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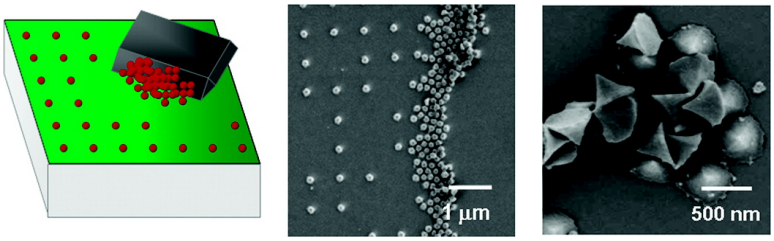

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Direct Fabrication and Harvesting of Monodisperse, Shape-Specific Nanobiomaterials

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Abstract: A versatile "top-down" method for the fabrication of particles, Particle Replication In Nonwetting Templates (PRINT), is described which affords absolute control over particle size, shape, and composition. This technique is versatile and general enough to fabricate particles with a variety of chemical structures, yet delicate enough to be compatible with sophisticated biological agents. Using PRINT, we have fabricated monodisperse particles of poly(ethylene glycol diacrylate), triacrylate resin, poly(lactic acid), and poly(pyrrole). Monodisperse particle populations, ranging from sub-200 nm nanoparticles to complex micron-scale objects, have been fabricated and harvested. PRINT uses low-surface energy, chemically resistant fluoropolymers as molding materials, which eliminates the formation of a residual interconnecting film between molded objects. Until now, the presence of this film has largely prevented particle fabrication using soft lithography. Importantly, we have demonstrated that PRINT affords the simple, straightforward encapsulation of a variety of important bioactive agents, including proteins, DNA, and small-molecule therapeutics, which indicates that PRINT can be used to fabricate next-generation particulate drug-delivery agents.

Particle devices that possess programmed responses to external stimuli and recognition events (i.e., "smart" particles) are necessary for many advanced delivery, targeting, and recognition applications.[1−4] Furthermore, the morphology of the particle (i.e., size and shape) needs to be engineered according to the function of the particle agent. Ideally, the particle morphology and composition should be co-designed to enhance the utility of the device. Most current techniques for particle fabrication are inherently incompatible with this seamless integration of form and function. For example, it is not straightforward to control morphology and structure using "bottom-up" self-assembly techniques, such as liposomal encapsulation,[5] heterogeneous polymerization,[6−10] and inorganic colloid synthesis.[11−13] Energetically driven assembly of these structures is sensitive to the chemical composition of the particle and could be adversely affected by the incorporation of functional agents.[14] On the other hand, "top-down" fabrication of particles using lithography suffers from a lack of compatibility with organic materials because it typically requires processing steps, such as reactive ion etching, baking, ultrasonication, and solvent processing, to define particle shape and remove residual "scum layers" or sacrificial films.[15−21] While these processing methods are well established in the semiconductor industry, where hard, robust, inorganic materials are the norm, they are incompatible with organic materials that are delicate or that contain biologically derived moieties. Rigorous processing techniques associated with the microelectronics industry, such as ion etching or high-temperature annealing, will often lead to the degradation of organic materials,[15] such as biologically derived particle cargos and molecular recognition elements. Heretofore, no single particle fabrication strategy to date has afforded the rigorous control of size, shape, composition, and

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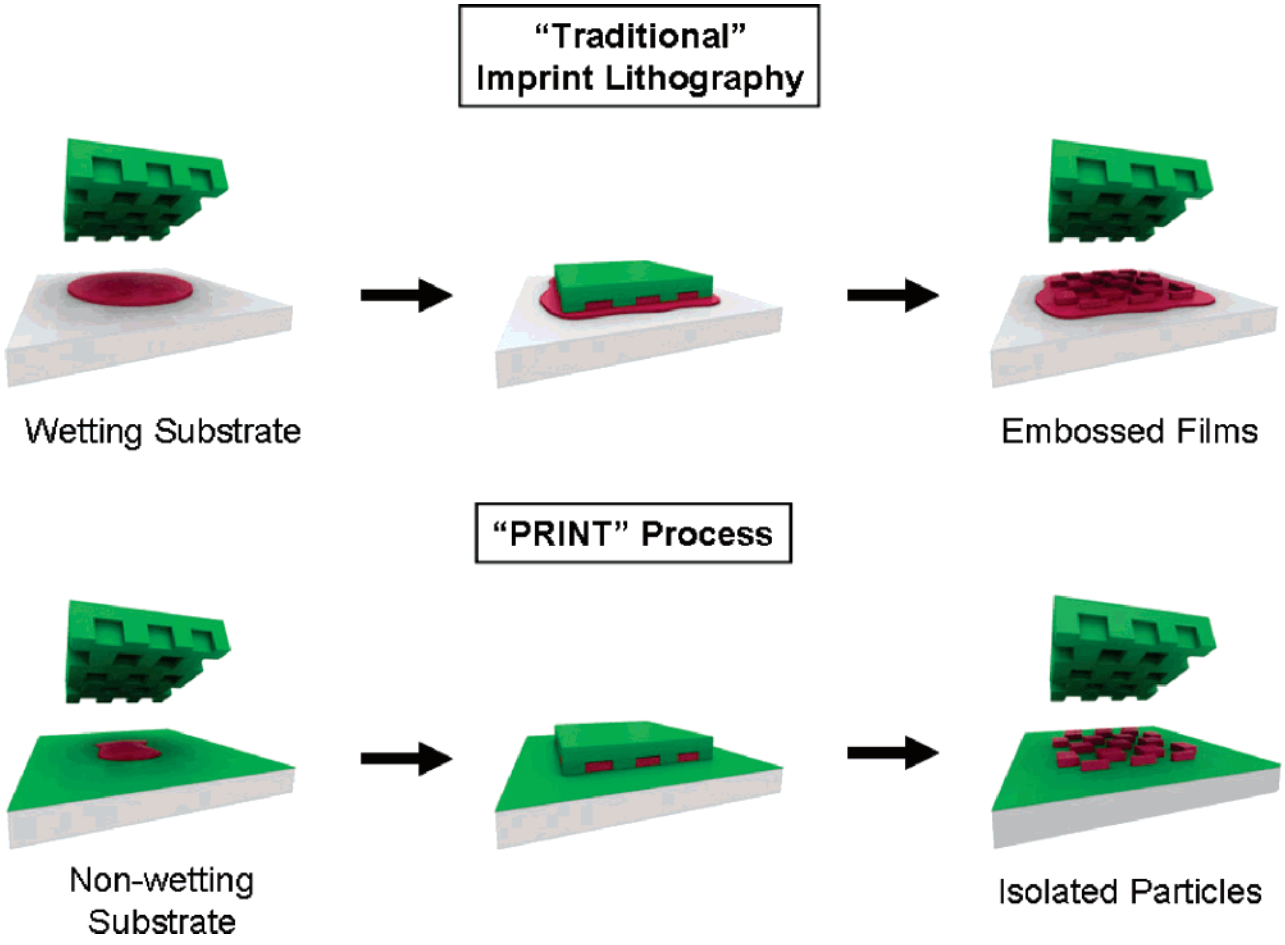

Figure 1. Illustration of the PRINT process compared to traditional imprint lithography in which the affinity of the liquid precursor for the surface results in a scum layer. In PRINT, the nonwetting nature of fluorinated materials and surfaces (shown in green) confines the liquid precursor inside the features of the mold, allowing for the generation of isolated particles.

chemical structure that would be necessary to fabricate and harvest organic particles.

Here, we report a very general technique, Particle Replication In Nonwetting Templates (PRINT), for the fabrication of monodisperse particles with simultaneous control over structure (i.e., shape, size, composition) and function (i.e., cargo, surface structure). To demonstrate the utility and generality of PRINT for particle generation, we have fabricated monodisperse, multifunctional particles from technologically relevant materials, including poly(ethylene glycol), poly(D-lactic acid), poly(pyrrole), and a triacrylate resin, with sizes below 200 nm and with precise shape control. The compatibility of PRINT with fragile biological cargos and recognition agents is demonstrated by incorporating DNA, proteins, and small-molecule therapeutics into sub-200 nm poly(ethylene glycol) nanoparticles using a simple and general encapsulation technique. The ability to independently control particle characteristics, such as size, shape, composition, cargo, and surface structure, makes PRINT uniquely suited to fabricate particles for many applications.

PRINT addresses many of the fundamental shortcomings of other particle fabrication methods. It enables the top-down fabrication to below 100 nm dimensions with orthogonal control of size, shape, and composition by taking advantage of significant advances in "soft lithographic" molding technology that were developed to achieve this goal. This scalable method to generate and harvest nanoparticles enables the possibility of a "particle foundry"—the functional equivalent of the continuous fabrication methodologies employed by the microelectronics industry—for fabricating delicate organic particles necessary for use in nanomedicine and other emerging nanotechnologies.

Here, we have used photocurable perfluoropolyether (PFPE) molds to emboss liquid precursor compounds using highly fluorinated surfaces that are nonwetting to organic materials, which enables the fabrication of isolated objects with superior shape and composition control but without harsh processing steps (Figure 1). We have previously demonstrated that these PFPE molds have superior replication properties for use in lithographic applications in the microelectronics industry, including sub-50 nm lateral resolution, clean surface release, and precise control over replication morphology.[22] Unlike other materials, such as poly(dimethylsiloxane) (PDMS), PFPE-based molds are both nonwetting and nonswelling to both inorganic and organic materials.[23] Though PRINT is akin to other soft lithographic molding methods, it is unique because it produces isolated particles instead of embossed films. This is especially important as one gets to nanometer size ranges because the dimensions of the desired objects approach the dimensions of the residual film, typically called a "scum layer" or "flash layer".[16−19] Other soft lithographic molding techniques typically produce scum layers because the material to be molded has

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significant interfacial interactions with the surface and the mold. PRINT is able to produce isolated, harvestable "scum-free" objects because the highly fluorinated elastomeric mold and the substrate are both nonwetting to the often hydrophobic, yet lipophilic, liquids to be molded. As such, a reversible seal can be formed between the mold and the substrate as slight downward pressure is applied, and the organic liquid to be molded is either confined within the shaped cavities of the mold or forced out due to the low-surface energy of both the mold and the surface. This is in contrast to traditional imprint lithography with PDMS, silicon, glass, or quartz molds, where it is difficult to eliminate this residual material between objects.

Results and Discussion

To demonstrate the utility and flexibility of PRINT, shape-specific organic particles composed of four different materials were generated from a variety of engineered silicon master templates. First, silicon master templates were either made by conventionally transferring a repetitive, uniform shape from a poly(methyl methacrylate) (PMMA) resist that was patterned using e-beam lithography into a silicon wafer or by acquiring commercially available master templates (Figures 2A and 3A,B, and Supporting Information). Second, elastomeric PFPE replica molds of the silicon master templates were generated by photochemically curing a dimethacrylate-functionalized PFPE oligomer that was pooled onto the various silicon master templates.[22] The PFPE replica molds were used to fabricate and harvest individual, monodisperse, nanometer scale particles in a variety of shapes by using the PRINT process.

Poly(D-lactic acid) (PLA) and derivatives thereof, such as poly(lactide-*co*-glycolide) (PLGA), have had a considerable impact on the drug delivery and medical device communities because they are bioabsorbable and nontoxic. To fabricate monodisperse PLA particles using PRINT, a small amount of (3*S*)-*cis*-3,6-dimethyl-1,4-dioxane-2,5-dione with stannous octoate catalyst was heated to 110 °C and molded in its liquid state. After polymerization was achieved, the PFPE mold and the flat, nonwetting substrate were separated to reveal monodisperse 200 nm trapezoidal particles and submicron cones (Figures 2B and 3C).

To further demonstrate the versatility and breadth of the PRINT technique, we chose to generate specifically shaped particles of 200 nm trapezoids from poly(pyrrole) (PPy). PPy has been used in a variety of applications, ranging from electronic devices and sensors[24] to cell scaffolds.[25] We fabricated PPy particles via one-step polymerization[26] by placing a drop of a 1:1 v/v solution of THF:pyrrole and perchloric acid into the molding apparatus, followed by vacuum evaporation of the solvent. Monodisperse 200 nm PPy particles were fabricated with good fidelity (Figure 2C).

Poly(ethylene glycol) (PEG) is a material of tremendous interest to the biotechnology community due to its commercial availability, nontoxic nature, and biocompatibility. Here, PRINT was utilized to produce monodisperse, nanometer scale PEG particles in a variety of shapes by molding a PEG−diacrylate liquid monomer followed by room temperature photopolymerization. Because the morphology of the particles is controlled by the master, it is possible to generate complex particles on a variety of length scales (Figure 4). Confirmation of the structural similarity between the silicon master and replicate PEG particles was confirmed via atomic force microscopy (AFM) and is illustrated in Figure 3B,D, respectively.

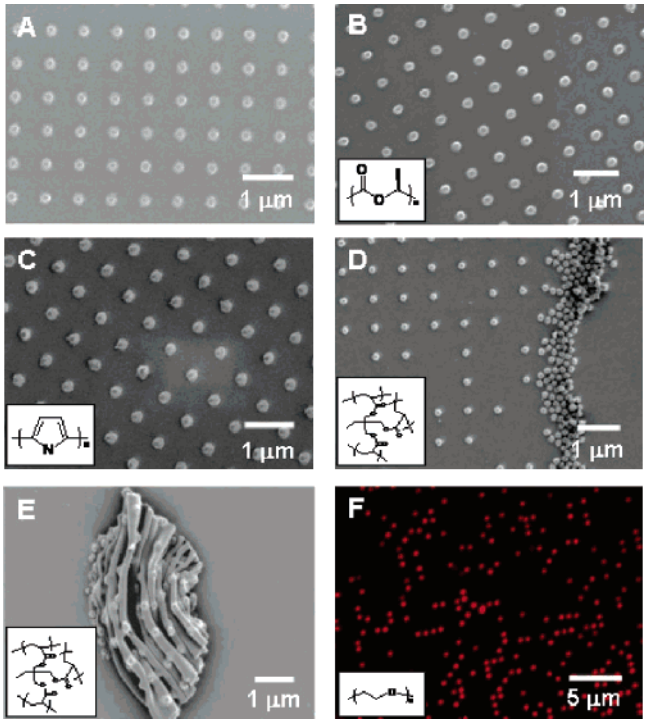

Figure 2. Trapezoidal-shaped particles fabricated using the PRINT process. (A) SEM image of the original trapezoidal silicon master (200 nm feature size) used to generate the PFPE mold that was used to generated the 200 nm trapezoidal particles. (B) SEM image of 200 nm trapezoidal PLA particles. (C) SEM image of 200 nm PPy trapezoidal particles. (D) SEM image of isolated 200 nm trapezoidal triacrylate particles and harvested mechanically using a doctor blade. The ability to harvest particles offers conclusive evidence for the absence of a scum layer. (E) SEM image of an attempted harvesting of 200 nm trapezoidal triacrylate particles that are not isolated but instead are interconnected with a scum layer, offering conclusive evidence that the particles in B, C, and D are isolated, shape-specific objects. (F) Fluorescent confocal micrograph of 200 nm trapezoidal PEG nanoparticles containing 24-mer oligonucleotides tagged with CY-3 fluorescent dye.

With the PRINT methodology, particle harvesting is straightforward and can be accomplished by simply gliding a doctor's blade across the flat substrate. Figures 2D and 3E show successful harvesting of triacrylate nanoparticles. This mechanical harvesting procedure lends itself to continuous fabrication processes using PFPE molds in a conveyor belt-like apparatus. Figure 2E shows an attempted harvesting of triacrylate particles that are interconnected by a scum layer, which prohibits the isolation of discrete objects. A comparison of Figure 2E with Figures 2D and 3E offers conclusive evidence that PRINT nanofabrication leads to truly isolated objects. Further evidence of the isolated nature of these particles is shown in Figures 2D,F, 3F,G, and 4B, where vacancies within the particle array and disruptions of its regular structure are observed. Vacancies arise from isolated particles remaining in the PFPE mold (most probably due to mechanical entrapment by the walls of the mold), and the disruptions of the regular array occur when limited adhesion between the particles and the mold causes the particles to be moved during mold release. We are currently

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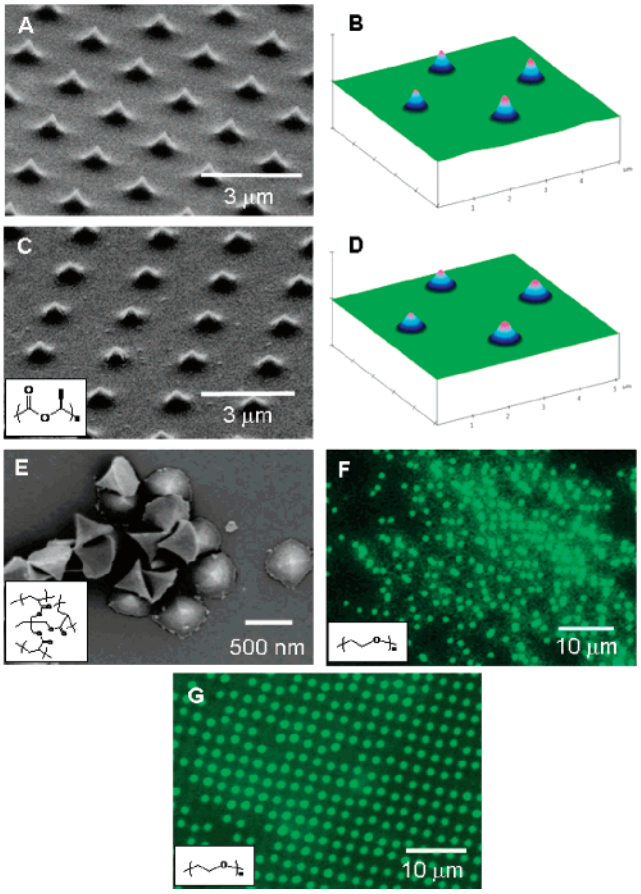

Figure 3. Conical-shaped particles fabricated using the PRINT process. (A) SEM image of the original conical-shaped silicon master (500 nm feature size) used to generate the PFPE mold that was used to generate the 500 nm conical-shaped particles. (B) Representative AFM image of silicon master cones. (C) SEM image of 500 nm conical-shaped PLA particles generated by using PRINT. (D) AFM image of replicate PEG particles. (E) SEM image of conical triacrylate particles that are <50 nm at the tip and harvested mechanically using a doctor blade. (F) Fluorescent confocal micrograph of 500 nm conical-shaped PEG particles containing avidin (68 000 Da) tagged with FITC fluorescent dye. (G) Fluorescent confocal micrograph of conical PEG particles containing doxorubicin.

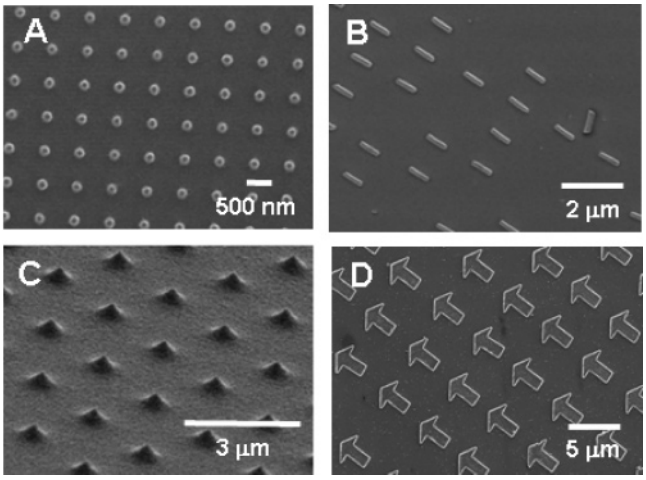

Figure 4. Manipulation of shape using PRINT: (A) 200 nm trapezoidal PEG particles; (B) 200 nm × 800 nm bar PEG particles; (C) 500 nm conical PEG particles that are <50 nm at the tip; (D) 3 μm arrow PEG particles.

developing methods to optimize the release of the molds to fabricate error-free particle arrays. Importantly, PFPE-based elastomeric molds are very robust; we have used the same mold for multiple molding experiments and have seen no degradation of the replicate morphologies.

Using PRINT, we are able to introduce a wide range of particle cargos, including oligonucleotides, proteins, and small-molecule pharmaceuticals, to generate functional nanobiomaterials. We have arguably generated DNA delivery vectors that are, themselves, first-generation "synthetic viruses" (monodisperse populations of shape-specific particles containing DNA). Furthermore, these biomolecule-containing particles could be used as nanoscale, shape-specific biosensors[27−30] or next-generation therapeutic agents.[1,31] Encapsulation of the fluorescently labeled, 24 bp oligonucleotide in 200 nm trapezoidal particles was accomplished by adding it to a PEG−diacrylate/water precursor solution and molding this mixture as described earlier. Using this methodology, therapeutic cargos and particle matrix precursors are co-sequestered within the patterned regions of the PFPE molds. We were able to confirm the encapsulation of the oligonucleotides by observing fluorescence from monodisperse particles using confocal microscopy (Figure 2F). Similarly, we have applied this method to other important compounds, including proteins and small-molecule therapeutics. The encapsulation of fluorescently labeled avidin (MW 68 000 Da) in 500 nm conical particles is shown in Figure 3F, and Figure 3G shows the encapsulation of doxorubicin, which is an important chemotherapy agent. PRINT has several distinct advantages over other vector fabrication techniques in that the particles are monodisperse and shape-specific. In addition, no surfactants, condensation agents, etc. are required for encapsulation using this simple methodology. Given the simplicity of the PRINT process, we anticipate that we can encapsulate a host of important agents (e.g., gene fragments, pharmaceuticals, cell-penetrating peptides, viruses). Because of the range of chemistries compatible with the PRINT process, we also expect to be able to decorate the surface of the particles with targeting ligands and other structures.[4] Furthermore, the simplicity of PRINT allows for the straightforward adjustment of particle properties, such as cross-link density, charge, and composition by the addition of other reagents.

One of the key advantages of the PRINT process is the intrinsic gentleness of the method, especially toward the delicate cargos encapsulated within the particles. We have confirmed that the biological activity of specific cargos can be maintained during PRINT encapsulation by performing biotin-binding experiments with avidin-containing particles. Briefly, fluorescently labeled avidin (CY-3 fluorescent dye) was encapsulated in 500 nm conical PEG−acrylate PRINT particles (70% PEG−diacrylate, 30% PEG−monomethacrylate) as described previously. The PEG−monomethacrylate was added to the particle formulation to increase the mesh size of the particles, which enhances the biotin diffusion rate. These avidin-containing PRINT particles were exposed to a fluorescein-labeled biotin solution for 30 min, washed with water, and observed using confocal microscopy. As shown in Figure 5, CY-3-labeled

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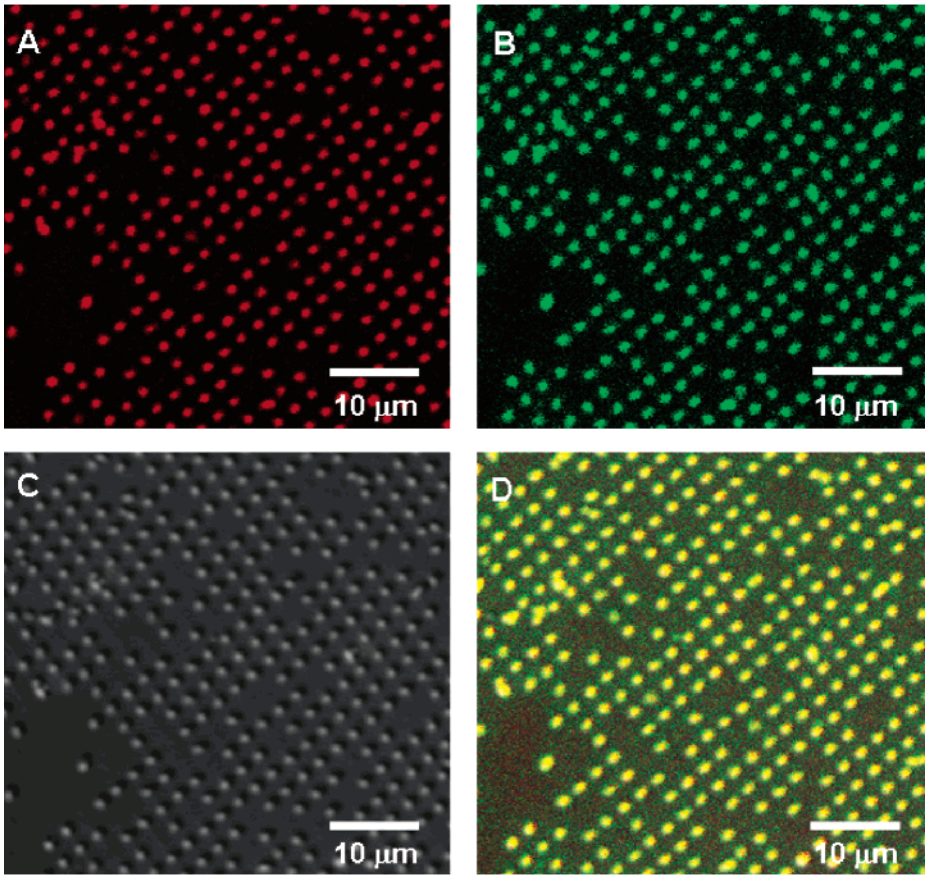

Figure 5. Confocal micrographs showing encapsulation and binding of fluorescently labeled avidin/biotin complexes within 500 nm conical PEG−acrylate particles. (A) Fluorescence image of particles with encapsulated CY-3-labeled avidin. (B) Fluorescence image of fluorescein-labeled biotin conjugates. (C) Differential image contrast micrograph showing the locations of the PEG−acrylate particles. (D) Overlay of the fluorescence images in A and B, showing co-localization of the avidin and biotin.

avidin (red) was co-localized with fluorescein-labeled biotin (green), which indicates preferential binding of biotin to the avidin-containing particles. Control experiments with PRINT particles that were identical in all regards, except that they did not contain encapsulated avidin, showed no binding of biotin (see Supporting Information). These results suggest that biological biotin/avidin recognition is preserved during PRINT photoencapsulation, which is consistent with a variety of previous studies which demonstrate the preservation of biological activity of proteins[27−30] and DNA[31] after UV photopolymerization within PEG−acrylate-based hydrogels.

PRINT is a highly versatile method for the production of isolated, monodisperse organic particles of nearly any size and shape that contain delicate organic functional agents. The shapes chosen in this work were engineered nonarbitrary shapes, but PRINT could easily be used to mold and replicate shapes found in nature, such as viruses, crystals, and proteins, or could be extended beyond the biological realm to fabricate monodisperse particles for taggants, imaging agents, and other applications. The fact that particles were made using three different synthetic schemes (free radical polymerization, metal-catalyzed high-temperature reaction, oxidative coupling using strong acid) demonstrates that PRINT is chemically flexible and tolerant. The facile, biocompatible encapsulation of DNA, proteins, and small-molecule therapeutics suggests that PRINT could be used to encapsulate many cargos simply by dispersing them into the precursor solution, followed by solidification. These studies demonstrate orthogonal control over size, shape, and composition in a relevant size range for drug delivery (<200 nm), sensing, and other applications. Finally, PRINT can be readily employed in a continuous manner (e.g., to enable larger quantities of particles for extended analysis, such as for clinical trials) through simple modifications to existing soft lithography roller technology[32] or silk screen printing methods.

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Supporting Information Available: Experimental methods, images of 200 nm trapezoidal masters, and detailed descriptions and images of the biotin/avidin binding control experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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