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Template synthesis of test tube nanoparticles using non-destructive replication

Jonathan Wagner^{1,2}, Jingyuan Yao², David Rodgers¹, and Bruce Hinds²

¹Department of Biochemistry and Center for Structural Biology, University of Kentucky, Lexington, KY, 40536

²Department of Chemical and Materials Engineering, University of Kentucky, Lexington, KY, 40506

Abstract

Nano test tubes are a promising delivery vehicle for a range of therapeutics including small molecule drugs and biologics. However, current template synthesis methods of producing nano test tubes are prohibitively expensive and time consuming. Here, non-destructive template replication was used to increase nano test tube yield from porous alumina by more than a hundredfold. We demonstrate how to produce nano test tubes of several sizes and compositions including hybrid tubes with different inner and outer surfaces for targeted surface chemistry. Nano test tubes were readily suspended and stored in aqueous solutions without the need for chemical treatment. These nano test tubes should find application as delivery vehicles for therapeutics, particularly for processive “bionanoreactors” loaded with enzymes.

1. Introduction

Nanoparticles have gained a great deal of attention as delivery vehicles for therapeutics including small drug molecules, peptides, DNA, proteins, and enzymes [1-4]. One promising type of nanoparticle under development for delivery of therapeutics is the nano test tube [5]. Nano test tubes have high surface areas and the ability to protect cargo from the environment by sequestering it within the tube. The presence of a single small opening at the end of elongated nano test tubes provides an opportunity for controlling diffusion of molecules into and out of the tubes. This makes nano test tubes a particularly attractive platform for enzyme immobilization since enzymes inside a nano test tube might be protected from the environment while still allowing diffusion of enzymatic substrates and products. This concept of a “bionanoreactor” has proven effective for the protection of enzymes inside spherical particles [6-9] as well as tubes in a two dimensional array [10]. Free floating nano test tubes should provide similar protection for enzymes, but facile synthesis of nano test tube particles remains a challenge.

Template-based synthesis methods have been devised using porous alumina templates (AAO) for test tube manufacture [11, 12]. AAO templates allow nm-scale control over pore diameter and length and thus over the dimensions of the tube. This is important since size is a critical parameter for nanoparticle behavior in the circulation. Particles between 10 nm and 200 nm in diameter are considered preferable for long-circulating particles [2, 3]. The elongated shape of the tubes insures they will have high surface area and tunable cell uptake dynamics [13, 14]. Template synthesis also allows for tubes with disparate chemical functionalization on the inner and outer surfaces [15]. For example, protein conjugation can

be targeted within the core, and the outer surface can be PEGylated to improve stability or inertness to immune response. Many different core-shell compositions are possible for template synthesized tubes including a wide variety of metals and biodegradable polymers [5, 16, 17]. Although template synthesis allows considerable control over nano tube parameters, current methods are limited in scale up for high volume manufacturing. Porous alumina templates of suitably uniform pore size and appropriate length are not commercially available. Synthesizing the templates is laborious and includes depositing thick layers of metal followed by long anodization in acid [18]. Once the desired materials are infiltrated, the alumina template is dissolved with strong etchants [5, 12], which are incompatible with many desired tube materials. Destroying the template means re-making a new template for every batch of tubes. The template synthesis method is essentially a 2-dimensional synthesis, requiring large amounts of surface area ($\sim 20 \text{ m}^2$) to produce gr. quantities. Therefore, the resources and labor that would be required to manufacture therapeutic quantities of nano test tubes is prohibitive.

In order to manufacture enough nano tubes to be therapeutically useful, it is necessary to find more efficient methods of nanotubule synthesis. Here, we describe the application of non-destructive template replication [19, 20] to quickly and easily synthesize test tube shaped nanoparticles (figure 1). In this new approach, the pores of a hard template are used to produce pillar like structures in a polymer material, which can subsequently be coated to make the nano tubes without alteration of the original template. These nanoparticles provide a customizable platform for drug delivery that can be manufactured in volumes sufficient for therapeutic use.

2. Experimental details

2.1. Template preparation

In order to engineer nano test tubes, we used a template synthesis method similar to solvent assisted micromolding (SAMIM) [21-23]. In contrast to previous work, however, we used a rigid template and a non-destructive replication approach (figure 2). For primary templates, we used several types of porous anodic aluminum oxide (AAO) representing a variety of pore sizes and depths. Templates were made by applying a modification of a well-established anodization technique [18] to aluminum deposited on silicon wafers. Porous alumina templates with pore size 50 nm were prepared using electron beam deposition [24] in a 6kW Torr International system (model #EB-4P-6kW). Aluminum was deposited on silicon wafers to a depth of 125 nm-1 μm on cut pieces of a silicon wafer. The coated wafers were anodized for 2 min in 0.3 M oxalic acid at 40 V at room temperature using a 40 V Topward power supply (model 3601D) and a 1 cm^2 platinum coated electrode. After anodization the aluminum was etched in 0.3 M phosphoric acid for 40 min to further open the pores. This procedure yielded templates with the expected 50 nm pore size (supp. figure 1a).

For larger 200 nm pore diameter templates, commercially available AnodiscTM filters (Whatman, # 6809-6022, 25 mm diameter) were used (supp figure 1b, supp figure 2). The filters were partially filled with cross-linked epoxy to control the length of resulting nano test tubes and utilize the region of uniform pore size near the top surface. Filters were placed on 1 mm thick glass supports and mounted on a spin coater (Chemat Technology model KW-4A). A mixture of EpoxicureTM heat curing epoxy (Buehler) was prepared using 1 g epoxy resin, 0.2 g hardener, and 0.2 g acetone (to thin the epoxy mixture). The epoxy was applied over the surface of the aluminum filter and allowed to permeate the filter pores. Excess epoxy was then spun off and the impregnated filters allowed to cure overnight. Remaining excess epoxy was removed using a Buehler Eco-met polisher with 1 μm aluminum polishing grit applied. After polishing the epoxy was selectively etched back to

produce replication wells of controlled depth in AAO pores using a Plasma Enhanced Chemical Vapor Deposition (PECVD) system (Tek Vac Industries model PECVD 60-R) operated at 140 W with 0.7 Torr pressure and 20.0 sccm air flow rate. The etch rate was approximately 250 nm/min and etching time was 2 min and 4 min to produce 500 nm and 1 μm deep replication wells, respectively.

2.2. Template replication

Approximately 2 cm^2 of either 200 nm or 50 nm porous aluminum oxide templates were wetted with a thin layer of acetone using a cotton swab. Cellulose acetate replicating tape 0.22 mm thick (Ted Pella, #44840) was touched to the wet surface with minimal pressure and capillary action was responsible for drawing the polymer down into the pore. After drying for 30 seconds the replicating tape was peeled off. Areas of the replication tape with pillars were apparent by becoming opaque compared to the clear tape in areas without pillars. For sputter film deposition, replicating tapes bearing the imprint from AAO template pores were mounted on a glass microscope slide using small pieces of carbon tape (Ted Pella, #16084-6). The tape was necessary in order to prevent curling of the replicas after peeling off of the AAO templates. The microscope slides were placed on a rotating stage perpendicular to the source and coated with gold, titanium, aluminum, or silicon using settings such that deposition rates were 0.01 - 0.03 nm/s. Power settings were in the 30 - 90 W range and pressures varied between 3 - 4 mTorr. A quartz crystal microbalance was placed beside the samples to measure thickness during deposition, and total metal deposition was kept to <10 nm.

2.2. Nano test tube suspension

The borders of the replicating tape that were not imprinted or that contacted carbon tape during coating were removed with a scalpel. Then the remaining metal coated pillars were immersed in de-ionized water in a 20 ml glass scintillation vial. The sample jar was placed in a 50-60 Hz table top cleaner (Sonicor model SC-50TH) and sonicated for 15 min in a 3 cm deep bath. Nano test tube release was quantified by area analysis of SEM micrographs of pillared surfaces after sonication. A micrograph of a representative area of post-sonication pillars was enlarged and printed as an 8.5 $\times$ 11 inch image. The printed image was analysed using the cut and weigh technique to estimate release area percentage. Areas of complete release accounted for 76% of the total area (figure 2). Thus, nano test tube yields were conservatively estimated at $1.9 \times 10^9/\text{cm}^2$ and $5.1 \times 10^8/\text{cm}^2$ and for 50 nm and 200 nm diameter pore size templates respectively.

2.2. Nano test tube characterization

Absorbance spectrums and settling of nano test tube samples was carried out using tubes with dimensions 50 nm $\times$ 1 μm composed of 5 nm gold (inner) and 3 nm Ti (outer). The sample was prepared from 36 cm^2 of pillared surface sonicated in 5 ml water for an estimated concentration of 6×10^9 tubes/ml. Settling half life was measured by monitoring absorbance at 600 nm. Spectra of pure titanium or gold/titanium tubes were done using suspensions at the same concentration but absorbances were measured in 96-well plate format (supp. figure 3). The absorbance of standard solutions of 5 nm gold colloid were taken in parallel for comparison (Ted Pella # 15702).

For scanning electron microscopy (SEM), suspended samples were filtered using 20 nm pore size Anodiscs to capture the particles. After test tube collection, the filters were broken into portions and mounted on SEM mounts with colloidal graphite. All SEM samples were sputter coated with ~15 nm of gold/palladium to reduce charging and imaged with a Hitachi S-4300 scanning electron microscope.

For transmission electron microscopy (TEM) nano test tubes were mounted on Lacey™ carbon grids (Ted Pella, #01881). The nano test tubes were mounted by submersing a Lacey grid in 5 uL of water then sandwiching the grid - together with the water droplet - between a piece of parafilm and a 2 cm² replicating tape imprinted with gold / titanium coated pillars / nano test tubes. Then the replicating tape was gently rubbed back and forth over the grid until the entire 2 cm² passed through the water droplet. The physical rubbing in a small volume of water acted as a substitute for sonication to release the small amount of test tubes required for TEM imaging. Transmission electron micrographs were obtained using a JEOL 2010fx microscope operated at 100 kV.

3. Results and Discussion

3.1. Template replication

Cellulose acetate replicating tape sold for the purpose of non-destructively replicating surface features for electron microscopy [25] proved to be an ideally suited and cost-effective material for creating small or large aspect ratio replicas of AAO templates. Consistent pillar formation was seen across the entirety of the replicated surface even at the highest aspect ratio of 20 (supp. figure 4). The templates were relatively robust allowing them to be replicated re-used more than one hundred times before being discarded due to wear. Production could potentially be scaled up even further by simply using larger AAO templates and applicators.

Once imprinted with a carpet of pillars corresponding to the pores of the AAO template, the replication tape was sputter coated with one or two thin layers of various metals including aluminum, silicon, titanium, and/or gold. Wetting of the polymer during replication caused the cellulose acetate replication tape to deform, requiring that the corners of the samples be affixed to a solid support prior to metal deposition (supp. figure 5). Mounting samples perpendicular to the metal source and spinning the sample stage during deposition produced the most uniform deposition, thus preventing formation of “nano canoe” shaped particles with one wall missing (supp. figure 6)

3.2. Test tube suspension optimization

Limiting the thickness of the metal deposited was critical to subsequent preparation of nano test tubes (supp. figure 7). Sonication of coated replicas in water proved sufficient to dislodge pillars of all sizes tested with lengths longer than 125 nm while shorter ones failed to release from the replication tape substrate during sonication. This limit was not investigated in detail and it may be possible to achieve even smaller aspect ratio tubes by thinning the metal deposited. For test tubes in the 250 nm - 1 μm length range dissociation from the polymer was only achieved when total metal deposition was kept to less than 10 nm total thickness. Initial attempts using metal layers thicker than 10 nm resulted in no dissociation of tubes during sonication. This was due to metal buildup at the base of the pillars causing the tubes to fuse into large sheets (supp. figure 7) that would delaminate. However, using appropriate thicknesses of metal resulted in greater than 76% of pillars breaking off at their base to become nano test tubes (figure 3). SEM micrographs of nano test tubes filtered from solution show a relatively homogenous population of cylindrical test tubes corresponding to the size of the alumina template used (figure 4). The polymer core could subsequently be removed by oxidative plasma etching as shown for 200 nm diameter tubes made from commercial AAO template (supp. figure 8)

Multiple washes are necessary for chemical conjugation of molecules to particle surfaces, so handling of the nano test tubes without sample loss is critical. We tested the ability to capture, wash, and resuspend nano test tubes of a variety of sizes and compositions using

porous alumina filters. In cases of gold tubes a thin layer of particles representing a negligible percentage of the total sample remained associated with the alumina filter after sonication. No sample loss was visible after resuspending gold lined test tubes with a negatively charged titanium outer layer (supp. figure 9).

3.3. Test tube layering

One of the primary advantages of the synthesis procedure reported here is that the nano test tubes can be prepared with different inner and outer surfaces. Titanium proved a good choice for the outside surface because it has a negative charge at neutral pH that aids in nano test tube suspension. Furthermore, titanium is well characterized as a biocompatible metal useful for biomolecule immobilization [26]. Gold is a useful inner surface because there is a large library of demonstrated chemistry to immobilize macromolecules on gold [27]. We also used aluminum and silicon successfully (data not shown) as the material for the outer lining of nano tubes. Transmission electron microscopy (TEM) clearly shows both gold and titanium layers of nano test tubes (figure 5). The gold appears as a densely packed layer of Au clusters due to island growth on low surface energy oxides. Interestingly, the sides of the tube are thinner relative to the base because of angle of incidence coverage effects during sputter coating. The thinner metal on the sides of tubes are likely important for pillar release during sonication. The polymer core is not seen in TEM due to poor contrast, but it is seen in SEM imaging. It should be possible to selectively remove the polymer inside nano test tubes. Then tethering chemistries could be used to direct macromolecules to either the inner or outer tube surfaces.

An advantage of gold lined nano test tubes is that gold absorbs light much more strongly than pure metal oxide tubes allowing spectrophotometric approximation of the concentration of test tubes after suspension (supp. figure 3). The absorbance of nano test tube suspensions per mg of gold was comparable to that expected based on the absorbance of standard gold colloid solutions indicating close to 100% suspension. Standard 5 nm diameter colloidal Au solutions had an adsorption of 472 abs/mg-cm while the tubes had 389 abs/mg-cm. The dispersed Au nano test tube mass (.003 mg) was calculated assuming 3 nm thick gold on tube tips and only 1 nm on sides with a yield of 76% pillars released. Nano test tube suspensions were observed to gradually settle with a half life of about 6 days (figure 6). Full re-suspension could be readily achieved by brief (30s) sonication of the particles.

These nano test tubes have the potential to demonstrate efficacy as delivery vehicles for biologics, with the delivery of enzymes being a particularly promising application. As catalysts, enzymes have the ability to convert large numbers of important metabolites over a long period of time. An approximation of the number of nano test tubes required for enzyme therapy can be calculated using Elaprase enzyme therapy for Mucopolysaccharidosis II (Hunter syndrome) as an example [28]. This therapy requires administration of 0.5 micromoles of idursulfase enzyme every two weeks. With a diameter of 4 nm² per molecule a dose would cover 13000 cm² of surface area. Projecting immobilization of idursulfase only on inner tube surfaces with dimensions 50 nm × 1 μm we would need 6400 cm² of AAO template per dose. Using such a large AAO template is impractical if the template must be remade after each synthesis. However, by using our non-destructive method to quickly turn out 100 secondary templates from a single AAO primary template we could get 6400 cm² from only 64 cm² of template. This replication process could be easily automated by passing acetone treated tape through rollers with AAO template grown on its surface. Furthermore, most enzyme therapeutics are quickly inactivated in the body. Idursulfase, for example, has an active half-life of only 114 minutes in the bloodstream. If enzymes could be stabilized through immobilization within nano test tubes they would exhibit active lifetimes beyond current enzyme therapies [7]. This, in turn, would allow much smaller doses requiring fewer nano test tubes.

Our nano test tube fabrication method produces tubes with very thin walls varying between 3-8 nm total thickness. Producing nano test tubes with very thin metal walls is attractive from a toxicity standpoint because it minimizes the amount of metal introduced into the body per surface area. For example, if a dose of idursulfase were immobilized on 3 nm thick nano test tubes the resulting injection would contain only 2 μ l of metal. Once diluted in the bloodstream it would be well within amounts deemed innocuous for gold particles in cell viability assays [29].

4. Conclusions

We report the use of non-destructive template replication to manufacture test tube shaped nanoparticles with diameters of 50-200 nm and lengths of 250-1000 nm. The ability to vary the aspect ratio with this technique may have advantages when using the particles as therapeutics or in industrial processes. The vacuum deposition method of forming nano test tubes is versatile allowing the incorporation of a number of useful materials including magnetic metals. These particles retain the desirable qualities of previously synthesized nano test tubes, but with a dramatically increased yield enabling practical delivery vehicles for therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of non-destructive replication of nano test tubes. (a) Replicate porous alumina template using replicating tape and (b) coating the resulting pillared surfaces with metal(s). (c) Immersion in water and sonication to (d) suspend nano test tubes into aqueous solution.

Figure 2.

(a) SEM micrograph of area of good release shows all pillars broken slightly above the surface. On average, 76% of sample area showed good release. (b) Micrograph of area of poor pillar release. On average, 24% of sample area showed poor release.

Figure 3. Non-destructive template replication. A thin coat of acetone is applied to the porous alumina (a) then replicating tape is touched to the wet surface. (b) After acetone evaporates the imprinted replica can be peeled off.

Figure 4.

Left panel - SEM micrographs of pillars replicated from 50 nm pore size AAO at different pore depths (a) 250 nm, (b) 500 nm, and (c) 1 μm. The right panel shows the corresponding samples after release as nano test tubes. Samples were mounted for imaging on 20 nm AAO filters.

Figure 5.

TEM micrograph of a layered tube sputter coated with 3 nm of gold (dark contrast) followed by 5 nm of titanium (light contrast). The sides of the tube are thinner relative to the base because of shadowing effects during sputter coating. Phase segregation caused the gold layer to take on a beaded appearance. 10 nm colloidal gold particles on lacey carbon and outer surface provide microscopy calibration.

Figure 6. Nano test tube suspen from 14 cm² of template area. 50 nm diameter 1 μ m long gold/titanium nano test tubes suspended in water (a). The absorbance of the suspension at 600 nm was measured over time (b).