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Kinetics of acoustic release of doxorubicin from stabilized and unstabilized micelles and the effect of temperature

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Abstract

Ultrasound is being investigated as a trigger mechanism to deliver high concentrations of chemotherapy drugs to cancerous tissues using polymeric micelles. In this paper, we examined the kinetics of acoustic release of doxorubicin using stabilized and non-stabilized micelles. Kinetic models were used to regress release and re-encapsulation time constants for three different compounds, namely non-stabilized Pluronic® P105 micelles, P105 micelles stabilized using an interpenetrating network of N,N-diethylacrylamide and micelles formed by PEO-b-poly(NIPAAmco-HEMA-lactate$_n$). Results showed that the kinetic release constant (k_r) depends on the micellar system under investigation. On the other hand, there is no statistically significant difference between re-encapsulation rate constants for stabilized and unstabilized micelles. We hypothesize that k_r depends on the degree of cross-linking or stabilization. r 2009 Published by Elsevier Ltd. on behalf of The Franklin Institute.

Keywords: Pluronic® P105; Polymeric micelles; Ultrasound; Kinetic constants; Stabilized micelles

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1. Introduction

Chemotherapy is one of the most effective methods used to eradicate cancer cells. Unfortunately, the administration of anti-neoplastic drugs affects both cancerous and healthy cells, which causes harmful side effects. Currently, doxorubicin (Dox) is one of the most commonly used chemotherapeutic agents. As an anthracycline drug, Dox intercalates with the DNA of malignant tissues, interfering with its functions and causing single- and double-strand breaks, which eventually lead to cell death. Its side effects include bone marrow depression, alopecia, gastrointestinal disturbances, cardiomyopathy and dermatological manifestations mainly at the site of injection. To combat Dox's toxicity, our research group has developed a micellar system with the intention of using nanotechnology (nanosize particles) to transport the chemotherapeutic agents to the cancerous site [1–14]. The micelle will then respond to ultrasound, where, on exposure to such stimulus, these nanostructures would release the drug. This localizes the effects of the drug onto the cancerous tissue, thus minimizing its undesirable side effects on other tissues.

Kinetics of release play an important role in the development of drug delivery systems. Previously, the kinetics of acoustic activation employed here were studied and modeled for unstabilized Pluronic® P105 micelles [6]. Our group has extended this research to include P105 micelles stabilized using an interpenetrating network of N,N-diethylacrylamide (NNDEA; NanoDeliv™) and PEO-b-poly(NIPAAm-co-HEMA-lactate$_n$) (PNHL) micelles [12]. The effect of temperature on drug release kinetics was also studied for unstabilized P105 micelles [12]. The mechanistic models used previously [6] were employed in this paper in an attempt to compare release and re-encapsulation timeframes for the different drug delivery vehicles under investigation. Additionally, the results would indicate whether fluctuations in temperature, both in vivo and in vitro, would affect the rate of drug delivery.

2. Materials and methods

2.1. Materials

Pluronic® P-105 was provided by BASF Corporation (Mount Olive, NJ); N,Ndiethylacrylamide was obtained from Polyscience (Warrington, PA); N,N-bis(acryoyl)cystamine (BAC) was obtained from Fluka (Milwaukee, WI); 2,2^0-azobis(isobutyronitrile) (AIBN) was obtained from Aldrich (Milwaukee, WI); 2-hydroxyethyl methacrylate, 3,6dimethyl-1,4-dioxane-2,5-dione, tin(II) 2-ethylhexanoate, methoxypoly(ethylene glycol), N-isopropylacrylamide, 4,4^0-azobis(4-cyanopentanoic acid) (ABCPA), 1,4-dioxane and thionyl chloride were obtained from Sigma-Aldrich (St Louis, MO) and used without further purification.

Dox was obtained from the University of Utah Hospital (Salt Lake City, UT) in a 1:5 mixture with lactose and from Pharmacia & Upjohn Company (Kalamazoo MI), in dosage form; it was dissolved in phosphate-buffered saline (PBS) and sterilized by filtration through a 0.2mm filter.

2.2. Drug encapsulation in Pluronic® unstabilized/stabilized micelles

Stock solutions of Pluronic® (BASF, Mount Olive, NJ) were prepared by dissolving P105 in a PBS solution to a final concentration of 10wt%. Dox was dissolved into the P105 solutions at

room temperature to produce a final Dox concentration of 10mg/ml in 10wt% Pluronic^s. The same drug concentration was also prepared in PBS [4].

To form an interpenetrating network of poly-NNDEA in Pluronic^s P105 micelles, the following procedure was used [2]. First, a 40ml aliquot of double distilled water containing 10wt% P105 was added to a round-bottom flask. NNDEA monomer was added to give a final concentration of 0.05wt% monomer. BAC was added as a cross-linking agent to give a BAC:NNDEA mole ratio of 1:20. AIBN was added as an initiator, to give an AIBN:NNDEA mole ratio of 1:100. The flask was then connected to a water condenser and purged with nitrogen for at least 1h without heat. The system was allowed to polymerize for 24h at 651C with magnetic stirring and a continuous nitrogen purge. Dox stock solution was added at room temperature to the resulting NanoDeliv^{TM} to make a final concentration of 10mg/ml.

Oligolactate esters of 2-hydroxyethyl methacrylate (HEMA-lactate$_n$), (n ¼ number of lactate units in oligolactate) were synthesized by ring-opening oligomerization of lactide using HEMA as the initiator and stannous octoate as a catalyst as described in [12]. In brief, a mixture of lactide and HEMA was stirred at 1101C under nitrogen purging until the lactide was molten. Subsequently, catalytic amounts of stannous octoate dissolved in toluene were added dropwise to the mixture. The mixture reacted for 1h. The target stoichiometry was 3 or 5 lactate units per HEMA.

Incorporation of PEO into the block copolymer required the synthesis of a PEO macroinitiator, which was produced in two steps. In the first step, 4,4^0-azobis(4cyanopentanoic acid) was treated with $SOCl_2$ at 1001C for 20min and converted to the corresponding acid chloride: 4,4^{0}azobis(4-cyanopentanoyl chloride) (ABCPC). In the second step, the PEO macroinitiator was prepared by a condensation reaction of ABCPC and PEO in dry dichloromethane in the presence of an excess amount of triethylamine for 24h [12].

PEO-b-poly(NIPAAm-co-HEMA-lactate$_n$) was obtained by radical polymerization of NIPAAm with HEMA-lactate$_n$ obtained above using the PEO macroinitiator. The copolymerization was conducted in 1,4-dioxane at 801C for 24h in a nitrogen atmosphere. The solution was then cooled to room temperature and concentrated under reduced pressure. The formed solid was dissolved in distilled water to form 100mg/ml solution and centrifuged for 30min (10,000rpm, Eppendorf 5415C) at 401C to separate the unreacted PEO. The precipitated copolymer products were dried under reduced pressure [12].

^{1}H NMR spectra of the copolymers were recorded using Varian Unity 300MHz instruments in DMSO-d_6. The molar composition of the block copolymer was determined from the relative integral intensities at 3.5ppm (CH_2CH_2O of PEO block), 3.8ppm (-NHCH$(CH_3)_2$ of PNIPAAm block) and 4.0–4.3ppm (OCHCHOCOCH(OH)CH_3 of P(HEMA-lactate$_n$) block). The data showed that the molar ratio of NIPAAm to HEMA-lactate$_n$ to PEO in the copolymer was the same as the feed ratio [12].

2.3. Measuring ultrasound-triggered release of Dox from Pluronic^s p105 micelles

A modification of the previous apparatus was used in these experiments [4]. The 488nm beam of an argon ion laser (Ion Laser Technology, 5500 A) was directed through a dielectric-interface beam splitter; the intensity of the split portion of the beam was measured by a photodetector (Newport 818-SL with 835 display) and used to monitor the laser power throughout our experiments (Fig. 1). The other portion of the beam was

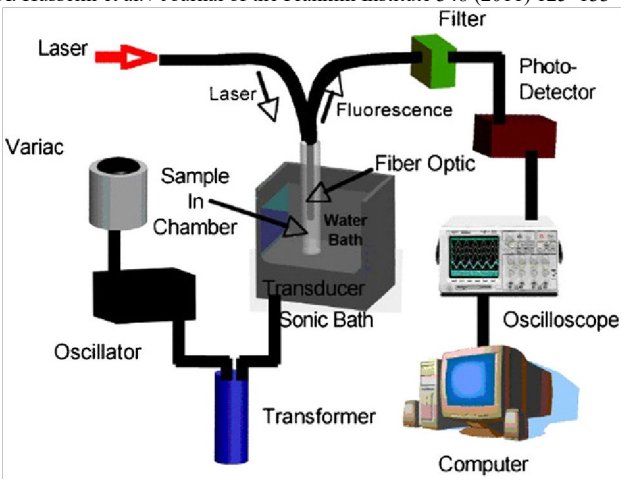

Fig. 1. Ultrasonic exposure chamber with fluorescence detection.

focused by a 20X microscope objective into one branch of a bifurcated fiber optic bundle (# DF13036M, Edmunds Optics, Barrington, NJ) that directed the light into an acoustically transparent plastic (cellulose butyrate, Tulox Plastics, Marion, IN) tube, with a diameter of 2.54cm, filled with the Dox solution. The laser light exited the common end of the bifurcated fiber optic bundle in a 0.09sr cone of light. Dox molecules within the cone of light absorb at 488nm and isotropically emit fluorescent light within a spectrum of about 530–630nm. In the same fiber optic bundle were fibers that collected and directed the fluorescence to a detector. Numerical integration of a model of isotropic fluorescence within the cone of excitation light showed that about 99% of the collected fluorescence originated from within 3mm of the fiber optic tip. The collected fluorescence signal was directed through the second branch of the fiber optic bundle through a dielectric bandpass filter (Omega Optical 535DF35) to a silicon detector (EG&G 450-1). The filter was used to reject any emissions with a wavelength below about 517nm, including any Rayleigh scattered laser light. The photodetector signal was captured on an oscilloscope (Tektronix TDS 3012) and subsequently stored on a computer for further processing.

This apparatus can measure the amount of acoustically activated Dox release from micelles because Dox exhibits a decrease in fluorescence when in contact with water molecules. Such is the case when Dox is released from Pluronic^{s} micelles, and it follows that the magnitude of the decrease in fluorescence intensity on application of ultrasound provides a quantifiable measure of drug release [4].

The decrease in fluorescence of the encapsulated drug solution was assumed to be directly proportional to the amount of drug released relative to a known baseline. The fluorescence of Dox in PBS, in the absence of Pluronic^{s}, was measured to simulate 100% release. Then, the percent release was calculated using the following [4]:

$$\%\ \text{release} = \frac{I_{P105} - I_{US}}{I_{P105} - I_{PBS}} \times 100\% \qquad (1)$$

where I_{US} is the fluorescence intensity upon exposure to ultrasound, I_{PBS} is the fluorescence

intensity in a solution of free Dox in PBS, and I_{P105} is the intensity recorded when the drug is encapsulated in Pluronic® P105 (which corresponds to 0% release or 100% encapsulation).

In these experiments, the fluorescence intensity of the drug in PBS was measured both with and without the application of ultrasound. Ultrasound was applied using a 70-kHz ultrasonicating bath (SC-40, Sonicor, Copiaque, NY) equipped with a single piezoceramic transducer that is driven at about 70kHz. The best description of the waveform is that of a 70-kHz wave amplitude modulated sinusoidally at about 0.12kHz. 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 ultrasound. To execute the experiments, the end of the fiber optic probe was positioned at an acoustically intense position in the ultrasonicating bath, as determined by a hydrophone [4]. The bath was filled with degassed water and the tube surrounding the fiber optic probe was filled with the solution of Dox in PBS. Fluorescence emissions were collected for different voltages applied to the ultrasonicating bath. Then, without changes in the experimental set-up, the Dox solution in PBS was carefully removed and replaced with a Dox solution of the same concentration in Pluronic® micelles. During insonation, fluorescence dropped due to Dox coming in contact with the surrounding aqueous environment.

3. Kinetic models

In analyzing data and obtaining kinetic rate constants, two models were used [6]. Model 1 assumes that both release and re-encapsulation follow first-order kinetics. The mechanism of release can be written as follows:

$$E \underset{ke1}{\overset{kr1}{\leftrightarrow}} F \tag{2}$$

During the ultrasound pulse, the kinetics of the system can be obtained by solving the following first-order differential equation:

$$\left.\frac{dE}{dt}\right|_{US} = -k_{r1}E + k_{e1}F \tag{3}$$

The variables E, F, k_{r1} and k_{e1} correspond to encapsulated drug concentration, free drug concentration, kinetic release and kinetic re-encapsulation rate constants, respectively. The subscript US indicates the presence of ultrasound.

To solve the above ordinary differential equation, the total drug concentration (T) is calculated and used as an initial condition, E(0)= T. Since the sum of encapsulated and free Dox is always constant, Eq. (3) reduces to

$$\left.\frac{dE}{dt}\right|_{US} = -k_{r1}E + k_{e1}(T-E) \tag{4}$$

The solution of the modified differential Eq. (4) becomes

$$\left.\frac{E(t)}{T}\right|_{US} = \left[1 - \left(\frac{k_{e1}}{k_{e1}+k_{r1}}\right)\right] e^{-(k_{e1}+k_{r1})t} + \left(\frac{k_{e1}}{k_{e1}+k_{r1}}\right) \tag{5}$$

Using the steady-state assumption (t =∞) and multiplying by T, Eq. (5) simplifies at steady state to

$$E(t)_{US_{SS}} = \frac{k_{e1}T}{k_{e1}+k_{r1}} \tag{6}$$

where the SS subscript indicates steady state. Eq. (6) provides a relationship between the release and re-encapsulation kinetic constants. If a plot of ln(E(t)/T) vs. time is constructed, the slope is equal to the sum of both rate constants.

One more relationship is needed to solve for k_e or k_r. This is accomplished by studying re-encapsulation data. In our models, we assumed that no drug is released when ultrasound is turned off. Therefore, in the absence of ultrasound, k_r ¼ 0 and Eq. (1) reduces to

$$\left.\frac{dE}{dt}\right|_{NoUS} = k_{e1}(T-E) \tag{7}$$

Using the initial condition E ¼ E(t_{off}), where E(t_{off}) is the steady value of encapsulated drug during ultrasonication (or 1–fraction of release), the solution to Eq. (5) becomes

$$\left.\frac{E(t)}{T}\right|_{NoUS} = \left[\left(\frac{E_{t_{off}}}{T}\right) - 1\right] e^{k_{e1}(t-t_{off})} + 1 \tag{8}$$

Thus, a plot of ln[(E(t)/T)1/(E(t_{off})/T)1] vs. (t–t_{off}) is a straight line with a slope of k_e.

Model 2 assumes that release and re-encapsulation follow zero-order and first-order kinetics, respectively. The differential equation that describes this system is given by

$$\left.\frac{dE}{dt}\right|_{US} = -k_{r2} + k_{e2}F$$

where k_{r2} and k_{e2} represent the rate of release and re-encapsulation, respectively. Using the same material balance on the drug as used to solve (2)–(4), the following equation is obtained:

$$E(t)_{US} = \left(\frac{k_{r2}}{k_{e2}}\right) e^{-2k_e t} - \left(\frac{k_{r2}}{k_{e2}}\right) + T \tag{10}$$

Since re-encapsulation follows a first-order path, Eq. (7) is applicable to Model 2 and k_{e1} ¼ k_{e2} ¼ k_e. Eq. (9) can then be used to solve for k_{r2}. For more details on the physical interpretation and derivation of these two models refer to our previous publication [6].

4. Results and discussion

We have previously investigated the extent of using ultrasound to release the anthracycline agent Dox from unstabilized and stabilized micelles [2,4,12]. Drug release was measured by fluorescence spectroscopy. The results of these experiments showed that optimal Dox release can be achieved at 561C using Pluronic® P105 at the highest power density attained during these experiments (0.76W/cm^2) (12).

In this paper the kinetics of drug release from three different micelles is reported [12]. Kinetic models were used to represent the ultrasonic release and subsequent reencapsulation of Dox from these micellar systems. Experiments employing 70-kHz ultrasound were then used to test the validity of these mechanistic representations.

Figs. 2 and 3 report the values of both release and re-encapsulation constants using two different kinetic models. Both figures show that the kinetic release constants k_{r1} and k_{r2} depend on the micellar system under investigation. We used Student-t statistics to compare the release and re-encapsulation rate constants between the various drug carriers. The p-values are 0.0187 and 0.0117 when we compare the 371C-release rate, k_{r1}, for nonstabilized P105 micelles with NanoDeliv™ and PNHL, respectively.

There is no statistically significant difference between re-encapsulation rate constants for stabilized and unstabilized micelles, NanoDeliv™ and PNHL, (p-values ¼ 0.064 and 0.114, respectively), indicating that the assembly of the polymeric molecules, after ultrasound is turned off, occurs within the same time frame for any of these carriers.

Both release and re-encapsulation time constants for non-stabilized micelles do not depend on temperature. The p-values for k_{r1} and k_{e1} at 371C and 581C were 0.157 and

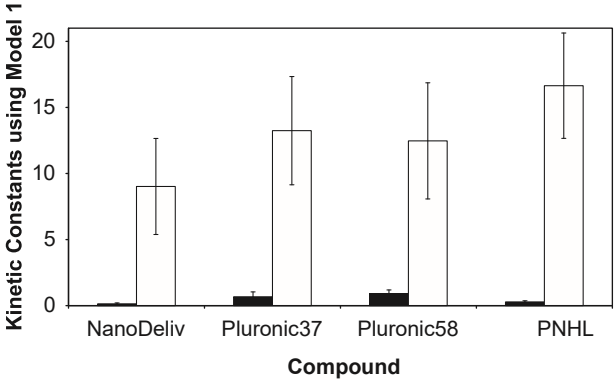

Fig. 2. Release (shaded) and re-encapsulation constants (k_{r1} ([¼] s^1) and k_{e1} ([¼] s^1)) for the three compounds under investigation: Pluronic^s P105 (at 371C and 581C), NanoDeliv^{TM} and PNHL (at 70kHz and 0.76W/cm^2) using model 1.

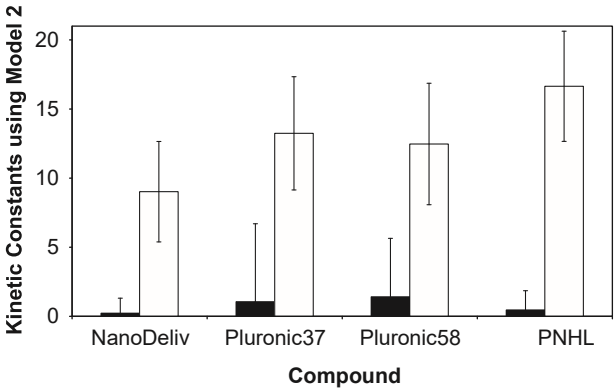

Fig. 3. Release (shaded) and re-encapsulation constants (k_{r2} ([¼]intensity units/s) and k_{e2} ([¼]s^1)) for the three compounds under investigation: Pluronic^s P105 (at 371C and 581C), NanoDeliv^{TM} and PNHL (at 70kHz and 0.76W/cm^2) using model 2.

0.727, respectively. More experiments need to be conducted in order to examine if the same trend exists with the stabilized micelles.

We hypothesize that the release rate constants depend on the degree of cross-linking or stabilization of the micelles. A strong correlation between acoustically activated drug release and cavitation events in the vicinity of drug loaded micelles has been reported previously [3]. Both NanoDeliv^{TM} and PNHL are highly crossed-linked; thus they should be more resilient to structural perturbation during ultrasound-related cavitation events.

The oscillatory expansion and contraction of gas bubbles in an oscillating pressure field, such as an ultrasonic field, causes high shear stresses in the near vicinity of the surface of the oscillating gas bubble. For example, shear rates in the boundary layer of cavitating bubbles have been calculated to be over $10^7 s^1$ for conditions very similar to those used in these experiments [15]. The shear stresses decrease with distance from the cavitating bubble. Such high shear stresses probably stretch and break apart unstabilized micelles such as the Pluronic^s micelles used herein. The rupture would expose the previously sequestered Dox molecules to the aqueous environment, where they may be taken up by nearby cells, or may diffuse back into nearby micelles. On the other hand, micelles that are stabilized by an interpenetrating network of thermally responsive

acrylamide (NanoDeliv^{TM}) or by strong hydrophobic interactions (PNHL) may require higher shear stresses for disruption. Because the magnitude of the shear stress drops quickly with distance from the oscillating bubble, at any given time, only a small fraction of Pluronic^{s} drug carriers may be sufficiently close to an oscillating bubble that the local stresses disrupt the micelle. For stabilized carriers with a higher threshold for disruption, an even smaller fraction may be sufficiently close for disruption.

We hypothesize that once the ultrasound is turned off in our in vitro experiments, the fractured micelles coalesce together to form new micelles. The released Dox then diffuses back into the nearby micelles. Because the diffusion of Dox is independent of the carrier from which it was released, it is not unexpected that the re-encapsulation rates are similar for all carriers.

Both release and re-encapsulation time constants for non-stabilized micelles do not depend on temperature. The p-values for k_{r1} and k_{e1} at 37 and 581C were 0.157 and 0.727, respectively. More experiments need to be conducted in order to examine if the same trend exists with the stabilized micelles.

5. Conclusions

In this paper we showed that kinetic constants for the release and re-encapsulation phenomena associated with ultrasonic drug delivery depend on the micellar system under investigation. Variations in temperature do not appear to affect the rate at which Dox is being released and re-encapsulated from non-stabilized Pluronic^{s} micelles.

In conclusion, we reiterate the advantages of this novel drug delivery system, mainly that it is non-invasive, and can focus on cancerous tissues without affecting the healthy cells in the vicinity. We also stress the importance of quantifying the kinetics of release and re-encapsulation in acoustically activated micellar drug delivery as it helps in optimizing the duration of therapy and the optimal drug concentration to be used clinically.

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