Stable and robust polymer nanotubes stretched from polymersomes

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We create long polymer nanotubes by directly pulling on the membrane of polymersomes using either optical tweezers or a micropipette. The polymersomes are composed of amphiphilic diblock copolymers, and the nanotubes formed have an aqueous core connected to the aqueous interior of the polymersome. We stabilize the pulled nanotubes by subsequent chemical cross-linking. The cross-linked nanotubes are extremely robust and can be moved to another medium for use elsewhere. We demonstrate the ability to form networks of polymer nanotubes and polymersomes by optical manipulation. The aqueous core of the polymer nanotubes together with their robust character makes them interesting candidates for nanofluidics and other applications in biotechnology.

cross-link | optical tweezers | nanofluidics | vesicles

Nanotubes are among the most promising structures in nanotechnology. Carbon nanotubes are already finding applications in composite materials requiring high strength-to-weight ratio, and many more applications are expected in the near future (1). Nanotubes also can naturally occur in biology. Transport of genetic material from phage viruses to bacteria can occur via protein nanotubes (2), and phospholipid nanotubes between live cells, purported to be a conduit for intercellular organelle transport, were recently observed (3). The use of nanotubes for transport of biological molecules is particularly exciting, with potentially significant applications in biotechnology. Gold nanotubules in polymer membranes have been used to separate small biological molecules on the basis of molecular size (4) and DNA fragments according to base recognition. Experiments on the movement of DNA in nanofabricated structures (5, 6) showed that new transport mechanisms occur when spatial confinement in one or more dimensions is less than the radius of gyration. Similar effects also have been observed with DNA fragments passing through multiwalled carbon nanotubes (7). All of these nanotubular structures have an interior aqueous environment suitable for biological molecules, but with the exception of ref. 6, the length of an individual channel is only on the order of 1 μm .

Extremely long, water-filled nanotubes can be formed from self-assembling amphiphilic molecules. For example, phospholipids can spontaneously form nanotubes under appropriate conditions (8). Vesicle membranes composed of self-assembled amphiphilic molecules can exhibit both elastic and viscoelastic properties, which, under the application of a localized force, can result in the formation of a nanotube. Pioneering work by Evans *et al.* (9) demonstrated the formation of phospholipid nanotubes by using a micropipette to pull on phospholipid vesicle (liposome) membranes. More recently, Orwar and coworkers (10) adopted the technique of Evans to create elaborate networks of liposomes interconnected by phospholipid nanotubes and demonstrated transport through these networks. Similarly, optical tweezers have been used to create phospholipid nanotubes by pulling directly on membranes of liposomes (11, 12) or by pulling on a microsphere attached to a lipid membrane (13). Alternatively, simultaneous hydration and suction-driven flow has been used to create extremely long phospholipid nanotubes in microfluidic devices (11, 14).

Phospholipid nanotubes, however, are transient and tend to collapse unless steps are taken to make them more stable. Attempts to cross-link self-assembled systems composed of lipids (or other low-molecular-weight amphiphiles) have met with limited success (9, 15). Conversely, supramolecular structures from the self-assembly of higher-molecular-weight amphiphiles, such as block copolymers, are more amenable to stabilization by cross-linking. Polymersomes (polymer vesicles) (16) have been chemically cross-linked to form structures that are stable indefinitely (17). Worm-like micelles of diblock copolymers with diameters as small as 10 nm and lengths exceeding 1 μm were cross-linked without any disruption of the cylindrical morphology (18). Recently, spontaneous formation and subsequent cross-linking of water-filled nanotubes from the self-assembly of amphiphilic triblock copolymers was reported (19).

Here, we report on the directed formation of water-filled nanotubes by pulling, using either optical tweezers or a micropipette, on bilayer membranes of polymersomes composed of amphiphilic diblock copolymers. Although the stretching of nanotubes from polymersomes using optical tweezers to pull on a microsphere, attached as a handle to the diblock copolymer membrane, has been reported (20),[§] generating sufficient forces to form a nanotube by pulling directly on the polymer membrane is much more difficult. An alternative to using more force is to modify the membrane such that it is easier to pull. It is well known that the elastic and viscoelastic properties of lipid membranes can be dramatically altered by the inclusion of surfactants such as detergents or charged lipids. Charged lipids have been used to increase the fluidity of lipid membranes such that optical tweezers can readily stretch nanotubes from lipid membranes (12). Detergent-like surfactants have also been used to alter the membrane properties of polymersomes, to increase water permeability and susceptibility to lysis (21). By incorporating a detergent-like, triblock copolymer in the formation of the polymersomes, we make the membranes sufficiently fluid to form nanotubes by pulling directly on the membranes with optical tweezers. We subsequently cross-link the polymers to form stable and robust nanotubes that can be removed for use elsewhere. We also demonstrate the formation of polymer vesicle–nanotube networks using optical tweezers.

Results and Discussion

Optical Pulling and Cross-Linking of Polymer Nanotubes. Fig. 1 is a sequence of images that shows a polymer nanotube being pulled

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Abbreviation: TEM, transmission EM.

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[§]The experiment of ref. 20 found that the intermonolayer friction of a polymersome membrane of a particular formulation was ≈ 1 order of magnitude higher than that of a typical phospholipid membrane. That is, the force required to pull a nanotube from their polymer membrane is ≈ 1 order of magnitude higher than the force required to pull a nanotube from a typical phospholipid membrane.

Fig. 1. Video images of the fluorescence from the membrane dye DiO-C16 showing the creation of a nanotube from a polymersome by directly pulling on the polymer membrane with optical tweezers. The nanotube was stretched from the membrane at a rate of $\approx 10 \mu\text{m/s}$. (Scale bar: $10 \mu\text{m}$.)

from the membrane of a giant polymersome[¶] using optical tweezers. We have been able to pull nanotubes up to $\approx 1 \text{ cm}$ in length and were limited by the travel of our microscope stage. Typically, the nanotube retracts back to the parent polymersome when the optical trap is switched off. The images of Fig. 1 were taken by using video fluorescence microscopy of the dye DiO-C16 in the polymer membrane. The apparent diameter of the nanotube is $\approx 250 \text{ nm}$, which is the diffraction limit of the optical microscope. The retraction of the nanotube to the parent vesicle, when the end of the nanotube is released, is indicative of the transient nature of pulled structures from self-assembled membranes; the system wants to relax to the lower-energy configuration. This behavior is readily observed for nanotubes pulled from lipid bilayer membranes (9). Although such nanotubes can be kept from retracting by attaching them to surfaces or other vesicles (10), the resulting structure is quite fragile and typically stable for only a few hours. For many applications, the nanotubes must be highly stable such that they can be moved from container to container. Although there have been attempts to stabilize pulled lipid nanotubes by imbedding, in the lipid bilayer, macromolecules that contain cross-linkable monomers (9), this strategy has not been successful for small ($< 200 \text{ nm}$) diameter lipid nanotubes. Conversely, polymers can be readily cross-linked to form stable structures.

We stabilize our pulled polymer nanotubes by free radical polymerization of the hydrophobic butadiene tails (17). We introduce the cross-linking reagents using a flow-cell device. The nanotubes became cross-linked and stable within 1 min of adding the reagents. Fig. 2*a* is a fluorescence image of a cross-linked polymer nanotube attached to the parent polymersome. The nanotube is curved because of the fluid flow as the cross-linking reagents were introduced. (Straighter, cross-linked nanotubes are produced by reducing the flow velocity.) A pulsed UV laser (optical scalpel) is used to cut the nanotube free from the similarly cross-linked, parent polymersome. Fig. 2*b* is a fluorescence image of the polymer nanotube after application of the UV pulse. The rigidity of the polymer nanotube is now evident. In contrast to the situation before cross-linking where the nanotube retracts to the parent polymersome (at a rate of $\approx 10 \mu\text{m/s}$) after release of the end of the nanotube from the optical trap, the cross-linked nanotube does not change shape after its connection to the polymersome is severed. Further evidence of the rigidity of the nanotube is seen in Fig. 2*c*, in which pulling on the end of the severed nanotube with optical tweezers (in the direction indicated by the arrow) displaces the nanotube without changing its shape. The cross-linked, polymer nanotubes are extremely robust, maintaining their shape even after several weeks of storage at room temperature. In contrast to lipid nanotubes pulled from liposomes, our cross-linked polymer

Fig. 2. Video fluorescence microscopy images of a pulled nanotube after cross-linking showing attachment to the parent polymersome (*a*), severing from the parent polymersome by the application of a short, high-energy UV laser pulse (*b*), and deflection (in the direction indicated by the arrow) of the rigid, cross-linked polymer nanotube by optical tweezers (*c*). The optical tweezers is not on in *a* and *b*, whereas in *c* the location of the optical tweezers is indicated by X.

nanotubes can be moved from the chamber in which they are formed to another container with a different buffer.

The rigidity of the nanotube suggests that the entire nanotube is cross-linked; however, the use of the pluronic F-127 may limit the maximum extent of cross-linking relative to a pure copolymer system. In a systematic study of cross-linking within worm-like micelles formed from a blend of cross-linkable and non-cross-linkable copolymers (22), percolation behavior (indicating complete cross-linking) was observed for mol fractions of cross-linkable copolymers > 0.15 . Because we only use 8 wt % ($\approx 1.7\%$ mol fraction) pluronic F-127 in the formation of our polymersomes, it seems reasonable that we should observe rigid nanotubes after cross-linking.

Nanotube Formation Using a Micropipette. Not surprisingly, we also can pull polymer nanotubes using micropipettes, similar to the technique demonstrated with lipid membranes (9, 10), and subsequently cross-link them as described earlier. Alternatively, we generate flow by expelling buffer containing the appropriate cross-linking reagents from a micropipette tip into a chamber containing a fairly high density of polymersomes in buffer. This flow causes the polymersomes to move past the micropipette tip, and occasionally one of the polymersome membranes sticks to the end of the tip. The flow continues to push the polymersome downstream pulling a nanotube out of the membrane, with the other end attached to the micropipette tip. Simultaneously, the cross-linking reagent flowing from the tip causes the resulting structure to become rigid over time. The process continues repeatedly as the polymersomes flow past the micropipette tip, generating many rigid and straight polymer nanotubes dangling from the end of the micropipette tip. As a final step, additional cross-linking reagents are added to the chamber to further stabilize all of the structures produced.

Nanotube Morphology and Diameter by Transmission Electron Microscopy (TEM) Imaging. To obtain a better measurement of the diameters and morphologies of our stable polymer nanotubes, we imaged them by TEM. Fig. 3 contains TEM images of a sample of cross-linked, polymer nanotubes. Fig. 3 *Left* shows a relatively uniform nanotube and one that is corrugated. A number ($\approx 80\%$) of cross-linked nanotubes imaged in this sample appeared corrugated. The corrugation appears only after cross-linking and depends on whether the nanotube is under some tension during the cross-linking.^{||} This result suggests that the corrugation is due to the cross-linking process and not due to polymer vesicles fusing together linearly (23), like a string of

[¶]We typically pulled nanotubes from giant polymersomes with diameters of $> 5 \mu\text{m}$, because these polymersomes adhered more readily to the coverslip surface and therefore did not move as we pulled on the membrane.

^{||}The corrugation also can be seen under fluorescence microscopy with nanotubes containing the membrane dye DiO-C16 and appears only after the addition of the cross-linking reagents. When care is taken to ensure that both ends of the nanotube are held securely and the nanotube is under some tension, the fraction of nanotubes that are corrugated, as measured with fluorescence microscopy, can be $< 10\%$.

Fig. 3. Low-resolution (*Left*) and high-resolution (*Right*) TEM images of cross-linked polymer nanotubes. The nanotubes, which were relatively straight just after cross-linking, were washed, dried, and stained with uranyl acetate before placing them in the TEM. This process of preparing the nanotubes for the TEM most likely caused them to bend and also bundle together. We observed that the majority of nanotubes in this sample appeared to be corrugated, which we later discovered occurred as a result of cross-linking. The wall of the nanotube is visible in the high-resolution image as the region along the edge of the nanotube that is slightly darker. A collapsed cross-linked polymersome is apparent in the upper portion of the low-resolution image.

beads. Corrugation of tubular lipid vesicles have been explained as a Rayleigh-type instability arising during front propagation because of a sudden increase in membrane tension (24). Corrugated patterns observed in cylindrical polymer gels (25) have been attributed to stress associated with the spatial changes in the polymer density across the thickness of the polymer boundary, which limits the diffusion of water. Similarly, periodic, corrugated patterns are observed in stiff polymer films supported on relatively soft, elastic media due to a strain-induced elastic buckling instability (26). Because we flow in chemicals to perform the free radical polymerization reaction, the polymers in the outer layer of the bilayer membrane structure will, most likely, cross-link before the molecules in the inner layer, possibly giving rise to stresses that may result in corrugation of the nanotube.

Also visible in Fig. 3 *Left* is a cross-linked polymersome that appears to have collapsed, possibly during dehydration of the sample. The rigidity of the cross-linked polymer membrane is apparent by the folds in the collapsed polymersome. Fig. 3 *Right* is a magnified view of another pair of nanotubes. Again, one of the nanotubes has a relatively uniform profile, whereas the other is highly corrugated. By measuring the outer diameter of the nanotube in this image and using a (unilamellar) membrane thickness of 8 nm,** we obtain an average inner diameter of ≈ 70 nm for the uniform nanotube and ≈ 25 nm at the constrictions of the corrugated nanotube. Similar diameters were obtained for nanotubes from other magnified TEM images.

Evidence for Aqueous Core. For applications of nanotubes as conduits for transporting biological material, it is necessary that the core of the nanotube contain water. Although our nanotubes are formed from amphiphilic copolymers, the question remains whether the core of the nanotube is aqueous or micellar, because

**We obtain a membrane thickness of 8 nm for our polymers using the curve in figure 3 of ref. 27. This value is consistent with measurements based on our TEM images (circled region of figure 3), which appear to give a membrane thickness of ≈ 12 nm but systematically overestimate the actual value because the images are not true cross-sectional views.

Fig. 4. Images illustrating the creation of polymer nanotube-vesicle networks. (*Left*) Sequence of images illustrating the pulling of a nanotube from the membrane of a polymersome using optical tweezers (shown in red) and the attachment of the nanotube to another polymersome. (*Right*) Composite image from video fluorescence microscopy of a network of polymer nanotubes and polymersomes, containing the membrane dye DiO-C16, assembled using optical tweezers. (Scale bar: 10 μm .) (*Inset*) Scanning confocal microscopy image of a nanotube pulled from a polymersome encapsulating sulforhodamine B dye in buffer. Variations in the intensity are due to movement of the nanotube during the scan. (Scale bar: 10 μm .)

giant worm-like micelles have been observed to form from the diblock copolymer PBd-PEO (18). To observe the water-filled core, we pulled nanotubes from polymersomes encapsulating buffer containing the water-soluble, fluorescent dye sulforhodamine B. Because the permeability of the polymer membrane to water is >1 order of magnitude less than the corresponding permeability for lipid membranes (21), it is a reasonable assumption that any water in the core of the pulled polymer nanotube comes from the water enclosed by the parent polymersome. Fig. 4 *Right Inset* is a fluorescence image, taken with a laser scanning confocal microscope, of a cross-linked, pulled polymer nanotube and the parent polymersome. The fluorescence from the excited sulforhodamine B dye evident in the parent polymersome also can be seen in the pulled nanotube, indicating that buffer containing dye is in the core of the nanotube.

Polymer Nanotube-Vesicle Network. The polymer membranes can be manipulated, with either optical tweezers or micropipettes, to form nanotube network structures such as Y-junctions, similar to those demonstrated with lipid membranes (9, 10). Fig. 4 *Right* contains a composite image of a network composed of polymer nanotubes and polymersomes. As illustrated in Fig. 4 *Left*, optical tweezers are used to grab onto the polymersome membrane and stretch out a polymer nanotube. The end of the nanotube, held by the optical trap, is pulled through the membrane of another polymersome and then released. Once released, the nanotube retracts until it reaches the polymersome membrane through which it was inserted, thus forming a structure composed of two polymersomes joined by a polymer nanotube. The two Y-junctions apparent in Fig. 4 *Right* were formed by pulling an additional nanotube from a polymersome that already had a nanotube attached to it. If the additional nanotube was pulled from the membrane at a location sufficiently close to the attachment point of the first nanotube, the attachment point of the additional nanotube would move along the polymersome membrane toward the attachment point of the first nanotube and then along the length of the first nanotube, thus forming a nanotube Y-junction. (The location of the attachment point of the additional nanotube to the first nano-

tube is such that all nanotubes emanating from the Y-junction experience only tension and no shear or bending force.) This process is the same mechanism of forming Y-junctions originally demonstrated with nanotubes pulled from liposome membranes using micropipettes (9, 10). We also have demonstrated the formation of a Y-junction by directly pulling on the wall of a nanotube to form an additional nanotube.

Conclusions and Outlook

We demonstrate the controlled formation of polymer nanotubes by directly pulling on a membrane composed of amphiphilic diblock copolymers using optical tweezers. We subsequently make them stable and robust by chemical cross-linking. The ability to pull directly with optical tweezers provides additional capabilities to form supramolecular structures of interest. For example, we have observed that the optical trap can be used to pull a nanotube directly from the wall of an existing nanotube to form a junction and that we can readily insert the end of a polymer nanotube through the membrane of a polymersome. We have been able to pull nanotubes up to ≈ 1 cm in length. We also have pulled polymer nanotubes using micropipettes, similar to the technique demonstrated with lipid membranes (9, 10), and subsequently cross-link them as described earlier. Alternatively, we generate flow by expelling buffer containing the appropriate cross-linking reagents from a micropipette tip into a chamber containing a fairly high density of polymersomes in buffer. This flow of reagents simultaneously directs the formation of nanotubes and cross-links them.

The cross-linked nanotubes we form appear to be quite rigid, suggesting that the entire structure is cross-linked. Although the use of a pluronic to make the polymersome membrane more flexible may limit the maximum extent of cross-linking, it is not clear whether the pluronic is still incorporated in the polymer membrane of the stretched nanotube or in the membrane, in general, after cross-linking. Further studies, for example, using fluorescently labeled pluronics, need to be performed to address this issue.

Although we have demonstrated stabilization of nanotubes by chemically induced cross-linking of our polymers, photo-induced cross-linking also should be possible and may, in some cases, be a more convenient and flexible technique to use. Direct exposure of the polymersomes to UV did not induce cross-linking.

We have observed that the chemical cross-linking process can produce corrugated nanotubes, and such structures may be useful for DNA sorting (5, 6). We also demonstrated the formation of polymer vesicle–nanotube networks that include Y-junctions. The ability to create stable, biologically compatible nanotubes of essentially arbitrary length in nontrivial geometries lends itself to many applications in biotechnology, such as nanofluidics, as well as opening up new opportunities for investigations of other systems, such as the behavior of long molecules effectively confined to one dimension.

Materials and Methods

Polymersome Preparation. Polymersomes were formed by using an electroformation technique (28). Diblock copolymer poly(butadiene-*b*-ethylene oxide) PBd(1800)-*b*-PEO(900) was purchased from Polymer Source (Dorval, PQ, Canada). A mixture of the diblock copolymer at 20 mg/ml solution in chloroform, 8 wt % pluronic F-127 (Sigma), and 10 mM 3,3'-dihexadecyloxycarbocyanine perchlorate (DiO-C16) membrane dye (Molecular Probes) was prepared. The detergent-like triblock copolymer PEO-poly(propylene)-PEO, Pluronic F-127, which incorporates into the membranes, was added to increase membrane fluidity. F-127 was chosen because very low concentrations (0.06 wt %) can permanently disrupt liposome membranes at room temperature (29). Because of the structural similarity between diblock PBd-PEO and triblock F-127, polymersome formulations incor-

Fig. 5. Illustration of the flow-cell device used for cross-linking polymer nanotubes. The sample chamber consists of a microscope slide with an ≈ 1 cm diameter hole drilled through the center. The bottom of the chamber is a glass coverslip (not shown) sealed to the microscope slide by silicone grease. The channel was formed by using two pieces of double-sided tape (each piece is ≈ 2 cm in length with a thickness of ≈ 70 μ m) spaced by 2 mm between the upper surface of the microscope slide and an additional glass coverslip. This coverslip only covered the channel region up to the edge of the sample chamber. The sample chamber was left uncovered to allow for evaporation to pull fluid to be pulled through the channel.

porating <10 wt % F-127 stably form vesicles (29). Fifty microliters of this polymer mixture was applied to ≈ 1 -cm length of two parallel platinum wires (1 mm diameter, spaced by a gap of 3 mm), dehydrated under nitrogen gas, and then vacuum dried for 1 h. The resulting polymer film on the platinum wires then was hydrated in an electroformation chamber, containing ≈ 1 ml of 0.3 M sucrose solution, at 10 V and 10 Hz for 18 h. Five hundred microliters of the polymersome suspension was transferred to a centrifuge tube containing 10 ml of buffer (10 mM Mops/50 mM NaCl, pH 7.0) and centrifuged at $672 \times g$ for 5 min. A sample from the bottom of the centrifuge tube was used for experiments. For some of the experiments, 1 mM sulforhodamine B (Sigma) dissolved in the 0.3 M sucrose solution was used, instead of the membrane dye DiO-C16.

Flow Cell and Cross-Linking. The polymersomes and nanotubes were cross-linked by free radical polymerization (17). A mixture of 90 μ l of potassium persulfate (12.5 mg/ml), 8 μ l of 9 mg/ml sodium metabisulfite solution, and 8 μ l of 130 mg/300 μ l ferrous sulfate heptahydrate solution was applied to the polymer structures by using a flow cell. The flow cell consisted of a channel constructed using two strips of double-sided tape that was sandwiched between a microscope slide, with a 1-cm-diameter hole drilled through the center, and a glass coverslip (Fig. 5). The 1-cm hole, sealed off on the lower side by another glass coverslip, using silicone high-vacuum grease (Dow-Corning), served as the sample chamber. The polymersomes, having a density greater than water because of the encapsulated sucrose, eventually (we typically wait 60 min) reside at the bottom of a chamber and stick to the coverslip surface, after which they are readily accessible for optical manipulation and imaging on an inverted microscope. The fluid channel, created by the double-sided tape and coverslip, is open to air at one end and empties into the top portion of the chamber at the other end. Cross-linking reagents are introduced at the open end of the channel and move toward and empty into the chamber region by capillary flow. Although the flow velocity in the channel is quite high, the flow velocity in the chamber is substantially lower because the cross-sectional area of the chamber is much larger than the cross-sectional area in the channel region. The low flow velocity in the chamber region allows the appropriate chemicals to reach the nanotube without destroying it. (The process of adding chemicals directly to the chamber by pipette turned out to be too violent and destroyed any nanotubes before stabilization by cross-linking could take place.)

Optical Tweezers and Scalpel. Optical manipulation was performed on a Zeiss Axiovert 100 inverted microscope, which was modified to include optical tweezers that can be moved across the field of view, and an optical scalpel (30). The trapping light is from a continuous-wave, ytterbium fiber laser (IPG Photonics, Oxford,