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Drug-Delivery Strategies by Using Template-Synthesized Nanotubes

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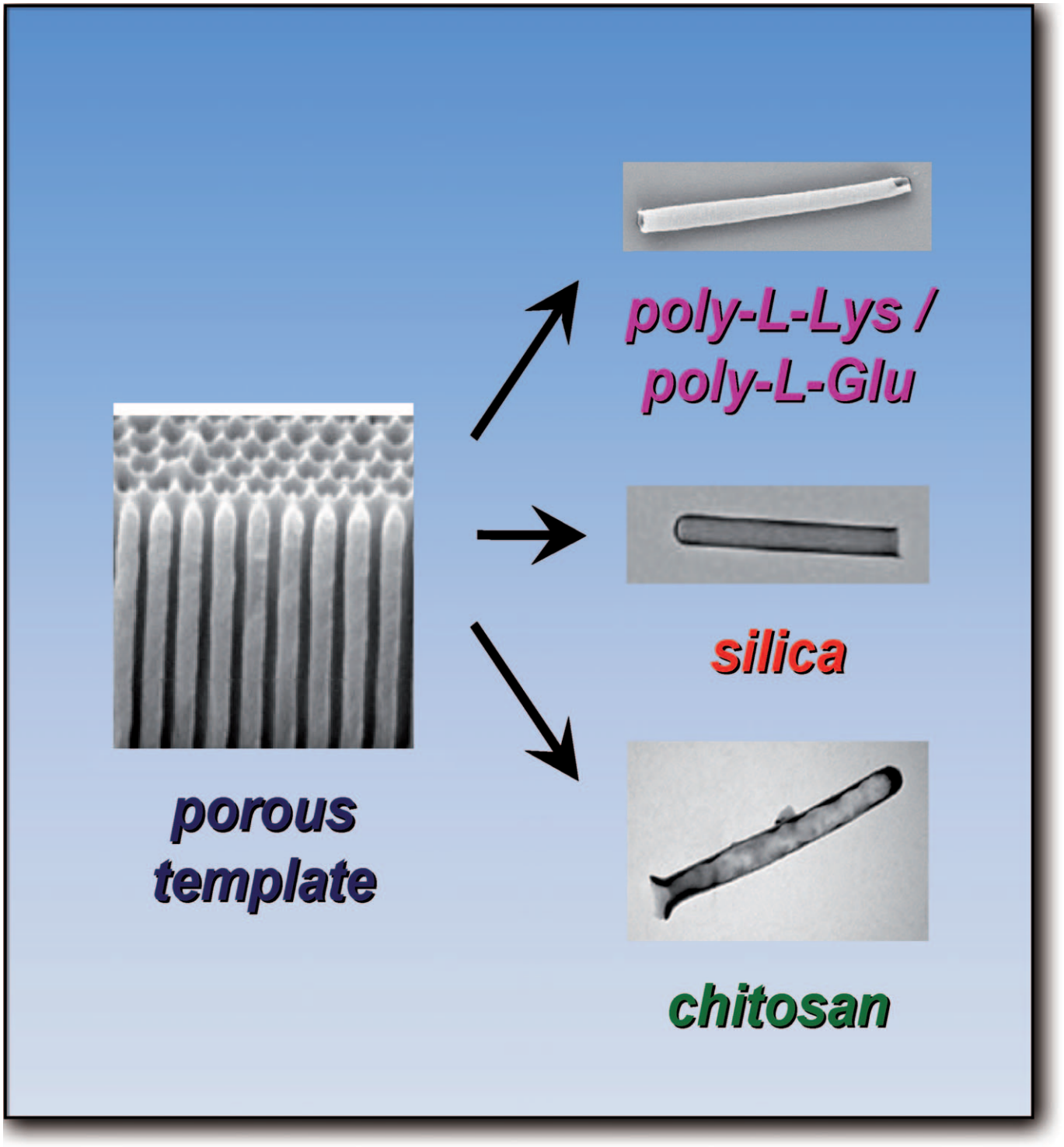

Abstract: Encapsulating drugs within hollow nanotubes offers several advantages, including protection from degradation, the possibility of targeting desired locations, and drug release only under specific conditions. Template synthesis utilizes porous membranes prepared from alumina, polycarbonate, or other materials that can be dissolved under specific conditions. The method allows for great control over the lengths and diameters of nanotubes; moreover, tubes can be constructed from a wide variety of tube materials including proteins, DNA, silica, carbon, and chitosan. A number of capping strategies have been developed to seal payloads within nanotubes. Combining these advances with the ability to target and internalize nanotubes into living cells will allow these assemblies to move into the next phase of development, in vivo experiments.

Keywords: drug delivery · nanotubes · template synthesis

Introduction

Once administered, the majority of current drugs do not accumulate selectively at the pathological site, but instead are distributed throughout the body. Furthermore, to reach the target site, therapeutic agents must often cross several biological barriers, while avoiding inactivation or clearance from circulation.[1,2] Maintaining a clinically relevant drug concentration at the pathological site often requires a large dose of drug, much of which interacts with healthy tissue. Encapsulating drugs within functionalized nano-vehicles not only shields them from enzymatic inactivation,[2,3] but also opens the potential for active targeting.

The ideal drug-delivery vehicle would be biocompatible and biodegradable, able to navigate the circulatory system to reach the target, and capable of releasing its therapeutic cargo only to the desired cells. In an effort to meet this goal, a variety of nanoscale structures have been developed by using a variety of different materials and shapes.[4] Spherical nanoparticles with a variety of diameters have been created from a diverse range of materials.[5–7] While spheres are simpler to synthesize, they suffer from several drawbacks as drug-delivery vehicles. Nanospheres remained in circulation for only a few hours,[7–9] whereas cylindrical-shaped carriers, such as nanotubes, were found in the blood of rats for up to one week after injection.[8] Moreover, DeSimone recently reported that cylindrically shaped PRINT particles (PRINT = particle replication in non-wetting templates) were internalized in HeLa cells at a rate four times faster than spherical particles of the same diameter.[10] Finally, cylindrical carriers can carry larger payloads than nanoparticles of the same diameter.[11] For these reasons, we will focus on template-synthesized cylindrical nanocarriers for biomedical applications. The use of fullerene carbon nanotubes for biomedical applications has been extensively reviewed elsewhere.[12–14]

Template Synthesis

Template synthesis allows a range of nanostructures to be prepared within porous membranes that are subsequently dissolved to leave the desired products behind.[15,16] In addition to controlling both size and shape, the method also allows for different surface chemistries on the inside and outside. While still embedded within the template, only the inner surface is accessible for functionalization. The outer surface becomes exposed only after the structures have been released by dissolving the template. Alumina, polycarbonate, polyethylene terephthalate, and polyester micro/nanoporous membranes have all been used to fabricate nanotubes and nanowires through template synthesis; however, because the range and uniformity of pores within commercially available membranes are limited, many studies have utilized alumina templates prepared by a two-step electrochemical procedure. Depending on the synthesis conditions, the alumina film can remain attached to the underlying aluminum or it can be detached. The former template yields nano test tubes (closed on one end), while the latter allows nanotubes (open at both ends) or solid nanowires to be fabricated.[16–21]

Nanotubes

Because only a limited quantity of DNA can be accommodated on the outer surface of nanotubes,[22–24] we fabricated layered nanotubes composed entirely of DNA using Mallouk's alternating α,ω-diorganophophonate (DOP)/Zr^{IV} chemistry and a commercially available alumina template membrane.[18,20] These structures contained an outer layer of α,ω-DOP/Zr^{IV} for structural integrity and an inner core of multiple double-stranded DNA layers held together by hybridization between the layers. After assembly, the alumina

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template was dissolved in phosphoric acid and the liberated tubes were collected by centrifugation (Figure 1). Heating the tubes above the melting point of the double-stranded DNA provided a route for releasing the DNA payload.

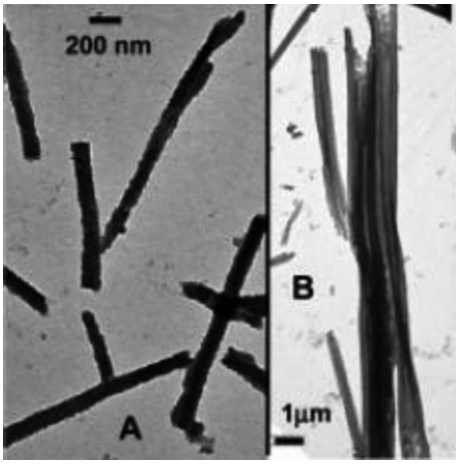

Figure 1. TEM images of five-layer DNA tubes. A) One layer of α,ω-DOP/Zr^{IV}. B) Three layers of α,ω-DOP/Zr^{IV}.[20] Reproduced with permission from the American Chemical Society.

Nanotubes have been functionalized with proteins on their inner or outer surfaces. As with the DNA example above, the nanotube carrier, rather than the protein, makes up most of the mass and volume. We have synthesized carrier-free glucose oxidase and hemoglobin nanotubes in a commercially available alumina template membrane using a layer-by-layer process.[21] After coating the alumina membrane with 3-aminopropylphosphonic acid, alternating solutions of glutaraldehyde and the desired protein were used to deposit successive protein layers. Dissolving the alumina template yielded free protein nanotubes (Figure 2).

In many cases, it is not desirable (or possible) to use the cargo itself to construct nanotubes. Silica provides an ideal material for proof of principle studies in drug delivery, since these nanotubes are simple to prepare, they can be suspended in aqueous solution, and their surfaces can be easily derivatized with a diverse range functional groups.

Chen investigated silica nanotubes, open at both ends, as gene-delivery vehicles.[25] A silica layer deposited by sol–gel chemistry on the surface of alumina with 200 nm diameter pores was given a positive surface charge by reacting with an amine-terminated silane. Red fluorescent CdSe/ZnS quantum dots were electrostatically adsorbed, then an additional silica layer was deposited and treated with an amine-terminated silane. After liberation from the template, plasmid-DNA-encoding green fluorescent protein was adsorbed to the inner surface by electrostatic interactions. Incubating monkey kidney COS-7 cells with these constructs led to green fluorescent protein expression in 10–20% of the cells. In addition, the plasmid DNA was resistant to DNase degradation while encapsulated within the silica nanotubes.[25]

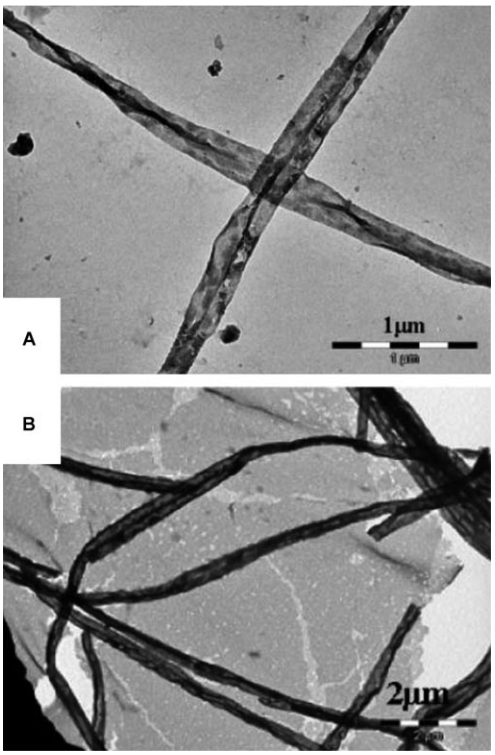

Figure 2. TEM images of protein nanotubes. A. Tubes prepared from glucose oxidase. B. Tubes prepared from hemoglobin.[21] Reproduced with permission from the American Chemical Society.

Li created chitosan/alginate nanotubes by depositing alternating layers of chitosan and fluorescently-tagged alginate within the pores of a commercially available polycarbonate template membrane (400 nm pore diameter × 10 μm thick) that had been modified with poly(ethyleneimine) to give a cationic surface.[26] Surface polymer layers were removed by polishing both membrane faces with alumina powder and free nanotubes were obtained by dissolving the template with CH_2Cl_2 (Figure 3). Their biodegradability was tested by overnight exposure to pancreatin, which produced defects and clumping of the tubes. AFM imaging showed a coarse surface and collapsed morphology.[26]

A similar layer-by-layer technique was used to fabricate nanotubes from alternating layers of poly-L-lysine and poly-L-glutamate (Figure 4).[27] As before, the dimensions of the free tubes matched those imposed by the template membrane with wall thicknesses of about 33 nm. While still membrane bound, the nanotubes were covered on their inner surfaces with citrate-coated Fe_3O_4 nanoparticles, which adsorbed by Coulombic interactions to the poly-L-lysine. After liberation from the template, their outer surfaces could be coated with FITC-labeled plasmid DNA (FITC = fluorescein isothiocyanate). The biodegradability of these nanotubes was probed by overnight treatment with α-chymotrypsin. As expected, the protease thinned the tube walls and led to a coarse surface and collapsed morphology.[27]

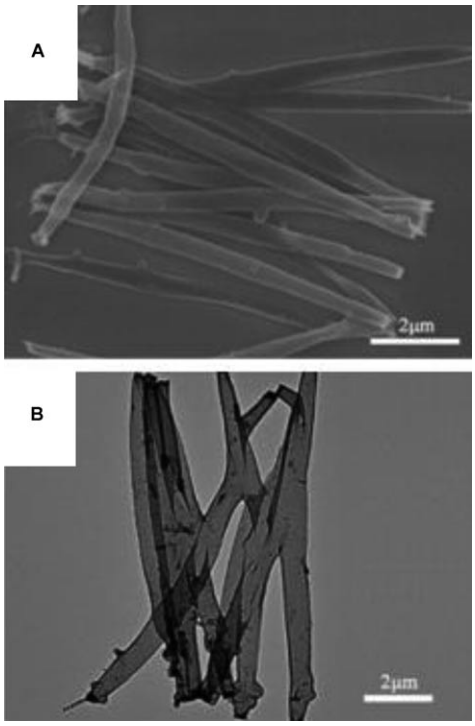

Figure 3. Alginate/chitosan nanotubes. A. SEM image. B. TEM image.[26] Reproduced with permission from Elsevier

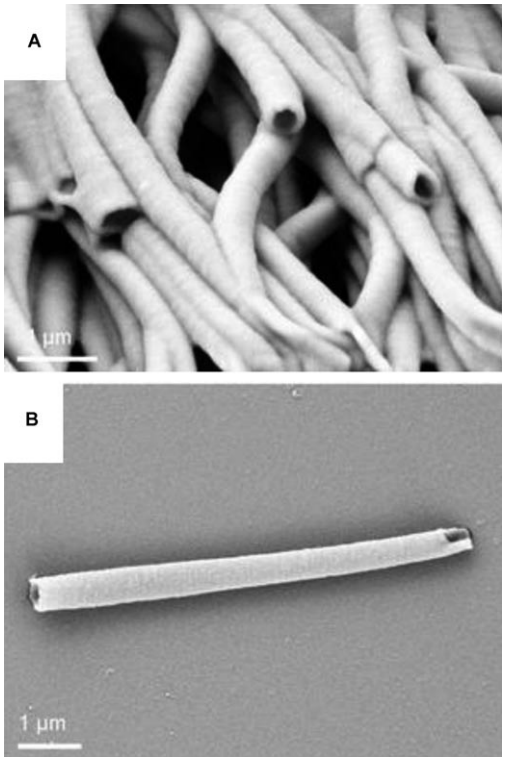

Figure 4. Poly-L-lysine/poly-L-glutamate nanotubes. A. Sample of multiple tubes. B. An individual tube.[27] Reproduced with permission from the Royal Society of Chemistry.

Nano Test Tubes

Covalent and/or electrostatic attachment to the walls of nanotubes is not always the most appropriate strategy for preventing premature release of the cargo. Filling the nanotubes with an aqueous solution of the payload, then capping the open ends would be the simplest and most general method for delivery. Of course, this approach also demands that uncapping and/or tube degradation must occur after the assemblies reach the desired target. This strategy requires that both ends of the nanotubes be capped; by contrast, nano test tubes (open only at one end) require only a single cap, which significantly simplifies the assembly problem. Alumina templates for producing nano test tubes can be prepared by the usual electrochemical strategy, except that the alumina layer is left attached to the underlying aluminum (Figure 5).

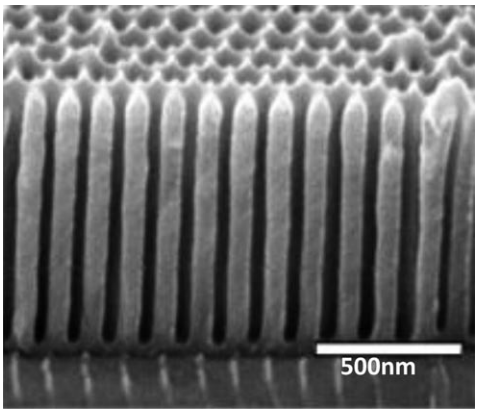

Figure 5. Cross-section SEM image of an alumina template membrane designed for nano test tube synthesis.[42] Reproduced from *Nanomedicine* **2010**, *5*, 1151–1160 with permission from Future Medicine Ltd.

Silica nano test tubes: Silica nano test tubes were prepared in alumina templates by both bulk sol–gel or a layer-by-layer deposition, although the latter allows more precise control over the wall thickness (Figure 6).[28] They can also be capped with latex nanospheres by electrostatic and/or covalent interactions.[29] While still template-bound, the

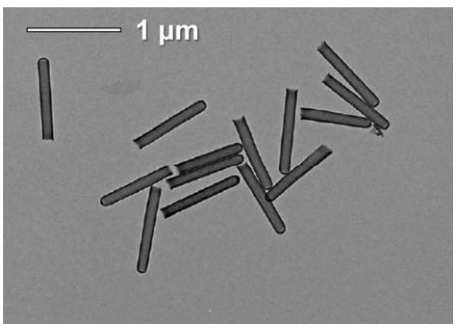

Figure 6. TEM image of hollow silica nano test tubes prepared by the layer-by-layer method.[32] Reproduced with permission from Elsevier.

inner walls (diameters of ca. 74 nm) were functionalized with an amine-terminated silane, then surfactant-free, polystyrene latex nanoparticles (mean diameters of 75–78 nm) with surface aldehyde groups were added. The cap size was deliberately chosen to prevent entry into the tubes and require that interactions be restricted to the edges near the mouth. Prior to dissolving the template, 95% of the tubes were capped. After this step, 80% of the isolated tubes retained their caps and a variety of control experiments were consistent with covalent bonds between tubes and caps.

Lee developed a different method for sealing the open ends of silica nano test tubes, based on seed-mediated gold growth.[30] While embedded in an alumina template, the inner surfaces of sol–gel-formed silica nano test tubes were modified with (3-trimethoxysilylpropyl) diethylenetriamine, which provided a positive surface charge. After dissolving the template, the test tubes were dispersed in water and incubated with 2 nm gold nanoparticles, the surfaces of which were covered with negatively charged isothiocyanate anions. Because bare silica has a negative charge above pH 2, the gold nanoparticles bound only to the interior tube surfaces where they served to seed localized gold deposition when chloroauric acid and ascorbate were subsequently added (Figure 7).

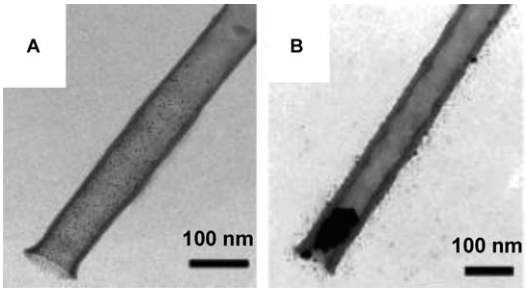

Figure 7. Sealing silica nano test tubes with gold. A. TEM image after incubation with 2 nm gold nanoparticles. B. TEM image after seed-mediated gold growth.[30] Reproduced with permission from the American Chemical Society.

An ingenious, purely mechanical strategy has also been used to seal the ends of silica nano test tubes with gold, silver, or polylactic *co*-glycolic acid.[31] In this method, a thin layer of the desired capping material was deposited onto the outside surface of silica nano test tubes within an alumina template by evaporation. This assembly was transferred to a microtube filled with alumina microbeads and hammered by shaking vigorously in a microtube vortex. After this treatment, most of the nano test tubes isolated after dissolving the template were closed, showing that vortexing had pushed the original surface layer down into the open ends of the silica nano test tubes (Figure 8). To demonstrate that this capping strategy could entrap a payload, the silica nano test tubes were filled with fluorescent dyes before vortexing. After capping, only a 15% dye release was observed from the membrane bound tubes. When the analogous experiment was carried out with isolated nano test tubes, their fluorescence intensity showed no apparent decrease after remaining in water for six weeks.

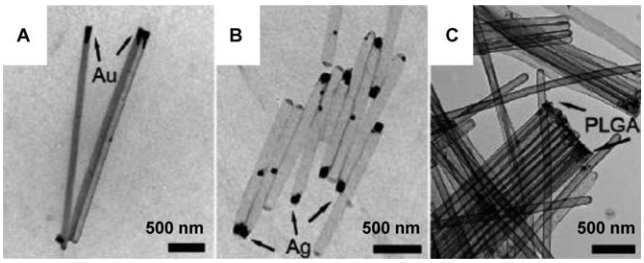

Figure 8. Sealing silica nano test tubes by mechanical means. A. Gold caps. B. Silver caps. C. Biodegradable polymer caps.[31] Reproduced with permission from the American Chemical Society.

We recently showed that antibody-functionalized silica nano test tubes could be targeted to breast cancer cells.[32] While still within the alumina template, the inner walls of silica nano test tubes were modified with a fluorophore, then the template was dissolved and the outer walls were treated with an aldehyde silane. These moieties served as handles to link IGF-1Rα (insulin-like growth factor 1 receptor) through Schiff base formation. As a control, analogous test tubes were also labeled with IGF-1Rβ, which interacts very weakly with the target cells. Fluorescence microscopy revealed that only the nano test tubes functionalized with IGF-IRα were taken up by the cells.

Magnetic particles have been studied extensively for drug delivery and contrast enhancement in magnetic resonance imaging (MRI).[33–36] Lee and co-workers created magnetic silica nano test tubes by exposing silica nano test tubes (60 or 200 nm diameters) within an alumina template to $FeCl_3$/$FeCl_2$ and NH_4OH, which formed a layer of magnetite nanoparticles on the inner surfaces.[37] When their interior surfaces were rendered cationic by an amine-terminated silane, these tubes could be used for controlled release of drug molecules.[37,38] Tubes were loaded with ibuprofen, *p*-nitrophenol, or 5-fluorouracil under non-aqueous conditions, then the amount of payload released after suspension in phosphate-buffered saline was monitored spectrophotometrically. While 10% of the ibuprofen ($pK_a = 4.8$) was released in 1 h, more than 90% of the *p*-nitrophenol ($pK_a = 7.15$) and 5-fluorouracil ($pK_a = 8.1$) dissociated during the same time period. These differences correlated with the expected strengths of Coulombic interactions with the cationic surfaces of the magnetic nano test tubes.

How the size and surface functionalization of magnetic silica tubes impacted cytotoxicity and cellular uptake has also been explored.[38,39] Nano test tubes were prepared with 50 nm diameter and either 200 or 500 nm lengths with exteriors either left bare or modified with amine-terminated silane. The measured viabilities of two different mammalian cells lines grown in the presence of varying levels of these nanotubes showed no differences between 200 and 500 nm tubes. Toxicity was insignificant for all nanotubes at low levels, although a dose-dependent effect was observed at

higher levels. At the highest level, the toxicity of amine-functionalized 200 nm tubes was significantly greater than the corresponding bare silica tubes, suggesting that the cationic tubes may have been taken up more efficiently due to interactions with negatively-charged cell surface.

Carbon nano test tubes: Kyotani and co-workers used chemical vapor deposition to prepare uniform carbon nano test tubes, the dimensions of which were controlled by the alumina template.[40] Unmodified carbon nano test tubes with lengths ≤ 5.0 μm proved to be water soluble. A small molecule cargo was sealed inside these tubes (35 nm diameter × 4.5 μm length) by adding a solution of the dye molecule eosin-Y to the carbon nano test tubes encased within the alumina template, then placing a polystyrene film over the surface.[41] Heating the assembly above the polymer's glass transition temperature (but below its melting point) caused polystyrene to be drawn into the open ends of the test tubes, where it solidified and formed a tight seal. After cooling the polystyrene film was detached, the excess surface layer of carbon was removed by oxygen plasma etching and the filled and sealed nano test tubes were liberated from the template. No significant dye release was observed when the tubes were dispersed in water or ethanol; however, the dye was released in acetone, which dissolves polystyrene.

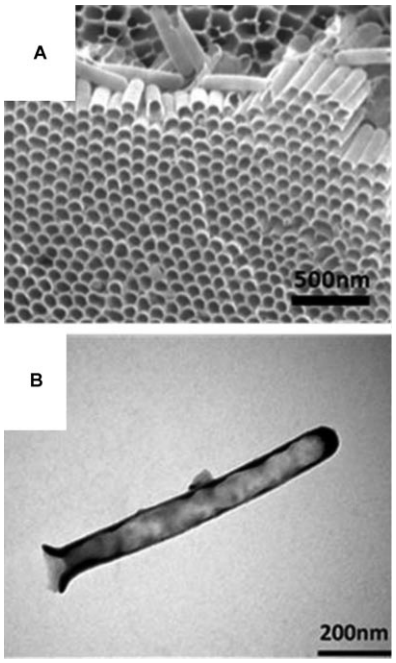

Figure 9. Chitosan nano test tubes. A. FE-SEM image of tubes following removal from the alumina template. B. TEM image of an isolated chitosan nano test tube.[42] Reproduced from *Nanomedicine* **2010**, *5*, 1151–1160 with permission from Future Medicine Ltd.

Chitosan nano test tubes: We fabricated chitosan nano test tubes by solvent casting within a surface-modified alumina template membrane.[42] Because bare alumina was poorly wetted by aqueous chitosan solutions, the template surface was made hydrophilic by first depositing a single layer of silica using Mallouk's method, then coating this layer with a PEG-modified silane.[28] Plasma etching was used to remove these materials from the template surface. The prepared template was exposed to a mixture of chitosan dissolved in an acidic solution and an amine-reactive imidoester cross-linker (dimethyl dithiobispropionimidate DTBP). The template membrane was dissolved in phosphoric acid to yield free chitosan nano test tubes, which were uniform in both size and shape (Figure 9). TEM showed that the wall thicknesses were about 15 nm, leaving internal diameters of approximately 70 nm. The tube lengths were determined by the template pore depths, and this could be manipulated by changing the anodization time during template preparation. As proof-of-principle, chitosan nano test tubes were prepared as described above in alumina template membranes that had been anodized at 50 V for 12, 10, 5, or 1 min. While the tube diameters were essentially unchanged, the tube lengths were 1.2, 1.0, 0.5, and and 0.1 μm, respectively.

Chitosan can be depolymerized by lysozymes, although reaction rates are slower for cross-linked versus linear chitosan.[43,44] To assess the biodegradability of our cross-linked chitosan nano test tubes, membrane-embedded tubes were incubated in the presence of lysozyme in phosphate-buffered saline at 37 °C.[45] Control samples were exposed to phosphate-buffered saline alone. Portions were removed daily and free nano test tubes were obtained by dissolving the alumina templates prior to imaging with FE-SEM. After lysozyme treatment for three days, the tube walls became perforated. After five days, the tubes largely lost their structural integrity and left only amorphous masses of chitosan. By contrast, control samples showed no signs of degradation during this time period.

Chemical reduction offers an alternative approach to degrading our cross-linked chitosan nano test tubes, since DTBP contains a disulfide bond. Moreover, because cells contain a high concentration of disulfide reducing agents, this strategy might allow in vivo intracellular degradation following drug delivery to the target cell. Proof-of-principle was demonstrated by treating samples of membrane-embedded chitosan nano test tubes with dithiothreitol (DTT) or reduced glutathione. Free tubes were isolated by dissolving the alumina template, and then they were studied by FE-SEM. After DTT exposure for 4 h, holes appeared in the tube walls, and the number of holes increased dramatically after 24 h. Similarly, tubes exposed to 5 mM reduced glutathione showed signs of degradation after 24 h with complete collapse after 120 h. Control samples not exposed to disulfide reducing agents showed no apparent changes over these time periods.

Conclusion

Template synthesis strategies provide excellent control over both the internal and external dimensions of nanotubes. Because these methods also allow internal and external surfa-

ces to display disparate functional groups, template-synthesized nanotubes are very attractive starting points for drug-delivery vehicles. Several key advances have recently been made that bring this goal closer to reality. First, nanotubes can now be prepared from a much broader range of materials that include biocompatible and biodegradable materials such as amino acid polymers, DNA, chitosan, and alginate. In addition, a number of capping strategies have been developed to retain drug payloads within nanotubes. Finally, nanotubes have been successfully taken up by target cells where their payload has been delivered. In the near future, these advances will be combined for drug-delivery studies in cell culture and more importantly, in whole animals.

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