

Targeted Worm Micelles

Paul Dalhaimer, Adam J. Engler, Ranganath Parthasarathy, and Dennis E. Discher*

*Department of Chemical and Biomolecular Engineering, University of Pennsylvania,
Philadelphia, Pennsylvania 19104-6391*

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Giant and stable worm micelles formed from poly(ethylene glycol) (PEG)-based diblock copolymer amphiphiles have the potential advantage compared to smaller assemblies for delivery of a large quantity of hydrophobic drugs or dyes per carrier. Here we show that worm micelles can be targeted to cells with internalization and delivery of nontoxic dyes as well as cytotoxic drugs. Constituent copolymers are end-biotinylated to mediate high affinity binding of worm micelles to both avidin-bearing surfaces and biotin-specific receptors on smooth muscle cells. Pristine worm micelles, that lack biotin, show much less frequent and nonspecific point attachments to the same surfaces. Biotinylated worm micelles prove stable in aqueous solution for at least a month and also prove capable of loading, retaining, and delivering hydrophobic dyes and drugs. The results thus demonstrate the feasibility of targeted delivery by polymeric worm micelles.

1. Introduction

Functionally targeted assemblies are of rapidly emerging importance for diagnostic and therapeutic applications since they maximize delivered dosage of contrast agents and drugs to select receptor-bearing cells.¹ In the case of poly(ethylene glycol) (PEG)-containing assemblies, much recent work has focused on attaching ligands or antibodies to the end hydroxyl group.^{2–8} Some relatively convenient and easily monitored covalent modifications have emerged.^{9,10} Here we take advantage of one such chemistry⁹ and biotinylate a neutral amphiphilic diblock copolymer. The diblock here has a molecular weight (MW) of ~ 4000 g/mol and forms worm micelles in aqueous solutions ranging from distilled water to phosphate buffered saline (PBS).^{11,12} Biotinylated copolymers are diluted with pristine/unmodified copolymers prior to forming micelles, which is accomplished by simple hydration of a dried copolymer film.¹¹ Fluorescent labeling of the worm micelles is done most simply with nontoxic hydrophobic fluorophores,¹¹ and the worms are seen to be highly stable. They are also shown to bind to both avidin-coated coverslips and cells.

Worm micelles have long been reported to form with small amphiphiles (≤ 1000 g/mol) that have a high critical micelle concentration (CMC) and thus exchange among other micelles as well as the unimer pool and other sinks in solution.¹³ Classical worm micelles are thus deemed “living” from a stability point of view; upon dilution, such as injection into the circulation of a large animal, the classical micelles will tend to disassemble.¹³ In contrast, the polymer-based worm micelles used here are formed from larger amphiphiles and are stable, nonliving assemblies. They are proving stable upon dilution in cell culture here as well as in the circulation of mice and rats.¹⁴

Based in part on tumor permeation of micron-long linear phages such as M13,^{15–17} recent advances in screening and

imaging have led to the identification of various ligands with specificity for cell surface receptors of interest.¹⁵ Proliferating tumor cells, in particular, overexpress a variety of receptor targets such as integrins,^{15–17} the iron transporter transferrin,¹⁸ and a cancer-specific type of high affinity folic acid receptor.¹⁹ Ligands that bind to these receptors are typically smaller than ~ 1000 Da and can in principle be conjugated to PEG through free amino groups by the chemistry used here. In this first study, however, we use the small vitamin biotin as a generic model for such targeting ligands.

Biotinylated macromolecules have previously been shown to undergo receptor-mediated endocytosis in both animal and plant cells.^{20,21} With living cells in culture, we show that biotinylated worm micelles both bind to cells and transfer cell-viable hydrophobic dye to intracellular organelles, the final destination when free dye is added to cells. This binding and delivery by worm micelles is extended to taxol, a well-known anti-proliferative drug. Taxol is clinically delivered by less than ideal emulsions (e.g., Cremophor) for anti-cancer therapy and is also coated onto vascular stents to block smooth muscle proliferation and arterial restenosis. Taxol is shown here to partition into the hydrophobic cores of biotinylated (and pristine) worm micelles. The results show that a combination of a targeting molecule (biotin) and a core-integrated drug (taxol) can selectively target and kill cells.

2. Materials and Methods

2.1. Copolymer Details and PEG Modification. Poly(ethylene glycol)–polyethylene (PEG–PEE) copolymers were prepared using anionic polymerization techniques described elsewhere.²² The hydroxyl end of the PEG–PEE copolymers (Figure 1) was made amine-reactive as described briefly below by reaction with nitrophenyl chloroformate (NPCF; Sigma; Supporting Information, Figure 1), which is the nitrophenyl ester of chloroformic acid.

* To whom correspondence should be addressed.

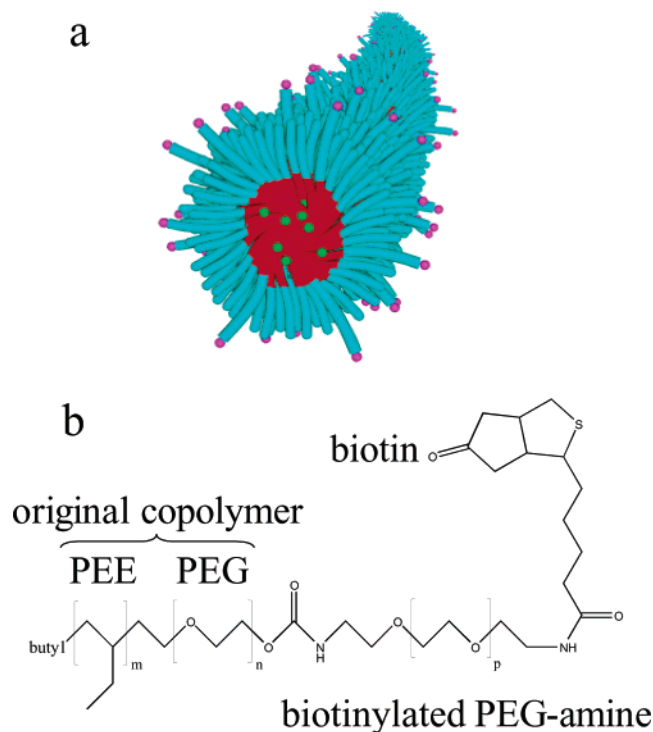


Figure 1. Biotinylated worm micelles. (a) Schematic illustration of a worm micelle where extra long chains terminating in magenta spheres represent PEG extensions with biotin end groups. Green spheres in the core of the micelle represent PKH26 fluorescent dye molecules (or hydrophobic drugs). (b) Chemical structure of a biotinylated PEG-PEE copolymer ($m \sim 37$, $n \sim 40$, $p \sim 9$), designated OEX.

Briefly, 0.2 g of PEG-PEE copolymer was dissolved in 1 mL of dimethylene chloride on ice. 8 μ L of pyridine and 30 mg of 4-NPCF were added to the mixture, and the solution was stirred overnight on ice. Next, 15 mL of an ice cold mixture of EtOH:HCl (40:1) was added, making the solution turbid. A precipitate formed upon equilibration for 15 min at -20 $^{\circ}$ C. The resulting mixture was spun down at 6000 rpm for 8 min at 4 $^{\circ}$ C, after which the pellet was broken up and 15 mL of an ice cold mixture of EtOH:HCl (160:1) was added. The centrifugation was repeated and 15 mL of ice-cold, pure ethyl alcohol was added to the solution. The PEE-PEG-NPCF copolymer was dried under vacuum and stored at -20 $^{\circ}$ C.

2.2. Copolymer Biotinylation. After preparing PEE-PEG-NPCF, biotin-PEG-amine was conjugated through the NPCF group.⁹ Biotin-PEG-amine (MW 720) was purchased from Sigma. For the biotinylation reaction, the PEE-PEG-NPCF copolymer was dissolved in 10 mM citrate at pH 4. Biotin-PEG-amine was added in molar excess to the copolymer and the pH was raised to 8.5 with NaOH, at which point the solution turned yellow due to liberation of the nitrophenylate anion. The solution was then incubated at room temperature for 2 h. The biotinylated copolymer was separated from the free biotin-PEG-amine by dialysis using a 2000 Da cutoff membrane. Diblock copolymers blended with 25% biotinylated copolymer were assembled into worm micelles at 60 $^{\circ}$ C via film rehydration with PBS.²³ Worm micelles were visualized with the hydrophobic dye, PKH26 (Sigma), which partitions into the cores of the preformed worms upon addition of the dye to the aqueous solution.^{11,24} Covalent labeling of PEE-PEG with

tetramethylrhodamine (TMR; Molecular Probes), as an alternative to PKH26, was also used to visualize the worm micelles in a fraction of the cell culture assays. The hydroxyl end of the PEG was covalently attached to TMR through a Curtius rearrangement of an acyl azide.²⁵ Briefly, a TMR acyl azide (Molecular Probes) was heated in toluene at 80 $^{\circ}$ C to cause rearrangement to an isocyanate. Simultaneously, 0.5 mg of PEG-PEE was added in a molar ratio of (10 molecules dye: 1 molecule copolymer) while keeping the temperature constant at 80 $^{\circ}$ C for 12 h. Finally, 20 mg of NH_3OH was added to the stirred solution for 2 h to deprotect the nonfluorescent urethane derivative, which turned the solution color from a pink to a deep red.

2.3. Avidin-Coated Surfaces. Fluorescein-streptavidin (Molecular Probes) was mixed in BSA at 0.1% (molar ratio) and 50 μ L of 1 mg/mL adsorbed to a glass cover slip. Excess protein was washed off with PBS. Imaging of fluorescein demonstrated homogeneous adsorption of avidin. A 50 μ L solution of 0.001 mg/mL PKH26-labeled worm micelles was then added to the surface, which was then carefully drained off without drying after 15 min. Rhodamine fluorescent images were then taken of the surface and the number of adsorbed worm micelles per area counted.

2.4. Cell Culture. Smooth muscle cells of the A7r5 lineage (rat aorta-derived) (ATCC, Manassas, VA) were maintained in polystyrene flasks between passages 2 and 15 and cultured in growth media: Dulbecco's Modified Eagle (DMEM), supplemented with 10% of fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin). Cells were passed every 3 days at $\sim 80\%$ confluency and plated on 35 mm dishes with a circular coverslip (Mattek Corp., Washington, DC) to enable in vitro microscopy. All cell culture products were purchased from Invitrogen. Prior to addition of drug, soluble reagent, or worm micelle, cells were plated at $\sim 1 \times 10^4$ cells/dish (25 mm) and grown for approximately 24 h. Worm micelles were incubated with the cells for 90 min and then analyzed by fluorescence microscopy for binding and internalization. For drug delivery assays, taxol (Molecular Probes) was loaded into the core of worm micelles in exactly the same manner as the addition of PKH26 dye mentioned above. Cells were tested for viability using a trypan blue assay.

2.5. Fluorescence and Atomic Force Microscopy. Fluorescent worm micelles were viewed with a 60 \times oil objective on an Olympus IX71 inverted microscope. Images were collected with a Cascade CCD camera and Image Pro software. A Digital Instruments Bioscope was used to image worm micelles adsorbed to a mica surface.

3. Results and Discussion

3.1. Functionalized Worm Micelle Structure. The schematic of Figure 1a shows a biotinylated worm micelle with biotin naively represented by magenta spheres and located at the hydrated ends of a minor fraction ($\sim 25\%$) of the copolymer chains. Because of the PEG-biotin conjugation through NPCF-copolymer, biotinylated copolymers have nine more ethyleneglycol groups than pristine copolymers

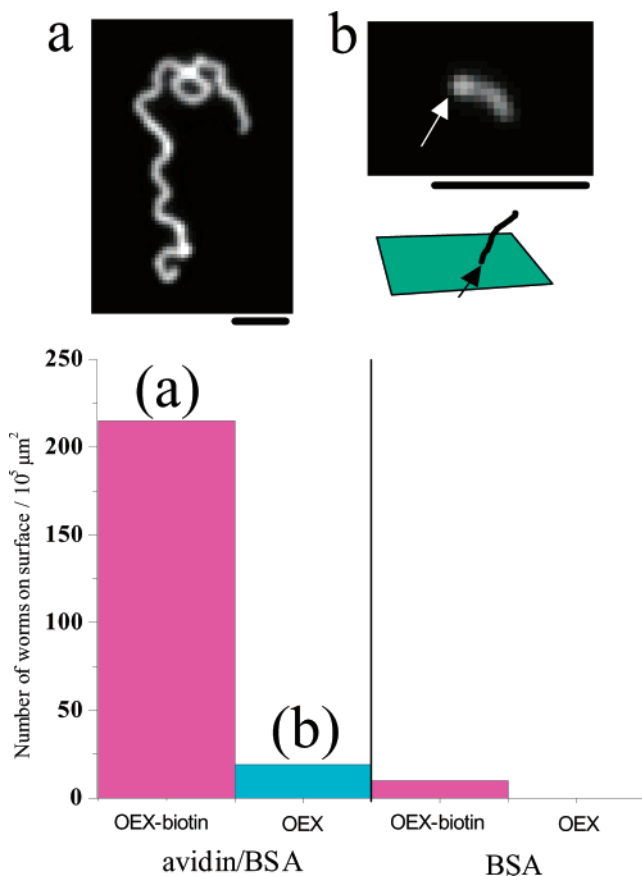


Figure 2. Biotinylated OEX worm micelles bind strongly to avidin-coated surfaces. (a) Worm with a 25% molar blend of PEE-PEG-biotin in PEG-PEE stuck on an avidin-coated coverslip. The entire contour of the worm micelle is in the focal plane indicating that it is fully attached. (b) Pristine worm micelle stuck at its end (indicated by arrow) to an avidin-coated coverslip. Pristine worms do not fully adsorb to the surface even after ~ 15 min. Scale bars are $2 \mu\text{m}$. Bar graph shows specificity of biotinylated worms versus equally dilute pristine worms to avidin coated surfaces. Biotinylated worms bind 2 orders of magnitude more frequently to dilute avidin/BSA-coated surfaces than pristine worms. Neither worm sample shows binding to BSA-coated surfaces.

(Figure 1b). The tiered brush architecture that results in the assembled micelle allows for more conformational freedom of the end-biotin and, consequently, more access to target receptors.

Physical properties of micron-long worm micelles made from the pristine copolymer have been described elsewhere.^{11,24} By visualizing the contours of worm micelles that are either surface-bound (e.g., Figure 2a) or confined between cover slips, biotinylated worm micelles appear to have the same persistence length, l_p , as the pristine micelles, regardless of mixing ratio. By use of the relationship $\langle R^2 \rangle = 2l_p^2[L/l_p - 1 + \exp(-L/l_p)]$ with R being the micelle end-to-end distance and L the micelle contour length $l_p = 500$ nm, this exceeds optical resolution and implies that the entire contour of a worm should be visible as observed.^{11,26} The lack of a detectable difference in l_p between pristine and PEG-biotin worms supports the idea that bending rigidity in these fluid cylinders is determined mainly by hydrophobic core diameter and interfacial tension²⁶ rather than brush length.

3.2. Worm Micelle Interactions with Avidin-Coated Surfaces. Biotinylated worm micelles show facile binding to avidin-coated surfaces. The bar graph of Figure 2

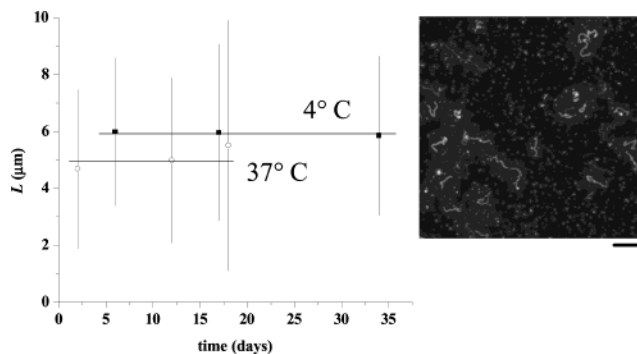


Figure 3. Biotinylated worm micelles are stable for at least a month. Plot shows average contour length (\pm S. D.) of biotinylated (25%) worm micelles in PBS. Contour lengths were measured by imaging with fluorescence microscopy after irreversible binding to an avidin coated surface. Image shows biotinylated worm micelles adsorbed to such a surface. Scale bar is $5 \mu\text{m}$.

demonstrates the binding specificity of biotinylated worm micelles versus pristine worm micelles to avidin/BSA-coated surfaces. The molar ratio of adsorbed avidin to BSA is intended to mimic the typical density of immobile receptors on a cell surface with a spacing of ~ 50 – 100 nm.

Biotinylated worm micelles “zip-up” on these avidin-coated surfaces (Figure 2a): full contour adsorption to the surface occurs in about 1 s. Pristine worm micelles, in contrast, either do not stick at all or attach only at or near one end of the micelle (Figure 2b). The difference is consistent with multivalent, high-affinity interactions. Although the worm micelles are fluid, the biotinylated fraction of copolymers does not segregate on the time scale of the zipping. Emerging measurements of lateral mobility along the micelle’s contour are indeed indicative of relatively slow but definitive diffusion.

3.3. Stability of Biotinylated Worm Micelles. Length distributions of biotinylated worm micelles formed by simple hydration of dried films prove stable for at least several weeks. For eventual application, such stability is not only crucial for storage but is also much longer than the time scale for in vivo circulation of related copolymer vesicles. Stability should thus not be limiting in such applications (ignoring shear effects). Figure 3 shows a plot of average contour length $\langle L \rangle$ as a function of time at 4 and 37 °C. Contour lengths were measured only for worm micelles with $L > 1 \mu\text{m}$ due to optical resolution limitations. The inset of Figure 3 shows biotinylated worm micelles bound to an avidin-coated surface after micelle storage for one week. Although worm micelles formed from small surfactants have long been known, the stability, measured by the CMC, for example, is exponential in the molecular weight of the hydrophobic segment; hence classic worm micelles prove to be much more dynamic and fragile.²⁷ The results here clearly demonstrate physical as well as chemical stability of the polymer-based worm micelles that are also functionalized.

3.4. Controlling Worm Micelle Length Distribution. It might be speculated that micron-long worm micelles will disrupt flow in the vasculature or be difficult for cells to internalize. We therefore anticipate a need for control over the contour lengths of the worm micelles. Figure 4 shows fluorescence and atomic force microscopy images of both biotinylated and pristine worm micelles adsorbed to surfaces

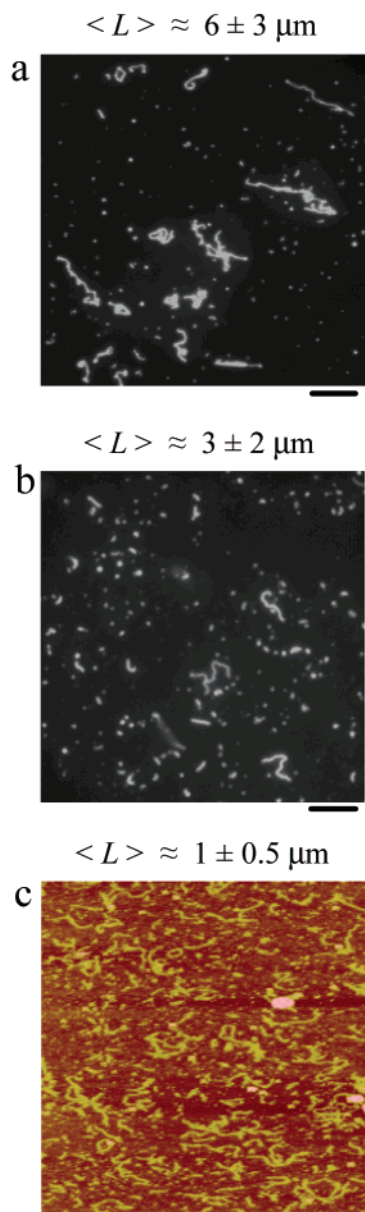


Figure 4. Contour length, L , reduction by mild sonication. (a) Biotinylated worm micelles adsorbed to a 1% (mol) avidin in BSA coated surface. (b) The same micelle sample after 2 min of sonication. (c) AFM image of worm micelles after 5 min of sonication revealing submicron length worms. Scale bars are 5 μm .

after brief intervals of sonication. An additional method (not shown) involves repeated extrusion of the worm micelles through nanoporous filters. This latter method for controlling the contour lengths of the worms also helps remove any micron-size aggregates that could obstruct circulation in vivo.

3.5. Cell Targeting with Functionalized Worm Micelles.

Since the vitamin biotin is internalized by many cells,^{20,21} we tested the cell-targeting ability of biotinylated worm micelles by adding the micelles to cultures of aorta-derived smooth muscle cells. It was first verified that biotin binds to these cells by showing that fluorescein-biotin labels these cells in standard culture (not shown). Biotinylated worm micelles were then incubated with smooth muscle cells for 90 min, after which time nonspecific adsorption is found to decrease the targeting efficiency of the biotinylated worm micelles.

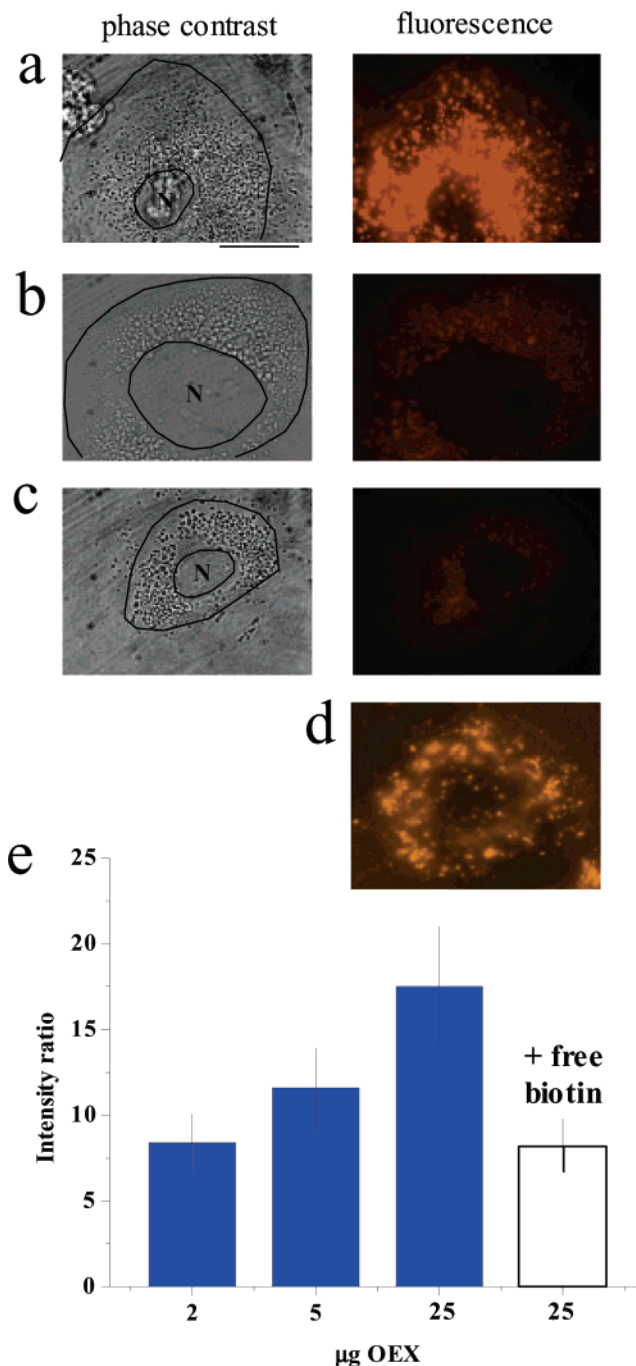


Figure 5. Biotinylated worm micelles preferentially bind to smooth muscle cells through a biotin receptor. Worm micelles prelabeled with fluorescent PKH26 hydrophobic dye were incubated in cultures of smooth muscle cells for 5 h. (a) Biotinylated worms. (b) Pristine worms. (c) No addition. "N" denotes nucleus. (d) Free PKH26 dye added to cells in culture showing that the final destination of the dye is the same whether free or encapsulated in biotinylated worm micelles. Scale bar is 20 μm . (e) Intensity measurements on at least 10 cells show that cells incubated with biotinylated worms appear brighter on average than cells incubated with an equal concentration of pristine worms at μg concentrations of copolymer OEX per ml in 25 mm culture dishes.

Figure 5 shows phase contrast and fluorescence images of smooth muscle cells that were either (a) incubated with biotinylated worm micelles, (b) incubated with pristine worm micelles, or (c) not exposed to worm micelles. The nucleus is generally devoid of fluorescence in cells incubated with labeled, biotinylated worm micelles, thus showing that the

biotin-targeted fluorescence is perinuclear. The final destination of the PKH26 dye in the cells proves the same when intercalated in biotinylated worms or when added as free dye to the cells in culture (Figure 5d). Importantly, biotinylated worm micelles prepared with copolymer that bears covalently bound rhodamine (also attached through PEF's terminal hydroxyl group) showed no qualitative difference in fluorescence distribution after incubation in cell culture. Such localization patterns provide relatively clear evidence of internalization rather than just surface binding.

Cells incubated with biotinylated worm micelles show a stepwise increase in mean intensity (normalized by the autofluorescence of control cells) relative to cells incubated with pristine worm micelles up to saturating copolymer concentrations ($\sim 25 \mu\text{g}$ per culture; Figure 5e). Addition of free biotin to cell culture reduces the binding of biotinylated worm micelles to the cells (Figure 5e). The concentration-dependence studies as well as the inhibition result thus show that the worm micelles themselves are being internalized through biotin-receptor associated endocytosis.

Since we see no large worm micelles in cell cultures with biotin-PEG-PEE, we conclude that micron-long worm micelles are being imported and perhaps fragmented as they enter these cells. Consistent with the latter conclusion, these cells show an increase in mean fluorescence after incubation with biotinylated worm micelles of reduced contour length: a 2-fold increase in cell fluorescence is seen per micron reduction in L from 3 down to $2 \mu\text{m}$.

3.6. Receptor-Targeting Delivery of Taxol with Worm Micelles. Therapeutic treatments of a wide-range of diseases involve the delivery of hydrophobic drugs to various cellular targets, beyond just membranes. Taxol is one such hydrophobic and cytotoxic drug. Taxol binds to and stabilizes microtubules, inhibiting cell division and proliferation as well as other microtubule-dynamics dependent processes.²⁸ Taxol is potent at cellular concentrations of $\leq 10 \text{ nM}$.²⁸ Since taxol is hydrophobic, it partitions into the cores of the worm micelles in a fashion similar to the hydrophobic PKH dyes used to visualize the micelles. HPLC separations show that the worm micelles can hold at least one molecule of taxol for every 10 copolymers.

Figure 6a shows toxicity results for smooth muscle cells incubated with taxol-loaded worm micelles that were either biotinylated or pristine. In this first simple test, biotinylated worm micelles containing taxol killed 75% of the cells in culture. Pristine, nonlabeled, worm micelles loaded with taxol did not kill any cells above control levels. These findings suggest that worm micelles with small ligands on their exterior can deliver hydrophobic drugs to receptor-expressing cells. A sketch of the delivery process (Figure 6b) summarizes the binding, internalization, and repartitioning pathways of worm micelles and taxol. Note that the l_p of the worms suggests an approximate length scale ($< 500 \text{ nm}$) needed for endocytic vesicles to package semiflexible worm micelles for entry.²⁹

4. Conclusion

The drug delivery capabilities of carrier vehicles can be significantly enhanced when specific targeting strategies are

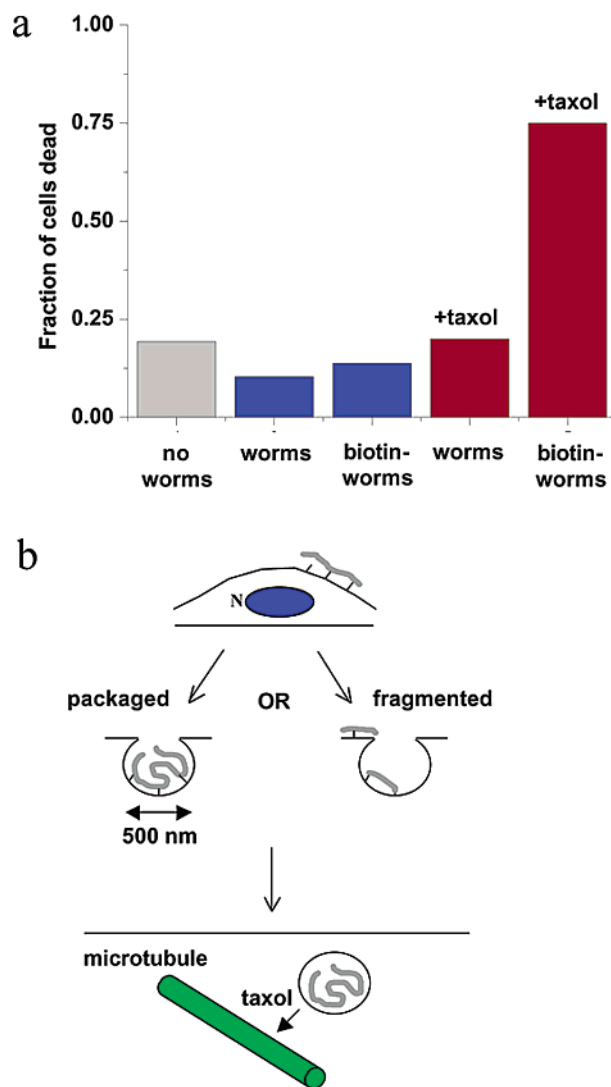


Figure 6. Biotinylated worm micelles loaded with the cytotoxic drug taxol selectively kill cells. (a) $5 \mu\text{g}$ of copolymer (when loaded, equivalent to $\sim 100 \text{ pM}$ free taxol) with and without biotin and taxol were incubated with A7r5 smooth muscle cells for 24 h. Cell viability was tested by a trypan blue exclusion assay. (b) Possible mechanism for dye/drug delivery. Biotinylated worm micelles bind to the cell's surface and are internalized by receptor mediated endocytosis either through packaging of the micelle or fragmentation. The hydrophobic contents are finally repartitioned to their destination, in this case, taxol binds to tubulin. "N" denotes nucleus.

used. We have shown here that polymeric worm micelles can be readily biotinylated for targeting and can readily transfer their hydrophobic cargos (a fluorescent dye or a cytotoxic drug) to cells. The same worm micelles were also found to bind to avidin-coated surfaces. In either case, pristine worm micelles that were not functionalized showed little interaction. These results strongly support the notion that worm micelles can be functionalized as target-specific carriers for delivery to and internalization by selected specific cell types. The results also demonstrate the successful delivery of drugs from these filamentous micellar carriers. Combined with the fact that these structures tend to orient and readily convect with blood flows at least in vitro,¹¹ the results begin to point to the perhaps surprising utility of phage-mimetic structures. The wide range of diblock copolymers that can be made, including those with known

biocompatibility and degradation pathways, suggest a promising new morphology in polymeric worm micelles for designed drug and dye delivery.

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Supporting Information Available. NMR spectra showing NPCF modification of PEE-PEG (Figure 1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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