Step and Flash Imprint Lithography for the Fabrication of Shape-specific, Enzymatically-triggered, Drug Nanocarrier

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ABSTRACT

Current concepts in the synthesis of nanocarriers involve the use of polymers or lipids to fabricate self-assembled or emulsion-based particles that are spherical, polydisperse, and release drugs through diffusion or hydrolysis. Top-down nanoimprinting techniques allows high-throughput fabrication of nanoparticles of precise geometries (shape and size). The ability to manipulate geometry and composition of nanoscale carriers is essential for controlling their *in-vivo* transport, bio-distribution, and release mechanism. Our objective is to fabricate monodisperse, shape-specific, biodegradable nanocarriers with an environmentally sensitive component incorporated into the polymer network using nanoimprint lithography. In this way, drugs can be released from the nanocarriers in the presence of an environmental signal. We have generated uniform nanocarriers sub-50nm in size and also demonstrate encapsulation and controlled release from these nanoparticles.

Keywords: shape-specific, nanocarriers, response-sensitive, nanoimprint lithography, controlled drug release

1 OBJECTIVES

Nanoparticle-based delivery of therapeutics has been extensively investigated for a variety of diseases and is considered to be an ideal platform for targeted intravenous and intracellular delivery of bioactive agents. Current concepts in the synthesis of drug nanocarriers primarily involve the use of polymers or lipids to fabricate self-assembled or emulsion based particles that are mostly spherical, polydisperse, and release drugs through diffusion or hydrolytic degradation. Although significant progress has been made in polymeric or liposomal drug delivery systems, there remain critical limitations in synthesizing nanocarriers with highly controllable architectures that can, at the same time, impart environmentally-triggered release mechanisms. Our ability to precisely manipulate size, shape, composition, and drug release mechanism of nanoparticles is essential for controlling their *in-vivo* transport, bio-distribution, and therapeutic efficacy [2-4].

While particles below 500 nm in size can be injected intravenously, only those below 200 nm are efficiently internalized by somatic cells. In fact, the efficiency of intravenous delivery of therapeutics into cells as well as to tissues and organs, especially tumors, increases with sub 100 nm particles. Recent reports also suggest that delivery into the lymphatic circulation requires particles that are even smaller in size (< 50nm) [5]. Besides size, mechanism of drug release from particle carriers is another important design parameter in nanomedicine. Stimuli-sensitive hydrogels have been widely studied as micro or macroscale "smart" delivery systems to release drugs in response to specific environmental stimuli [6-9]. Similar nanoparticles that deliver their cargo to target cells primarily in response to disease-specific environmental signals could significantly improve therapeutic care of complex diseases. For example, most chemotherapeutic drugs that are toxic to normal cells would have significantly less morbidity and mortality if they could be released primarily in response to a tumor-specific, pathological signal. The design of carriers at the nanometer length scale that incorporate such triggered-release mechanisms has remained elusive because of the lack of flexible fabrication methods that can incorporate responsive bio-molecules within the nanoparticle matrix.

Size, material chemistry, and particle surface characteristics have so far been the primary variables used to fabricate nanocarriers. However, recent reports suggest that particle shape could play a significant role in the *in-vivo* performance of delivery vehicles [2, 10]. Specifically, shape and shape-related form factors, like aspect ratio or edges, affect particle transport characteristics, influence cell-particle interactions, and alter drug release kinetics [2, 4]. Ferrari and colleagues have shown through theoretical modeling that shape can significantly affect how particles interact with tumor capillaries during transport [11]. Discher and colleagues demonstrated that self-assembled, filamentous particles with very high aspect ratios (less than 100 nm diameter with several microns in length) have unique long-circulating properties compared to traditional spherical liposomes; however, the micron-sized dimension of these particles is not suitable for intracellular delivery [10]. Until recently, particle shape has been an unexplored area of research in drug delivery due to our inability to reliably synthesize nano or microparticle carriers with

precise and pre-designed geometry. There are in fact only a few methods that have been developed to fabricate particles with varying shape, especially at the nanoscale. Recently, Mitragotri and colleagues reported unique solvent-based methods to generate polystyrene micro and nanoparticles of various shapes [2] with feature sizes as low as 60 nm. It remains to be evaluated how these methods are translated to biopolymers and whether other features, like stimuli-responsiveness, can be incorporated.

DeSimone and colleagues demonstrated the ability to form nanosize particles on silicon wafers using a top-down particle nanoreplication (PRINT) method [12-14]. Synthesis of shape-specific particles as small as 160 nm was demonstrated using this process. However, methods to generate particles with precise size and shape that also incorporate environmentally-triggered drug release are yet to be reported, especially at the sub 100 nm range. In addition, a major limitation in current nanofabricated delivery devices is the process of particle harvesting. For example, the PRINT particles are harvested from the wafer either using physical scraping with surgical blades [13] or by shear force using a glass slide, [14] both of which could damage the particles and may not be suitable for large scale manufacturing.

Advances in nanoscale fabrication methods could provide unique solutions to manufacture stimuli-sensitive nanocarriers for drug delivery in a high throughput manner. Our objective is to use Step and Flash Imprint lithography (S-FIL) to fabricate stimuli-responsive, easily harvestable nanoparticles (as small as 50 nm) of precise sizes, shapes, and compositions. Applying S-FIL technology, our group has fabricated a variety of shape and size nanoparticles (down to 50nm length scale) that are environmentally response sensitive [1]. The particle matrix incorporates enzymatically-degradable acrylated peptides and acrylated polyethylene glycol macromers and can provide triggered release of encapsulated drugs or contrast agents in response to specific physiological or pathophysiological conditions.

S-FIL is a versatile, high-throughput, commercially available nano-molding process that utilizes the topography of a quartz template to mold UV cross-linkable macromers into patterns on a substrate[15]. The feature size of this method is essentially limited by the electron beam lithography (EBL)-based template fabrication process. S-FIL has demonstrated ability to reproducibly imprint sub 50 nm structures with sub 20 nm features,[16] is capable of 10 nm in-liquid alignment accuracy[17] and patterns can be imprinted on top of existing topography.[18]

2 METHOD AND RESULTS

2.1 Fabrication of shape-specific nanocarriers

We have investigated the use of S-FIL based fabrication of nanocarriers using photo-crosslinkable macromers; PEG diacrylates (PEGDA) as previously reported [1]. A combination of PEGDA and acrylated, enzymatically degradable GFLGK-DA was used to produce stimuli-responsive particles, such that the cross-linked nanoparticle-matrix is degraded in the presence of tumor-specific enzymes. S-FIL was performed using the IMPRIO 100 S-FIL system (Molecular Imprints, Austin TX) For efficient, one-step particle harvesting from the imprinting surface, a layer of water soluble poly(vinyl alcohol) (PVA, Fluka, Mw ~30,000 Da) is applied prior to imprintation. A critical aspect of using such imprinting techniques to generate nanocarriers is our ability to efficiently and easily harvest intact particles from the silicon wafer. S-FIL fabricated imprints were plasma etched, incubated in filtered dH_2O, and gently pipetted to dissolve the PVA layer thus releasing the nanoparticles.

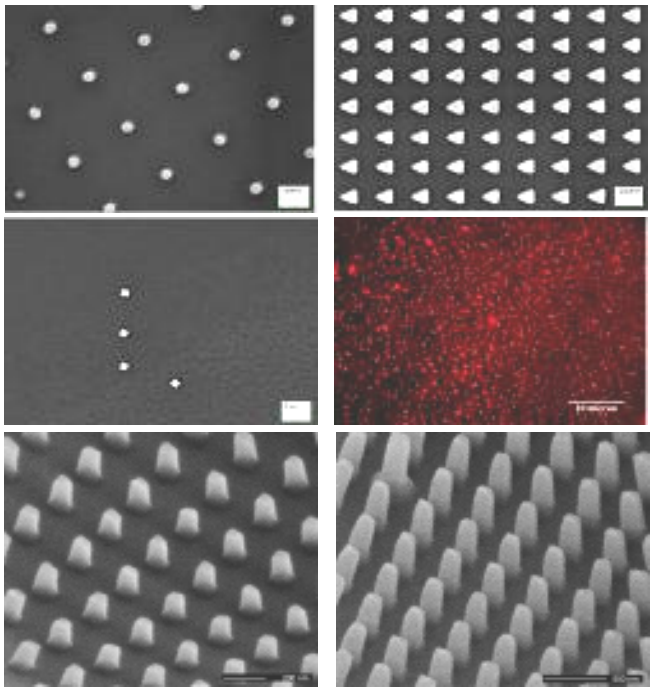

Figure 1: S-FIL Fabricated PEGDA nanoparticles: (A-C) SEM images (A) 50nm squares, 100nm scale bar; (B) 200nm triangles, 200nm scale bar; (C-D) Released nanoparticles (C) 400nm, 1μm scale bar, (D) Fluorescence image of 400nm particles with encapsulated fluorescently labeled goat anti-mouse IgG, 1μm scale bar; (E-F) SEM image at 52°D angle (E) 100nm squares, 200nm scale bar; (F) 200nm squares, 500nm scale bar [1]

Figure 1 A-F shows S-FIL generated arrays of PEGDA (Mw 700) nanoparticles. The various panels underscore the power of this method in generating highly monodisperse nanoparticles of precise sizes and geometries. As shown, particles with square (a, c, d), triangular (b) as well as particles with high aspect ratio (e, f) profiles can be easily generated using nanoimprinting. In addition, particles of specific lateral dimensions, 50 nm (a), 200 nm (b), 400 nm (c, d) and 400 nm squares with ~500nm height (e), and 200nm squares with ~500nm height (f), were fabricated [1]. The heights of the features can be controlled by the

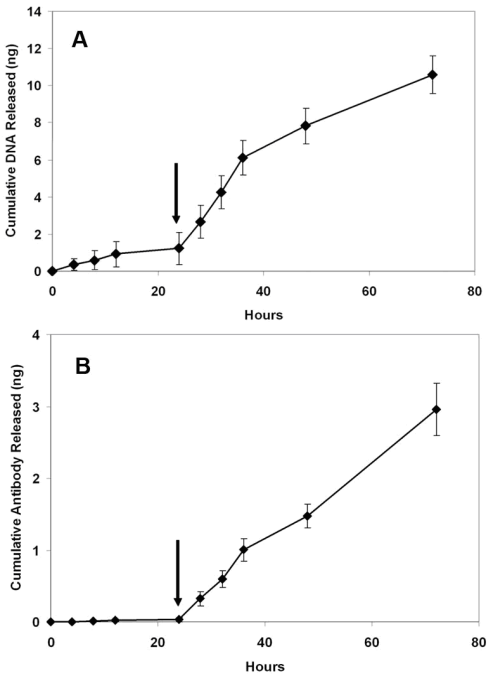

Figure 4: Stimuli-responsive release of biological agents encapsulated within imprinted PEGDA-GFLGK-DA particles in response to 20U/mL Cathepsin B over time: release profile of plasmid DNA encapsulated nanoparticles (A), and release profile of fluorescently-labeled goat anti-mouse IgG encapsulated nanoparticles (B). Arrows indicate time points where Cathepsin B was added to the particles. [1].

drop pattern, volume of the macromer, the imprint force, and the etching depth of the template. **Figure 1 C-D** shows 400 nm square particles after release in water and 400 nm squares loaded with a fluorescently labeled antibody [1]. Similar release layers have been previously described for micron scale soft-lithography particles but have not been implemented in nanoimprinting [19].

2.2 Triggered release of encapsulated agents from response-sensitive nanocarriers

To be able to incorporate the peptide GFLGK into a polymer network the amine groups on the alpha-amine and the lysine amine residues were acrylated based on previously reported method by Healy and colleagues [20] [1]. ^{1}H NMR was used to evaluate acrylation (results not shown here). GFLGK-DA was simply added in an equimolar mixture of PEGDA imprinting solution. Photo initiation of polymer solution causes cross linking between the acrylated groups to form an enzyme sensitive hydrogel.

As a proof of disease responsive, controlled release, we fabricated imprinted nanoparticles using an equimolar mixture of PEGDA (Mw 700 Da) and GFLGK-DA [1]. Fluorescently labeled antibodies (Alexa Fluor 594 labeled IgG, Invitrogen) and plasmid DNA encoding for beta galactosidase (gWiz Beta-gal, Aldevron Inc.) was successfully incorporated during S-FIL into PEGDA-GFLGK-DA nanocarriers. The enzyme-triggered release kinetics of encapsulated plasmid DNA from imprinted PEGDA-GFLGK-DA (75% (w/v)) nanoparticles was evaluated *in vitro [1]*. Stimuli-responsive release of encapsulated antibodies (Alexa Fluor 594 labeled goat anti–mouse IgG, Invitrogen) from imprinted PEGDA-GFLGK-DA (100% w/v) nanoparticles was evaluated in PBS. As shown in **Figure 2 A-B**, efficient enzyme triggered release was achieved. A minimal amount of antibody (~1%) and some amount of DNA (~11%) was released prior to Cathepsin B addition indicating some baseline DNA leakage from the matrix. This is likely due to the highly hydrophilic nature of DNA compared to the antibody. However, most of the encapsulated cargo was released only after exposure to the enzyme indicating possible means to achieve highly controlled, disease responsive or intracellular release of drugs and contrast agents.

2.3 Surface functionalization of nanocarriers

Prior to imprinting, methacrylic acid (MAA) was added to the PEGDA macromer solution at a 1:4 ratio as detailed by us before [21]. Photoploymerizations of the PEGDA:MAA solution leads to the carboxylic acid of the MAA to tether from the surface of the nanoparticles. The imprints were then exposed to 20 seconds of oxygen plasma etching, and then rinsed in MES buffer. Imprints were then exposed to 1-ethyl-3-(3-dimethylaminopropyl) (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) to activate the carboxylic acid group on the surfaces of the nanoparticles to be amine reactive. The imprints were then incubated with FITC labeled streptavidin for 4 hours. Imprints were then triple rinsed in PBS to remove any un-bonded streptavidin. Fluorescent microscopy of imprints demonstrated that streptavidin had attached to the surface of the nanoparticles **Figure 3.**

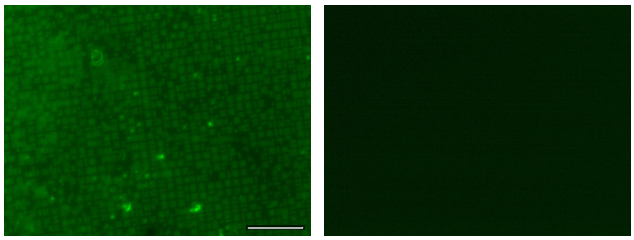

Figure 3: Fluorescent Microscopy of 400nm PEGDA:MAA Imprints**:** (A) etched imprints exposed to EDC Sulfo-NHS, then incubated with FITC labeled streptavidin and (B) imprints exposed to streptavidin

3 CONCLUSION

In conclusion, we have demonstrated a unique method for creating nanocarriers of precise sizes and shapes for

drug and contrast agent delivery. We have achieved particle size as small as 50 nm along with efficient stimuli-responsive release of encapsulated agents. The imprinted particles can be directly harvested into aqueous buffers using a simple, biocompatible process. In addition, the method is mild and does not require high temperature, high shear, organic solvents or long UV light exposure, allowing for efficient encapsulation of biological agents. This process also uses commercially available instruments and thus has the potential to be a translatable, high-throughput technique. A single 4 inch wafer could generate $1.8x10^{11}$ 50 nm particles and $2.6x10^{10}$ 200 nm particles using a 12.5 x 12.5 mm imprint template pattern. S-FIL has the capability to imprint whole wafers at a time, imprint on top of existing nanofeatures, generate sub 50 nm structures and allow for ~10 nm alignment accuracy. Thus, we envision that the process could fabricate, in a high throughput manner, even smaller nanoparticles as well as multi-layered particles with varying composition and release mechanisms. In addition, the material chemistry used here is conducive of readily attaching specific ligands to the particle surface[22] thus providing opportunities of cell targeted, disease-triggered delivery of drugs and imaging agents.

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