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Immobilization of Biomolecules on Cycloolefin Polymer Supports

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Recent trends in the development of microfluidic and biondiagnostic chips favor polymer materials over glass, primarily for optical and economical reasons. Therefore, existing chemical methods to prepare biomolecule microarrays on glass slides have to be adapted or replaced in order to suit polymer substrates. Here we present a strategy to immobilize DNA and antibodies on cyclic polyolefin slides, like Zeonor. This polymer represents a class of new polymeric materials with excellent optical and mechanical properties. By plasma and liquid chemical treatment followed by coating with polyelectrolytes, we have succeeded in immobilizing DNA onto the polymer substrate, yielding stable and versatile biosensor surfaces. We demonstrate the stability and usage of the coated Zeonor substrates not only in DNA chip technology but also in protein chip technology with DNA-directed immobilization of proteins.

In biomedical diagnostics, drug discovery, genomics, and proteomics, biochips are essential for fast, sensitive, reliable, and simultaneous detection of many different analytes present in a biological mixture.^{1–6} Consequently, much effort has been expended in developing new, and in optimizing existing, strategies to immobilize functional biomolecules onto glass slides for multiplexed detection of proteins and nucleic acids.^{7–10} Of the variety of developed sensing methods, optical techniques are widespread, and within these, fluorescence detection is typically the method of choice when minute amounts of material need to be analyzed.^{11–13}

Polymeric supports provide a suitable, versatile, and cost-saving alternative to the use of glass substrates for optical components. The possibility of mass replication technologies, such as injection molding and hot embossing, favor highly transparent polymer materials over glass in precision optics today.^{14,15} For analytical chemistry and molecular biotechnology, a new class of amorphous thermoplastics, cycloolefin polymers and copolymers, has received attention, primarily because of chemical stability and optical transparency. These materials can be produced by chain copolymerisation of cyclic monomers such as norbornene with ethane (Topas, Ticona) or by ring-opening metathesis polymerization of various cyclic monomers followed by hydrogenation (Zeonor, Zeonex, Zeon Corp.).¹⁶ Zeonor exhibits such properties as high transparency, high purity, optical clarity, low autofluorescence, absence of UV absorption, good chemical resistance, low shrinkage, good insulating properties, low birefringence, and good resistance to polar solvents.^{17–20} The outstanding optical properties make Zeonor resins particularly well-suited for sophisticated microchip designs that include optical detection in biomedical and molecular diagnostics.^{15,16,21}

Hydrophobicity and chemical resistance are desired properties of Zeonor in applications such as medical vials, syringes, or packaging¹⁷ but hamper standard methods for coating and immobilization of biomolecules. As coatings suitable for biosensor applications are increasingly required on plastic optics, a detailed protocol is presented here that allows the use of Zeonor substrates in bioanalytical systems, in particular the detection of DNA and proteins.

Polymer surfaces can be activated and hydrophilized, without destroying their optical properties, by low-pressure gas plasma or slightly basic treatment.^{14,18} There are four concurrently

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occurring factors involved in the modification of the surface: introduction of functional groups, cross-linking of near-surface groups, degradation of polymer molecules, and etching of the surface.²² These processes affect only the top few molecular layers (<100 nm) and result in a charged surface. However, these surfaces have been reported to be unstable for storage.^{22,23} In the work presented here, we overcome this instability by coating the polymer substrate with polyelectrolytes. This process introduces defined functional surface groups and provides wash-stable, as well as storage-stable, hydrophilic surfaces. Zeonor substrates functionalized in this way with carboxylic acid allow covalent attachment of antibodies and amino-labeled DNA by carbodiimide activation. Although Rucker et al. recently reported the adsorption of antibodies onto plasma-treated polymer surfaces,²⁴ covalent attachment of biomolecules is preferred to hydrophobic adsorption as it is more controllable and minimizes protein denaturation. Analytical surface studies have been performed to characterize the surface properties of uncoated and coated Zeonor slides and to optimize the supports for the immobilization of biomolecules. Within these studies, standard glass slides were always used as reference and therefore treated similarly to Zeonor slides.

While DNA chips are already widely used for applications in biomedical and molecular diagnostics,^{1–3} protein arrays have not yet realized their full potential, because of fabrication, storage, as well as protein orientation, conformation, and stability issues.^{4,6,10,25,26} By DNA-directed immobilization (DDI) of proteins in complex arrays, these problems can be largely circumvented:^{27,28} First, stable and robust arrays of unique sequences of immobilized single-stranded (ssDNA) are created, followed by sequence-specific hybridization of complementary DNA strands linked to proteins. Thus, by the use of protein–DNA conjugates, a DNA surface is converted into a specific and sensitive biosensor surface for the detection of protein–protein interactions. This technique provides the potential to build DNA microarrays on protein chips. Furthermore, immobilization of antibodies by DDI is a useful alternative to printing or using PDMS channels for creating patterns. Using this technique, we demonstrate the feasibility of Zeonor as a substrate for combined DNA and protein microarrays to be used in biondiagnostic applications.

EXPERIMENTAL SECTION

Poly(acrylic acid) (PAC) Coating. Commercial glass coverslips purchased from Sigma-Aldrich, and Zeonor slides Zr1060R, provided by Ämic AB, were cleaned in an ultrasonic bath with 2% aqueous surfactant solution (Micro90, Sigma-Aldrich) for 1 h at 65 °C, followed by rinsing with bidistilled water, and then dried under a nitrogen flow.

Surface activation was carried out afterward by wet chemistry, as well as by plasma treatment. The former method was performed

by sonicating both types of slides for 1 h at 65 °C in an aqueous ammonia solution (10–20% NH₄OH and 5% H₂O₂ in bidistilled water, Sigma-Aldrich) to create hydrophilic surfaces via etching. Prior to PAC coating, the slides were extensively rinsed with bidistilled water to pH neutrality and dried afterward under a nitrogen flow.

After the initial cleaning procedure, plasma treatment of the Zeonor and glass slides was performed in an oxygen plasma for 5 min (Harrick Plasma PDC-002, 30 W, 0.1 Torr O₂, 3 sccm) immediately before the coating step. The clean and activated slides were immersed in a solution of 2 mg/mL polyethyleneimine (PEI; Sigma-Aldrich) with 0.5 M NaCl for 10 min, followed by rinsing with water. They were then placed in a solution of 2 mg/mL poly(acrylic acid) (PAC; Sigma-Aldrich) with 0.5 M NaCl for 10 min.^{29,30} This coating technique was repeated three times. After washing with bidistilled water and drying under a nitrogen flow, the coated slides could be used immediately or stored in a desiccator for ~1 month.

Surface Characterization. The contact angle of bidistilled water on the treated solid supports was measured under air at room temperature using a video-based optical contact angle measuring device (FTA200, First Ten Ångströms) with an electronic syringe unit. Uniform drops with volumes of 2 µL were deposited on the surface and after 30 s, which allowed the drops to reach a quasi-stable configuration, digital images of the drops were taken. Using the FTA32 Video 2.0 software, left and right contact angles and dimension parameters of the drops were calculated.

DNA Immobilization. To immobilize single-stranded receptor-DNA on PAC-coated Zeonor and glass slides, first an adhesive silicone mask (Silicone RTV 14 RST, Bauer Handels GmbH) with an array of 3 × 3 reaction chamber of 10-µL volumetric capacity was fixed onto the PAC-coated substrates. Afterward, 5'-amino-modified capture oligonucleotides (adaptors A (24 bp) and B (26 bp), Chimera Biotec and custom-tailored 23 mer, TIB Molbiol) were covalently bound to the carboxyl groups by carbodiimide activation.

Carboxylamine conjugation activated by carbodiimide has been optimized elsewhere.³¹ Briefly, aminated ssDNA was diluted in 10 mM 2-(*N*-morpholino)ethansulfonic acid buffer (MES buffer, pH 6; Sigma-Aldrich) containing 10 mM MgCl₂ (Sigma-Aldrich), 5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; Sigma-Aldrich), and 0.33 mM *N*-hydroxysulfosuccinimide (NHSS; Sigma-Aldrich). Ten microliters of ~10^{−6} M DNA solution was pipetted into the reaction chambers and incubated for 1 h at room temperature. Before hybridization of complementary DNA strands, residual carboxylic groups on the PAC surface were blocked. This was achieved by immersing the slides in a solution of either 10^{−4} M ethanolamine in 10 mM MES buffer, pH 6, containing 5 mM EDC and 0.33 mM NHSS, or blocking buffer (Chimera Biotec), or a solution of 5% BSA in PBS (Sigma-Aldrich). After 1 h at room temperature, washing and drying procedures were then performed as described above.

Hybridization, Protein Binding, and Detection. Sequence-specific hybridization of complementary DNA strands, linked to rabbit-IgG or Cy5 molecules (Cy5 is a trademark of Amersham

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Biosciences Ltd. or its subsidiaries, Chimera Biotec and TIB Molbiol, respectively), to immobilized ssDNA was performed in hybridization buffer for PCR/DIG ELISA (Roche) at a concentration of 10^{-7} M. After 1 h at room temperature, the slides were washed four times with hybridization buffer. After drying under a nitrogen gas flow, the successful hybridization reaction of Cy5-labeled oligonucleotides was detected by an array scanner (GMS 418, Genetic Micro Systems, λ_{exc} 633 nm).

To apply Cy5-labeled anti-rabbit-IgG (Cy5 AffiniPure goat anti-rabbit IgG (H+L), Stratech) solution at a concentration of 1.5 mg/l in PBS buffer (pH 7.4, Sigma-Aldrich) to the surface, the slides were washed and immersed in antibody solutions of precise concentrations for an additional hour. After washing four times with buffer and drying under a nitrogen gas flow, the slides were scanned by an array scanner.

RESULTS AND DISCUSSION

The controlled immobilization of biomolecules on cyclic polyolefin substrates requires the development of a precise method for introducing functional groups onto the surface. One possibility is the chemical transformation of free substituents of the cyclic monomer. However, due to the fact that the properties of the cycloolefin polymers are mainly determined by these substituents,¹⁷ these components are protected by the producer and only limited information is available. A commissioned microanalysis of Zeonor 1060R, undertaken at the Microanalytical Laboratory, University College Dublin, indicated a chemical composition of $88 \pm 2\%$ carbon and $11 \pm 2\%$ hydrogen with an absence of nitrogen, sulfur, and chloride. In the recently published ^{13}C NMR investigations of Zeon's cyclic olefin polymers,¹⁶ Shin et al. concluded that the polymer microstructure contains mainly ethylene/norbornanediyl units. Hence, an alkene structure for the side groups of Zeonor 1060R was assumed.

The lack of detailed information regarding the chemical composition of the substrate made it necessary to test the surface properties after liquid chemical and plasma treatments. ζ -Potential measurements provide evidence of a negatively charged polymer surface after exposure of Zeonor to oxygen plasma or basic treatment.^{15,22,23} However, as the source of the surface charge was unclear,¹⁵ the substrates were coated with polyelectrolytes in order to introduce defined functional surface groups to bind biomolecules covalently.

According to the information supplied by Zeon Corp., Zeonor is resistant against 50% caustic soda and 10% aqueous ammonia, respectively. A slightly higher concentration of the basic solutions will slightly etch the polymer surface, increasing the wettability and allowing polyelectrolyte coating of the polymer substrate. Figure 1 shows the equilibrium water contact angles of the plain, aqueous ammonia-, caustic soda-, and plasma-treated surfaces, which were subsequently coated with PEI and PAC. Basic treatment of plain Zeonor reduces the contact angle and increases the wettability of the surface. Afterward, the surface can be coated alternately with a positively and a negatively charged polyelectrolyte, here PEI and PAC, to yield a homogeneous surface film. As reference, the procedure was also carried out on conventional glass slides. For both coated glass and coated polymer slides the water–air–solid contact angles are of the same magnitude.

In addition to being a time-saving and waste-reducing method, the most obvious result of the air plasma treatments is the

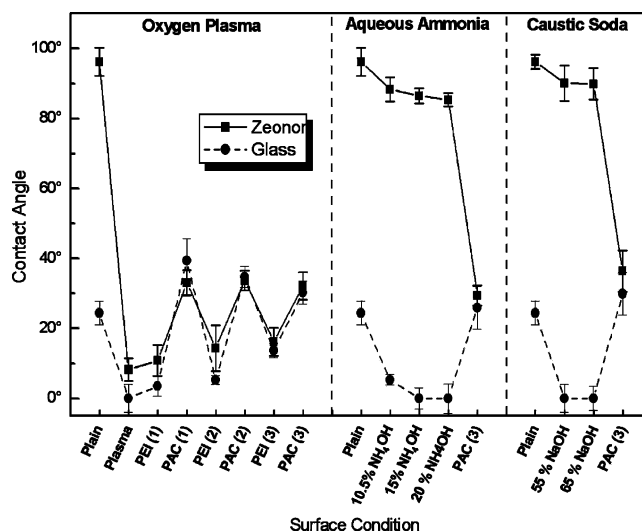


Figure 1. Equilibrium water contact angles of oxygen plasma- and liquid chemical-treated Zeonor and glass surfaces followed by coating of alternating PEI and PAC films. Number in parentheses gives the number of layers of each polyelectrolyte. Oxygen plasma treatment decreases the contact angles dramatically and, hence, increases the surface wettability significantly. Liquid chemical treatments reduce the contact angle slightly. The alternate superimposition of PEI and PAC films can be followed by the alternating water–air–solid contact angles between the typical degrees of each polyelectrolyte. For the liquid chemical-treated substrates, only the contact angles after three layers PEI and three layers PAC are shown. Coating with three layers of PAC provides constant contact angles on each treated substrate.

improved wettabilities of surfaces.²² Before oxygen plasma treatment, the surfaces of Zeonor and glass had equilibrium water contact angles of approximately 95° and 25° , respectively. Plasma treatment decreases the contact angles of both to lower than 10° , significantly changes the hydrophilicity of the polymer, and, consequently, makes polyelectrolyte coating possible.

With a power/gas flow ratio of 10 W/sccm, relatively mild low-flow gas plasma conditions were used, and this will affect only the top few molecular layers.²² Although the equilibrium water contact angles are stable toward washing with 70% 2-propanol, over time much of the wettability of the plasma-treated polymers is lost during dry storage. The storage instability is attributed to a mechanism whereby high-energy groups at the surface of the polymer are replaced by low-energy groups from the interior through a reorganization of the structure.³² Figure 2 shows the significant increase of the contact angles over just 6 days. These results are in good agreement with those of Mela et al.,¹⁵ who reported that the ζ -potential decayed over a period of days. Larsson et al. reported recently on a method using high-flow plasma conditions to yield permanent hydrophilic Zeonor surfaces, but at the cost of strong alteration of the chemical structure and increasing surface roughness.^{14,32} Surface roughness is a critical factor for high-sensitivity, surface-based optical biosensors and methods that increase that should be avoided. Surface reorganization was not observed after PAC coating or after liquid chemical treatment (Figure 2). Therefore, Zeonor slides should be coated immediately after either plasma activation or liquid chemical treatment. PAC-coated slides, regardless of substrate material,

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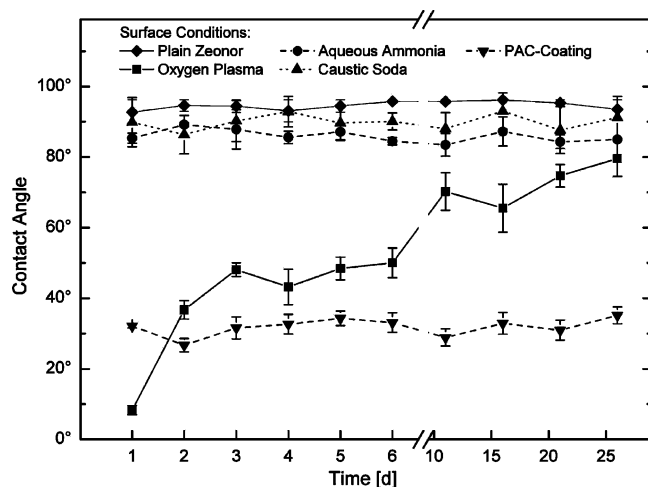


Figure 2. Storage stability of oxygen plasma-treated, liquid chemical-treated, and PAC-coated Zeonor slides. While the equilibrium water contact angle of plasma-treated Zeonor changes significantly over only 6 days, liquid chemical and PAC-coated Zeonor slides remain stable for 30 days of storage.

remained stable and could be used within 1 month for the immobilization procedure. Furthermore, we have been able to perform binding experiments with coated slides stored under appropriate conditions for up to 6 months.

Coating substrates with polyelectrolyte multilayers constructed by a layer-by-layer deposition technique results not only in thin, homogeneous, and stable films but also eliminates the electrostatic effects of the substrate material. Consequently, by providing a solid support with a thin coating, the properties of the particular surface depend mainly on the properties of the coating material. At a minimum of three polycation–polyanion bilayers, we found out that the surface properties of PAC-coated glass and PAC-coated Zeonor slides are similar. Surfaces functionalized with carboxylic acid are very suited for covalent binding of amino-modified ssDNA, because of the good reaction efficiency and the very low nonspecific binding of nonaminated DNA.^{8,29} Oligonucleotide probe density is a controlling factor for the efficiency of target capture and kinetics of the target/probe hybridization.³³ Therefore, optimized protocols for carbodiimide activation were taken from refs 8 and 31 to yield appropriate receptor densities of the ssDNA–receptor.³³ Hybridization reactions with Cy5-labeled oligonucleotides with complementary sequence to surface-tethered receptor ssDNA were successfully performed at different concentrations on both polymer and glass surfaces, with no significant differences concerning receptor densities. Additionally, the results on PAC-coated glass are in good agreement with the results of Krieg et al.²⁹ In Figure 3 the average fluorescence signals ($n = 5$) measured after target/probe hybridization onto carboxylic acid-functionalized substrates are plotted against the analyte Cy5-labeled ssDNA concentrations. PAC-coated slides without surface-tethered ssDNA were used as reference and to investigate nonspecific adsorption. At an analyte concentration of $\sim 3 \times 10^{-7}$ M, the fluorescence signal reaches a plateau, which represents the intensity level of maximum occupation of the receptor sites. Hence, we determined the density of the immobilized oligonucleotides to be ~ 10 fmol/

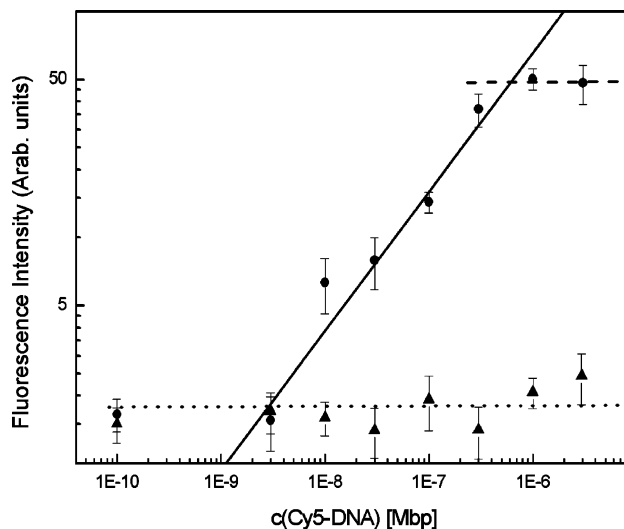


Figure 3. Dependence of Log (fluorescence signal) upon Log (analyte concentration) on PAC-coated substrates measured by hybridization reaction between Cy5-labeled oligonucleotides and surface-tethered complementary ssDNA (circles). The same analyte concentrations were also used to measure nonspecific binding of ssDNA onto plain PAC coatings (triangles). The dashed line corresponds to the intensity level of maximum occupation of receptor sites, the dotted line to the average level of background fluorescence, and the solid line is a linear fit to experimental values. The statistics for the linear fit: $\log(y) = (3.90 \pm 0.63) + (0.62 \pm 0.08) \times \log(x)$, $R = 0.9741$.

mm². For such a low probe density, Peterson et al. reported in ref 33 an essentially 100% hybridization efficiency and a fast binding reaction.

Poly(acrylic acid) coatings without immobilized nucleic acids, show, indeed, a low interaction with the analyte ssDNA in solution (Figure 3). Probably the repulsion of the negatively charged surface at neutral pH prevents oligonucleotides from adsorbing.^{8,29} The amount of surface-tethered molecules, and especially the higher nonspecific binding of DNA, favor PAC-coated substrates over others for biosensors detecting surface-generated fluorescence.^{12,13,34}

Carboxylic acid-functionalized and DNA-immobilized slides are robust and remain active after dry storage for up to 1 month. In conditions appropriate for biomolecules, DNA–DNA binding is likewise stable for several weeks. Stability tests of surface-tethered Cy5-labeled DNA stored in hybridization buffer over 1 week result in constant fluorescence signals (Figure 4). Thus, the binding can be accepted as strong under used conditions. However, a controlled denaturation of double-stranded DNA molecules allows regeneration of the surface. By either chemical or thermal treatments, the DNA double helix can be dehybridized and DNA–DNA (or DNA–protein) conjugates can be removed from the surface.²⁵ Here, 100% 2-propanol and 50 mM NaOH solution have been used to denature the double-stranded DNA after preliminary hybridization (Figure 4). With only a single washing with NaOH or twice with 2-propanol, the fluorescence originating from hybridized Cy5-labeled oligonucleotides decreases significantly. After regeneration, the surface can be used a second time for hybridization reactions recovering nearly the same fluorescence intensity (Figure 4).

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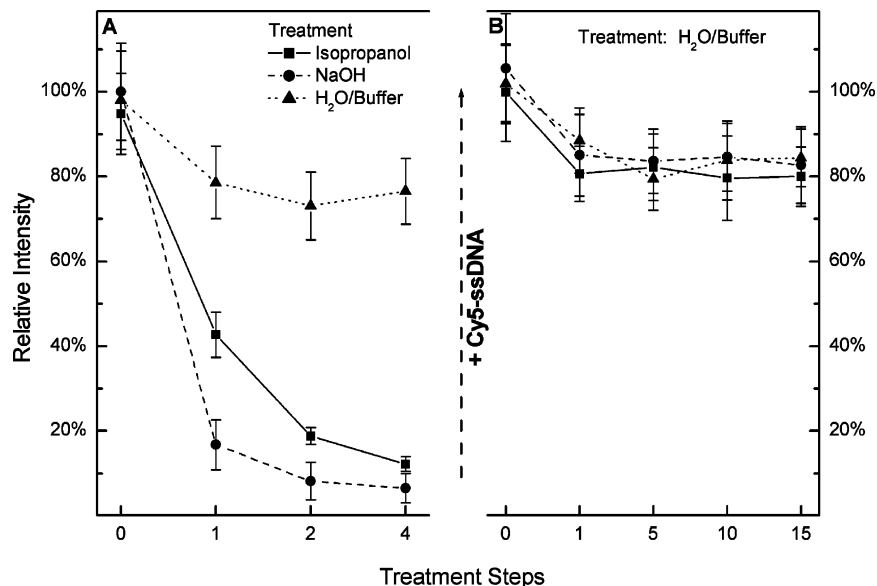


Figure 4. (A) Stability of surface-tethered double-stranded DNA against H₂O/buffer, 2-propanol (100%), and NaOH (50 mM) solution. Due to dehybridization of the Cy5-labeled DNA helix, the intensity of the fluorescence signal at the surface is reduced by ~90% within only 2 treatment steps of NaOH and 4 treatment steps of 2-propanol, respectively. (B) After denaturation of double-stranded DNA, complementary Cy5-labeled oligonucleotide can be again hybridized to the remaining receptor single-stranded DNA. The new DNA–DNA conjugate is again stable against washing with H₂O/buffer.

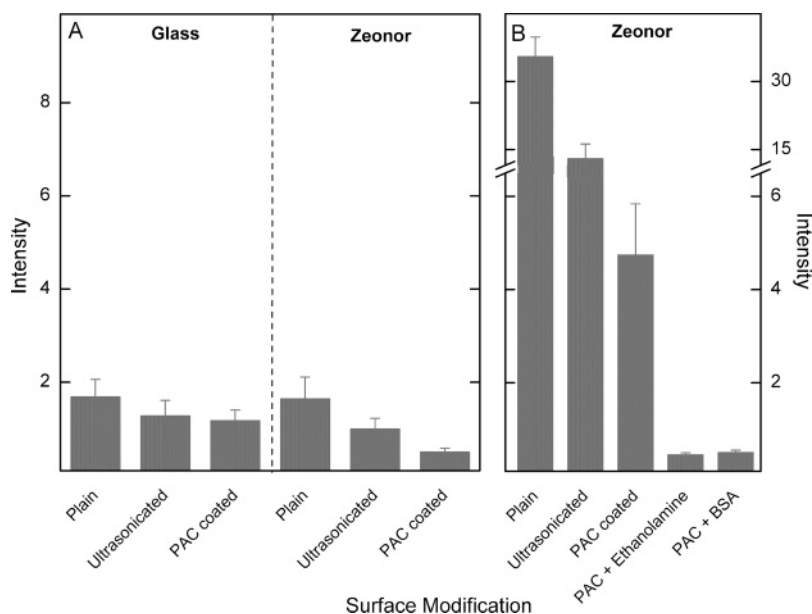


Figure 5. Nonspecific binding studies of oligonucleotides and antibodies onto different treated substrates. Comparison of surface-generated fluorescence intensity after immobilization of (A) 100 nM Cy5-ssDNA onto plain, ultrasonicated and PAC-coated glass and Zeonor slides, and (B) 100 nM Cy5-anti-rabbit onto plain, ultrasonicated, and blocked and unblocked PAC-coated Zeonor slides.

In order to convert a DNA chip into an protein array by DDI of proteins, the surface requires additional criteria other than sufficient receptor density and stability, for example, minimization of background signals of nonspecific binding and ensuring proper placement of the antibody onto the sensor chip. While the first can be achieved by the use of appropriate blocking agents, the second is given by sequence specificity of analyte oligonucleotides to unique surface-tethered DNA sequences.

The results of an adsorption study of DNA, as well as for anti-rabbit-IgG, are summarized in Figure 5B, wherein the bars give the average fluorescence intensities obtained from a 100 nM biomolecule concentration without any linker in the reaction

solution. As mentioned above, PAC coatings show a significantly decreased tendency to adsorb oligonucleotides nonspecifically compared to plain substrates (Figure 5A). Whereas the unspecific binding of DNA onto Zeonor can be easily prevented by cleaning the surface with 100% 2-propanol, a dramatic increase of adsorbed antibodies is detected. However, when the substrate is cleaned in an ultrasonic bath, as described in the Experimental Section above, the polymer surface shows a lower tendency for antibody adsorption.

While DNA repulsion from the negatively charged PAC surface is held responsible for the low unspecific DNA adsorption^{8,29}

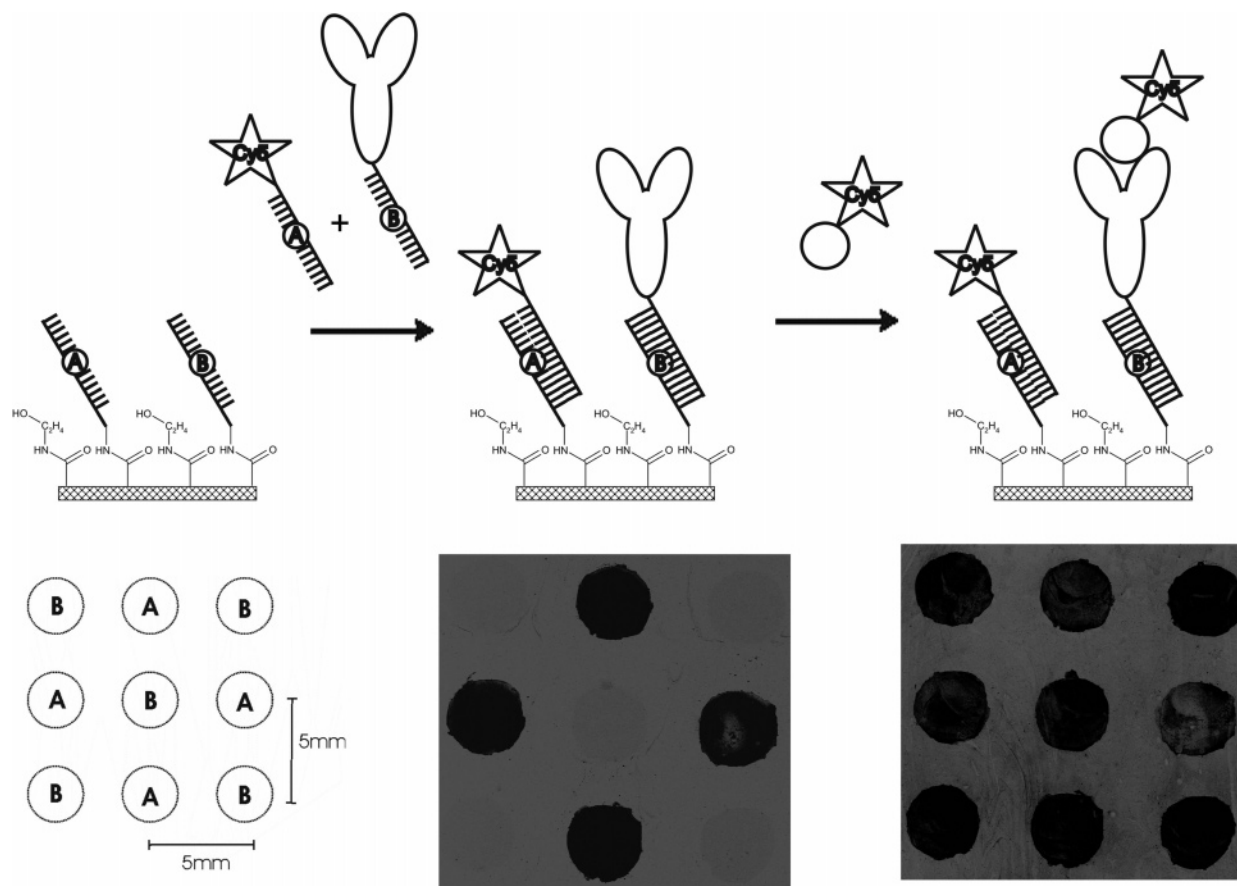


Figure 6. Sequence specificity control after immobilization of two different receptor sequences (A and B) onto PAC-coated Zeonor substrates. First, Cy5-labeled oligonucleotide complementary to sequence A and afterward rabbit-IgG conjugated oligonucleotide complementary to sequence B was hybridized. While first only fluorescence from the sequence A spots can be detected, incubation with Cy5-labeled anti-rabbit-IgG allows detection of the complete pattern at the chip.

(Figure 5A), proteins are mainly attracted to carboxylic groups (Figure 5B).

In general, BSA or agents with hydroxyl or poly(ethylene oxide) groups are used to prevent nonspecific protein adsorption in assays where an analyte at low concentration must be detected in the presence of a much larger amount of nonspecific adsorbing molecules.^{25,35,36} The usage of EDC to activate carboxylated supports for fixation of amino-modified oligonucleotides suggests blocking of the remaining activated functional groups with ethanolamine. Both agents, BSA and ethanolamine, reduce the unspecific binding onto the PAC slides sufficiently (Figure 5B), but due to the smaller molecule size, and the same binding chemistry of the receptor and the blocking agent associated with the same stability, ethanolamine is favored.

Sequence-specificity control experiments were performed based on the previous results: Zeonor 1060R substrate was activated by low-flow plasma and coated immediately with PAC. Amino-modified oligonucleotides with two different sequences, sequences A and B, were spotted onto the coated polymer in a pattern shown in Figure 6. Afterward, residual activated carboxylic groups were blocked by ethanolamine, and Cy5-labeled DNA

complementary to sequence A was hybridized. The fluorescence-labeled oligonucleotide will only bind to the receptors with the complementary sequence by hybridization. Immobilization of noncomplementary sequences did result only in negligible fluorescence signals from the surface.

Finally, the DNA–rabbit-IgG conjugate complementary to sequence B was hybridized onto the surface, and after incubation of a Cy5-labeled anti-rabbit-IgG solution, the corresponding spots at the surface could be detected. These control experiments clearly demonstrate that immobilization of the DNA constructs is controlled exclusively by sequence-specific hybridization. Ethanolamine prevents the protein segment from nonspecific binding, and hybridization specificity ensures the proper placing of the conjugate onto the surface.

CONCLUSION

In this report, we have presented the successful immobilization of both DNA and antibodies on cyclic polyolefin substrates. Detailed protocols for coating Zeonor substrates with polyelectrolytes are provided. Characterization and optimization of these coated substrates with respect to their receptor density, stability, protein resistance, and specific binding of biomolecules were performed. Therefore, the outcome of this study can be used for both DNA and protein arrays. As a result, it should be possible to use this surface patterned with specific oligonucleotides to detect

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a specific biomolecule from a cocktail of different DNA–biomolecule conjugates by sensors detecting surface-generated fluorescence.

ACKNOWLEDGMENT

The authors thank T. Ruckstuhl for his advice, A. Krieg and H. McEvoy for useful discussions, Åmic AB for making

Zeonor 1060R slides available, and the Microanalytical Laboratory at University College Dublin for microanalysis of Zeonor 1060R.

Received for review December 22, 2006. Accepted June 1, 2007.

AC062420Y