Photolithographical Immobilization of Biomolecules onto Non-Biofouling Surfaces

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Received June 11, 2005

Key Words : Aryl azide, Pattern generation, PEG film, Photolithography

Poly(ethylene glycol) (PEG)- and oligo(ethylene glycol) (OEG)-based materials have extensively been used in bionanotechnological and biomedical applications because of their ability to prevent nonspecific protein adsorption and cell adhesion as well as their nontoxicity and nonimmunogenicity.[1] Surfaces, such as gold, SiO_2, and polymers, modified with PEG or OEG are biologically inert surfaces (surfaces that resist adsorption of proteins and adhesion of cells).[2] A consensus on the mechanism of non-biofouling ability of PEG or OEG is not yet established, but theoretical considerations suggest that the PEG chains are highly mobile and attain extremely large exclusion volumes in solution due to the steric repulsion to incoming particles.[1,3]

Many research groups have generated inert surfaces based on the formation of self-assembled monolayers (SAMs) to study the interactions of proteins on surfaces, to control the adhesion of cells and the shape and size of attached cells using well-defined patterns of ligands on surfaces, and to fabricate surfaces that resist bacterial adhesion.[4] These tools facilitate the investigation of interactions between cells and surfaces and the study of cell biology on man-made surfaces. A variety of techniques have been developed for creating micrometer-sized patterns of inert surfaces on substrates in order to pattern proteins and cells. These techniques include the SAMs of EG-terminated alkanethiols and surface-initiated polymerization of EG-acrylate on gold combined with microcontact printing (μCP),[5a,b] fabrication of PEG hydrogel using photolithography,[5c] and formation of PEG microwells through three-dimensional microfluidic channels.[5d]

Our approach to the simple generation of patterns of biomolecules (*e.g.* biotin) and cells on a non-biofouling PEG film is based on aryl azide chemistry (Figure 1a).[6] Photolysis of aryl azide yields highly reactive aryl nitrene resulted from the elimination of nitrogen and the generation of a reactive uncharged singlet or triplet nitrene. The nitrenes react with hydrocarbons by abstraction of hydrogen and insertion into a single bond (usually C-H) and C=C double bonds. Generation of aryl nitrene from the photolysis of aryl azide has been used as a synthetic platform of various photoactivatable cross-linkers.[6] In this study, as a proof-of concept we used a commercially available biotin-linked nitrophenyl azide (**1**) [*N*-(4-azido-2-nitrophenyl)-*N'*-(*N*-D-biotinyl-3-aminopropyl)-*N'*-methyl-1,3-propanediamine from Pierce] to modify a non-biofouling PEG film.

Figure 1b shows a schematic illustration of the procedure for photolithographical patterning of biotin on a PEG film. In brief, the glass slide was modified with a chlorosilane-based adhesion promoter (3-acryloxypropyl trichlorosilane).[5c] The adhesion promoter enables the covalent linkage of the PEG film to the glass slide. The self-assembled adhesion

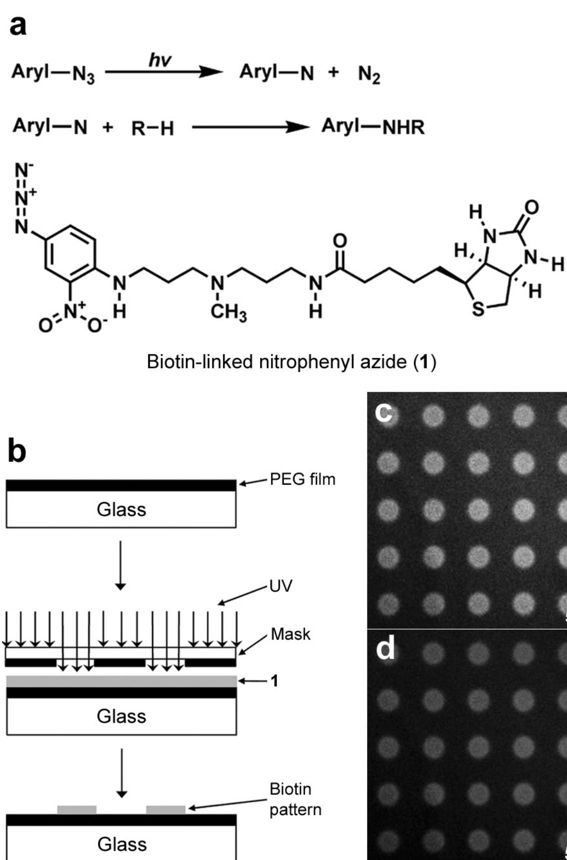

Figure 1. (a) Schematic representation of the photolysis of aryl azide, reactions of aryl nitrene, and structure of biotin-linked nitrophenyl azide. (b) Schematic representation of the procedure for photolithographical patterning of biotin on a PEG film. (C) Fluorescence micrographs of FITC-conjugated streptavidin bound to patterned biotin and (d) TRITC-conjugated biotin-GRGRSPK bound to patterned streptavidin/biotin.

promoter was employed to introduce terminal acrylate functional groups, which could participate in the photoinitiated free radical polymerization and the covalent anchoring PEG film to the glass slide. For the formation of a PEG film on the glass slide, the solution of PEG-diacrylate (MW: 575) containing photoinitiator (2,2-dimethoxy-2-phenylacetophenone, 1% (w/v)) was spin-coated onto the silane-treated glass slide (3000 rpm for 60 sec) and then the sample was placed under a UV lamp (254 nm, 4 W/cm^2) for 2 h for cross-linking (the thickness of PEG film: ~1.5 μm).[5c,d] A 20-μL aliquot of an aqueous solution of **1** (1 mg/mL) was pipetted onto the PEG film. The substrate was covered with a glass cover slip and dried in a vacuum oven at 37 °C for 4 h. The **1**-coated PEG film was mounted onto the imaging system and the photoactivation reaction was performed for 5 min. The quartz mask was placed directly onto the **1**-coated PEG film and then the resulting sample was exposed to 365-nm UV light (25 mW/cm^2) using a mask aligner. Finally, the sample was rinsed with distilled water to remove any unreacted compound **1**.[7] After patterning biotin, the sample was incubated in a phosphate-buffered saline (PBS) solution of fluorescein (FITC)-conjugated streptavidin (0.1 mg/mL) containing bovine serum albumin (BSA, 0.1%) and Tween 20 (0.02%).[9] Successful patterning of biotin was verified by fluorescence microscopy after a complexation with FITC-conjugated streptavidin (Figures 1c and 2a).

We used a RGD peptide, biotin-conjugated Gly-Arg-Gly-Asp-Ser-Pro-Lys (biotin-GRGDSPK), to study a spatially directed cell attachment onto the streptavidin-patterned PEG film.[8] Tripeptide RGD (Arg-Gly-Asp) is well known as an active sequence of adhesive proteins of the extracellular matrix (ECM) that binds to cell surface receptors (CSRs) (*e.g.* integrins).[9] In order to promote cellular adhesion, the RGD-containing peptide was immobilized onto the streptavidin-patterned PEG film. Self-orientation was induced by simply immersing a streptavidin-patterned sample in a solution of the biotin-GRGDSPK peptide. Surface-bound biotin-GRGDSPK peptide was marked with TRITC-fluorophore (TRITC-conjugated biotin-GRGDSPK). The TRITC-conjugated biotin-GRGDSPK peptide was found to be bound to only the areas of streptavidin-patterned surface (Figure 1d). The RGD peptide-patterned sample was incubated with a suspension of human epidermoid carcinoma A431 cells in cell culture media at a density of 1 $\times 10^5$ cells/mL. The cells were attached onto the GRGDSPK peptide-patterned surface (the lateral dimension of 50 μm separated by 50 μm) and cultured for 24 h. Figure 2b shows a phase contrast image of the 24-h cultured cells: the cells are confined only in the areas presenting GRGDSPK peptides.

In summary, we reported a simple photochemical method for the surface modification of non-biofouling surfaces: pattern generation of biotin and cells on a PEG film based on the photochemical reaction of aryl azide. The biotin-linked nitrophenyl azide was chosen as a model ligand because the attachment of biotin could be easily detected by a complexation with a fluorophore-labeled (strept)avidin. The strategy reported can be generalized to surface modification of any polymer-based biomaterials and be extended to the introduction of biologically-active ligands (peptides, proteins, or nucleic acids) by subsequent photochemical reactions.

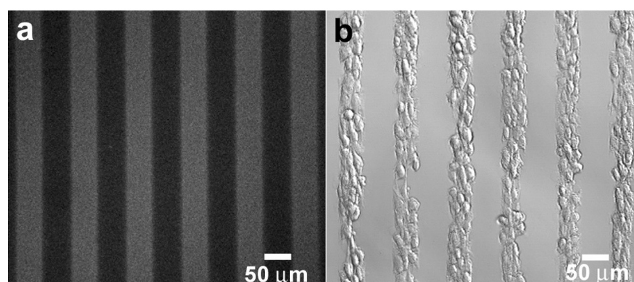

Figure 2. (a) Fluorescence micrograph of biotin-GRGDSPK/streptavidin (FITC)/biotin-patterned PEG film. (b) Optical micrograph of A431 cells seeded for 24 h on a RGD peptide-patterned PEG film.

Acknowledgment. This work was supported by the R&D Program of Fusion Strategies for Advanced Technologies (I.S.C.) and by Korea Research Council of Fundamental Science & Technology (KRCF, Z.-W.L.).

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