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Fabrication of Micropatterned Polymeric Nanowire Arrays for High-Resolution Reagent Localization and Topographical Cellular Control

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Abstract

Herein, we present a novel approach for the fabrication of micropatterned polymeric nanowire arrays that addresses the current need for scalable and customizable polymer nanofabrication. We describe two variations of this approach for the patterning of nanowire arrays on either flat polymeric films or discrete polymeric microstructures and go on to investigate biological applications for the resulting polymeric features. We demonstrate that the micropatterned arrays of densely packed nanowires facilitate rapid, low-waste drug and reagent localization with micron-scale resolution as a result of their high wettability. We also show that micropatterned nanowire arrays provide hierarchical cellular control by simultaneously directing cell shape on the micron scale and influencing focal adhesion formation on the nanoscale. This nanofabrication approach has potential applications in scaffold-based cellular control, biological assay miniaturization, and biomedical microdevice technology.

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ASSOCIATED CONTENT

Supporting Information

Includes additional information on methods, quantification of AAO membrane pore diameters and density, and further characterization of micropatterned nanowire arrays and drug localization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interest.

Keywords

Nanofabrication; bio-MEMS; bioscaffolds; drug loading; nanowires; polycaprolactone

Substrates that contain micro- and nanoscale features are important for a number of biological applications. For example, topographical cues at the micro- and nanoscale can direct cellular behavior,^{1,2} micro- and nanoarrays provide high-throughput biological analysis,^{3–5} and micro- and nanoscale devices can enhance drug uptake and localization.^{6–9} However, nanofabrication techniques are restricted in either pattern customization or throughput, limiting their application and/or scalability in biotechnology.² While direct-write fabrication approaches, including electron beam, focused ion beam, and dip pen lithography, provide custom polymer patterning on the nanoscale^{10,11} with electron beam lithography capable of sub-10 nm resolution under ideal conditions,¹² these techniques require sequential formation of individual nanoscale features, limiting throughput and scalability and increasing cost.² These drawbacks are mitigated in the technique of nanoimprint lithography (NIL) in which multiple resists or substrates can be mechanically deformed by a nanopatterned mold, thereby increasing throughput.¹³ However, molds for NIL are typically fabricated with low-throughput direct-write techniques and have limited lifetimes.^{14,15} Conversely, bottom-up nanofabrication approaches, including block copolymer self-assembly, nanosphere lithography, and nanoporous membrane- based templating, provide high-throughput, scalable fabrication of polymeric nanofeatures with tunable dimensions but do not typically allow for custom patterning.^{16–18} Therefore, there is an unmet need for scalable, customizable fabrication techniques for cost-effective application of nanofabrication to biotechnology.

Efficient approaches to polymer nanofabrication may be particularly advantageous in the scale-up of nanotopographical tissue scaffolds. Because micro- and nanotopography influence cellular adhesion, alignment, shape, proliferation, and differentiation,^{1,2,19} topographical cues incorporated into cellular scaffolds are capable of controlling a wide range of cellular behaviors. Many studies have found that anisotropic and/or hierarchical patterning is essential in achieving the intended cellular effects.^{19–26} The enhanced control provided by hierarchical structures is not surprising, as cells are influenced both on the microscale by contact guidance and on the nanoscale through direct interaction of cellular receptors with external physical cues.^{27–29}

Hierarchical substrates may also have utility for micron-scale reagent and drug loading of miniaturized biological assays and biomedical microdevices. A number of techniques have been utilized to load micron-scale reservoirs, including photolithography, inkjet printing, and supercritical polymer impregnation,^{30–33} but no approach is capable of both highly scalable and low-waste drug loading.³⁴ As a result of dramatically increased surface area, densely packed nanowires are capable of providing superhydrophilic surfaces with extreme wettability.^{35,36} Superwetable nanowire arrays have been used to enhance drug loading capacity^{6,37–39} but have not been applied for custom, spatially controlled drug localization. We hypothesize that micropatterned nanowire arrays can provide rapid, low-waste reagent and drug localization with micronscale resolution.

To address the need for customizable, scalable nanofabrication, we developed a nonsequential approach to fabricate polymeric nanowire arrays with custom micropatterns and tunable nanowire dimensions. We utilized two variations of this approach to pattern nanowire arrays over either flat polymeric films, for applications in tissue engineering and microarray technology, or on discrete polymeric microstructures, for applications in biomedical microdevice technology. We went on to investigate the ability of these micropatterned nanowire arrays to (1) provide efficient drug/reagent localization with micron-scale resolution and (2) influence cellular behavior through both micro- and nanoscale interactions.

Our fabrication approach employed polymer templating, a rapid and inexpensive nanofabrication technique that involves extruding a polymer into a nanoporous membrane and subsequently etching the membrane to expose polymeric nanowires.^{17,40,41} Previous studies have micropatterned templated nanowire arrays by scraping with a microstructured tool to remove nanowires in regions of contact and create grooved arrays 400 μm in width separated by 60 μm gaps,⁴² but no techniques are currently available for custom two-dimensional patterning of nanotemplated arrays. For custom nanowire array micropatterning and enhanced resolution, we combined templating and photolithographic techniques. As shown in Figure 1, two approaches were used to fabricate polycaprolactone (PCL) nanowire arrays on either a flat poly(methyl methacrylate) (PMMA) film or on discrete PMMA microstructures. PMMA is a common material in FDA-approved orthopedic implants,⁴³ and PCL is a polymer used in FDA-approved sutures and drug delivery devices and has been shown to facilitate cellular adhesion and growth.^{44,45}

To form micropatterned PCL nanowire arrays on PMMA films (Figure 1A), a nanoporous anodized aluminum oxide (AAO) membrane (GE Healthcare, Piscataway, NJ) was spun-cast with Microposit S1818 positive photoresist (MicroChem, Westborough, MA) and patterned via photolithography with grooves of 10, 20, 40, or 80 μm widths and equal spacing or other various micropatterns. The patterned side of the AAO membrane was then brought into contact with a silicon wafer spun-cast with a PMMA base layer and an overlying layer of PCL 5, 10, or 15 μm in thickness and heated to 80 $^{\circ}\text{C}$, which is above the melting temperature of PCL but below that of PMMA. After uptake of melted PCL into pores of the AAO membrane in regions not coated with photoresist, the AAO membrane and photoresist were selectively dissolved in a 0.5 M sodium hydroxide solution for 1 h to expose the PCL nanowires.

To form nanowire arrays on discrete PMMA microstructures (Figure 1B) PMMA and an overlying photoresist layer were spun-cast onto a silicon wafer, and the photoresist was patterned with arrays of squares with 10, 20, 40, or 80 μm edge lengths and equal spacing or other various micropatterns via photolithography. The photoresist pattern was then transferred to the PMMA layer by reactive ion etching with oxygen plasma to form PMMA microstructures. After chemically stripping the remaining photoresist, the PMMA features were heated to 80 $^{\circ}\text{C}$ and brought into contact with PCL spun-cast onto a separate wafer at thicknesses of 5, 10, or 15 μm . Upon separation of the wafers, the PCL lifted off onto the PMMA features. Finally, the PCL-coated features were templated with an AAO membrane at 80 $^{\circ}\text{C}$, and the membrane was etched in 0.5 M sodium hydroxide for 1 h.

These fabrication approaches resulted in micropatterned arrays of densely packed PCL nanowires on either flat PMMA films or discrete PMMA microstructures (Figure 2). Both fabrication approaches had adequate resolution for all feature sizes tested (10–80 μm). Nanowires formed clusters approximately 1–10 μm in width, possibly as a result of capillary force during drying in preparation for SEM. The micropatterned nanowire arrays on PMMA films had well-defined borders, but the nanowire arrays on PMMA microstructures had rounded corners and edges overhanging the PMMA base layer (Figures 2 and 3A), likely due to beading of molten PCL during the lift-off step and/or compression during templating.

After demonstrating custom patterning of nanowire arrays, we proceeded to investigate approaches to tune nanowire dimensions. We first examined an approach to adjust nanowire length. In previous studies, PCL templating was performed without a PMMA base layer, and templating time and temperature were adjusted to control nanowire length.^{17,44,46} For the approach used in this study, we hypothesized that templating would occur until the AAO membrane contacted the PMMA base layer, allowing for control of nanowire length by adjusting PCL thickness. For film-based nanowire arrays, nanowire lengths roughly matched respective PCL thicknesses for PCL layers 5, 10, and 15 μm thick (Figure 3 A). Nanowires coating PMMA microstructures also scaled in length with PCL thickness but were shorter than nanowires of film-based arrays fabricated with identical PCL thicknesses, indicating only partial adhesion of the PCL layer during the lift-off step. We also investigated control over nanowire diameter through selection of AAO membranes of varying pore sizes. As shown in Figure 3B, templating PCL-coated microstructures with AAO membranes with mean pore diameters of 120 ± 40 , 200 ± 60 , and 290 ± 50 nm (Supporting Information Figure S1) resulted in mean nanowire diameters of 140 ± 30 , 190 ± 30 , and 320 ± 50 nm, respectively. Thus, in addition to customizable nanowire array patterning, nanowire dimensions can also be tuned for length and diameter by adjusting PCL thickness and AAO pore size, respectively.

We hypothesized that once wetted with drug/reagent solution micropatterned nanowire arrays would facilitate high-resolution drug/reagent localization as solvent evaporated. The wettability of a nanorough surface is highly dependent upon the entrapment of air within the nanofeatures with entrapped air reducing liquid–solid contact and thereby reducing surface wettability.^{47,48} To compare the wettability of PCL nanowire arrays to nontemplated PCL films under conditions relevant to the localization approach proposed in this study, contact angles were measured for nontemplated PCL, nontemplated NaOH-treated PCL (to

investigate the effects of NaOH exposure during AAO etching), and PCL nanowire arrays under prewetted conditions. Films were prewetted by submerging in water and then spinning at 2000 rpm for 5 s to remove excess water. Droplets added to PCL nanowires were taken into the nanowire arrays and could not be measured for contact angle while the contact angles of nontemplated PCL and nontemplated NaOH-treated PCL were 78 ± 2 and $73 \pm 4^\circ$, respectively (Supporting Information Figure S2). The dramatically enhanced wettability of PCL nanowires over nontemplated PCL under prewetted conditions suggested that micropatterned nanowire arrays could potentially draw in drug/reagent solution as solvent evaporated, providing high-resolution drug/reagent localization.

To investigate this approach 40 μm PMMA microstructures coated with either PCL nanowires or nontemplated PCL lacking nanowires as a control were wetted with solutions of the hydrophilic reagents FITC-BSA and FITC-dextran (average MW = 10 kDa) in water or the hydrophobic reagents Oregon Green 488 - paclitaxel and Nile red in ethanol at 5 $\mu\text{g}/\text{cm}^2$, inverted, and allowed to dry. The thin film of water or ethanol dried within 10 min (Supporting Information Figure S3). The features were then imaged with confocal microscopy to determine the localization of the fluorescently labeled drug/reagent. While features coated with nontemplated PCL demonstrated drug/reagent localization to regions between the PMMA microstructures (Figure 4A,D), features coated with PCL nanowires facilitated localization to the surface of the microstructures (Figure 4B,E) indicating that the nanowire arrays mediated drug/reagent localization. Within the nanowire arrays, drug/reagent fluorescence intensity patterns showed clustered regions approximately 1–10 μm in width (Figure 4C,F), similar to the PCL nanowire folding/clustering pattern observed in nanowire arrays (Figures 2 and 3). This intensity pattern indicated that drug/reagent localized to clustered nanowires, suggesting that localization was mediated by capillary action between nanowires as solvent evaporated. Furthermore, time-lapse fluorescence imaging demonstrated that drug/reagent solutions collected over nanowire-coated features over time as solvent dried (Supporting Information Figure S3), further suggesting that nanowires enhanced drug/reagent localization by drawing in drug solution. While many drug loading techniques are only compatible with water-soluble drugs,^{30,31} micropatterned nanowire arrays provided efficient localization of both hydrophilic, water-soluble and hydrophobic, water-insoluble reagents through selection of solvents to maximize solubility. To quantify drug/reagent localization to microfeatures, the confocal imaging Z-stacks were merged by mean intensity values, and localization efficiency was calculated as the ratio of fluorescence intensity integrated over the microstructures to fluorescence intensity integrated over the entire analyzed region. The presence of nanowires dramatically increased localization efficiencies for all reagents tested (Figure 4G). Further investigation of FITC-BSA localization demonstrated efficient localization onto nanowire arrays coating both PMMA films and PMMA microstructures for all feature sizes tested (Supporting Information Figure S4). Together, these results demonstrated a rapid, low-waste drug/reagent localization approach. Furthermore, because this drug localization is nonsequential, this approach could be easily scaled for a larger number of features.

The ability to concentrate reagents onto high-resolution patterns could be employed to enhance biological analysis. For example, micropatterned nanowire arrays could be utilized to miniaturize biological assays into a microarray format while providing rapid, low-waste

localization of reagents or samples. In addition to efficient localization, the concentration of luminescent samples to microscale regions may also increase local signal intensity, thereby enhancing sensitivity. Furthermore, based on a pore density of 13 ± 1 pores/ μm^2 for the 290 nm pore size AAO membranes (Supporting Information Figure S5), nanowires fabricated from these membranes to 15 μm in length and 320 nm in diameter will provide greater than a 1000-fold increase in surface area available for conjugation of biomolecules or reagents, also potentially enhancing signal intensity.

This localization approach may also have applications to biomedical microdevice technology. Microfabricated devices loaded with a drug can significantly increase the uptake of the drug in vitro and in vivo.^{32,49–51} However, the task of loading the drug into the micron-scale reservoirs of these devices is a major challenge to the scale-up of this technology. For example, relatively high-throughput loading approaches such as photolithography suffer from wasted materials as a result of the spin-casting and development steps,^{31–33} which is a major drawback when loading expensive drugs. Conversely, low-waste approaches such as inkjet printing allow for quasi-zero-waste loading but require sequential loading of individual drug reservoirs, limiting throughput and scalability.^{30,31,34} Here, we present the first example of polymeric, nanowire-coated microparticles and demonstrate an inherent mechanism for rapid, low-waste drug localization. Such structures may be used in the development of more cost-effective and scalable biomedical microdevices and drug delivery systems.

We also investigated the application of micropatterned nanowire arrays to provide hierarchical topographical control over cellular behavior. Microscale topography influences cell growth through the alignment of cells with topographical features, a cellular behavior known as contact guidance.²⁸ This influence over cellular shape and elongation can alter cytoskeletal tension, resulting in altered signal transduction.⁵² Nanoscale features, which approach the macromolecular scale, interact more directly with integrins, transmembrane receptors that allow cells to recognize and bind to their external environment, leading to the formation of focal adhesion complexes.^{2,53} Both the nanoscale distribution of integrin receptors and the micron-scale size and shape of focal adhesions influence cellular behavior through downstream signaling pathways.^{27,29} In vivo, cells reside in niche environments with tissue-specific micro- and nanotopography. Skin, bone, tendon, neural tissues, skeletal muscle, and blood vessels all present hierarchical micro/nanostructures of specific dimensions.^{2,54} Scaffolds designed to mimic the micro- and nanotopography of cellular niche environments have been used to decrease fibrosis and enhance regeneration for wound healing,^{23,25,55,56} maintain stem cell pluripotency in vitro,^{29,57–59} and direct stem cell growth and differentiation for therapeutic applications.^{20,21,26,60,61}

To investigate the ability of micropatterned nanowire arrays to simultaneously influence cells on both the microscale and nanoscale, 3T3 fibroblast cells were grown on films consisting of nontemplated PCL, PCL nanowires, micropatterned nontemplated PCL (with grooves 10 μm in width and 5 μm in height), and micropatterned nanowire arrays (with grooves 10 μm in width and nanowires 5 μm in length) (Figure 5A–D). After 2 days of culture, fibroblasts were fixed, permeabilized, and stained to visualize nuclei, actin, and vinculin, a focal adhesion protein (Figure 5E–L). While fibroblasts cultured on films without

micropatterns showed isotropic morphology as indicated by actin staining (Figure 5E,F), fibroblasts cultured on micropatterned films extended along the microgrooves (Figure 5G,H). However, fibroblasts cultured on micropatterned nanowires showed a significantly higher degree of elongation than cells cultured on nontemplated PCL microgrooves lacking nanotopography. Vinculin staining, which visualized the effects of scaffold topography on cellular focal adhesion formation, provided a possible explanation for this enhanced cellular elongation. The extensions of cells grown on nanowire arrays (Figure 5J,L) showed increased vinculin localization relative to cells grown on films lacking nanotopography (Figure 5I,K). This observation suggested that nanowires enhanced focal adhesion formation, which agrees with previous studies demonstrating that polymeric nanowire arrays promote cellular adhesion.^{46,62} It is possible that when micropatterned to direct cellular alignment and the direction of cellular growth, the enhanced focal adhesion provided by nanowires in turn increases cellular elongation.

Taken together, these results demonstrate that the micropatterned nanowire arrays influenced cells through both microgroove-mediated contact guidance and nanowire-mediated focal adhesion formation to provide a unique cellular morphology not achievable through micro- or nanotopographies alone. With customizable micropatterning and tunable nanowire length and diameter, this fabrication approach could be used to create scaffolds designed to mimic different cellular niche environments with specific nanoscale topographies and microscale patterns. As PCL can be functionalized and matrixloaded with chemical factors,^{63,64} future studies may also incorporate signaling molecules into these scaffolds to further recapitulate cellular niche environments.

Hierarchical micro/nanostructures have a number of biological applications, but the lack of nanofabrication approaches that are scalable while also allowing for custom patterning is a limiting factor in the application of these technologies. We have presented an approach to fabricate micropatterned arrays of polymeric nanowires without the need for sequential-write nanofabrication approaches. Furthermore, the techniques utilized in this approach have been shown to be highly scalable; roll-to-roll anodization approaches allow for high-throughput, relatively inexpensive fabrication of AAO membranes for templating,⁶⁵ and many automated photolithography approaches are capable of processing more than 60 wafers per hour.⁶⁶ Therefore, this approach could be easily scaled up in a cost-effective manner. In addition, the templating utilized in this approach creates nanowires with dimensions not easily achievable through other nanofabrication techniques. Specifically, the high pore density of AAO membranes and the ability to etch away the membrane after templating allow for creation of densely packed nanowires with aspect ratios >100 ,¹⁷ features likely to increase the number of contact regions with cellular receptors and enhance drug/reagent loading capacity. Two variations of this approach provide fabrication of micropatterned nanowire arrays on either flat films or discrete microstructures, both with tunable nanowire dimensions and an inherent drug/reagent localization mechanism. This scalable nanofabrication approach has a number of possible biological applications in tissue scaffold fabrication, bioassay miniaturization, and biomedical microdevice technology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fabrication approaches to create micropatterned PCL nanowire arrays. (A) Fabrication of micropatterned PCL nanowire arrays on PMMA films. (i) A nanoporous AAO membrane (gray) is spun-cast with positive photoresist (red) and patterned by exposure to UV light through a computer-designed photomask. Photoresist is cleaved in regions exposed to UV light, and these regions are selectively dissolved upon chemical development. (ii) The AAO membrane is inverted, and its micropatterned side is brought into contact with a layer of PCL (blue) deposited over a PMMA base layer (white) under heat, allowing PCL to melt and extrude into membrane pores in regions not coated with photoresist. (iii) The AAO and resist are selectively dissolved in an alkaline solution to expose the PCL nanowires. (B) Fabrication of PCL nanowire arrays on PMMA microstructures. (i) A layer of PMMA (white) spun-cast onto a silicon wafer (black) is spun-cast with positive photoresist (red), which is exposed to UV light through a computer-designed photomask. Photoresist is cleaved in regions exposed to UV light, and these regions are selectively dissolved upon chemical development. (ii) The photoresist pattern is transferred to the PMMA layer by anisotropic reactive ion etching with oxygen plasma. (iii) After stripping the photoresist, the PMMA microstructures are heated and brought into contact with PCL (blue) spun-cast onto a separate silicon wafer, bonding PCL in regions of contact. (iv) Upon separation of the wafers, the PCL lifts off onto the PMMA microstructures. (v) The PCL is melted and templated with an AAO membrane (gray). (vi) The AAO membrane is selectively dissolved in an alkaline solution to expose the PCL nanowires.

Figure 2.

Nanowire array fabrication approaches demonstrate sufficient resolution to pattern features as small as 10 μm . SEM micrographs of PCL nanowire arrays on PMMA films (A–D) and PMMA microstructures (E–H) with 10 μm (A,E), 20 μm (B,F), 40 μm (C,G), and 80 μm (D,H) feature sizes. Scale bars are 20 μm .

Figure 3.

Templating parameters can be adjusted to tune nanowire dimensions. (A) PCL thickness controls nanowire length. 45° SEM micrographs of nanowire-coated films and microstructures fabricated with PCL thicknesses of 5, 10, and 15 μm demonstrate that nanowire length increases with PCL thickness. Scale bars are 2 μm . (B) AAO membrane pore size controls nanowire diameter. As shown in SEM micrographs and histograms, diameters of PCL nanowires fabricated on PMMA microstructures correlated with AAO membrane diameter. Templating with mean membrane pore diameters of 120 $\pm$ 40, 200 $\pm$ 60, and 290 $\pm$ 50 nm yielded mean nanowire diameters of 140 $\pm$ 30, 190 $\pm$ 30, and 320 $\pm$ 50 nm, respectively. Scale bars are 500 nm. *Indicates statistically significant difference between average nanowire diameter with $p < 0.001$.

Figure 4.

Nanowires mediate drug/reagent localization. Three-dimensional confocal imaging reconstructions of $5 \mu\text{g}/\text{cm}^2$ Oregon Green 488 - paclitaxel (A,B) and FITC-BSA (D,E) loaded onto features with either nontemplated PCL (A,D) or PCL nanowires (B,E) demonstrate that nanowires dramatically enhance drug/reagent localization to array features. Two-dimensional confocal imaging slices of loaded nanowire arrays (C,F) show clustered localization patterns, suggesting that drug/reagent collects between nanowires as a result of capillary action. (G) Localization efficiencies of drugs/reagents were quantified as the ratio of fluorescence intensity integrated over the microstructures to the fluorescence intensity integrated over the entire analyzed region. All scale bars are $20 \mu\text{m}$.

Figure 5.

Micropatterned nanowire arrays simultaneously influence cellular behavior on both the micro- and nanoscales. Fibroblasts were cultured on nontemplated PCL (A), PCL nanowire (B), micropatterned nontemplated PCL (C), and micropatterned PCL nanowire (D) films (imaged with SEM). Staining of actin (green) and nuclei (blue) merged with brightfield images (E–H) demonstrates that micropatterned films (G,H) promote cellular alignment to microgrooves with micropatterned nanowires (H) providing enhanced cellular elongation relative to micropatterned PCL lacking nanotopography (G). Vinculin (red) and nuclei (blue) staining (I–L) demonstrates that nanowires increase vinculin localization to cellular extensions (J,L) relative to cells cultured on nontemplated PCL (I,K), indicating that nanowires enhance focal adhesion formation. Scale bars are 50 μm .