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Covalently Assembled Bifunctional Copolymer Layers as a Matrix for Immobilization of Oligonucleotides

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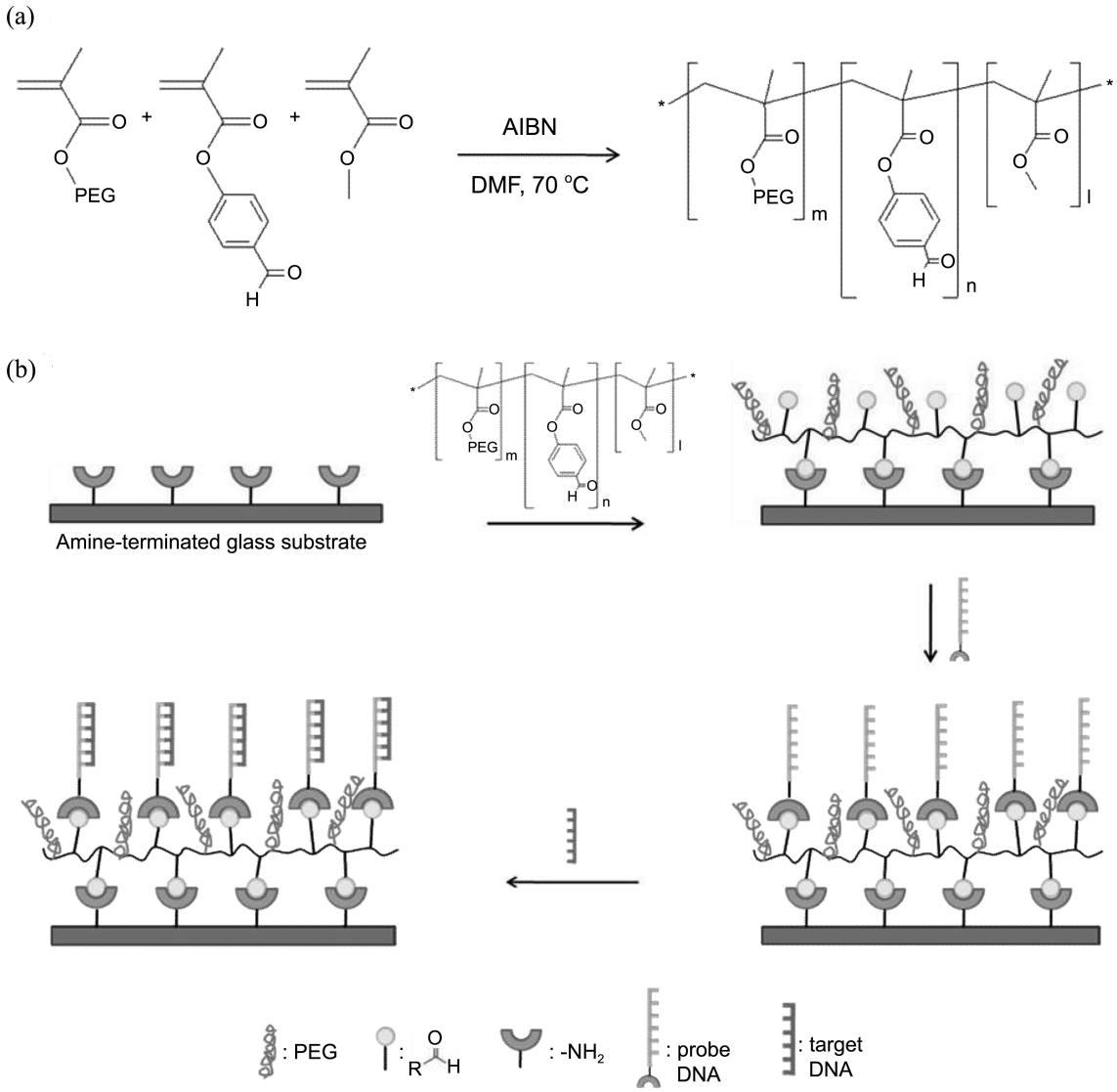
It is of particular interest to immobilize oligonucleotides on glass substrates for construction of DNA microarrays, because of extensive employment of the microarrays for diagnostic purposes and in the different areas of research in genomics.[1] Immobilization of oligonucleotides requires proper functionalization of the glass substrates to introduce two specific functionalities, *i.e.* bio-inertness and bio-reactivity, which provide effective resistance to bio-fouling and high binding ability for specific immobilization of oligonucleotides, respectively. The functionalization of glass surfaces generally relies on the formation of self-assembled monolayers (SAMs) of silanes.[2] However, the silanization process often results in poor reproducibility of surface functionalization because of unavoidable generation of defects and polymerized siloxanes on the glass surface.[3] Therefore, many researchers have proposed to use polymeric materials for the surface functionalization of glass substrates. For example, the employment of random copolymers have been reported to construct polymeric functional layers on the glass surfaces.[4] Surface-initiated polymerization has also been proposed to form polymer brushes with tailoring of surface properties of the substrates.[5]

Herein, we report the synthesis of random copolymers with bifunctionalities, *i.e.* bio-inertness and bio-reactivity, by radical polymerization and their use for specific immobilization of amine-modified oligonucleotides on glass surfaces. The random copolymers comprised of three monomeric units, *i.e.* poly(ethyleneglycol) methyl ether methacrylate (PEGMA) as a bio-inert part, *p*-formylphenyl methacrylate (FPMA) as a bio-reactive part, and methyl methacrylate (MMA) as a spacing part. We have also compared the bio-fouling resistance and binding ability of the random co-polymers, which we denote as poly(PEGMA-*co*-FPMA-*co*-MMA), with respect to different molar ratios of the mono-mers.

Scheme 1(a) represents the chemical structure of the poly(PEGMA-*co*-FPMA-*co*-MMA) synthesized from three kinds of monomers with different molar ratios. The *p*-form-ylphenyl methacrylate (FPMA) was synthesized as reported previously[6] (Supporting Information, Figure S1) and used for the radical polymerization of poly(PEGMA-*co*-FPMA-*co*-MMA) with the other two commercially available mono-mers. We synthesized a series of the poly(PEGMA-*co*-FPMA-*co*-MMA) with five different contents of FPMA as shown in Table 1, based on the assumption that the func-tionalities of the copolymers could be simply tailored by controlling initial feed ratios of each specific monomer during the synthesis of the polymer.[4a] The resulting content of FPMA in each copolymer was evaluated on the basis of peak areas corresponding to each monomer in the ^{1}H NMR spectra (Figure 1) and was found to be 30.43, 42.63, 49.51, 54.56, and 63.23% for copolymer **1**, copolymer **2**, copolymer **3**, copolymer **4**, and copolymer **5**, respectively. This result indicates that the composition of the copolymers was in relative agreement with the initial feed ratios of the mono-mers. The molecular weight of the synthesized five copoly-mers was also found to be in the range from 8300 to 12000 with a polydispersity index (PDI) of ~1.6 as determined by gel permeation chromatography (GPC) (Supporting Infor-mation, Figure S2). These results indicate that the synthesis of the poly(PEGMA-*co*-FPMA-*co*-MMA) was controllable by simply changing the initial feed ratios of each specific monomer with resultant production of relatively monodis-perse copolymers.

After confirmation of the successful synthesis of the poly(PEGMA-*co*-FPMA-*co*-MMA) by radical polymeri-zation, we carried out covalent assembling of the series of random copolymers on amine-terminated glass substrates and subsequent immobilization of amine-modified probe oligonucleotides onto the polymeric layers for downstream hybridization experiments (Scheme 1(b)). The covalent assemblies of the poly(PEGMA-*co*-FPMA-*co*-MMA) were prepared by incubating amine-terminated glass substrates (NSB Amine Slide, NSB POSTECH, Inc., Korea) in the copolymer solutions (20 wt % in THF) for 24 h at 60 ^{o}C via formation of covalent bonds between aldehyde moieties, *i.e.* FPMA, of the copolymers and amine groups on the glass surfaces. Then, we immobilized 5'-amine-modified probe oligonucleotides onto the copolymeric layers through Schiff base formation and subsequent treatment with sodium boro-hydride, as described in the Experimental Section. The primary amine groups of the oligonucleotides attacked the available aldehyde moieties on the copolymeric layer to form Schiff bases, which were further stabilized by the



Scheme 1. (a) Synthesis of the poly(PEGMA-*co*-FPMA-*co*-MMA). (b) Illustration of covalent assembling of the poly(PEGMA-*co*-FPMA-*co*-MMA) on an amine-terminated glass substrate and subsequent immobilization of amine-modified probe oligonucleotides onto the polymeric layer for downstream hybridization experiments.

Table 1. A series of the poly(PEGMA-*co*-FPMA-*co*-MMA) with five different FPMA contents

Copolymer	PEGMA : FPMA : MMA	
	Feed ratio	Observed ratio
1	1.00 : 1.00 : 0.40	1.00 : 0.63 : 0.44
2	1.00 : 2.00 : 0.40	1.00 : 1.33 : 0.79
3	1.00 : 3.00 : 0.40	1.00 : 2.04 : 1.08
4	1.00 : 4.00 : 0.40	1.00 : 2.75 : 1.29
5	1.00 : 5.00 : 0.40	1.00 : 3.13 : 0.82

treatment with sodium borohydride. Furthermore, the treatment with sodium borohydride reduced unreacted aldehyde groups into non-reactive primary alcohols. The resultant probe oligonucleotide-immobilized glass substrates were exposed to a solution of fluorescently labeled target oligonucleotides, which were either complementary or non-complementary to the probe sequence. Figure 2(a) shows fluorescence micrographs of the glass surfaces after the hybridization reaction and subsequent washing. As expected, significant fluorescence signals were observed from the complementary target oligonucleotides hybridized on the probe-immobilized copolymeric surfaces, which indicates successful immobilization of probes on the series of copolymeric layers. In contrast, we observed no detectable fluorescence when the probe-immobilized surfaces were exposed to the fluorescent but noncomplementary target oligonucleotide with subsequent washing; this observation indicates that the immobilized probe oliogonucleotides were functional in a sense that they could exclusively hybridize with their complement. We also observed no detectable non-specific adsorption of fluorescently labeled oligonucleotides on the copolymeric layers, where all the available aldehyde moieties were reduced to non-reactive alcohol groups without the probe oligonucleotides. It is presumed that such a behavior

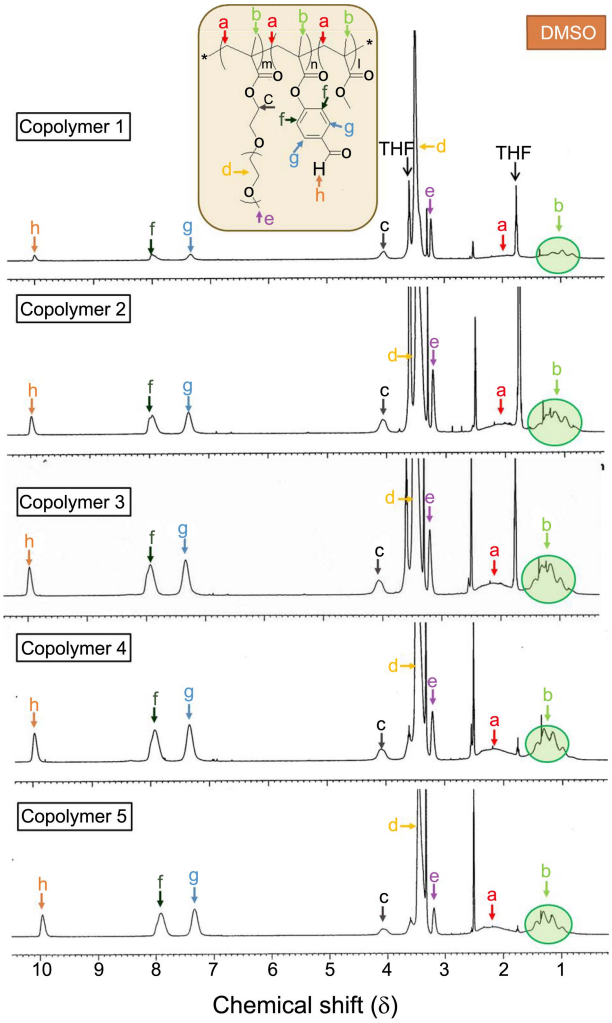


Figure 1. ^{1}H NMR spectra of the poly(PEGMA-*co*-FPMA-*co*-MMA) (DMSO).

could be due to the presence of bio-inert PEGMA on the copolymeric layers (Supporting Information, Figure S3). Interestingly, the immobilization of oligonucleotides on the series of copolymeric surfaces was found to be dependent on the compositions of the copolymeric layers. Figure 2(b) indicates that the composition of the copolymeric layers affected the fluorescence intensities from the hybridized fluorescent target oligonucleotides, *i.e.* the efficiencies for hybridization of target oligonucleotides and thus efficiencies for immobilization of probe oliogonucleotides. It was observed that with an increase in the contents of FPMA (the bio-reactive groups of the copolymers) on the copolymeric layers, there was a gradual increase in the hybridization efficiencies of target oligonucleotides; such a behavior could be presumably due to increased amount of available aldehyde moieties in the copolymeric layers with the resultant presence of large number of immobilized probe oligonucleotides. However, further increase in the contents of FPMA led to decrease in the hybridization efficiency, which can be understood in terms of presence of too dense probe layers on the polymeric surfaces with high FPMA content.[7] We achieved

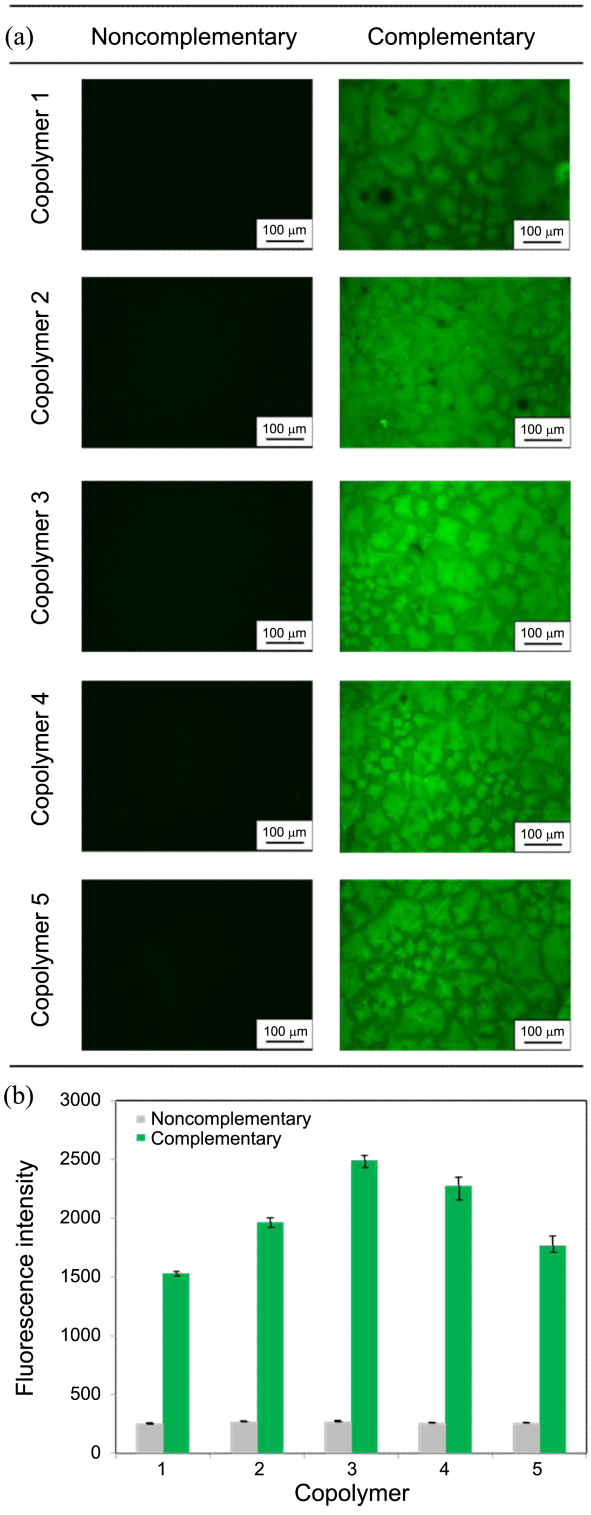


Figure 2. (a) Fluorescence micrographs of probe oligonucleotide-immobilized copolymeric surfaces after exposure to fluorescently labeled target oligonucleotides (either noncomplementary or complementary to the probe sequence) and subsequent washing. (b) Fluorescence intensities of target oligonucleotides, both noncomplementary and complementary to the probe sequence, hybridized on the immobilized probe oligonucleotides depending on the series of copolymeric layers. Experimental conditions: The probe-immobilized copolymeric surfaces were exposed to target oligonucleotide solutions (20 μM in 5X SSC buffer containing 0.1% SDS) for 16 h at 42 °C and subsequently washed.

an optimized hybridization response on the copolymer **3** layer with 49.51% FPMA content. The obtained results indicate that we can control the density of bio-reactive groups by changing the initial feed ratio of each monomer during the synthesis of the poly(PEGMA-*co*-FPMA-*co*-MMA) and thus optimize the covalently assembled copolymer layers as a matrix for immobilization of oligonucleotides.

In summary, we demonstrated the synthesis of a series of poly(PEGMA-*co*-FPMA-*co*-MMA) by radical polymerization and their subsequent covalent assembling onto amine-modified glass substrates for forming bifunctional (bio-inert and bio-reactive) surfaces. Amine-modified oligonucleotides could be immobilized on the series of copolymeric layers and the immobilized oligonucleotides could exclusively hybridize with their complement with minimal non-specific adsorption. The density of bio-reactive moieties in the poly(PEGMA-*co*-FPMA-*co*-MMA) was controllable by changing the initial feed ratio of each monomer during the radical polymerization, which allowed optimization of the covalently assembled copolymer layers as a matrix for immobilization of oligonucleotides.

Experimental Section

Synthesis of FPMA and Radical Polymerization of poly(PEGMA-*co*-FPMA-*co*-MMA). The FPMA was prepared based on a synthetic procedure reported previously.[6] Briefly, 45 mmol of TEA was added to 30 mL of a dichloromethane solution containing 4-hydroxybenzaldehyde (41 mmol). The mixture was cooled to 0 °C and 45 mmol of methacryloyl chloride was added dropwise to the mixture with stirring. After 1 h, the mixture was stirred at room temperature for 12 h under N_2 atmosphere. Subsequently, the mixture was filtered to remove salts and was sequentially washed several times with sodium hydroxide (5 wt %) and water. The organic layer was dried over $MgSO_4$ and the solvent was evaporated under vacuum to obtain the crude product, which was crystallized from aqueous ethanol to obtain the final yellowish product (Supporting Information, Figure S1).

Radical polymerization of poly(PEGMA-*co*-FPMA-*co*-MMA) copolymers was performed as previously reported, but with certain modifications.[4a] Briefly, a mixture solution of different molar concentrations of PEGMA, FPMA, and MMA in DMF was transferred to a 100 mL three-neck flask containing 10 mL of DMF solvent pre-heated to 70 °C under a dry Ar blanket. The polymerization reaction was initiated by adding 1 mL of 2,2'-azobis isobutyronitrile (3% molar ratio to monomers, in DMF) and carried out at 70 °C for 24 h under Ar atmosphere. After evaporation of solvent under vacuum, the copolymers were obtained in the form of a viscous yellowish liquid.

Preparation of Probe-immobilized Copolymeric Layer. The 5'-amine-modified probe oligonucleotides were immobilized on the poly(PEGMA-*co*-FPMA-*co*-MMA)-coated glass slides as follows: the probe solution (20 μM in 50 mM sodium phosphate buffer, pH 8.5) was spotted onto the glass slide using a micropipette. The spotted slide was incubated in a chamber in which the humidity was controlled with saturated NaCl solution at room temperature for 30 min. This resulted in formation of covalent bonds (Schiff bases) between the primary amino groups of the probes and the aldehyde groups of the copolymers. The attachment of oligonucleotides on the substrate was stabilized through a dehydration reaction by baking the probe-spotted slide at 120 °C for 60 min. The slide was then washed with 0.2% SDS and diH_2O in a sequential manner. 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, which resulted in irreversible covalent immobilization of the probes. After washing with 0.2% SDS and diH_2O, the slide was blown dry by N_2 stream for subsequent hybridization experiments.

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