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Direct Fabrication of Functional and Biofunctional Nanostructures Through Reactive Imprinting

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Reactive imprinted surfaces: One-step reactive imprinting of a protected maleimide polymer in a provides a nanopatterned maleimide surface. These patterned surfaces provide scaffolds for the generation of functional and biofunctional structures

Supporting information available: NMR, Bright field, fluorescence images, XPS data, TGA for the polymer and AFM images of surfaces. This material is available online at Wiley InterScience or from the authors.

Nanoimprint lithography (NIL) ^[1] is an important tool for generating two- and three-dimensional structures beyond the resolution of optical lithography. NIL is a scalable technique, and can be adapted to high throughput roll-to-roll processing. The versatility of the NIL has made it an attractive strategy for a wide range of applications such as organic electronics,^[2] photonics,^[3] magnetic devices^[4] and biological applications^[5].

Reactive imprint lithography (RIL) provides an efficient tool for nanostructure formation, combining the imprinting step with chemical activation and transformation^[6]. RIL provides a potential means of generating chemically activated polymer patterns with minimal processing, providing increased efficiency and reduced cost. Facile post-functionalization onto polymer patterns, however, requires reactive functionalities on the surface to provide efficient derivatization under mild conditions,^[7] generating challenges including undesired side reactions. Recently, polystyrene-*block*-poly(*tert*-butyl acrylate) films have been used to create patterns with carboxylic acid functionalities, demonstrating the potential of this method.

Among various chemical functionalities, the maleimide is a versatile functional group for “click” functionalization^[8]. This reactive functionality provides a facile derivatization, for example thiol-ene conjugation under mild and often reagent-free conditions. Significantly, the highly reactive maleimide group can be reversibly deactivated/activated using Diels-Alder/retro-Diels Alder (retro-DA) reactions. The retro-DA of the maleimide-furan adducts via thermal activation affords maleimides with quantitative yields, and has been applied to the generation of thermal responsive dendrons,^[9] polymers with a tunable crosslinking density,^[10] segmented block dendrimers,^[11] self-healing polymers,^[12] and reversible covalent assemblies.^[13] The unmasking of the maleimide functionality via retro-DA provides an attractive strategy for activating dormant polymer film into “clickable” reactive polymer patterns by processing through thermal NIL.

We report here the activation of inert furan-masked maleimide polymer films into highly reactive maleimide-functionalized nanopatterned materials using a thermal RIL strategy. These patterns have then been used for the generation of functional and biofunctional structures. In this strategy, reactive NIL patterning of a furan-protected maleimide polymer (Polymer **1**) in a single step provides maleimide patterns via retro-DA reactions. These reactive patterns were then successfully post-functionalized to create functional magnetic structures and biofunctional structures (Scheme 1). Furthermore, these biofunctional structures were effectively used to align cells, showing the potential for RIL-generated materials in the field of tissue engineering.

Initially, we determined the temperature for retro-DA activation of Polymer **1** using thermogravimetric analysis (see Fig. S2 supporting information). As expected, the furan-maleimide adducts in the polymer were quantitatively converted to maleimide groups above 120 °C.^[14] After establishing our retro-DA conditions, thermal NIL was performed at 175 °C (the polymer *T_g* is 60 °C, see Fig. S3 supporting information) under 400 PSI for 5 minutes onto a spin cast Polymer **1** film, resulting in the transfer of the mold pattern into the Polymer **1** layer. Simultaneously, the high temperature and pressure trigger the retro-DA reaction to yield the maleimide patterned surface. The imprinted pattern was analyzed by

using an attenuated total reflectance infrared spectrometer (ATR-IR) to confirm maleimide functional group formation. The decrease in the distinct peak for the C=O stretch of the furan-maleimide adduct^[15] at 1770 cm⁻¹ after imprinting clearly indicates the occurrence of retro-Diels-Alder reaction. The increase in intensity at 1150 cm⁻¹ (C-N-C in maleimide ring) further supports this conclusion (Figure 1a).

The efficiency this reaction was calculated by comparing the peak height decrease at 1770 cm⁻¹ after imprinting with a non-changeable peak at 1125 cm⁻¹ (C-O-C in the tetraethylene glycol pendant chain), indicating a yield of 68% (Figure 1a). The efficient derivatization of the maleimide functionality on the patterned surface was demonstrated by the immobilization of the fluorescent thiol Bodipy-SH via thioether formation. Figure 1b shows the bright field image of the imprinted surface, while Figure 1b (inset) and Figure 1c show the fluorescence images before and after the reaction with the green fluorescent Bodipy-SH respectively. The effective attachment of the dye via thioether formation is clearly evident due by the strong green fluorescence of the patterned surface (Figure 1c). The green fluorescence was only seen on the patterns indicating the absence of polymer in the trenches, presumably via a de-wetting process^[16]. The patterns were also analyzed for the absence of residual layer using phase imaging^[17] mode in the AFM. The phase image shows a sharp contrast, indicating no residual polymer layer on the trenches (see Fig. S4 supporting information).

The reactive maleimide functionalized patterns can be used as scaffolds to generate functional structures via post-functionalization. To create functional structures, we deposited nanoparticles (NPs) onto the reactive patterns due to the tunable surface properties of NPs. This post-functionalization utilizing NPs onto patterned surfaces provides a highly modular approach that can be used to tune the electronic, magnetic, optical, and biological properties of these surfaces.^[18] The covalent functionalization of patterns with iron oxide NPs was carried out providing discrete magnetic structures. Initially, we attached a heterobifunctional tethering linker (mercaptoundecanoic acid) onto the maleimide patterns, and then used the free carboxylate to capture 6 nm core diameter iron oxide NPs. The successful immobilization of the tethering linker was confirmed by using ATR-IR, showing an increase in the peak intensity at 1700 cm⁻¹ (C=O stretch, see Fig. S5 supporting information). To analyze the immobilization of magnetic NPs on the surface, we used atomic force microscopy and magnetic force microscopy (MFM). Figure 2a shows the topology of the pattern, a concurrent increase in the feature height from 72 to 80 nm (Figure 2c) consistent with a deposition of a monolayer of particles on the patterned surface (NPs size - 6 nm). As before, AFM imaging showed no residual layer on the trenches. Figure 2b shows the MFM image, indicating the formation of magnetic nanostructures.

We next used the RIL-generated maleimide-functionalized patterns to generate biofunctional structures^[19] engineered to dictate cell surface interactions,^[20] an important criteria in tissue engineering scaffolds. We have chosen 300 nm reactive patterns (see Fig. S6 supporting information) to achieve efficient cell directionality^[21] and attached RGD peptide via thioether formation (Figure 3). XPS analysis revealed the effective immobilization of RGD onto the maleimide patterned surface, showing an increased N1s and S2p after immobilization of RGD (see Fig. S7 supporting information). We then investigated the

utilization of these biofunctional structures for cell culturing CDBgeo model cells. These cell culturing experiments revealed that the cells were highly elongated and spread along the direction of pattern, indicating the potential application of these surfaces in the field of tissue engineering (Figure 3c). As expected, the unpatterned surface with immobilized RGD, however, showed good cell adhesion but no orientation of cells (Figure 3b). The unpatterned RGD-functionalized surface showed excellent adhesion but no directionality (Figure 3a). Patterned surfaces without RGD showed poor cell adhesion and modest directionality due to the presence of poly(ethylene glycol) side chain on the polymer (Figure 3d).

In summary, we have demonstrated a facile method to generate maleimide functional patterns using reactive nanoimprinting. These reactive patterns provide versatile scaffolds for the fabrication of both chemically functional and biofunctional structures, providing direct access post-functionalizable surfaces. These surfaces provide scaffolds for the creation of both functional and biofunctional materials.

Experimental

Materials and methods

Synthesis of Polymer 1—Monomer FMMA was prepared as reported previously.^[22] To a solution of FMMA (0.2 g, 0.68 mmol), MMA (0.068 g, 0.68 mmol) and PEGMA (0.102 g, 0.34 mmol) in dry CHCl_3 (10 mL), was added AIBN (0.009 g, 0.056 mmol). The mixture was degassed and then heated to reflux for 12 h. At the end of the reaction, CHCl_3 was evaporated under vacuum, the residue dissolved in minimum amount of dichloromethane and added to cold MeOH to precipitate the Polymer **1** as a white solid (52% yield). $M_n=4K$, $PDI=1.45$. While the furan-protected maleimide groups provide the reactive maleimide units for efficient functionalization of the structures, the PEG units provide the desired antibiofouling characteristics, and the hydrophobic methyl methacrylate units provide stabilization of these structures to aqueous environments necessary for applications such as biofunctionalization. *Film preparation:* Solutions consisting of 50 mg Polymer **1** was dissolved in 10 mL of chloroform. The solution was filtered and spin-coated at 3000 rpm for 60s onto a silicon substrate, yielding a thin film of Polymer **1** (~65nm).

Nanoimprint lithography (NIL)—Nanoimprinting of the Polymer **1** was performed using a Nanonex NX-2000 nanoimprinter using a patterned silicon mold that contained test patterns of various feature sizes. Imprinting was performed at 175 °C and a pressure of 400 PSI for 5 minutes. The silicon NIL mold (line width 303 nm, period 606 nm and Groove depth 190 nm) was purchased from Lightsmyth technologies, and was used in the cell patterning. The silicon mold used for the magnetic structures has the dimensions (line width 2 μm , period 4 μm and groove depth 600 nm). Both molds were treated with Heptadecafluoro-1,1,2,2-tetrahydrodecyl)dimethyl- chlorosilane at 75°C for 2 days in vacuum chamber.

RGD immobilization

The patterned surface was reacted with RGD thiol for 2 hrs at room temperature in methanol solution (1 mL of 0.1 mg/mL) with a triethylamine (2 μ L) base. The substrate was then washed with methanol and dried by using argon gas.

Nanoparticle immobilization via tethering linker—10 mg of mercapto-undecanoic acid was dissolved in a solvent of methanol with triethylamine base to adjust the pH of the solution to 7–7.5. The imprinted surfaces/patterns were incubated for 3 hrs to allow for thiol-maleimide click reaction. After the reaction the patterned surface was thoroughly washed using methanol. Iron oxide NP immobilization was carried out by the surface place exchange reaction of carboxylic acid on the patterned surface and Iron oxide NPs. 20 μ L of NP (100 μ M NP solution) was added and mixed well with the 1 mL of hexane. The patterns were incubated for 3 hrs to allow for surface place exchange reaction, resulting in NP immobilization. The substrate was washed with hexane and methanol multiple times then dried under an argon gas flow.

NP synthesis

Iron oxide NP were prepared as described in previous reports.^[23]

Cell culture

BALB/c mouse breast epithelial cells CDBgeo (normal immortalized) were chosen as a model cell line. CDBgeo cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, 4.5g/L glucose) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) at 37°C in a 5% CO₂ environment. For cell growth experiments, cells were trypsinized, centrifuged, and suspended in culture media. Surfaces were placed in 6 well plate and ~100,000 cells in 1mL media were placed in each well. After 5 days, optical micrographs were captured after staining the live cells with calcein AM (2 μ M), which is cleaved into a fluorescent green product by intracellular esterases found only in viable cells.

Characterization

Bright field images and fluorescence were detected using an Olympus IX51 microscope with excitation wavelength of 470 nm and 535 nm. AFM imaging of surfaces was done on a Dimensions 3000 (Veeco) in tapping mode using a RTESP7 tip (Veeco). A Veeco Dektak Stylus profilometer was used to measure the thickness of the polymer films. Attenuated total reflectance infrared (ATR-IR) spectroscopy was used to follow the chemical functionalization. XPS studies were carried on a Physical Electronics Quantum 2000 spectrometer using a monochromatic Al K α excitation at a spot size of 10 mm with pass energy of 46.95 eV at 158 take-off angle to probe the topmost layer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

a) ATR-IR spectrum for Polymer **1** before and after imprinting, showing the decrease in the C=O stretch (1770 cm^{-1}) and increase in the C-N-C (1150 cm^{-1}) band upon imprinting. b) Bright field image; inset is fluorescence image of imprinted pattern before bodipy-SH conjugation. c) Fluorescence image after bodipy-SH conjugation.

Figure 2.

a) AFM imaging of reactive pattern after immobilization of magnetic NPs. b) MFM imaging of pattern 3a. c) Representative horizontal AFM crosssection of topography before and after immobilization of NPs.

Figure 3.

Fluorescence micrographs of cell cultured surfaces, stained with Calcein AM. a) Fluorescence image of the unpatterned maleimide surface. b) Fluorescence image of RGD functionalized unpatterned surface. c) Fluorescence image of patterned RGD functionalized surface. d) Fluorescence image of patterned surface without RGD. Scale bar 20 μ m. (insets: bright field image of a patterned surface, scale bar 2 μ m)

Structures of the protected polymer and maleimide formation via thermal imprinting, the fabrication of patterned surfaces *via* thermal NIL of protected polymer (Polymer **1**), immobilization of iron oxide (Fe₃O₄) NPs, and RGD peptide via thiol-maleimide click reaction