The Covalent Immobilization of Biomolecules to Polymer Surface by Deep-UV Lithography Using N-Hydroxysuccinimidyl Azidobenzoate.

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ABSTRACT

We synthesized N-Hydroxysuccinimidyl (NHS) azidobenzoate as a cross-linking reagent for immobilization of peptide onto the solid surface. Thin polystyrene(PS) films spin-coated with a NHS azidobenzoate solution were exposed with ultraviolet light at 245nm(3.3 mW/cm^2) for 5 min. The NHS active ester groups became covalently attached to the polymer via photogenerated, highly reactive nitrene intermediates derived from NHS azidobenzoate. Using this technique, it is demonstrated that well-defined surface regions can be functionalized with a minimum observable feature size of 1mm for UV exposure. Through reaction of this functionalized PS surface with primary amine-containing biomolecules, biological molecule had been immobilized on the polymer surface.

INTRODUCTION

Surface modification by the introduction of functional groups has been of recent interest for the development of biosensor[1] and active devices. The combination of surface modification with photo-lithography offers precise control over the position of the surface-bound chemically reactive species that enables the immobilization of peptide, proteins[2], cell[3], and other biomolecules in spatially defined fashion.

On the other hand, the phenyl azido group is easily photolyzed upon UV irradiation to generate a highly reactive phenyl nitrene, which is so reactive that it induces various reactions, including insertion of C-H bonds, addition to C=C double bonds, and abstraction of hydrogen atoms with neighboring compounds. These complex reactions lead to photochemical fixation of phenyl azido-derivatized polymer and biomolecules[4, 5].

In this paper, we present the photoinduced surface immobilization technique based on phenyl azido chemistry. In addition, a new method for photoinduced immobilization of an activated platelet adhesive peptide with micro-scale-patterned arrays on PS film surface are discussed.

METHODS AND MATERIALS

Synthesis of NHS azidobenzoate

A solution of dicyclohexylcarbodiimide(DCC) in tetrahydrofuran(THF) was added dropwise into a solution of N-Hydroxysuccinimide and 4-azido-benzoic acid in THF cooled in an ice bath with stirring. After 3h, the reaction mixture was warmed slowly to room temperature and stirring was continued for one night. A white solid was formed and was filtered off and the solvent was removed under reduced pressure. The yellow residue obtained was crystallized from isopropyl alcohol.

Electron Impact(EI) Mass spectroscopy

Hewlett-Packard 5988 MS instrument was used for electron impact mass spectrometry. This was measured under the 70eV of electron energy, 200℃ of source temperature, and 300μA of trap current conditions.

Fast Atomic Bombardment(FAB) Mass Spectroscopy

FAB source was the 35keV Cs^+ ion beam and the glycerol was used for the metrix of this mass spectrometry.

Analytical High Performance Liquid Chromatography (Analytical-HPLC)

Reverse phased high performance liquid chromatography of phenyl azido substituted GGGRGDF was measured with μBondapack-C_{18} column by acetonitrile gradient mobile phase.

Preparation of Micro-Scale Patterned Arrays on PS Coated Glass

The surface modification scheme was studied on thin films of polystyrene(PS). Cover slide glass was spin-coated with a solution of 5% PS (MW 125000) in xylene at 1000 rpm for 1 min to produce a PS film that was < 1 μm thick. A solution of 1 % wt of NHS azidobenzoate in nitromethane was then spin-coated on top of the PS film at 1000 rpm for 1min. The film was baked in an oven at 60℃ for 20 min. The NHS azidobenzoate coated PS film was exposed under the UV light at 254nm (3.3mW/cm^2) for 5 min. To study the immobilization of primary amine-containing reagents in well-defined patterns, a fluorescent dye was immobilized on the functionalized PS film. The PS film was immersed in a solution of 5-(aminoacetamido)fluorescein in ethanol at 25℃ for 1h. After washing it in a pure nitromethane, the patterns were viewed with an optical microscope equipped with a fluorescein filter set (excitation wavelength 450-490nm, emission wavelength > 510 nm).

RESULTS AND DISCUSSION

NHS azidobenzoate was synthesized by the N-hydroxysuccinimide and 4-azidobenzoic acid with DCC as catalyst. NHS azidobenzoate has two functional groups. One is a N-acyl succinimide group for an amide bond formation with the free amino group of biomolecules and the other is a photoreactive phenyl azido group. For chemical structure analysis, we used the methods such as ^{1}H-NMR, and electron impact mass spectroscopy. Figure 1 shows the ^{1}H-NMR spectrum of N-Hydroxysuccinimidyl azidobenzoate at 500MHz. This spectrum consists of three sharp peaks corresponding to the phenyl group and the succinimidyl group. The phenyl protons appear to down field compared to succinimidyl protons. Thus, for succinimidyl protons, δ (4H, CH_2) = 2.9ppm and δ (4H, phenyl) = 7-8ppm. Acyl derivatives of N-hydroxysuccinimide are also useful for synthesis of peptides and other types of amides. The N-hydroxysuccinimide that is liberated is easily removed because of its solubility in dilute base. The relative stability of the anion of N-Hydroxy-succinimide is also responsible for the acyl derivative being reactive toward nucleophilic attack by an amino group. On the other hand, NHS azidobenzoate gives the EI mass spectrum shown in Figure 2. From these two spectra, we could know that this compound is the NHS azidobenzoate.

To examine the photoreactivity of NHS azidobenzoate, the decomposition of azide upon photolysis was monitored by FTIR using KBr disk as the support. The FTIR spectrum showed strong absorption at 2122 cm^{-1} due to the azide group after NHS azidobenzoate was spin-coated on the PS film. This absorption completely disappeared after the film was irradiated with 254nm lamp for 5 min.

After fabrication of micro-scale patterned arrays by UV lithography, the film was immersed in a solution of 5-(aminoacetamido)fluorescein to detect the arrays by fluorescence microscope. Figure 4 shows the arrays. However we need the arrays with less size than 4 micrometer to platelet study related diabetics. Finally, we tried to immobilize the platelet adhesive peptide (GGGRGDF) on the PS coated cover glass. So, we synthesized the phenyl azido group derivatized GGGRGDF. We used the Dimethylformamide as the reaction solvent[6]. After the synthesis, HPLC was used to separate the phenyl azido group derivatized GGGRGDF from the mixture. Five peaks appeared in

the case used 0.1% TFA in acetonitrile as mobile phase. To know which compound is phenyl azido-derivatized GGGRGDF, positive FAB mass spectrum was measured. From those results, we could know that the forth peak fraction is the phenyl azido derivatized GGGRGDF. From these results, we can suggest that the photoreactive GGGRGDF peptide enables to make micro-scale patterned by Deep-UV lithography.

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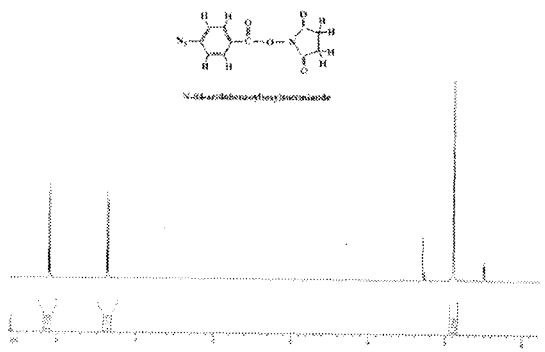

Figure 1. 1H-NMR spectrum of NHS azido-benzoate at 500 MHz.

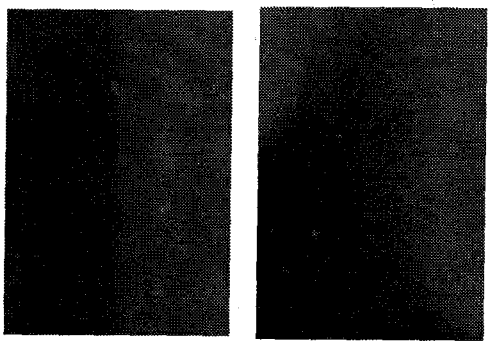

figure 4. Micrograph taken under a fluorescence microscope with a fluorescein filter set showing the patterned PS film after photolysis

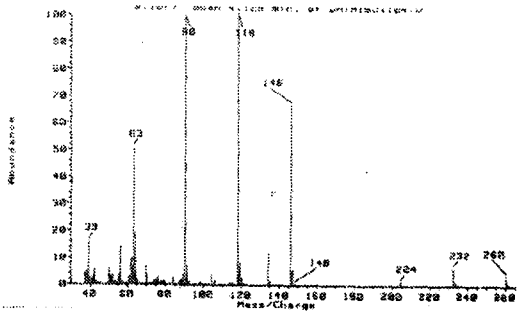

Figure 2. EI mass spectrum of NHS azido-benzoate

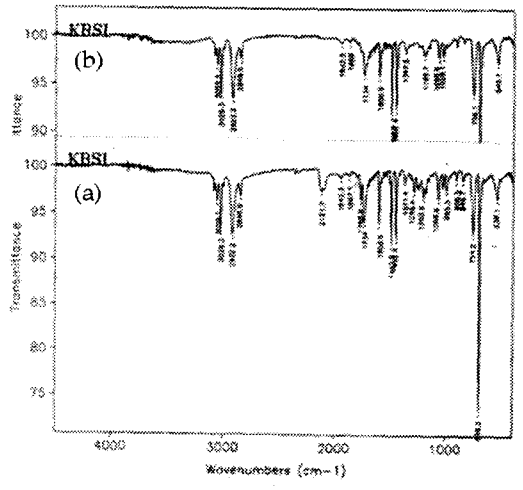

Figure3. FTIR spectra of (a) NHS azido-benzoate coated PS film. (b) The NHS azidobenzoate coated PS film photolyzed with 254nm for 5min.