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Preparation of photolithographically patterned inverse opal hydrogel microstructures and its application to protein patterning

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ABSTRACT

Protein pattern has played an important role in biosensors, bioMEMS, tissue engineering, fundamental studies of cell biology, and basic proteomics research. Here, we developed a straightforward and effective protein patterning technique using macroporous poly(2-hydroxyethyl methacrylate) (PHEMA) hydrogel micropatterns as a three-dimensional (3D) template for protein immobilization. Micropatterns of macroporous hydrogels with inverse opal structures were prepared on poly(ethylene glycol) (PEG)-coated silicon substrates by combining a colloidal crystal templating method with photopatterning. The resultant inverse opal hydrogel (IOH) micropatterns were modified with 3-aminopropyltriethoxysilane using the hydroxyl groups in PHEMA for the covalent immobilization of proteins. Proteins were selectively immobilized only on the hydrogel micropatterns, while the PEG regions served as an effective barrier to protein adsorption. Because of their highly ordered and interconnected 3D macroporous structures and large internal surface areas, protein loading in the IOH micropattern was about six times greater than that on a non-porous hydrogel micropattern, which consequently improved the protein activity. The porosity of the hydrogel micropatterns could be controlled using different sizes of colloidal nanoparticles, and using smaller nanoparticles produced hydrogel micropatterns with higher protein loading capacities and activities. To demonstrate the potential use of IOH micropatterns in biosensor systems, biotin was micropatterned on the hydrogels and the specific binding of streptavidin was successfully assayed using IOH micropatterns with better fluorescence signals and sensitivity than that of the corresponding non-porous hydrogel micropatterns.

1. Introduction

Since MacAlear and Wehrung (1978) created protein patterns to integrate protein molecules into bioelectronic microcircuits, protein patterning has become critical to basic biological research involving cell biology, high-throughput proteomic arrays and combinatorial library screening as well as conventional biosensor applications (Ito, 1999; Kane et al., 1999; MacBeath and Schreiber, 2000; Mitchell, 2002; Ostuni et al., 2001; Templin et al., 2003; Veiseh et al., 2002; Zhou et al., 2001; Zhu et al., 2000). Protein patterning is characterized by protein immobilization at specific locations in a two- or three-dimensional space at a micro- or nano-meter scale. Protein patterning has been performed by the selective attachment of proteins within designated regions in a substrate surrounded by a background region that is resistant to the adsorption of proteins and other molecules (Blawas and Reichert, 1998). A variety of methods have been explored previously for protein localization, including photolithography (Orth et al., 2003; Veiseh et al., 2002), photochemical fixation (Balakirev et al., 2005; Hengsakul and Cass, 1996), soft lithography (Hyun and Chilkoti, 2001; Whitesides et al., 2001), dip-pen lithography and related scanning probe patterning methods (Hyun et al., 2002; Lee et al., 2002), focused-ion-beam patterning and nanoimprint lithography (Hoff et al., 2004; Jiang et al., 2008). Most protein patterns have been generated on two-dimensional (2D) hard substrates such as glass, silicon, and gold through non-specific adsorption or covalent binding to the monolayer of tethered functional groups on the surfaces. However, the limited surface area of simple planar substrates limits the amount of protein that can be attached. Furthermore, immobilized proteins may become dehydrated and denatured due to rapid evaporation of the liquid environment and close contact with hard substrates, eventually losing their native structures and functions.

Several approaches were used to increase the amount of immobilized proteins by developing three-dimensional (3D) surfaces using porous substrates, nanofibers or the assembly of micro/nanoparticles (Kang et al., 2005; Lee et al., 2011; Son et al., 2011; Stewart and Buriak, 2000; Yang et al., 2009; Zhang, 2006).

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Protein denaturation could be minimized by modifying the surfaces of solid substrates with a soft material layer such as dendrimer and polymeric multilayers, which can provide a "cushioning effect" as well as a large hydration volume at the surface (Ajikumar et al., 2007; Doh and Irvine, 2004; Pathak et al., 2004). Hydrogels, 3D crosslinked polymers that swell in water or other biological fluids, are another attractive support material for protein immobilization, because the soft and hydrated environment of a swollen hydrogel can provide proteins with near-physiological conditions that minimize denaturation and help them to carry out their full biological functions (Zhang, 2004). Protein patterns were generated by encapsulating proteins within hydrogel micropatterns or immobilizing proteins onto the surfaces of hydrogels (Kim et al., 2008; Kiyonaka et al., 2004; Revzin et al., 2001; Russell et al., 1999; Tanase et al., 2011; Zourob et al., 2006). In the former case, diffusion limitation can interrupt the interactions between encapsulated proteins and other molecules; while in the latter case, the amount of immobilized protein cannot be dramatically increased compared to conventional hard materials.

To overcome above problems related with hydrogel-based protein patterning, we fabricated macroporous poly(2-hydroethyl methacrylate) (PHEMA) hydrogel micropatterns and used their surfaces as templates for protein patterning with the expectation that porous hydrogel could provide higher immobilization capacity and a more aqueous environment for immobilized proteins as compared to a two-dimensional hard surface. By combining a colloidal crystal templating method with nanoparticles and photopatterning, we fabricated hydrogel micropatterns that had three-dimensional (3D) inverse opal structures with highly ordered and interconnected pores of uniform size. After confirming that macroporous hydrogel micropatterns accommodated more proteins and exhibited better protein activity compared with nonporous hydrogel micropatterns, the feasibility of macroporous hydrogel micropatterns for biosensor applications was investigated.

2. Experimental

2.1. Materials

Styrene, potassium persulfate (KPS), 2-hydroethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), 2-hydroxy-2-methylpropiophenone (HOMPP), 3-aminopropyltriethoxysilane (APTES), bovine serum albumin (BSA) and BSA conjugated with fluorescein isocyanate (FITC-BSA) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Ethyl alcohol, chloroform and aqueous ammonia were purchased from Duksan Chemicals (Seoul, Korea). Glutaraldehyde (GA) (25% in solution) was purchased from Junsei Chemical (Tokyo, Japan). Biotinyl-3,6,9-dioxaoctanediamine (biotin-NH_2), prostate-specific antigen (PSA), anti-PSA antibody (anti-PSA), and a micro-BCA protein assay kit were obtained from Pierce (Rockford, IL, USA). FITC-labeled streptavidin (FTIC-SA) was purchased from Biosource (Camarillo, CA, USA). A chrome sodalime photomask for photolithographic patterning of hydrogels was designed to contain 200 μm circles arranged into a 50 × 50 array format and was purchased from Advanced Reproductions (Andover, MA, USA).

2.2. Equipment

Photopolymerization of the hydrogel precursor was performed using a 365 nm, 300 mW/cm^2 UV light source (EXFO Omnicure UV spot lamp, Mississauga, Ontario, Canada). Surface modifications were monitored by X-ray photoelectron spectroscopy (XPS) (Kratos Analytical Inc., Chestnut Ridge, NY, USA). Absorbance was measured using a VersaMax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Pattern morphology was observed with scanning electron microscopy (S-800, Hitachi, Tokyo, Japan). A microscope equipped with an integrated color CCD camera (BX-51, Olympus, Tokyo, Japan) was used to obtain optical and fluorescence images. Image analyses were performed using commercially available image analysis software (TOMORO Capture 2.0, Olympus). Confocal slice images were obtained using confocal microscopy (LSM 510 META, Carl Zeiss Inc., Thornwood, NY, USA). The *z*-average size of the PSNPs was measured suing a nanometer sized test instrument (model 2S, NanoS, Malvern Instruments, UK).

2.3. Synthesis of polystyrene nanoparticles

Polystyrene nanoparticles (PSNPs) were synthesized by dispersion polymerization. Water (400 mL), NH_3 stabilizer and styrene monomer (20 mL) were added to the double jacket reactor under a stirring speed of 500 rpm. After a solution of KPS initiator (0.25 g) pre-dissolved in water (25 mL) was added to the reactor, the polymerization proceeded for 8 h. The solid content of the synthesized polystyrene (PS) latex was 2.77 wt%. The PS latex was washed by centrifugation and dispersion with water, and was adjusted to a concentration of 20 mg/mL before use. The size of the PSNPs could be tuned in the range of 160–400 nm by varying the amount of NH_3 stabilizer. In this study, PSNPs with average diameters of about 160 nm, 180 nm, 240 nm and 400 nm were used. The amount of NH_3 stabilizer to tune PSNPs and size dispersion of each PSNP was shown in Table S1 and Fig. S1, respectively (see Supplementary material).

2.4. Micropatterns of inverse opal hydrogel

Fig. 1 describes the overall process for preparing micropatterns of inverse opal hydrogel (IOH) based on PHEMA. First, PSNPs (160 nm, 180 nm, 240 nm or 400 nm) were self-assembled onto silicon wafers (1.5 cm × 1.5 cm) via a meniscus evaporation procedure. To form close-packed colloidal crystal templates, we evaporated the aqueous solvent in the colloidal suspension at 80 °C for 40 min in a thermostatic chamber after 400 μL of colloidal suspension were deposited onto the silicon wafers. Silicon substrates were surface-modified with PEG by a previously described method to minimize non-specific adsorption (Papra et al., 2001). After that, 100 μL of PHEMA hydrogel precursor solution, consisting of HEMA, 1 wt% of EGDMA as a crosslinker and 2 wt% of HOMPP as an initiator, were dropped onto the colloidal templates, and were then spin-coated for 40 s at 3000 rpm to generate a thin layer of precursor solution with uniform thickness. This layer was covered with the photomask we designed and exposed to UV light for 25 s through the photomask. Upon exposure to UV light, only the exposed regions underwent free-radical-induced gelation and became insoluble in water. As a result, the desired microstructures were obtained by washing away the unreacted precursor solution with water. Finally, substrates containing hydrogel micropatterns were immersed in chloroform for 3 min to completely remove the hydrogel-embedded PSNPs and create inverse opal structures within the hydrogel micropatterns. As a control experiment, nonporous PHEMA micropatterns were fabricated using the same photolithography process except for the steps involving the deposition and removal of PSNPs.

2.5. Protein immobilization on the hydrogel micropatterns

A silanization reaction was used for surface modification of PHEMA hydrogel micropatterns (both non-porous and inverse opal hydrogels) by utilizing the hydroxyl groups in the PHEMA. Hydrogel micropatterns were immersed in 3% (v/v) APTES in 95% (v/v) ethanol under a nitrogen environment for 2 h at room