statistically significantly higher rate compared to samples that were not exposed to ultrasound. These results confirm that ultrasound, in addition to allowing the release of Dox from stabilized micelles on a short time scale as reported previously, has a deteriorating effect on the cross links in an NNDEA network. Since MI is a measure of the probability and intensity of inertial cavitation, the observation that the degradation rate does not differ between 70-kHz and 476-kHz ultrasound at the same MI may suggest that inertial cavitation plays a role in the structural degradation of NanoDeliv™.

The results of these experiments give further merit to our technique that combines Dox, ultrasound and polymeric carriers as a controlled drug delivery mechanism.

Acknowledgments

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