Video Article

Preparation and Characterization of Individual and Multi-drug Loaded Physically Entrapped Polymeric Micelles

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Abstract

Amphiphilic block copolymers like polyethyleneglycol-*block*-polylactic acid (PEG-*b*-PLA) can self-assemble into micelles above their critical micellar concentration forming hydrophobic cores surrounded by hydrophilic shells in aqueous environments. The core of these micelles can be utilized to load hydrophobic, poorly water soluble drugs like docetaxel (DTX) and everolimus (EVR). Systematic characterization of the micelle structure and drug loading capabilities are important before *in vitro* and *in vivo* studies can be conducted. The goal of the protocol described herein is to provide the necessary characterization steps to achieve standardized micellar products. DTX and EVR have intrinsic solubilities of 1.9 and 9.6 µg/ml respectively Preparation of these micelles can be achieved through solvent casting which increases the aqueous solubility of DTX and EVR to 1.86 and 1.85 mg/ml, respectively. Drug stability in micelles evaluated at room temperature over 48 hr indicates that 97% or more of the drugs are retained in solution. Micelle size was assessed using dynamic light scattering and indicated that the size of these micelles was below 50 nm and depended on the molecular weight of the polymer. Drug release from the micelles was assessed using dialysis under sink conditions at pH 7.4 at 37 ^{o}C over 48 hr. Curve fitting results indicate that drug release is driven by a first order process indicating that it is diffusion driven.

Video Link

The video component of this article can be found at https://www.jove.com/video/53047/

Introduction

Amphiphilic block copolymers with repeating structure composed of hydrophilic and hydrophobic domains can spontaneously self-assemble to form three dimensional macromolecular assemblies known as polymeric micelles. These structures have an inner hydrophobic core surrounded by a hydrophilic shell. The hydrophobic core has the ability to incorporate hydrophobic drugs either by physical entrapment through hydrophobic interactions or by chemical conjugation on to the polymer backbone.[1] Many advantages exist to using these block copolymers to form micelles for drug delivery. These include incorporation of poorly soluble drugs, improving pharmacokinetics of the incorporated drugs, and the biocompatibility and/or biodegradability of the polymers makes them a safe alternate to conventional solubilizers.[2] Another advantage of using polymeric micelles is their colloidal particle size, between 15–150 nm[3], making them attractive for parenteral delivery. Therefore, over the last 20 years polymeric micelles have emerged as viable drug delivery systems for poorly water-soluble drugs especially for cancer therapy.[3,4]

Currently there are five polymeric micellar formulations for cancer therapy undergoing clinical trials.[4] Four of the micelles in the clinical trials are PEG-based diblock copolymers while the last is a triblock copolymer containing polyethyleneoxide. The size of these micelles varied from 20 nm to 85 nm. The advantage of using PEG based polymers is their biocompatibility and depending on the second block can also be biodegradable. Recently new drug delivery systems based on polyethyleneglycol-*block*-polylactic acid (PEG-*b*-PLA) polymeric micelles have been developed for the concurrent delivery of multiple anticancer drugs. The PEG-*b*-PLA micelles are both biocompatible and biodegradable. These multi-drug loaded micelles have shown a synergistic inhibition of different cancers models *in vitro* and *in vivo*[2,5,6] and fit into the current paradigm of utilizing multiple drugs in chemotherapy to prevent resistance and lowering toxicity. Therefore, there is a great deal of interest in preparing and characterizing these micellar drug delivery systems for use in cancer and other disease states.

In the work below we have outlined a step-by-step process by which such micelles can be prepared and characterized before evaluating them in disease states of interest. For the purpose of this work two poorly-soluble anti-cancer agents, docetaxel (DTX) and everolimus (EVR) have been chosen. Both DTX and EVR are poorly water-soluble compounds with intrinsic water solubilities at 1.9 and 9.6 µg/ml respectively.[7,8] Two PEG-*b*-PLA polymers with different molecular weights were used in this protocol as the building blocks for the formulated polymeric micelles, these polymers are PEG_{2000}-*b*-PLA_{1800} (3,800 Da) and PEG_{4000}-*b*-PLA_{2200} (6,200 Da). PEG-*b*-PLA micelles can therefore provide a unique platform as a nanocarrier for DTX and EVR individually and in combination. The required Reagents/Materials and Equipment needed to prepare and characterize these micelles are listed in **Table 1**.

Protocol

1. Preparation of Individual and Multi-drug Loaded Micelles by Solvent Casting Method

1. Weigh out DTX 1 mg or EVR 1 mg or both drugs at 1 mg each for the dual drug micelles (DDM).
2. Weigh out 15 mg of PEG_{2000}-*b*-PLA_{1800} or PEG_{4000}-*b*-PLA_{2200} for either individual or DDM.
3. Dissolve the drug/ drugs and the polymer in 0.5 ml of acetonitrile and place in a 5 ml round bottom flask.
4. Form a thin drug distributed polymer film by evaporating the drug(s)-polymer acetonitrile solution under reduced pressure using a rotary evaporator. Set the rotary evaporator to 100 rpm, the water bath temperature of 40 ^{o}C and a vacuum pressure of 260 mbar for 5 min followed by a reduction to 100 mbar for 3 more min.
5. Rehydrate the drug-polymer film with 0.5 ml of deionized water at 50 ^{o}C and gently shake the flask to form the micelles.
6. Filter the resulting micellar solution through a 0.2 µm nylon filter to remove any un-dissolved drug or contaminants into a 1.5 ml centrifuge tube.

2. Assessment of Drug Loading and Stability in Micelles Using Reverse-phase High Performance Liquid Chromatography (RP-HPLC)

1. Perform RP-HPLC analysis with a C8 column equilibriated at 40 °C in an isocratic mode with a mobile phase of acetonitrile/water (62/38) containing 0.1% phosphoric acid and 1% methanol at a flow rate of 1 ml/min and an injection volume of 10 µl.
2. Dilute freshly prepared micelles (section 1) 1:100 in mobile phase prior to analyzing by RP-HPLC to determine initial drug loading. Store undiluted individual micelles and DDM at room temperature (25 ^{o}C) for 48 hr and prepare fresh 1:100 diluted samples in mobile phase to re-assess by RP-HPLC and determine drug(s) stability in micelles over 24 hr.
3. Monitor DTX and EVR peaks at 227 and 279 nm respectively with retention times of 1.7 and 5.7 min respectively. Perform all measurements in triplicate. Present data as Mean ± SD drug loading.

3. Assessment of micelle Particle Size by Dynamic Light Scattering (DLS)

1. Dilute freshly prepared micelles (as described in section 1) in deionized water at a ratio of 1:20 to yield a final polymer concentration of 1.5 mg/ml.
2. Measure the intensity of He-Ne laser (633 nm) at 173° to determine scattering. Perform all measurements at 25 °C following pre-equilibration for 2 min.
3. Perform all measurements in triplicate. Present data as the Mean Z-average size ± SD along with the polydispersity index (PDI) of the distribution.

4. Assessment of *In Vitro* Drug Release from Individual Micelles and DDM

1. Prepare individual micelles and DDM as described in section 1. Load 2.5 ml of the micelles into a 3 ml dialysis cassette with a molecular weight cut-off (MWCO) of 7,000 g/mol.
   NOTE: This MWCO was chosen to enable the free drug(s) along with the unassociated polymer molecules to diffuse freely out of the cassette and thereby ensure sink conditions.
2. Place the cassettes in 2.5 L of 10 mM pH 7.4 phosphate buffer (prepared by diluting stock 200 mM solution) and change the buffer every 3 hr to ensure sink conditions. Maintain the temperature of the buffer at 37 ^{o}C throughout the duration of the experiment.
3. At 0, 0.5, 1, 2, 3, 6, 9, 12, 24, and 48 hr, withdraw 150 µl of the solution in the cassettes and replace with 150 µl of fresh buffer.
4. Analyze the samples using RP-HPLC as established in section 2 to determine the drug concentration. Curve-fit the drug(s) release data based on a simple diffusion model with a one phase exponential association using stastitical software.
5. Calculate the time needed to reach 50% of drug release ($t_{1/2}$) of each drug in individual micelles or DDM based on the curve fitting. Perform all measurements in quadruplet.

Representative Results

Individual DTX or EVR micelles and DTX and EVR DDM in PEG-*b*-PLA micelles are successfully formulated in either PEG_{4000}-*b*-PLA_{2200} or PEG_{2000}-*b*-PLA_{1800} (**Figure 1**).

DTX, EVR, and the DDM showed similar stability in PEG_{4000}-*b*-PLA_{2200} or PEG_{2000}-*b*-PLA_{1800} over 48 hr (**Figure 2**). Initial drug loading of EVR in PEG_{4000}-*b*-PLA_{2200} and PEG_{2000}-*b*-PLA_{1800} is 1.86 and 1.87 mg/ml respectively. While initial DTX loading in PEG_{4000}-*b*-PLA_{2200} and in PEG_{2000}-*b*-PLA_{1800} is 1.85 and 1.78 mg/ml. The initial loading of both DTX and EVR in DDM micelles using each of the polymers is similar to individual micelles. All the micelles retained 97% or more of the initial loading at 48 hr at room temperature.

Micelle size is assessed by DLS and based on the results all micelles showed a unimodal distribution with PDI values of less than 0.2. The z-average mean sizes for DTX, EVR and DDM in PEG_{2000}-*b*-PLA_{1800} is approximately 18.05 ± 0.06 nm (PDI = 0.079 ± 0.013) while in the PEG_{4000}-*b*-PLA_{2200} the size is approximately 34.09 ± 0.24 nm (PDI = 0.137 ± 0.004) (**Figure 3**).

To represent the utility of using both polymers the release experiments are performed using PEG_{4000}-*b*-PLA_{2200} for EVR micelles or DDM and PEG_{2000}-*b*-PLA_{1800} for the DTX micelles. The *in vitro* drug(s) release from micelles is assessed in pH 7.4 buffer at 37 °C by dialysis under sink conditions for 48 hr. Based on the data, DTX release from individual micelles and DDM is approximately 60% over 48 hr (**Figure 4**). EVR release

from individual micelles and DDM was 60% and 50% respectively (**Figure 4**). The $t_{1/2}$ for each drug from individual micelles and DDM and the goodness of fit data is presented in **Table 2**. The goodness of curve-fitting (r^2) for all micelles except EVR individual micelles is above 0.950 which means that the assumption of first-order release is a good approximation to explain drug release from individual micelles and DDM.

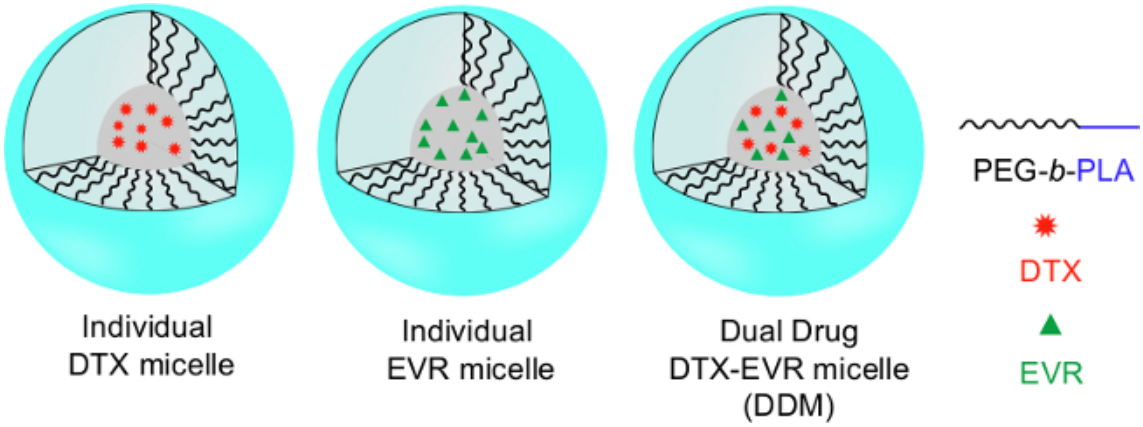

Figure 1: Schematic representation of individual DTX or EVR PEG-*b*-PLA micelles and DDM loaded with DTX and EVR.

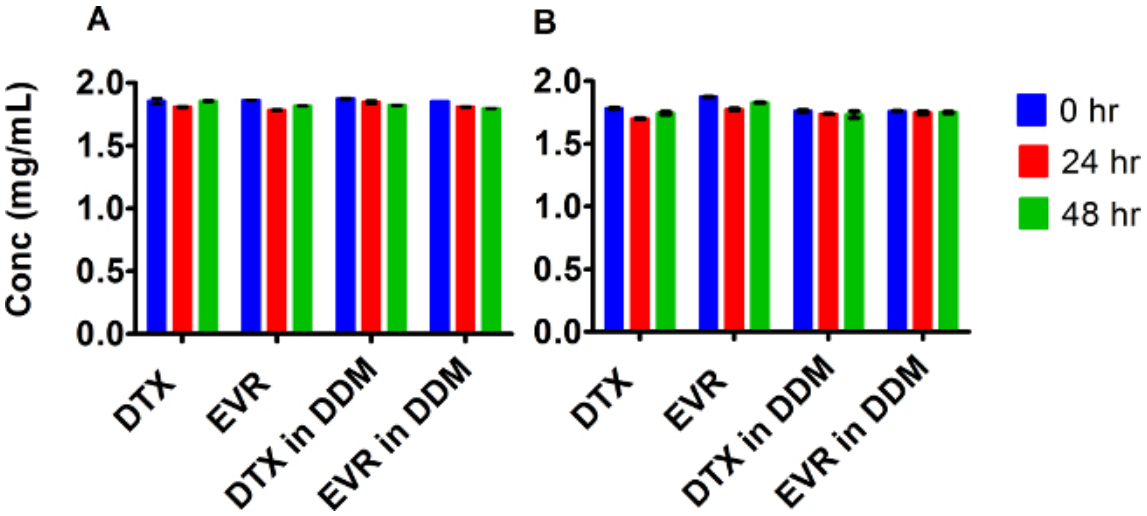

Figure 2: Drug(s) loading and stability of DTX and EVR individual micelles and DDM in PEG_{2000}-*b*-PLA_{1800} (**A**) or PEG_{4000}-*b*-PLA_{2200} (**B**). The drug concentration in the micelles is quantified by RP-HPLC at 0, 24, and 48 hr. Data represented as Mean ± SD of triplicate runs. Please click here to view a larger version of this figure.

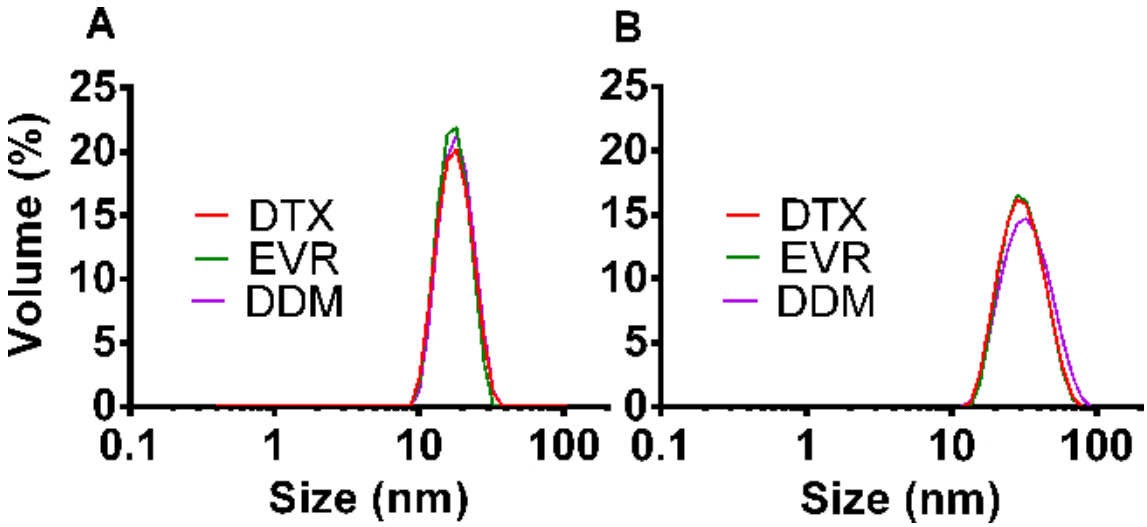

Figure 3: Individual micelle and DDM size determination by DLS in PEG_{2000}-*b*-PLA_{1800} (**A**) or PEG_{4000}-*b*-PLA_{2200} (**B**). The micelle size is assessed by Dynamic Light Scattering. Data is presented is a representative of the distribution for the individual micelles and DDM in the two polymers. Please click here to view a larger version of this figure.

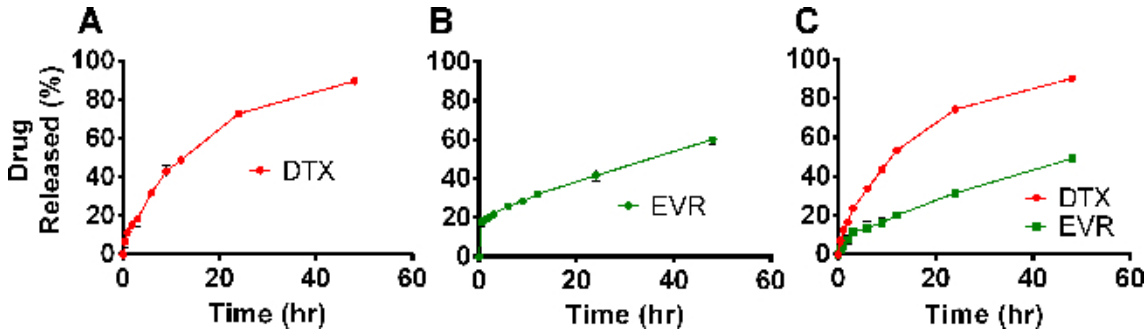

Figure 4: Drug(s) release of DTX (**A**) and EVR (**B**) from individual micelles and DDM (**C**). Drug release studies are performed by dialysis and under sink conditions while maintaining the temperature if the system at 37 °C. The data presented is Mean Drug Release ± SD of 4 replicates. Please click here to view a larger version of this figure.

Micelle	**$t_{1/2}$ (hr)**	**r^2**
DTX	10	0.986
EVR	35	0.82
DDM	DTX – 8.86	DTX – 0.987
	EVR – 48	EVR – 0.955

Table 2: The time needed for 50% drug release ($t_{1/2}$) and goodness of curve fitting (r^2) from *in vitro* release study.

Discussion

The use of polymeric micelles for drug delivery continues to expand due to their versatility and ability to deliver hydrophobic drugs for various disease states. Therefore, the techniques needed to prepare and characterize these formulations prior to use in cell culture or animals is a critical first step to determine the best pairing between the drug and the polymer. PEG-*b*-PLA are excellent amphiphilic block copolymers for drug delivery purposes. However, the block length of the hydrophilic and hydrophobic sections plays an important role in drug encapsulation and stability and are specific to the drug(s) that are chosen to be incorporated. Therefore, initial studies with multiple block copolymers may be necessary to identify the best paring of drug(s) and polymer prior to initiating other studies.

The solvent casting method of micelle preparation is well documented and a reproducible method. The actual conditions for the rotary evaporator need to be optimized for the mixture by changing the water bath temperature, time on the rotary evaporator, the speed of rotations and the vacuum pressure. The end result should be a clear film that is continuous and free of bubbles and should quickly solubilize in the deionized water.

A uniform micellar solution when assessed by DLS should result in a unimodal distribution as seen in the data presented. The size of the micelle is dictated by the molecular weight of the polymer and not by the number of drugs loaded. This is corroborated with the data presented for the DLS where the higher molecular weight PEG_{4000}-*b*-PLA_{2200} has a larger size than the lower molecular weight PEG_{2000}-*b*-PLA_{1800}.

The *in vitro* release of the physically entrapped drugs in polymeric micelles usually follows first-order release kinetics with a one-phase association, as the release tends to be diffusion driven. This been demonstrated by our results here as well as others.[9-11] The curve fitting data gives an approximate half-life but caution should be used in anticipating similar half-lives *in vivo* as the *in vitro* study does not take into account the effect plasma proteins may have on micelle stability. In general, expect shorter half-lives *in vivo*.

Nanoscale systems like micelles, liposomes, and nanoparticles are being extensively explored for their versatility as drug delivery systems. The methods outlined in the manuscript while focused on characterizing polymeric micelles can be adapted to other nanoscale systems. Thus, the applications of this protocol go beyond just drug loaded polymeric micelles. The limitations of this protocol are that they do not address the specifics of preparation for the other nanoscale systems and only provide some guidance of their characterization.

Disclosures

The authors have nothing to disclose.

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