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Immobilization of Amine-modified Oligonucleotides on Bifunctional Polymer Brushes Synthesized by Surface-initiated Polymerization

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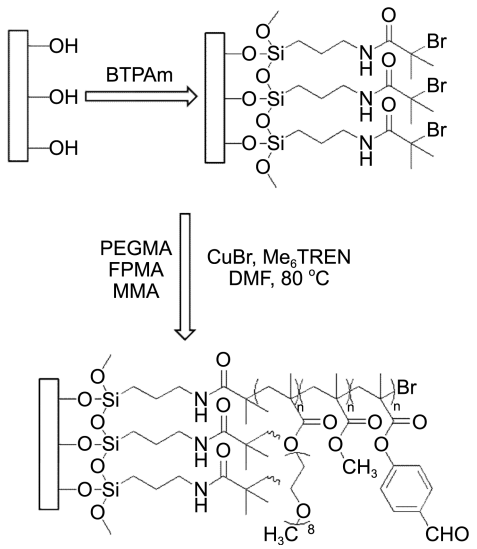
Polymer brushes on solid substrates formed by surface-initiated polymerization can tailor the substrates' surface properties,[1] allowing for example, the functionalization of glass surfaces for the construction of DNA microarrays used in the diagnosis of diseases and the study of genomics.[2] Glass surfaces are often functionalized by the formation of self-assembled monolayers (SAMs) of silanes containing functional groups, such as amine, alcohol, or carboxylic acid, at their terminal ends.[3] However, silanization is difficult to control for the formation of high-quality SAMs, resulting in poorly reproducible surface functionalization.[4] Therefore polymer brushes, which can be grown as high-density brushes in a controllable manner, have been studied for the surface functionalization of glass substrates. For example, polymer brushes based on polyethylene glycol methacrylate (PEGMA) have been used for surface coatings because they can limit the nonspecific adsorption of biomolecules onto the surfaces.[5]

Here, we report the synthesis of a bifunctional polymer brush by surface-initiated polymerization and its application in the immobilization of oligonucleotides. The polymer brush possessed two functional moieties, bio-inert polyethylene glycol (PEG) and bio-reactive aldehyde groups, which respectively provided effective resistance to biofouling and high binding ability for the specific immobilization of amine-modified oligonucleotides.

The surface-initiated polymerization of the bifunctional polymer brush is outlined in Scheme 1. 2-Bromo-2-methyl-*N*-(3-triethoxysilylpropyl)-propionamide (BTPAm) was synthesized by a synthetic procedure reported previously[6] (Supporting Information, Figure S1) and a SAM of BTPAm was formed by immersing a freshly cleaned and O_2 plasma-treated glass substrate in a toluene solution of BTPAm (0.3 w/v %) with subsequent curing at different temperatures. The resulting SAM was confirmed by the appearance of peaks characteristic of NH-CO at 1650 cm^{-1}, N-H bending at 1530 cm^{-1}, and Si-O stretching at 1112-1026 cm^{-1} in the ATR-FTIR spectrum of the BTPAm initiator film (Figure 1, spectrum b) compared with the spectrum of bare surface (Figure 1, spectrum a).[6b] The surface-tethered BTPAm presented a terminal bromoisobutyrate moiety, which allowed surface-initiated atom transfer radical polymerization (SI-ATRP) on the glass substrates.[7] SI-ATRP can provide control over chain length and surface density of polymers under ambient conditions.[8] The surface-initiated polymerization was then conducted in an oxygen-free environment using CuBr/Tris[2-(dimethylamino)ethyl]amine (Me_6TREN) cata-



Scheme 1. Surface-initiated polymerization of poly(PEGMA-FPMA-MMA).

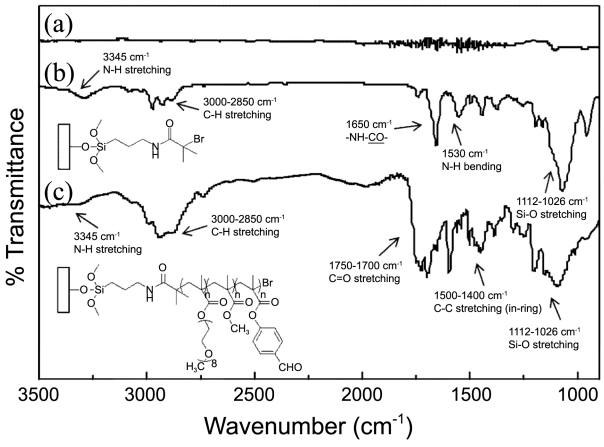


Figure 1. ATR-FTIR spectra of (a) bare, (b) BTPAm-coated, and (c) poly(PEGMA-FPMA-MMA)-coated surfaces.

lysts in a DMF solution of three monomers: bio-inert poly(ethylene glycol) methyl ether methacrylate (PEGMA), bio-reactive formylphenyl methacrylate (FPMA), and methyl methacrylate (MMA) as a spacer (See Experimental Section for details). The use of MMA allowed more efficient polymerization, presumably due to its alleviating steric hindrance. After polymerization, the substrate was ultrasonicated in DMF and blown dry by a N_2 stream. Formation of the polymer brush, which we denominated as poly(PEGMA-FPMA-MMA), was confirmed by the increased characteristic peaks of such as C=O stretching at 1750-1700 cm^{-1} and C-C stretching at 1500-1400 cm^{-1} in the ATR-FTIR spectrum of the polymer brush (Figure 1, spectrum c).

After confirming the successful growth of the poly(PEGMA-FPMA-MMA) polymer brush by surface-initiated polymerization, we assessed the feasibility of the poly(PEGMA-FPMA-MMA)-coated substrate as a bifunctional (bio-inert/bio-reactive) surface. We immobilized 5'-amine-modified probe oligonucleotides onto the poly(PEGMA-FPMA-MMA)-coated surface through the formation of a Schiff base and subsequent treatment with sodium borohydride (See Experimental Section for details). The primary amino groups (NH_2) on the oligonucleotides attacked the aldehyde moieties, *i.e.* FPMA, of the polymer brushes to form covalent bonds (Schiff base). The attachment was stabilized by subsequent treatment with sodium borohydride. The resulting probe oligonucleotide-immobilized glass substrate was incubated with a solution of fluorescently labeled target oligonucleotides complementary to the probe sequence. Figure 2 shows fluorescence micrographs of the glass surface after the hybridization reaction and subsequent washing. As expected, significant fluorescence was observed exclusively from the complementary target oligonucleotides hybridized on the probe-immobilized area, indicating the successful immobilization of the probes on the poly(PEGMA-FPMA-MMA)-coated substrate. In contrast, no significant fluorescence was detected after the probe-immobilized surface was exposed to fluorescent but noncomplementary target oligonucleotides and subsequently washed, indicating that the immobilized probe oligonucleotides were functional in that they hybridized only their complement exclusively (Supporting Information, Figure S2). It is also notable that no detectable nonspecific adsorption of fluorescently labeled oligonucleotides was observed on the poly(PEGMA-FPMA-MMA)-coated substrate that had only blocked polymer brushes without the probe oligonucleotides, presumably due to the presence of the bio-inert PEGMA (Supporting Information, Figure S3). Interestingly, the immobilization of oligonucleotides on the polymer brushes was found to be dependent on the integrity of surface-tethered BTPAm initiator. The curing temperature for the formation of the BTPAm initiator films on the glass substrates affected the fluorescence intensity from the hybridized fluorescent target oligonucleotides, *i.e.* the hybridization efficiency of the target oligonucleotides and thus the efficiency of the immobilization of the probe oligonucleotides (Figure 3). As the curing temperature increased to 100 °C, the hybridization efficiency of the complementary target was improved, presumably due to the improved integrity of the initiator film, resulting in the efficient formation of the poly(PEGMA-FPMA-MMA) polymer brush and greater immobilization of probe oligonucleotides.

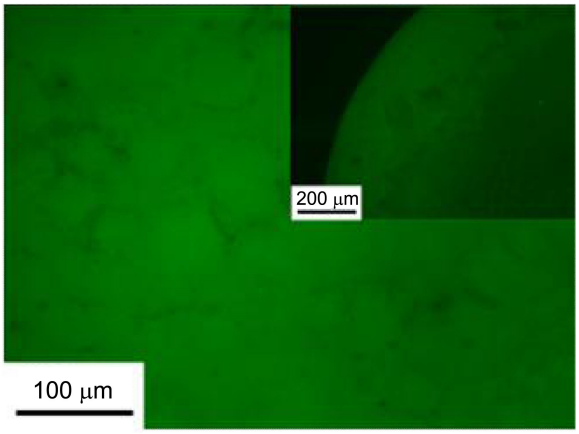


Figure 2. Fluorescence micrographs of a probe oligonucleotide spot immobilized on the poly(PEGMA-FPMA-MMA)-coated surface after exposure to a fluorescently labeled target oligonucleotide complementary to the probe sequence and subsequent washing. Inlet shows an enlargement of the probe oligonucleotide spot.

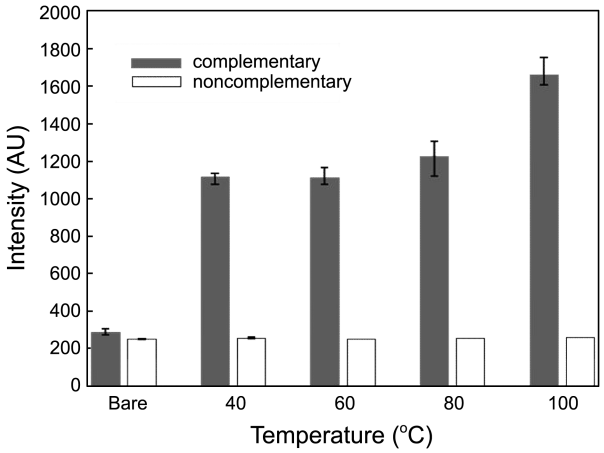


Figure 3. Fluorescence intensities of target oligonucleotides, both complementary and noncomplementary to the probe sequence, hybridized on the immobilized probe oligonucleotides according to the curing temperature for the formation of the BTPAm initiator film on the glass substrates. For comparison, background fluorescence intensities, obtained from bare glass slides after exposure to the fluorescently labeled target oligonucleotides and subsequent washing, were also presented.

In summary, surface-initiated polymerization was used to form a bifunctional poly(PEGMA-FPMA-MMA) polymer brush that could immobilize oligonucleotides. The polymer brush showed specific binding ability to amine-modified oligonucleotides, through the bio-reactive aldehyde (FPMA) and effective biofouling resistance, through the bio-inert polyethylene glycol (PEG). The integrity of the BTPAm initiator film was important to the formation of the polymer brush by surface-initiated polymerization.

Experimental Section

Chemicals and Materials. Copper(I) bromide (CuBr), toluene (anhydrous, 99.8%), 2-bromoisobutyryl bromide, (3-aminopropyl)triethoxyamine (APTES), methacryloyl chloride, and poly(ethyleneglycol) methyl ether methacrylate (PEGMA, M_n ~476) were purchased from Sigma-Aldrich (MO, USA). Tris(2-aminoethyl) amine was obtained from Lancaster (UK). Sodium hydroxide was received from Oriental Chem (Korea). Formaldehyde and formic acid were obtained from Kanto Chem (Japan) and Junsei Chem (Japan), respectively. Methylene chloride (MC, 99%) and methyl methacrylate (99%) were purchased from Deajung, Inc. (Korea). Triethylamine (TEA, 97%) was obtained from TCI (Japan). 4-Hydroxybenzaldehyde (99%) was received from Acros (Belgium). DNA oligonucleotides modified with amine or fluorescein were used as received from Bioneer Corp. (Daejeon, Korea). The sequences of the 5'-amine-modified probe and the 5'-fluorescein-labeled targets were as follows: ssDNA probe, 5'(amine)AGA AAG AGG AGT TAA TCC ATG CAA CTC TAA 3'; ssDNA complementary target, 5'(FAM)TTA GAG TTG CAT GGA TTA ACT CCT CTT TCT 3'; ssDNA noncomplementary target, 5'(FAM)CCG TGT CGT TTG ACC GCC CAA TTT ACC GTC 3'.

Synthesis of BTPAm, Me_6TREN, and FPMA. BTPAm was synthesized as previously reported. Briefly, 1.48 mL of 2-bromoisobutyryl bromide (1.20 eq) in toluene was added dropwise to a toluene mixture containing APTES (1.00 eq) and TEA (1.18 eq) at 0 °C under nitrogen atmosphere. The mixture was stirred for 3 h at 0 °C and then for another 10 h at room temperature. The mixture was filtered to remove salts and evaporated to remove the unreacted TEA under reduced pressure. A thick yellowish liquid product was obtained after vacuum drying (Supporting Information, Figure S1).

Me_6TREN was synthesized by a previously reported procedure.[9] Briefly, a mixture of tris(2-aminoethyl)amine (0.07 M, 10.5 mL), formic acid (66%, 100 mL), and formaldehyde (34%, 50 mL) was refluxed at 120 °C until the evolution of carbon dioxide stopped. After cooling to room temperature, volatile fractions were removed by vacuum distillation. 200 mL of sodium hydroxide (10%) was then added and an oily yellow layer formed that extracted into the methylene chloride. The extract was re-extracted with ether and dried over $MgSO_4$. After distillation, the final liquid product was obtained (Supporting Information, Figure S4).

FPMA was prepared by a modification of a previously reported method.[10] Briefly, 3.3 mL of TEA (1.10 eq) was added to 50 mL of a methylene chloride solution containing 4-hydroxybenzaldehyde (5 g, 1.00 eq). The mixture was cooled to 0 °C and 4.53 mL of methyacrloyl chloride (1.10 eq) was added dropwise to the stirred mixture. After 30 min, the mixture continued being stirred at room temperature for 10 h under nitrogen. It was then filtered to remove salts and washed several times with sodium hydroxide (5%) and water. The organic layer was dried over $MgSO_4$ and distilled to give the final yellowish liquid product (Supporting Information, Figure S5).

Surface-initiated Polymerization. A mixture of copper(I) bromide (0.07 g) catalyst, PEGMA (1.1 mL, 5 eq), FPMA (1.9 g, 20 eq), and MMA (1.06 mL, 20 eq) was transferred to a 100 mL three-neck flask containing Me_6TREN (158 μL, 1 eq), free BTPAm (0.18 g, 1 eq), and a BTPAm-functionalized glass slide. Surface-initiated polymerization was then carried out at 80 °C for 20 h under nitrogen. For detailed characterization of the polymerized poly(PEGMA-FPMA-MMA), free BTPAM was added to the reaction mixture, which led to the accompanying polymerization of free poly(PEGMA-FPMA-MMA) in the solution phase. The ratio of the three monomer units incorporated in the free poly(PEGMA-FPMA-MMA) was found to be (1:5:5) by ^{1}H NMR spectroscopy (Supporting Information, Figure S6). The free poly(PEGMA-FPMA-MMA)'s average molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC) to be M_n = 7000, PDI = 1.25, respectively (Supporting Information, Figure S7). No significant adsorption or precipitation of the free poly(PEGMA-FPMA-MMA) was observed on the glass substrates although weak adsorption or precipitation of the free polymer is still a possibility; it was polymerized in the solution in the presence of the free BTPAm initiator.

Preparation of Probe-immobilized Polymeric Layer. 5'-Amine-modified probe oligonucleotides were immobilized on the poly(PEGMA-FPMA-MMA)-coated glass slides as follows. Probe solution (20 mM in 50 mM sodium phosphate buffer, pH 8.5) was spotted onto the glass slide using a micropipet. The spotted slide was incubated in a chamber with humidity controlled using saturated NaCl solution at room temperature for 30 min. This resulted in the formation of covalent bonds (Schiff bases) between the primary amino groups of the probes and the aldehyde groups of the polymer brush. The attachment was stabilized through a dehydration reaction by baking the probe-spotted slide at 120 °C for 60 min. The slide was then sequentially washed with 0.2% SDS and diH_2O. Next, the probe-immobilized slide was placed in a blocking solution (0.25% sodium borohydride, 25% ethanol in 50 mM sodium phosphate buffer, pH 8.5) at room temperature for 15 min to remove unreacted aldehyde moieties. This treatment with sodium borohydride also reduced the double bond between the probe and surface, resulting in the irreversible covalent immobilization of the probes. After washing with 0.2% SDS and diH_2O, the slide was blown dry by a N_2 stream for the subsequent hybridization experiments.

Instruments. ^{1}H NMR and FTIR spectra were recorded with a Bruker spectrometer (Bruker Ultrashield 400 MHz) and a Thermo Mattson spectrophotometer (Infinity Gold FTIR Series), respectively. Gel permeation chromatograms were obtained using a Waters 515 HPLC system equipped with a MINI DAWN refractometer (Wyatt Technology Corp., USA) after calibration with standard polystyrene samples (Daelim Corp., Korea). Fluorescence micrographs were obtained with a fluorescence microscope (Nikon ECLIPSE Ti-U, Nikon Co., Japan) equipped with a mercury lamp

(Nikon INTENSELIGHT C-HGFI, Nikon Co.) and a CCD camera (CoolSNAP EZ, Photometrics Ltd., USA).

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