Nanotechnology **16** (2005) S1–S8 doi:10.1088/0957-4484/16/0/000

Polymeric worm micelles as nano-carriers for drug delivery

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Ascii/Word/NAN/
nano193485/SPE
Printed 29/4/2005
Issue no
Total pages
First page
Last page
File name
Date req
Artnum
Cover date

Received 19 January 2005, in final form 18 March 2005
Published
Online at stacks.iop.org/Nano/16/S1

Abstract
Nanoscale carriers of active compounds, especially drugs, need not be spherical in shape. Worm micelles as blends of degradable polylactic acid (PLA) and inert block copolymer amphiphiles were prepared for controlled release and initial study of carrier transport through nano-porous media. The loading capacity of a typical hydrophobic drug, Triamterene, and the release of hydrophobic dyes were evaluated together with morphological changes of the micelles. Degradation of PLA by hydrolysis led to the self-shortening of worms and a clear transition towards spherical micelles, correlating with the release of hydrophobic dyes. Perhaps equally important for application is the flexibility of worm micelles, which we show allows them to penetrate nanoporous gels where 100 nm sized vesicles cannot enter. Such gels have served as tissue models, and so the results here collectively suggest a new class of hydrophobic drug nano-carriers that are capable of tissue permeation as well as controlled release.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Q.1 Spherical micelles formed from various amphiphilic copolymers are being used as nanoscale drug delivery vehicles to (1) increase the solubility of hydrophobic drugs, (2) reduce toxicity to healthy tissue caused from excess dosage, and (3) achieve more sustained release profiles of drugs [1, 2]. The assembly of amphiphilic diblock copolymers into other microstructures such as vesicles or cylinders primarily depends on the weight fraction of the hydrophilic block relative to the total copolymer molecular weight [3, 4]. Cylindrical worm micelles made with such amphiphilic diblock copolymers may not only offer the advantages of being bio-inert and stable, but also of being flexible. Q.2 We have recently shown that PEO-based worm micelles have limited adhesion in flow to cells in human blood [20], and we have also shown that, by modifying the hydrophilic termini of diblocks, it is possible to target specific cells [4]. Worm micelles thus seem to have significant potential as drug carriers, as they can take up drugs and possibly permeate tissues as shown here with the use of model systems.

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One such hydrophobic drug is Triamterene, which is used clinically to treat high blood pressure and fluid retention caused by heart disease and various other conditions. The multi-ring structure (figure 1(a) inset) and low molecular weight (232 Da) makes it typical of hydrophobic drugs. Triamterene is especially convenient for model drug studies because its intrinsic fluorescence makes its whereabouts relatively easy to assess, even allowing the imaging by fluorescence of drug that is intercalated into our worm micelles (figure 1(a)).

Broad interest in biodegradable and biocompatible polymers has led to the synthesis of an increasingly wide range of amphiphilic diblock copolymers. Various types of hydrophobic polymers, including poly L-lactic acid (PLA) [6] and poly ε-caprolactone (PCL) [7] as well as others [8, 9], have been used as the hydrophobic core blocks of copolymer micelles. For the hydrophilic corona of micelles or vesicles, polyethylene oxide (PEO) has been used extensively to yield stealthy, stable structures in aqueous solution. By Q.3 blending different diblock copolymers, we recently introduced self-porating bilayered vesicles composed of PEO–PLA or PEO–PCL for controlled degradation and release [17].

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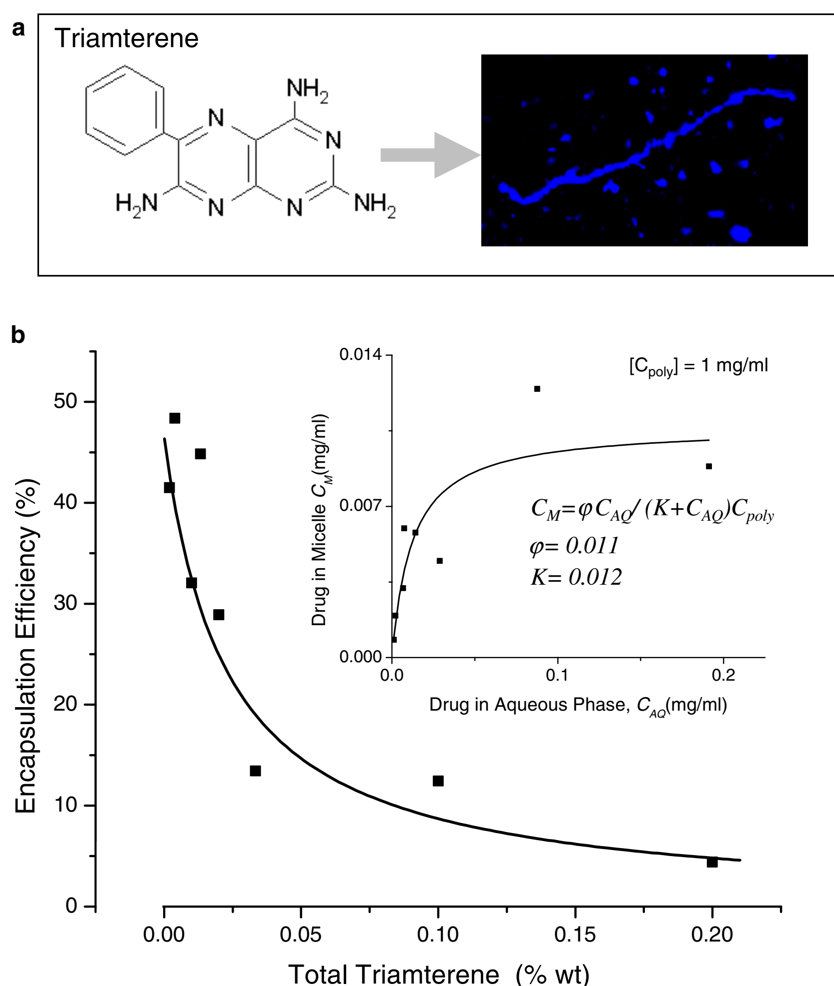

Figure 1. Drug partitioning into worm micelles. (a) The fluorescent drug, Triamterene, was loaded into OE7′ worm micelles, which were then imaged by fluorescence microscopy. (b) Encapsulation efficiency versus total added Triamterene and the Langmuir binding isotherm (inset).

Table 1. Physical properties of various diblock copolymers.

Copolymer name	Formula A_m–B_n	M_h^a (kg mol^{-1})	M_n (kg mol^{-1})	PD	w_{EO}
OB1	EO_{128}–BD_{46}	2.31	8.1	1.13	0.72
OB16	EO_{50}–BD_{54}	2.95	5.2	1.1	0.43
OB18	EO_{80}–BD_{125}	6.8	10.4	1.1	0.29
OE7′	EO_{44}–EE_{40}	2.13	3.9	1.1	0.45
OL2	EO_{109}–LA_{56}	4.0	10.0	1.16	0.50

[a] $M_h \sim n \times M_{monomer}$.

Spherical micelles also produced by amphiphilic diblock copolymers have been studied for at least a decade as drug carriers [10]. For the amphiphilic micelles of interest here, hydrophobic drug loading sites include both the core and core–corona interface [11–13]. In typical studies at a single drug concentration, micelles are equilibrated at excess drug concentration in a closed system [13]. While release times vary between different diblock copolymer systems, PEO–PCL micelles have been reported to show constant release over 30 days [9]. In this study, diblocks of PEO–PLA and of two inert diblock copolymers (see table 1) were blended to make worm micelles. Triamterene was loaded directly into worm micelle suspensions as was done previously with hydrophobic dyes [20] to study practical loading limits. The release behaviour from these degradable, self-shortening worm micelles was then studied. This study is the first that applies such a treatment to interpret morphological change and its effect on drug release.

Here, we have begun to study the transport of worm micelles through tortuous environments, in the hope of creating a vehicle that can deliver agents to tissue of similar porosity. Nano-porous gel structures such as agarose are popular as biomimetics of tissues [14]. Serwer *et al* (1988) showed that the pore size of agarose gels could be precisely controlled [15]. The loading, release, and permeation properties of worm micelles elucidated here will thus suggest the application of worm micelles as drug carriers into porous tissues.

2. Materials and methods

2.1. Materials

The copolymers used in this study are listed in table 1. PEO–polyethylethylene (PEO–PEE) and PEO–polybutadiene (PEO–PBD) block copolymers were synthesized by anionic polymerization as described elsewhere [16]; PEO–PLA block copolymers are commercially available and their properties have been studied recently [17]. Polystyrene beads were purchased from Polysciences Inc. (Warrington, PA) and dialysis tubing was purchased from Spectrum (Rancho Dominguez, CA). THF (tetrahydrofuran), DCM (dichloro methane) and chloroform were purchased from Fisher Scientific (Suwanee, CA). Absolute alcohol, L-lactide monomer, PKH26 and PKH67 cell tracking dye, phosphate buffered saline (PBS), Silane-prep slide glass and Triamterene were all purchased from Sigma-Aldrich (St. Louse, MO).

2.2. Worm micelle preparation and drug/dye loading

Worm micelles form spontaneously upon hydration of copolymer films at 50–60 °C for up to ∼16 h. Aqueous hydrating solutions range from pure water to 290 mOsm PBS. Polymeric concentrations in aqueous solutions ranged from 0.6 to 1.0 mg ml^{-1}. Worm micelles synthesized from blends of PEO–PLA and PEO–PEE and were used immediately after 60 °C hydration to measure micellar physical properties. Triamterene was dissolved in ethanol and loaded into worm micelles from a 1 mg ml^{-1} stock solution; the excitation/emission maxima of Triamterene are 370/434 nm. Hydrophobic fluorescent dyes (PKH26 or PKH67) were either loaded directly or diluted with a small amount of ethanol and added to a worm micelle suspension. These hydrophobic dyes accumulate and remain stable in the hydrophobic core of the worm micelles for weeks [4]. Excess dye from worm micelle suspensions was removed either by one hour batch adsorption into polystyrene (PS) beads or by extensive dialysis under isotonic conditions.

2.3. Visualization

Worm micelles and beads were imaged with an Olympus IX71 inverted fluorescent microscope with a 60× oil objective and a Cascade CCD camera [4]. The hydrophobic fluorescent drugs/dyes that have partitioned into the micelle's cores allow the imaging of worm micelles with contour lengths >1 μm. To prevent shear fragmentation, worm micelles were imaged while they were moving freely under low flow conditions.

2.4. Characterization

Fluorescence images were used to measure the change of worm micelle contour lengths and fluorescence intensity over time. The fluorescent intensity of Triamterene was measured with spectrofluorimetry. Gel permeation chromatography (GPC) using a Breeze system (Waters Co.) with reflective index (RI) detector was used to characterize PLA degradation. For GPC analysis, worm micelles were prepared in DI water, and after preparation, incubated in 37 °C water bath. At each time point, worm micelles were then dissolved with THF, and the solutions were completely dried with nitrogen to remove all water. Samples were filtered with a 0.4 μm pore size syringe filter. Peaks were identified via GPC calibration with PEG and L-lactide monomer.

2.5. Gel and 100 nm diameter vesicles

Various concentrations (up to 2.0 wt%) of agarose were used to make gels that were tested for permeation. The gels were polymerized between cover glass and silanized slide glass to remove any void space. A short gel width was maintained to allow a short permeation length, and the ends of the gel were sealed with parafilm. 100 nm diameter vesicles were prepared via sonication, freeze and annealing, and extrusion as described elsewhere [17]. Worm micelles were prepared and diluted in DI water. Permeation of worm micelles into gel was induced by solute density difference by placing 10× PBS solution against DI water on the other side of the gel. Unwanted channelling through void space was observed as a rapid mixing flow was clearly noticed by comparison with the slow and tortuous flow through the gel. Gel collapse by excessive flow was prevented by varying permeation length.

3. Results and discussion

3.1. Drug integration into worm micelles

To determine the encapsulation efficiency (e) of a drug, and to investigate any concentration dependence in the integration process, loading experiments were performed in PBS buffer solution over a range of drug concentrations and at fixed copolymer concentration. Free drug in solution was measured after extraction into polystyrene beads, which are aromatic and an excellent sink for Triamterene. The amount of drug incorporated was measured by fluorimeters after extracting free drug into polystyrene beads. For simple partitioning, integration of a drug into a worm micelle would be proportional to the drug concentration. If the interaction were more specific, each copolymer would provide a fixed number of binding sites for hydrophobic molecules [18]. While integration is obvious in figure 1(a), the amount of drug incorporated increases only up to saturation (figure 1(b)), consistent with specific binding.

We denote C_{M} and C_{AQ} as the concentrations of drug in either micelles or free in aqueous solution, respectively. Thus, results show $e = C_{\mathrm{M}}/(C_{\mathrm{M}} + C_{\mathrm{AQ}}) \times 100$ decreases from 50% to <2% at high drug concentrations to copolymer. The inset of figure 1(b) shows a Langmuir-type isotherm fit for drug binding to the micellar phase (C_{M}). Encapsulation efficiency versus total added Triamterene in figure 1(b) with parameters from the Langmuir binding isotherm (inset) also shows a saturation profile.

Simple partitioning would give a linear relationship rather than saturation [19]. At the point $C_{\mathrm{AQ}} = K \approx 0.012$ mg ml^{-1}, drug uptake is half-saturated and e is already very low. In contrast, in the low C_{TOT} range the drug incorporation is nearly linear. Many past reports of encapsulation efficiency into copolymer systems have only studied a narrow range of drug and not clarified the mechanisms (partitioning versus binding) [13].

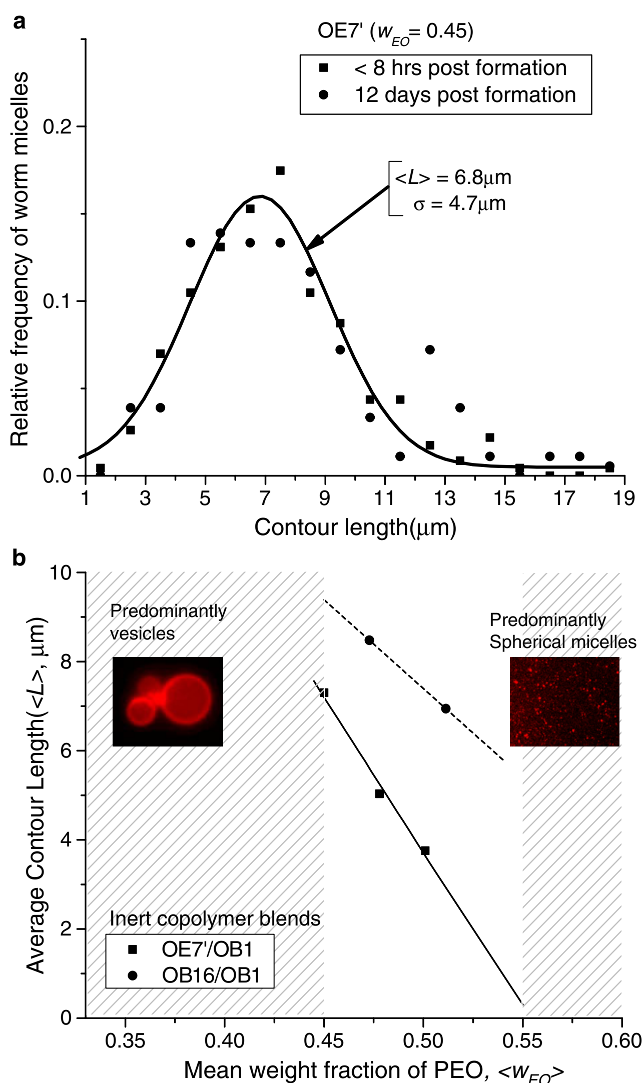

Figure 2. The worm micelle contour length is stable in inert blends and is set by hydrophilic fraction. (a) The contour length distribution of worm micelles. The near-Gaussian length distribution of inert worm micelles shows no change over 12 days when stored in solution at room temperature. (b) The average contour lengths of two copolymer blending pairs. OE7′ was blended with OB1 ($w_{\rm EO} = 0.72$) at 0, 5, and 10 wt%. Also, OB16 ($w_{\rm EO} = 0.43$) was blended with OB1 at 5 and 10 wt%. In both cases, the contour length decreased as $\langle w_{\rm EO}\rangle$ increased.

3.2. Worm micelle length control and self-shortening through PLA hydrolysis

Worm micelles composed of inert block copolymer are extremely stable assemblies, as indicated by the contour length distributions over time (figure 2(a)). The contour lengths of worm micelles are measured from fluorescent microscopy images. The distributions are generally Gaussian, with mean contour lengths $\langle L\rangle$ that are typically 10–50% greater than the standard deviations, σ. The OE7′ worm micelles measured here persist for at least 12 days at 37 °C, in PBS solution. Without adding fluorescent dyes, the distributions stay stable for more than a month at room temperature, confirming that worm micelles have consistency in their morphology and physical properties on such timescales [4].

The most recent demonstration that the morphology of a copolymer aggregate depends mainly on $w_{\rm EO}$ in a diblock copolymer appears in the phase diagrams of Jain and Bates [21]. However, a dependence of contour length on $w_{\rm EO}$ was not suggested. To show the dependence here with the same chemistry, two blends of copolymers were studied (see table 1). As shown in figure 2(b), OB1 block copolymer ($w_{\rm EO} = 0.72$) and OE7′ block copolymer ($w_{\rm EO} = 0.45$) were blended at 0%, 5%, and 10% OB1 by weight to form worm micelles and observe the effect on morphology. With increasing $\langle w_{\rm EO}\rangle$, the mean contour length of blended worm micelles clearly decreases and fits to a linear function. The slope of $-70\Delta w_{\rm EO}$ (μm) neglects any explicit effect of molecular weight, which differs (in number-average, $M_{\rm n}$) by a factor of two. Because $M_{\rm n}$ skews the phase diagram [21], a more similar $M_{\rm n}$ diblock, OB16 ($w_{\rm EO} = 0.43$), was blended with OB1 at 10% and 20% by weight. The contour length also decreased linearly in this case as the average $\langle w_{\rm EO}\rangle$ ratio increased. For this pair of blends, the slope is about half that of the other example here. However, in the results that follow for degrading copolymers, it is likely that changes in $\langle w_{\rm EO}\rangle$ dominate changes in $M_{\rm n}$.

The blending of hydrolysable PEO–PLA (OL2) copolymer with inert copolymer (OE7′) before hydration provides a dynamic means of length control. Blending followed by incubation at 50–60 °C yields worm micelles containing both **Q.4** inert and degradable copolymer. Note that PLA is generally considered hydrophobic, provided it is of a sufficiently high molecular weight [22]. In a prior study, PEO–PLA was blended with inert, vesicle-forming diblock copolymers to give self-porating, controlled release vesicles [17]. By a similar scheme here, blends will be shown to yield self-shortening worm micelles for controlled release [5, 23].

In figure 3, the mean contour length of worm micelles formed from blends of OE7′ and OL2 is seen to shorten with time. The transition to sub-optical assemblies (contour lengths <1 μm) is complete in <12 days. Rates of self-shortening are linear and depend on blend ratio: for a 50% blend, the rate is 19 nm h^{-1}, while for a 75% blend the rate is 25 nm h^{-1}. Note that pure OE7′ worm micelles did not show any change over 15 days. The small initial length difference between figures 3(a) and (b) probably comes from the incubation time in preparation: 12 h for samples in figure 3(a), 15 h for figure 3(b). The conversion time to spherical micelles can be extrapolated to ∼6.5 days for the 75/25 blend and ∼9 days for the 50/50 blend. Since samples were stored without any shear or stirring, the shortening of contour lengths is likely due only to the hydrolysis of the hydrophobic PLA block.

Although *polymerized* lactides can be sufficiently hydrophobic to drive self-assemblies, lactic acid monomers or dimers are water soluble. It is likewise well known that even the hydrophobic core of a lipid bilayer is permeated by water molecules [24]. Water molecules no doubt permeate the hydrophobic core of worm micelles, which leads to the hydrolysis of PLA to generate lactic acid monomer and increase $\langle w_{\rm EO}\rangle$. Indeed, figure 4 shows by gel permeation chromatography the generation of L-lactic acid monomer (or dimer) and an increase in the weight fraction of remaining PEO hydrophilic block (for the 50/50 blend). The rate of $\langle w_{\rm EO}\rangle$ increase was calculated from the linear regression of

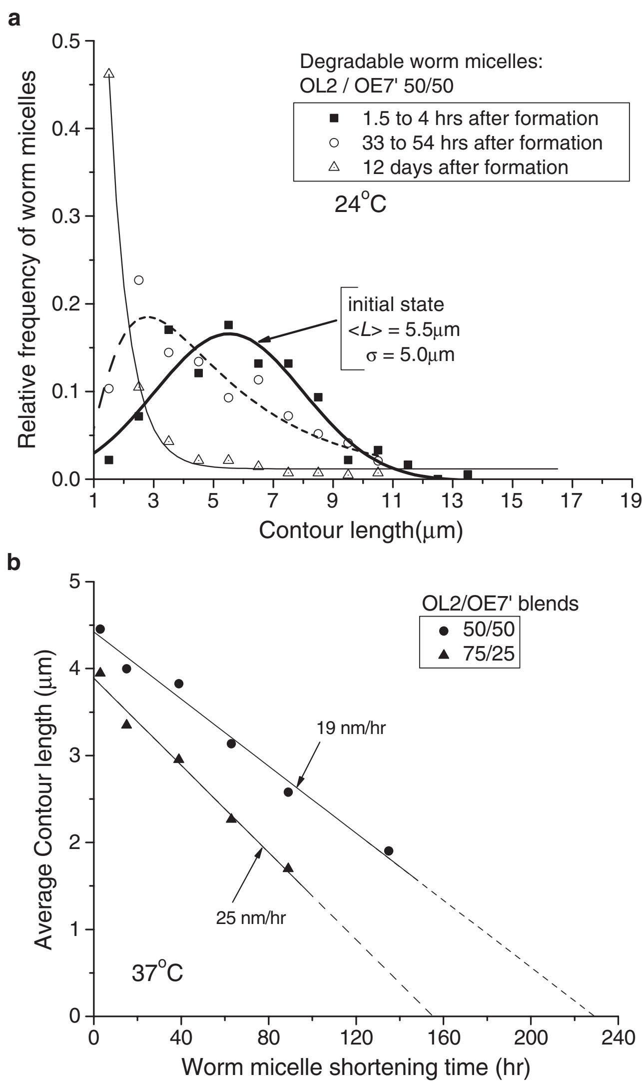

Figure 3. Hydrolytic shortening of worm micelles composed of blends of OE7′ with OL2. (a) The distributions of OE7′/OL2 blends worm micelle contour lengths over time. The initial distribution of worm micelle contour lengths is Gaussian, which shifts toward zero over time. Non-Gaussian fits only guide the eye. (b) The worm shortening rate increases with increasing blending ratios of OL2. The time to shorten to half the initial length is ∼110 h for the 75/25 blend and ∼155 h for the 50/50 blend.

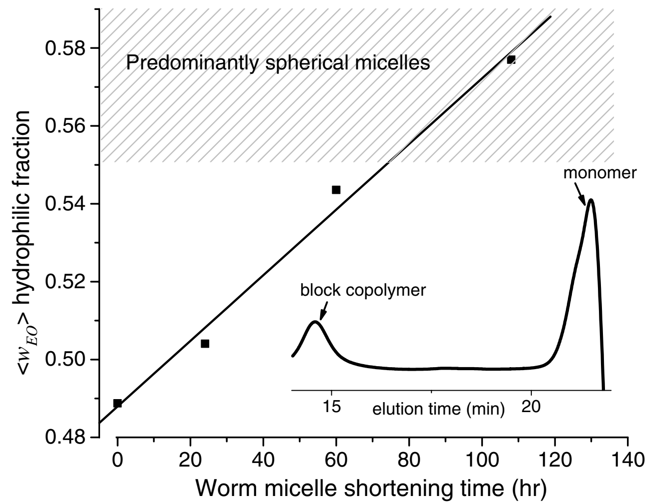

Figure 4. Hydrophilic fraction of copolymer, $\langle w_{\mathrm{EO}}\rangle$, for 50/50 PEO–PEE / PEO–PLA blended worm micelles as analysed by gel permeation chromatography (GPC). (Inset) The GPC chromatogram shows considerable monomer, 60 h after preparation and after storage at 37 °C.

GPC analysis, $\frac{\mathrm{d}\langle w_{\mathrm{EO}}\rangle}{\mathrm{d}t} = 0.084/100$ h starting from an initial average weight fraction $\langle w_{\mathrm{EO}}\rangle = 0.49$. Considering the shortening rate of 2.5 μm/100 h for the same blended worm micelles, the change in weight fraction per change of length can be calculated as $-\frac{\mathrm{d}\langle w_{\mathrm{EO}}\rangle}{\mathrm{d}L} = 0.034/\mu$m. This increasing $\langle w_{\mathrm{EO}}\rangle$ plays an integral part in the mechanism of worm micelle self-shortening through contour length changes. Also in figure 3(b), the initial length changes with blend ratio because of different $\langle w_{\mathrm{EO}}\rangle$. Moreover, given the narrow range of w_{EO} that allows worm micelle formation, it seems likely that the 0.034 μm change in w_{EO} per length drives copolymer segregation along the worm micelles to pinch off spheres.

Clearly, by increasing the ratio of PEO–PLA in worm micelles, degradation rates increase and contour lengths shorten at faster rates, as shown in figure 3(b). Hydrolysis of PLA in worm micelles therefore destabilizes the micelle structure and hydrophobic core, which affects the loading ability and controls the release of hydrophobic drugs, dyes, or other loadable compounds.

3.3. Release kinetics from inert and degradable worm micelles

The release of hydrophobic dye from inert (OE7′) and degradable (OE7′/OL2) worm micelles was measured in two different ways. Dye release from worm micelles was again studied by using the hydrophobic polystyrene beads (∼6 μm diameter) as sinks for free hydrophobic fluorophore in aqueous medium. With this method, hydrophobic fluorophore could be removed more quickly (by centrifugation) than by the second method of extensive dialysis to an aqueous reservoir. Figure 5 shows the release kinetics of inert and self-shortening worm micelles over 15 days. An excess of beads was used to ensure that the beads were not saturated by dye. The initial release kinetics are linear for the inert and two degradable systems studied, and show no burst release. With time, all three types of worm micelle lose nearly all of their dye, and the beads show a maximum intensity. The release time constants decrease from 210 h for inert to 140 h and 80 h, respectively, for 50/50 and 75/25 (OL2/OE7′) blends. Similar trends for release and similar timescales (within 15–25%) were found after extensive dialysis into PBS. Past work on spherical micelles [25], using extensive dialysis, has shown, by zeta potential analysis [26], that the release of hydrophobic molecules from spherical micelles is an interfacial desorption phenomenon, with the interface being between the hydrophobic core and the hydrophilic corona. Such a mechanism would be consistent with the binding site results of figure 1(b).

To prove that the dye released from worm micelles does not relabel other micelles, two-colour mixing experiments were undertaken. Inert worm micelles were labelled with red dye or green dye and dialysed against PBS solution at 4 °C to remove excess dye. Red and green worm micelles were

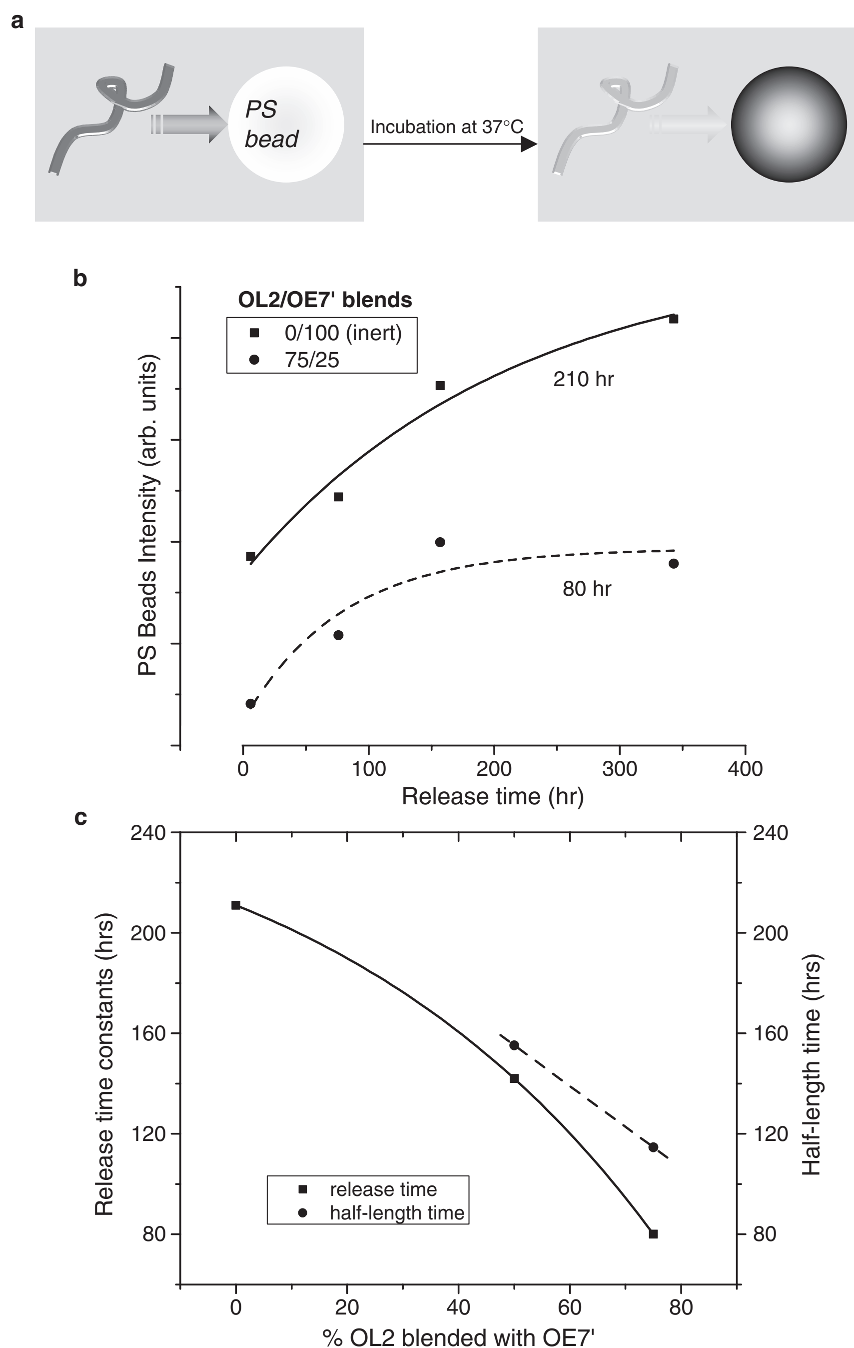

Figure 5. Fluorescent dye transfer from worms to polystyrene beads. (a) Schematic of transfer experiment. PKH-dye loaded worms were mixed with PS beads and stored at 37 °C. (b) Fluorescence intensity was measured by fluorescence microscopy at various time points. Fluorescence intensity profile of PS beads over time. Bead intensity indicates the amount of dye released from the worm micelles. The intensities are fitted to saturation kinetics. (c) Release time constants and shortening times (figure 3(b)) versus OL2 blend ratio show near proportional decays in both release and shortening times with increased blending of OL2.

mixed at a 1:1 ratio (0.08 wt% copolymer) and stored in PBS at 37 °C. Images were then taken at various time points to measure the number of micelles that were *both* red and green. After up to 5 days of observation, no significant mixing was observed. This result again confirms the consistent physical properties of worm micelles on such timescales. The blend-dependent release kinetics are compared in figure 5(c) to the self-shortening half-lives, where very similar times and trends are apparent. This comparison suggests, of course, hydrolysis-coupled release. Moreover, the predictable release behaviour of hydrophobic molecules from degradable worm micelles suggests that carriers of hydrophobic drug can be designed for specific release times.

3.4. Permeation of flexible worm micelles through porous structure

Worm micelles are ‘nano’ in cross-section and flexible in structure (e.g. figure 1(a)), which should foster the effective delivery of hydrophobic drugs into porous tissues. The ability of worm micelles to permeate nanoporous structures was tested here using agarose gels of controlled pore sizes. **Q.5**

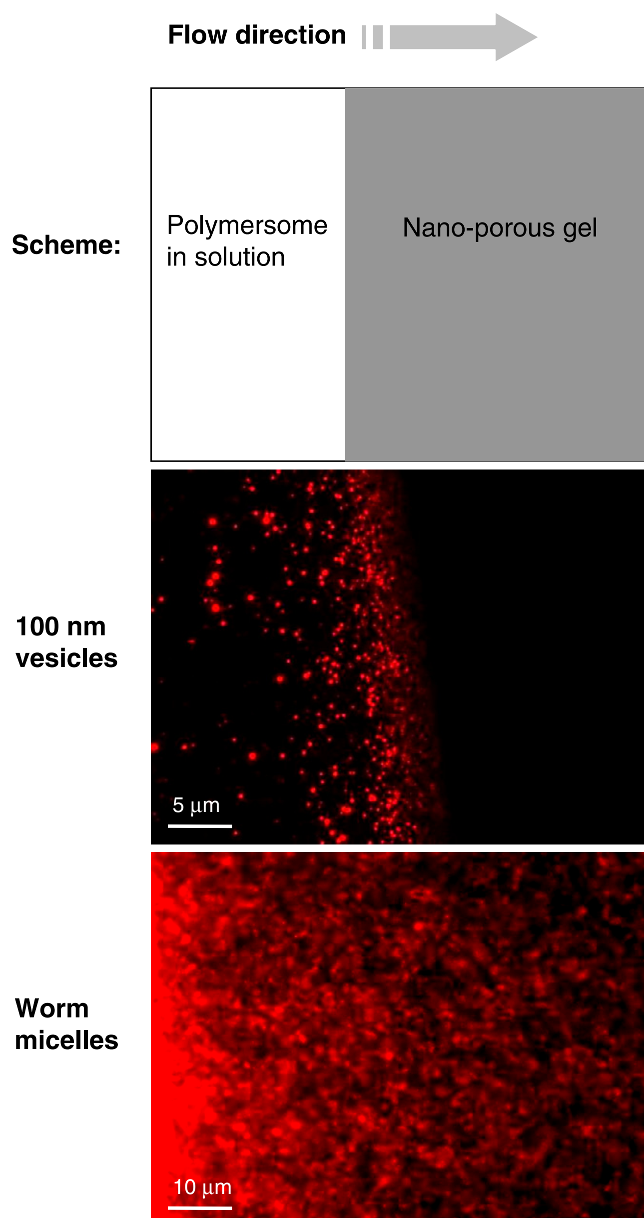

Figure 6. Gel permeation of vesicles and worm micelles through 2.0% agarose gels (100 nm effective pore size). Worm micelles (OE7′ copolymer) were able to permeate through the agarose gel, while 100 nm diameter vesicles (OB18 copolymer) remained at the entrance of the gel with no permeation.

Pores in agarose range from a few hundred to a few thousand nanometres; 1.3% and 2.0% gels respectively give 300 and 100 nm effective pore sizes [15]. Agarose gels are commonly used to separate DNA strands by length, making them an effective material for the study of worm micelle permeation. Here, the permeation of these gels by worm micelles was compared to that of 100 nm sized polymer vesicles, which have been studied as carriers of both hydrophilic and hydrophobic compounds [27].

To make the desired agarose gels, a thin gel layer between two microscope slides was polymerized at room temperature. For permeation of the gel, worm micelles or polymer vesicles (OB18 copolymer) were mixed with 10× PBS solution and placed on the opposite side of the gel as DI water. The solute density gradient was used to drive flow through the gel. Q.6

After applying the worm micelle solution for 20 min, flow through the thin gel was almost complete and permeation was assessed by imaging. With the 0.7% gel medium (pore size ≫300 nm), both 100 nm sized vesicles and worm micelles permeated the gels (data not shown). With 1.3% gels, however, the permeation of 100 nm sized vesicles was slight, while worm micelles still showed good permeation. The difference was most dramatic with the 2.0% gel medium, where 100 nm vesicles were stuck at the entrance of the gel while worm micelles permeated through the porous gel structure (figure 6). After the driving force diminished, worm micelles remained in the porous gel structure. These permeation results are very promising for tissue permeation by these novel drug carriers.

4. Conclusion

Worm micelles, whether inert or degradable, can be used as novel carriers for hydrophobic drugs and dyes by allowing such compounds to partition into the hydrophobic core. The loading efficiency was shown to be in a range that does not give a toxic initial burst of drug. The controlled release kinetics of copolymer-blended worm micelles reveal the potential of self-shortening (at ∼nm h^{-1}) worm micelles as drug delivery vehicles over periods of days. The self-shortening of PEO–PLA-containing worm micelles results from hydrolysis of the PLA hydrophobic block, which increases the weight fraction of the hydrophilic block (w_{EO}) and proves consistent with the transition to spherical micelles. Lastly, we showed the permeation of worm micelles through nanoporous gels, which suggests an effectiveness to deliver drug deep into tissues.

Acknowledgments

This work was supported by an NIH R21 from the National Institute for Biomedical Imaging and Bioengineering (NIBIB), the NanoTech Institute (NTI), and NSF-Materials Research Science & Engineering Centers (MRSEC).

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