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Broderick, Adam H.; Lockett, Matthew R.; Buck, Maren E.; Yuan, Yuan; Smith, Lloyd M.; and Lynn, David M., "*In situ* Synthesis of Oligonucleotide Arrays on Surfaces Coated with Crosslinked Polymer Multilayers" (2012). Chemistry: Faculty Publications, Smith College, Northampton, MA.
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Chem Mater. Author manuscript; available in PMC 2013 March 13.

Published in final edited form as:
Chem Mater. 2012 March 13; 24(5): 939–945. doi:10.1021/cm202720q.

In situ Synthesis of Oligonucleotide Arrays on Surfaces Coated with Crosslinked Polymer Multilayers

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Abstract

We report an approach to the *in situ* synthesis of oligonucleotide arrays on surfaces coated with crosslinked polymer multilayers. Our approach makes use of methods for the 'reactive' layer-by-layer assembly of thin, amine-reactive multilayers using branched polyethyleneimine (PEI) and the azlactone-functionalized polymer poly(2-vinyl–4,4'-dimethylazlactone) (PVDMA). Post-fabrication treatment of film-coated glass substrates with D-glucamine or 4-amino-1-butanol yielded hydroxyl-functionalized films suitable for the Maskless Array Synthesis (MAS) of oligonucleotide arrays. Glucamine-functionalized films yielded arrays of oligonucleotides with fluorescence intensities and signal-to-noise ratios (after hybridization with fluorescently labeled complementary strands) comparable to those of arrays fabricated on conventional silanized glass substrates. These arrays could be exposed to multiple hybridization-dehybridization cycles with only moderate loss of hybridization density. The versatility of the layer-by-layer approach also permitted synthesis directly on thin sheets of film-coated poly(ethylene terephthalate) (PET) to yield flexible oligonucleotide arrays that could be readily manipulated (e.g., bent) and cut into smaller arrays. To our knowledge, this work presents the first use of polymer multilayers as a substrate for the multi-step synthesis of complex molecules. Our results demonstrate that these films are robust and able to withstand the ~450 individual chemical processing steps associated with MAS (as well as manipulations required to hybridize, image, and dehybridize the arrays) without large-scale cracking, peeling, or delamination of the thin films. The combination of layer-by-layer assembly and MAS provides a means of fabricating functional oligonucleotide arrays on a range of different materials and substrates. This approach may also prove useful for the fabrication of supports for the solid-phase synthesis and screening of other macromolecular or small-molecule agents.

Keywords

layer-by-layer; reactive assembly; polymer multilayers; thin films; oligonucleotide arrays; *in situ* synthesis; Maskless Array Synthesis

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Introduction

Array-based methods for solid-phase synthesis—in which large numbers of molecules are synthesized directly on a solid support in a spatially patterned and/or addressable manner—are broadly useful because the resulting arrayed compound libraries are well-suited for high-throughout screening in a parallel and multiplexed manner.[1–5] Approaches to solid-phase synthesis and screening are now well-established and are used extensively for the fabrication of DNA microarrays used in genome-wide studies to (i) identify and/or quantify RNA transcripts present under different experimental conditions,[6] (ii) identify DNA modifications such as methylated bases,[7] and (iii) monitor DNA-protein interactions.[8] The *in situ* synthesis of oligonucleotides on appropriately functionalized planar substrates can be used to fabricate high-density arrays composed of large libraries of oligonucleotides (e.g., with densities greater than 100,000 features/cm^2).[9–11] In comparison to methods for the immobilization or 'spotting' of arrays of pre-synthesized oligonucleotides on surfaces,[12,13] *in situ* synthesis has the general advantage that each individual oligonucleotide feature can be synthesized directly, in parallel, and in a base-by-base manner.[14] Phosphoramidite-based chemistries[15,16] are widely used for this purpose and permit the synthesis of immobilized sequences as long as ~150 bases in length in either a random or a directed manner (i.e., as n-mers of all possible base combinations or as *n*-mers of a specific base sequence).[17]

The types of chemical reactions and environments used during *in situ* synthesis often restrict the types of substrates that can be used for array fabrication. In many approaches to the synthesis of oligonucleotide arrays, for example, substrates are exposed repeatedly to organic solvents, ultraviolet light, oxidizing agents, etc. An additional design requirement specific to the development of substrates for use with phosphoramidite chemistry is the need for free hydroxyl groups on the surface of a substrate to enable coupling of the first nucleoside phosphoramidite. Finally, in addition to stability *during* synthesis, substrates should also, ideally, be able to withstand exposure to the range of solvents, reagents, and other physical/mechanical challenges associated with downstream use of the arrays (e.g., in subsequent biochemical/screening assays, etc.). A number of approaches have been developed to functionalize the surfaces of glass,[18] nanocrystalline diamond,[18,19] and amorphous carbon[20,21] substrates with terminal hydroxyl groups to permit *in situ* synthesis, improve array performance, and address other issues that can arise during subsequent studies.

The work reported here was motivated by our interests in the design of reactive, polymer-based interfaces[22–27] and expanding the range of substrates that are compatible with *in situ* oligonucleotide synthesis.[18,28–30] Polymer-based substrates could present attractive alternatives to glass and carbon-based substrates used in past studies because they are low cost, durable, and easily processed. Approaches based on polymer thin films could also be attractive because they can often be used to functionalize the surfaces of non-planar (i.e., curved) objects and porous/flexible substrates that could provide practical advantages during synthesis or subsequent screening. One drawback common to many conventional polymer-based materials, however, is that they can exhibit poor resistance to organic solvents commonly used during synthetic reactions (e.g., they either dissolve or swell upon prolonged exposure). Conversely, many of the more chemically- and mechanically stable polymer-based materials are, inherently, more resistant to facile chemical functionalization. Here, we report a step toward the design of polymer-based thin films as substrates for the *in situ* synthesis of oligonucleotide arrays. Our approach is based on methods developed for the 'layer-by-layer' fabrication of polymer thin films on surfaces.

Methods for the layer-by-layer assembly of polymer thin films[31] provide precise (and often nanometer-scale) control over the chemical composition, surface chemistry, and physical/

mechanical properties of thin films fabricated from a broad range of materials.[32–35] This approach does not involve high-temperature processing steps and is also well suited for the fabrication of thin, conformal films on the surfaces of objects with complex surface features. In comparison to polyelectrolyte-based multilayers stabilized by weak interactions (e.g., ionic or hydrogen-bonding interactions)[31,35–37] that can be disrupted under a variety of conditions, covalently cross-linked multilayers can exhibit increased stability in both organic and aqueous environments. Covalently crosslinked multilayers can be fabricated either by (i) post-fabrication crosslinking of pre-assembled multilayers (e.g., by treatment with chemical crosslinking agents),[38–42] or (ii) by direct layer-by-layer deposition of mutually reactive polymers.[43–47]

Our group recently reported a 'reactive' approach to the layer-by-layer fabrication of crosslinked multilayers that exploits reactions between amine-reactive, azlactone-functionalized polymers [e.g., poly(2-vinyl-4,4-dimethylazlactone) (PVDMA)] and primary amine-containing polymers [e.g., poly(ethylene imine) (PEI)] (Figure 1).In addition to providing a convenient route for the design of covalently-crosslinked thin (and ultrathin) films, this reactive approach (Figure 1C) yields films containing residual, amine-reactive azlactone groups that can be used to introduce additional chemical or biological functionality to film-coated surfaces (e.g., by simple post-fabrication treatment with a range of different amine-functionalized agents; see Figure 1D).[22,24,25,27,48]

In this paper, we demonstrate that these crosslinked and reactive thin films can be used as platforms for *in situ* solid-phase synthesis and array-based screening. We demonstrate that hydroxyl-functionalized multilayers fabricated from PEI and PVDMA (referred to hereafter as PEI/PVDMA films) are compatible with phosphoramidite chemistry and the range of chemical and photochemical conditions encountered during the *in situ*, base-by-base maskless array synthesis (MAS) of oligonucleotide microarrays (Figure 1E).[10,49] This approach can be used to fabricate stable and reusable oligonucleotide arrays on the surfaces of rigid planar substrates (e.g., on film-coated glass slides) and on the surfaces of flexible polymer-based substrates [e.g., on thin sheets of poly(ethylene terephthalate) (PET)]. We note here that the fabrication of DNA microarrays on surfaces coated with conventional, polyelectrolyte-based multilayers (PEMs) by the printing of pre-synthesized oligonucleotide sequences has been reported previously.[50] To the best of our knowledge, however, this current report is the first demonstration of the *in situ* and multi-step synthesis of arrays of complex molecules on multilayer-coated surfaces.

Materials and Methods

Materials

Branched poly(ethylene imine) (PEI; MW ~25,000), reagent grade solvents, and all chemicals used were purchased from Sigma Aldrich (Milwaukee, WI) and used without further purification, unless otherwise noted. 2-Vinyl-4,4-dimethylazlactone (VDMA) was a kind gift from Dr. Steven M. Heilmann (3M Corporation, Minneapolis, MN). Poly(2-vinyl-4,4-dimethylazlactone) (PVDMA; M_W = 20,400; PDI = 3.3) was synthesized as described previously.[25] Thin sheets of PET film (0.004 inches thick) were purchased from McMaster Carr. Compressed air used in all drying steps was filtered through a 0.2 μm membrane syringe filter to remove particulates.

Substrate Preparation

Glass and poly(ethylene terephthalate) (PET) substrates were cut to dimensions (25 mm × 35 mm) suitable for MAS oligonucleotide array synthesis. Prior to the fabrication of PEI/PVDMA multilayers, each substrate was prepared as follows: Glass slides were first

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silanized in a 1% (v/v) solution of (3-aminopropyl)triethoxysilane (APTES) in anhydrous toluene for 1 hour. The slides were then rinsed with ~10 ml of toluene and ~10 ml of ethanol and heat cured for 1 hour at 100 °C. PET films were rinsed with methanol, dried under a stream of compressed air, and placed in a solution of PEI (1 mg/ml in methanol) overnight at 37 °C prior to use. Hydroxyl-terminated glass slides, used as references for oligonucleotide array fabrication, were modified with *N*-(3-triethoxysilylpropyl)-4-hydroxy-butyramide, as described previously.[18]

Fabrication and Functionalization of PEI/PVDMA Multilayers

PEI/PVDMA multilayers were fabricated on glass and PET substrates using the following general procedure: (i) substrates were submerged in a solution of PVDMA (20 mM in acetone with respect to the polymer repeat unit) for 30 s; (ii) substrates were removed and immersed in an acetone bath for 15 s and then rinsed with ~10 ml of acetone; (iii) substrates were submerged in a solution of PEI (20 mM in acetone with respect to the polymer repeat unit) for 30 s; and (iv) substrates were removed and rinsed again using the procedure outlined under step (ii). This cycle was repeated four times to fabricate multilayers consisting of 4 PEI/PVDMA layer pairs (referred to hereafter as 'bilayers'). A final layer of PVDMA was then deposited (using steps (i) and (ii) of the above procedure) to provide additional reactive azlactone groups on the top surface of the film. Films having this general structure are referred to hereafter as being 4.5 bilayers thick. The multilayers were washed with ~25 ml of acetone and then dried under a stream of compressed air. These films were functionalized with D-glucamine or 4-amino-1-butanol by incubating film-coated substrates in solutions of either molecule (110 or 200 mM, respectively) in DMSO for 1 hour.[22,24] These functionalized films were then rinsed with DMSO, methanol, and RO water (~25 ml of each) and dried under a stream of compressed air.

Synthesis of Oligonucleotide Arrays

Each oligonucleotide array was synthesized directly on hydroxyl-terminated PEI/PVDMA film-coated substrates using a previously described ultraviolet light-directed photolithographic method known as Maskless Array Synthesis (MAS).[10] Briefly, individual oligonucleotide sequences were synthesized, in a base-by-base manner, directly on the multilayers using 3'-nitrophenylpropyloxycarbonyl (NPPOC)-protected nucleosides and a digital micromirror-based Biological Exposure and Synthesis System (BESS) connected to a Perseptive Biosystems Expedite Nucleic Acid Synthesis System. Table 1 summarizes the oligonucleotide sequences used in this work. Probes 1 and 2 (19 and 23 nt, respectively) were separated from the surface by a 10 thymidine (dT) spacer. This length of spacer has been shown in past studies to increase hybridization efficiency.[51]

Characterization and Analysis of Oligonucleotide Arrays

Fluorescently labeled complementary oligonucleotide sequences (Complements 1 and 2, Table 1) were synthesized by Integrated DNA Technologies (Coralville, IA) with a 3'-fluorescein (36-FAM) or a 3'-Cy3 (3Cy3Sp) moiety. Oligonucleotide arrays synthesized on PEI/PVDMA multilayers were hybridized by placing 40 μl of 1X SSPE buffer containing the complementary oligonucleotide(s) (2 μM total oligonucleotide concentration, 1X SSPE buffer contains 150 mM NaCl, 10 mM Na_2HPO_4, and 1 mM EDTA, pH = 7.4) on the surface, covering the surface with a glass cover slip to ensure the liquid was equally spread across the surface, and then incubating the array for 30 minutes at room temperature in a humid chamber. Nonspecifically bound complementary oligonucleotides were removed from the surface by placing 100 μl of 1X SSPE buffer on the surface and incubating the array for 5 minutes at 37 °C in a humid chamber, and then rinsing the surface with approximately 10 ml of 1X SSPE buffer. Fluorescence images of the hybridized arrays were acquired using a GeneTAC UC 4×4 scanner (Genomic Solutions). To keep the hybridized

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arrays wet while fluorescence images were acquired, 20 μl of 1X SSPE buffer was placed on the array and a cover slip was applied to provide a uniform layer of buffer covering the surface. Excess liquid was removed before imaging.

The hybridization density of each array (i.e., the density of oligonucleotides on the surface that are accessible for hybridization with complement, under the above hybridization conditions) was determined using a previously reported wash-off method.[52,53] Each array was hybridized with fluorescein-labeled complements (Table 1), the nonspecifically bound oligonucleotides were removed, and the array was then dehybridized in 2 ml of 8 M urea. The fluorescence intensity of the urea solution was compared to a calibration curve (10^{-11} to 10^{-8} M) of the fluorescein-labeled complement in 8 M urea, and the hybridization densities were calculated.

The stability of the oligonucleotide arrays synthesized on film-coated substrates was determined over five consecutive hybridization and dehybridization cycles. Each array was hybridized with fluorescently labeled complement and a fluorescence image was obtained, as described above. The arrays were dehybridized by placing 250 μL of 8 M urea onto the array, incubating the array for 30 minutes at room temperature in a humid chamber, and then rinsing with ~25 ml of RO water. Fluorescence images of the dehybridized arrays were acquired to verify that the complementary oligonucleotides were removed from the surface. This general procedure was repeated up to five times (see text). The values reported in this work are averages from n = 3 arrays, unless otherwise noted.

Results and Discussion

Fabrication and Functionalization of Reactive Multilayers

Glass substrates coated with reactive PEI/PVDMA multilayers were fabricated using a layer-by-layer procedure (Figure 1C) optimized previously for the fabrication of these materials on glass and silicon substrates.[22] For all experiments described below, we used glass silanized with APTES, a primary amine-containing silane reagent, to improve adhesion of the multilayers to the underlying substrate and prevent delamination during oligonucleotide synthesis. Films were fabricated by the sequential deposition of 4.5 layer pairs (or ‘bilayers’) of PEI and PVDMA (see Materials and Methods for additional details related to film fabrication). Otherwise identical films fabricated on reflective silicon substrates exhibited linear film growth profiles and were ~35 nm thick, as determined using ellipsometry. These results are consistent with those of our past studies.[22]

After deposition, PEI/PVDMA films were treated with solutions of D-glucamine or 4-amino-1-butanol (structures shown in Figure 1B) to immobilize terminal hydroxyl groups required for phosphoramidite-based oligonucleotide array synthesis (Figure 1). 4-Amino-1-butanol was selected because it is similar in structure to motifs used previously to functionalize substrates for MAS;[18,28] glucamine was selected on the basis of our past studies on the functionalization of PEI/PVDMA films.[24,27] In that work it was demonstrated that treatment of PEI/PVDMA films with solutions of glucamine for one hour is sufficient to react exhaustively with residual azlactone functionality (as determined by FTIR spectroscopy) and immobilize the hydroxyl functionality (or other groups).[24] These past studies also revealed that glucamine and other amine-based agents immobilized in this manner are accessible at the surfaces of the treated films (e.g., as evidenced by large changes in water contact angles of treated films, etc.).[22,24,26,27] We note, however, that this treatment procedure likely also results in the installation of hydroxyl functionality in the interior (i.e., the lower layers) of these films, and that these additional hydroxyl groups could also react with phosphoramidite monomers during oligonucleotide synthesis. We return below to a discussion of this possibility.

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Fabrication and Characterization of Oligonucleotide Arrays on Film-Coated Glass Substrates

Oligonucleotide arrays were *in situ* synthesized on hydroxyl-functionalized films in a base-by-base manner using 3'-nitrophenylpropyloxycarbonyl (NPPOC)-protected nucleosides and a Maskless Array Synthesizer (see Materials and Methods and reference 18 for additional details). Table 1 shows the sequences of the two different oligonucleotides (Probes 1 and 2) used in this study. Control arrays were also synthesized on hydroxyl-terminated (uncoated) glass substrates, the conventional substrate used for this application,[18] to provide a direct comparison to established methods and materials. We note here that each iterative cycle of MAS (during which a single new nucleoside is added) exposes the solid-phase substrate to multiple different chemical processing steps (including flowing and static exposure to different organic and drying media, exposure to activator or base pair solutions, photo-irradiation steps, and oxidation procedures).[18] The complete multi-step synthesis of the oligonucleotide arrays used in the studies described below thus requires the exposure of a surface to ~450 individual (albeit iterative) chemical processing steps. The fabricated arrays were then hybridized with fluorescently labeled complementary oligonucleotides and imaged using a fluorescence scanner to characterize both the fidelity of patterning and the ability of the immobilized sequences to pair with complementary oligonucleotide sequences.

We performed an initial series of MAS experiments to evaluate the suitability of glucamine- and 4-amino-butanol-treated PEI/PVDMA films as substrates for the *in situ* synthesis of arbitrary, but well-defined, patterns of oligonucleotides. Figure 2 shows images of a glucamine-treated film (Figure 2A) and a 4-amino-1-butanol-treated film (Figure 2B) presenting patterns of Probe 2 (hybridized with Cy3-modified Complement 2 prior to imaging). The large areas of uniform red fluorescence in these images (scales are in millimeters) provide visual evidence that the thin, crosslinked multilayer films do not peel or delaminate significantly under the conditions used during synthesis (or during subsequent hybridization and imaging). Visual inspection of these images also reveals that levels of non-specific, background adsorption of Complement 2 are significantly higher on 4-amino-1-butanol-functionalized films than for arrays fabricated on glucamine-functionalized films [that is, areas not patterned with Probe 2 during MAS appear dark on the glucamine-treated film (2A), but significant levels of red fluorescence are observed in these same locations on the 4-amino-1-butanol-treated film (2B)].

We next fabricated oligonucleotide arrays containing checkered patterns of square-shaped features of Probes 1 and 2 on each substrate (individual features are 144×144 μm in size). Figure 2C shows a representative image of an array [hybridized with fluorescein-modified Complement 1 (shown in green) and Cy3-modified Complement 2 (shown in red) prior to imaging] synthesized on a glucamine-treated film. The average fluorescence intensities were measured, and the average signal-to-noise values for both the fluorescein and Cy3 containing complements were determined using Eqn 1. The background signal is defined here as any area of the array that does not contain a feature of interest (i.e., the background for fluorescein-containing features includes all areas of the array that contain no oligonucleotides and all features of the array that were hybridized with Cy3 Complement 2).[28] Table 2 summarizes the average fluorescence intensities and signal-to-noise values for the checkered pattern oligonucleotide arrays synthesized on film-coated glass substrates and control silanized (uncoated) glass substrates. The values reported in Table 2 are the average values collected from three separate arrays. Arrays on substrates functionalized using 4-amino-1-butanol resulted in higher fluorescence background intensities for both fluorescein and Cy3 modified complements, indicating a higher amount of non-specific adsorption of oligonucleotides to the surfaces of these films as compared to arrays fabricated on glucamine-functionalized films.

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$$\frac{S}{N}=\frac{(\text{avg. feature signal, RFU})-(\text{avg. background signal, RFU})}{(\text{std. dev. background signal, RFU})} \quad \text{Eqn 1}$$

The hybridization density (i.e., the number of oligonucleotides on the surface of the films accessible for hybridization per cm^2) for each of the oligonucleotide arrays used above was also determined (Table 2). Each array was hybridized with fluorescein-modified complement, rinsed to remove non-specifically adsorbed oligonucleotides, and then dehybridized in a known volume of 8 M urea.[52,53] The urea solutions were collected and their fluorescence intensities were compared to a calibration of known concentrations of the fluorescein-modified oligonucleotide in 8 M urea. The hybridization densities measured for the silanized glass substrates ($1.45 \pm 0.39 \times 10^{12}$ oligonucleotides/cm^2) were comparable to those previously reported in the literature.[18,28] The hybridization densities for arrays synthesized on both glucamine and 4-amino-1-butanol functionalized films were comparable to those of arrays synthesized on silanized glass (although the signal-to-noise ratios were higher for 4-amino-1-butanol functionalized films, as described above).

We note again that the presence of hydroxyl functionality in the bulk of the films (i.e., in the layers below the surface) could also lead to the synthesis of 'buried' oligonucleotides (or short fragments of oligonucleotides) in the interiors of these films. While our current results do not establish the extent to which this occurs, it is unlikely that such buried sequences would be readily accessible to the complement strands used in the hybridization assays described above. We therefore interpret the hybridization densities of the arrays on film-coated substrates shown in Table 2 to reflect the density of immobilized oligonucleotide present and accessible at or near the topmost surfaces of these films.

Characterization of Oligonucleotide Array Stability

We conducted a series of experiments to characterize the ability of arrays synthesized on film-coated substrates to withstand the chemical and mechanical challenges associated with repeated hybridization/dehybridization cycles. Physically and chemically stable substrates enable the reuse of arrays,[18,28] and can both alleviate the cost of obtaining multiple high-density arrays and allow multiple different experiments to be performed using a single array (e.g., to eliminate concerns associated with array-to-array variation).[54] The stability of oligonucleotide arrays synthesized on glucamine-functionalized films was compared to the stability of arrays fabricated on silanized glass. Fluorescence intensity images of the arrays (and corresponding signal-to-noise ratios) were used to characterize the stability of oligonucleotide arrays exposed to a series of five hybridization/dehybridization cycles. Figure 3 shows the average fluorescence intensity of Cy3-containing features as a function of hybridization cycle. Fluorescence intensity images were also acquired after each dehybridization procedure to verify that all of the oligonucleotides were removed from the surface.

As shown in Figure 3, the fluorescence intensity of arrays synthesized on glucamine-treated films was relatively constant over the first three hybridization/dehybridization cycles. These arrays, however, lost 22% of their initial fluorescence intensities by the fifth hybridization (arrays on silanized glass substrates retained their initial fluorescence intensity). This loss of fluorescence intensity correlates with a decrease in the hybridization density of the surface (or the number of oligonucleotides accessible for hybridization) and could result from (i) a decrease in the number of oligonucleotides that remain attached to the surface or (ii) changes in interactions between the oligonucleotides and the film surface (e.g., during dehybridization steps) that restrict their accessibility during subsequent re-hybridization. With respect to this first possibility, we note that the treatment of our azlactone-containing

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films with glucamine results in the immobilization of glucamine through the formation of an amide/amide linkage[55] (Figure 1D) that should be hydrolytically stable under the conditions encountered during the hybridization/rehybridization conditions used here.

Decreases in oligonucleotide density over multiple dehybridization cycles could also result from the loss or leaching of small numbers of oligonucleotide-functionalized polymer chains that are physically entrapped (as opposed to covalently crosslinked) in the topmost layers of a film. We note, however, that images of these rehybridized films did not reveal changes in the individual features of these arrays (e.g., feature broadening or blurred edges) that would also be expected to result from the lateral diffusion of free polymer chains (data not shown). We also did not observe large-scale physical delamination of films during any of these experiments (e.g., by optical or fluorescence microscopy), and such delamination would be more likely to result in complete, rather than gradual, loss of signal as observed here. Finally, as outlined above, the decreases in fluorescence intensity shown in Figure 3 could also result from changes in the extent to which surface-bound oligonucleotides are physically accessible after multiple treatment cycles. For example, repeated chemical and physical manipulation of these films could result in the exposure of segments of PEI (present in underlying layers of the films) that could interact with and sequester negatively charged oligonucleotides through ionic interactions. Additional characterization will be necessary to understand the extent to which such changes could occur in these experiments. In the context of this current study, however, we conclude that arrays fabricated on these film-coated substrates are stable and robust, and that they can be reused for at least three hybridization/rehybridization cycles without significant deterioration of signal.

Fabrication of Oligonucleotide Arrays on Flexible Polymer Sheets

One potential practical advantage of the layer-by-layer approach to film-fabrication used here is that it can be used to deposit thin, amine-reactive films on a broad range of substrate materials, including objects with complex surface features and a range of physical/mechanical properties.[25,27] We sought to determine whether the approach used above to fabricate arrays on film-coated glass substrates could be used to synthesize functional arrays on the surfaces of soft/flexible substrates. Arrays of molecules synthesized on soft, flexible, and topographically complex or patterned substrates could offer practical advantages over traditional arrays fabricated on rigid substrates, including the potential for new assay formats. Arrays fabricated on soft substrates can also be cut or separated into smaller pieces using simple laboratory equipment (e.g., a razor blade or dissection scalpel) more easily than arrays fabricated on glass, silicon, or carbon substrates and could thus offer advantages from both manufacturing/processing and point-of-use perspectives.

To investigate the feasibility of this approach, we performed a series of experiments using thin sheets of flexible PET as a model transparent polymer substrate. PEI/PVDMA films were fabricated on PET sheets and then functionalized by treatment with glucamine using procedures identical to those described above for the preparation of film-coated glass substrates (see Materials and Methods for additional details related to preparation of PET substrates). Figure 4a shows images of four oligonucleotide arrays synthesized, in a checkered pattern of Probes 1 and 2, on a film-coated PET substrate. The hybridization densities for these arrays were similar to those measured for arrays synthesized on film-coated glass substrates (Table 2), but the average signal-to-noise ratio was approximately 2-fold lower. The stability of the PET arrays upon multiple hybridization/dehybridization cycles was comparable to that of their film-coated glass analogs (Figure 3b) with an average fluorescence intensity loss of $30 \pm 6\%$ after five hybridizations (as compared to the $22 \pm 3\%$ average fluorescence intensity loss for arrays on film-coated glass).

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The flexible nature of the underlying PET films did not have an apparent influence on the fluorescence intensities or the average signal-to-noise ratios of the hybridized oligonucleotide features. We performed two experiments to investigate the possible influence of substrate flexing either before or after hybridization. In the first experiment, a PET array was hybridized, a fluorescence image was obtained, and the curvature of the hybridized array was temporarily altered by manual bending of the substrate for one minute (each substrate was flexed such that the opposite ends of the surface were brought in contact, but a crease was not formed). No difference in the average fluorescence intensity or signal-to-noise ratio was observed as a result of bending. In a second set of experiments, we compared properties of PET arrays that were not bent prior to hybridization to those of arrays that were bent prior to use. The hybridization densities, average fluorescence intensities, and signal-to-noise ratios for arrays subjected to these treatments were statistically indistinguishable (data not shown). While we did not characterize the effects of flexing these array substrates surface during hybridization, several reports suggest that the curvature of an array surface can influence the density of hybridization.[56,57] The ability to deposit PEI/PVDMA multilayers on a variety of different soft and flexible substrates could thus provide new tools to investigate the effects of curvature and dynamic flexing on hybridization.

Figure 4b shows a section of the oligonucleotide array shown in Figure 4a that was cut and separated from the original array using a razor blade. For this experiment, the intact array was (i) hybridized and imaged (Figure 4a), (ii) dehybridized in 8 M urea, (iii) physically cut in half, and then (iv) rehybridized and imaged again (Figure 4b). The signal-to-noise ratio for this smaller section of the array decreased marginally after cutting the substrate into pieces (e.g., a signal-to-noise decrease from 59 to 51 for the Cy3-containing features). We attribute these small increases in background fluorescence, at least in part, to difficulties associated with keeping the hybridized arrays on these cut substrates wet during imaging. (Each fluorescence image was acquired by wetting the array with 1X SSPE buffer and then placing a microscope cover slip over the array to evenly disperse the water across the surface and keep the array wet, as used in the studies described above on glass substrates. Roughness at the edges of the cut arrays used in these experiments, however, made it more difficult to maintain uniform contact with the cover slips and resulted in higher background fluorescence intensity signals.) We did not observe significant peeling, delamination, or large-scale cracking of oligonucleotide arrays fabricated on film-coated PET during synthesis or as a result of any of the physical manipulations (e.g., cutting or bending) described above.

We used PET in this study as a model transparent/flexible polymer substrate to demonstrate proof-of-concept. In a broader context, however, the ability to fabricate PEI/PVDMA multilayers on a wide variety of other types of substrates (including inorganic and organic surfaces, ‘hard’ and ‘soft’ materials, and chemically/topographically patterned substrates)[22,25,27,58,59] could permit the solid phase synthesis of libraries of both large and small molecules in new formats and enable the development of new approaches to compound screening. In particular, we have demonstrated in past studies that functionalized PEI/PVDMA films can be removed from the substrates on which they were fabricated to produce free-standing films that can be transferred readily to the surfaces of other objects.[48] In combination with new approaches to on-film synthesis, this multilayer-based approach could thus be used to transfer arrays of molecules onto substrates, or to install them in locations, that are otherwise difficult (or impossible) to use with MAS or other methods of synthesis.

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Summary and Conclusions

We have reported an approach to the *in situ* synthesis of oligonucleotide arrays on surfaces coated with covalently crosslinked polymer multilayers. Our results demonstrate that amine-reactive PEI/PDVMA multilayers can be functionalized with hydroxyl-containing molecules suitable for the phosphoramidite-based Maskless Array Synthesis (MAS) of oligonucleotides. Film-coated glass substrates treated with D-glucamine yielded arrays of model oligonucleotide probes with average fluorescence intensities and signal-to-noise ratios (after hybridization with fluorescently labeled complement strands) that were comparable to those of arrays fabricated on conventional hydroxyl-terminated silanized glass substrates. The versatility of the layer-by-layer approach used to fabricate PEI/PVDMA multilayers also permitted the synthesis of oligonucleotide arrays directly on thin bulk sheets of polymer (e.g., PET). The ability to fabricate multilayers on the surfaces of soft and flexible substrates creates opportunities for post-fabrication processing (e.g., cutting) and manipulation (e.g., bending) steps that are more difficult using arrays fabricated on rigid substrates. Finally, our results demonstrate that these PEI/PVDMA films are able to withstand exposure to the range of chemical and physical processing conditions used during MAS and the subsequent hybridization, imaging, and dehybridization of the arrays (including repeated exposure to concentrated solutions of urea). To our knowledge, this work represents the first use of polymer multilayers as a solid-phase substrate for the *in situ* and multi-step synthesis of complex molecules. With further development, this approach to surface functionalization could also prove useful for the design of media for the solid-phase synthesis, characterization, and screening of other macromolecular or small-molecule agents.

Acknowledgments

Financial support to D.M.L. was provided by the NSF (DMR-0520527) through a grant to the Materials Research Science and Engineering Center (MRSEC) at the University of Wisconsin. Financial support for L.M.S., M.R.L., and Y.Y. was provided by the National Science Foundation under Grant No. CHE-0809095 (co-funded by the MPS/CHE and BIO/MCB Divisions). A.H.B. is a NSF Graduate Research Fellow. M.E.B. was funded in part by an NIH Chemistry-Biology Interface Training Grant (NIGMS T32 GM008505).

References

1. Geysen HM, Meloen RH, Barteling SJ. Proc. Natl. Acad. Sci. U. S. A. 1984; 81:3998–4002. [PubMed: 6204335]
2. Frank R. Tetrahedron. 1992; 48:9217–9232.
3. Lam KS, Renil M. Curr. Opin. Chem. Biol. 2002; 6:353–358. [PubMed: 12023117]
4. Blackwell HE. Curr. Opin. Chem. Biol. 2006; 10:203–212. [PubMed: 16682247]
5. Hook AL, Anderson DG, Langer R, Williams P, Davies MC, Alexander MR. Biomaterials. 2010; 31:187–198. [PubMed: 19815273]
6. Lockhart DJ, Winzeler EA. Nature. 2000; 405:827–836. [PubMed: 10866209]
7. Fouse SD, Nagarajan RP, Costello JF. Epigenomics. 2010; 2:105–117. [PubMed: 20657796]
8. Warren CL, Kratochvil NCS, Hauschild KE, Foister S, Brezinski ML, Dervan PB, Phillips GN, Ansari AZ. Proc. Natl. Acad. Sci. U. S. A. 2006; 103:867–872. [PubMed: 16418267]
9. Lipshutz RJ, Fodor SPA, Gingeras TR, Lockhart DJ. Nat. Genet. 1999; 21:20–24. [PubMed: 9915496]
10. Singh-Gasson S, Green RD, Yue YJ, Nelson C, Blattner F, Sussman MR, Cerrina F. Nat. Biotechnol. 1999; 17:974–978. [PubMed: 10504697]
11. Nuwaysir EF, Huang W, Albert TJ, Singh J, Nuwaysir K, Pitas A, Richmond T, Gorski T, Berg JP, Ballin J, McCormick M, Norton J, Pollock T, Sumwalt T, Butcher L, Porter D, Molla M, Hall C, Blattner F, Sussman MR, Wallace RL, Cerrina F, Green RD. Genome Res. 2002; 12:1749–1755. [PubMed: 12421762]

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12. Chrisey LA, Lee GU, Oferrall CE. Nucleic Acids Res. 1996; 24:3031–3039. [PubMed: 8760890]
13. Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N, Conroy J, Hamilton G, Hindle AK, Huey B, Kimura K, Law S, Myambo K, Palmer J, Ylstra B, Yue JP, Gray JW, Jain AN, Pinkel D, Albertson DG. Nat. Genet. 2001; 29:263–264. [PubMed: 11687795]
14. Heller MJ. Annu. Rev. Biomed. Eng. 2002; 4:129–153. [PubMed: 12117754]
15. Chambers RW, Moffatt JG, Khorana HG. J. Am. Chem. Soc. 1957; 79:3747–3752.
16. Caruthers MH, Barone AD, Beaucage SL, Dodds DR, Fisher EF, McBride LJ, Matteucci M, Stabinsky Z, Tang JY. Methods Enzymol. 1987; 154:287–313. [PubMed: 3431460]
17. LeProust EM, Peck BJ, Spirin K, McCuen HB, Moore B, Namsaraev E, Caruthers MH. Nucleic Acids Res. 2010; 38:2522–2540. [PubMed: 20308161]
18. Phillips MF, Lockett MR, Rodesch MJ, Shortreed MR, Cerrina F, Smith LM. Nucleic Acids Res. 2008; 36 Art. No E7.
19. Strother T, Knickerbocker T, Russell JN, Butler JE, Smith LM, Hamers RJ. Langmuir. 2002; 18:968–971.
20. Sun B, Colavita PE, Kim H, Lockett M, Marcus MS, Smith LM, Hamers RJ. Langmuir. 2006; 22:9598–9605. [PubMed: 17073485]
21. Lockett MR, Weibel SC, Phillips MF, Shortreed MR, Sun B, Corn RM, Hamers RJ, Cerrina F, Smith LM. J. Am. Chem. Soc. 2008; 130:8611–8613. [PubMed: 18597426]
22. Buck ME, Zhang J, Lynn DM. Adv. Mater. 2007; 19:3951–3955.
23. Fredin NJ, Broderick AH, Buck ME, Lynn DM. Biomacromolecules. 2009; 10:994–1003. [PubMed: 19290643]
24. Buck ME, Breitbach AS, Belgrade SK, Blackwell HE, Lynn DM. Biomacromolecules. 2009; 10:1564–1574. [PubMed: 19438231]
25. Buck ME, Lynn DM. ACS Appl. Mater. Interfaces. 2010; 2:1421–1429. [PubMed: 20402471]
26. Buck ME, Schwartz SC, Lynn DM. Chem. Mater. 2010; 22:6319–6327. [PubMed: 21151704]
27. Broderick AH, Azarin SM, Buck ME, Palecek SP, Lynn DM. Biomacromolecules. 2011; 12:1998–2007. [PubMed: 21504222]
28. Lockett MR, Smith LM. Anal. Chem. 2009; 81:6429–6437. [PubMed: 20020675]
29. Chen SY, Smith LM. Langmuir. 2009; 25:12275–12282. [PubMed: 19821627]
30. Negrete OD, Onses MS, Nealey PF, Cerrina F. J. Vac. Sci. Technol., B. 2009; 27:3082–3087.
31. Decher G. Science. 1997; 277:1232–1237.
32. Peyratout CS, Dahne L. Angew. Chem., Int. Ed. 2004; 43:3762–3783.
33. Ariga K, Hill JP, Ji QM. Phys. Chem. Chem. Phys. 2007; 9:2319–2340. [PubMed: 17492095]
34. Hammond PT. Adv. Mater. 2004; 16:1271–1293.
35. Bertrand P, Jonas A, Laschewsky A, Legras R. Macromol. Rapid Commun. 2000; 21:319–348.
36. Schonhoff M. Curr. Opin. Colloid Interface Sci. 2003; 8:86–95.
37. Quinn JF, Johnston APR, Such GK, Zelikin AN, Caruso F. Chem. Soc. Rev. 2007; 36:707–718. [PubMed: 17471396]
38. Yang SY, Rubner MF. J. Am. Chem. Soc. 2002; 124:2100–2101. [PubMed: 11878948]
39. Richert L, Boulmedais F, Lavalle P, Mutterer J, Ferreux E, Decher G, Schaaf P, Voegel JC, Picart C. Biomacromolecules. 2004; 5:284–294. [PubMed: 15002986]
40. Zelikin AN, Quinn JF, Caruso F. Biomacromolecules. 2006; 7:27–30. [PubMed: 16398494]
41. Schneider A, Vodouhe C, Richert L, Francius G, Le Guen E, Schaaf P, Voegel JC, Frisch B, Picart C. Biomacromolecules. 2007; 8:139–145. [PubMed: 17206799]
42. Boudou T, Crouzier T, Auzely-Velty R, Glinel K, Picart C. Langmuir. 2009; 25:13809–13819. [PubMed: 20560550]
43. Bergbreiter DE, Liao KS. Soft Matter. 2009; 5:23–28.
44. Serizawa T, Nanameki K, Yamamoto K, Akashi M. Macromolecules. 2002; 35:2184–2189.
45. Such GK, Quinn JF, Quinn A, Tjipto E, Caruso F. J. Am. Chem. Soc. 2006; 128:9318–9319. [PubMed: 16848452]

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46. Seo J, Schattling P, Lang T, Jochum F, Nilles K, Theato P, Char K. Langmuir. 2010; 26:1830–1836. [PubMed: 19761256]
47. Zhang YJ, Yang SG, Guan Y, Cao WX, Xu J. Macromolecules. 2003; 36:4238–4240.
48. Buck ME, Lynn DM. Langmuir. 2010; 26:16134–16140. [PubMed: 20857952]
49. Beier M, Hoheisel JD. Nucleosides Nucleotides. 1999; 18:1301–1304.
50. Zhou XC, Wu LY, Zhou JZ. Langmuir. 2004; 20:8877–8885. [PubMed: 15379521]
51. Guo Z, Guilfoyle RA, Thiel AJ, Wang RF, Smith LM. Nucleic Acids Res. 1994; 22:5456–5465. [PubMed: 7816638]
52. Peelen D, Smith LM. Langmuir. 2005; 21:266–271. [PubMed: 15620313]
53. Lockett MR, Phillips MF, Jarecki JL, Peelen D, Smith LM. Langmuir. 2008; 24:69–75. [PubMed: 18047381]
54. Hu ZY, Troester M, Perou CM. Biotechniques. 2005; 38:121–124. [PubMed: 15679094]
55. Heilmann SM, Rasmussen JK, Krepski LR. J. Polym. Sci., Part A: Polym. Chem. 2001; 39:3655–3677.
56. Cederquist KB, Keating CD. ACS Nano. 2009; 3:256–260. [PubMed: 19236058]
57. Kira A, Kim H, Yasuda K. Langmuir. 2009; 25:1285–1288. [PubMed: 19132834]
58. Saurer EM, Flessner RM, Buck ME, Lynn DM. J. Mater. Chem. 2011; 21:1736–1745. [PubMed: 21383867]
59. Buck ME, Lynn DM. Adv. Mater. 2010; 22:994–998. [PubMed: 20217827]

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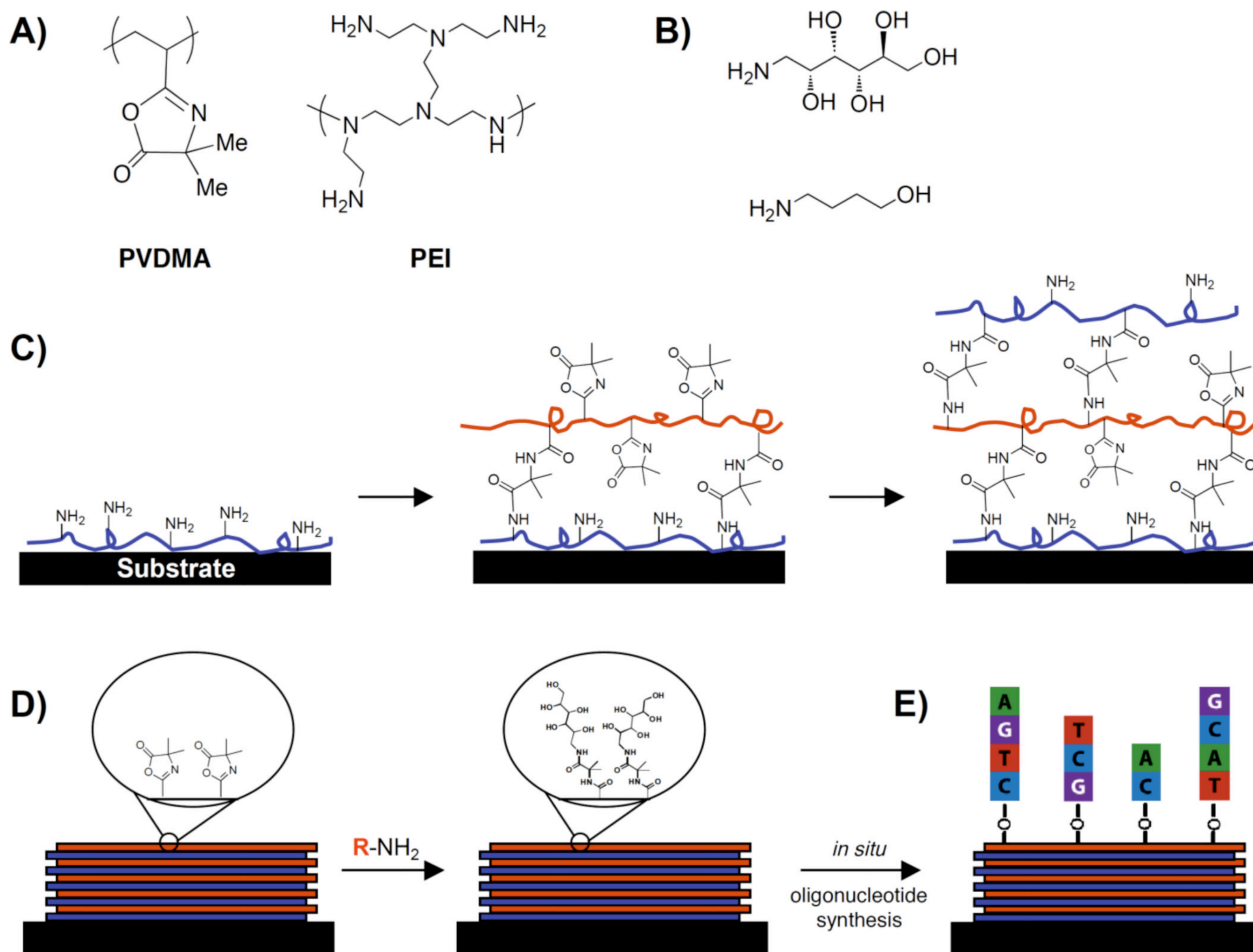

Figure 1.

Schematic illustration of the structures, processes, and procedures used to fabricate reactive, crosslinked polymer multilayers on planar supports as substrates for *in situ* oligonucleotide array synthesis. (A) Chemical structures of poly(2-vinyl-4,4-dimethylazlactone) (PVDMA) and poly(ethylene imine) (PEI). (B) Chemical structures of D-glucamine and 4-amino-1-butanol. (C) Schematic of the reactive layer-by-layer film fabrication process. PEI and PVDMA are deposited sequentially to provide azlactone-functionalized multilayers (D) that can be readily functionalized post-fabrication by treatment with primary amine-functionalized nucleophiles (a glucamine-treated film is shown as an example). Films functionalized to display hydroxyl functionality (D) provide surfaces suitable for the *in situ*, base-by-base Maskless Array Synthesis (MAS) (E) of arrays of oligonucleotides.

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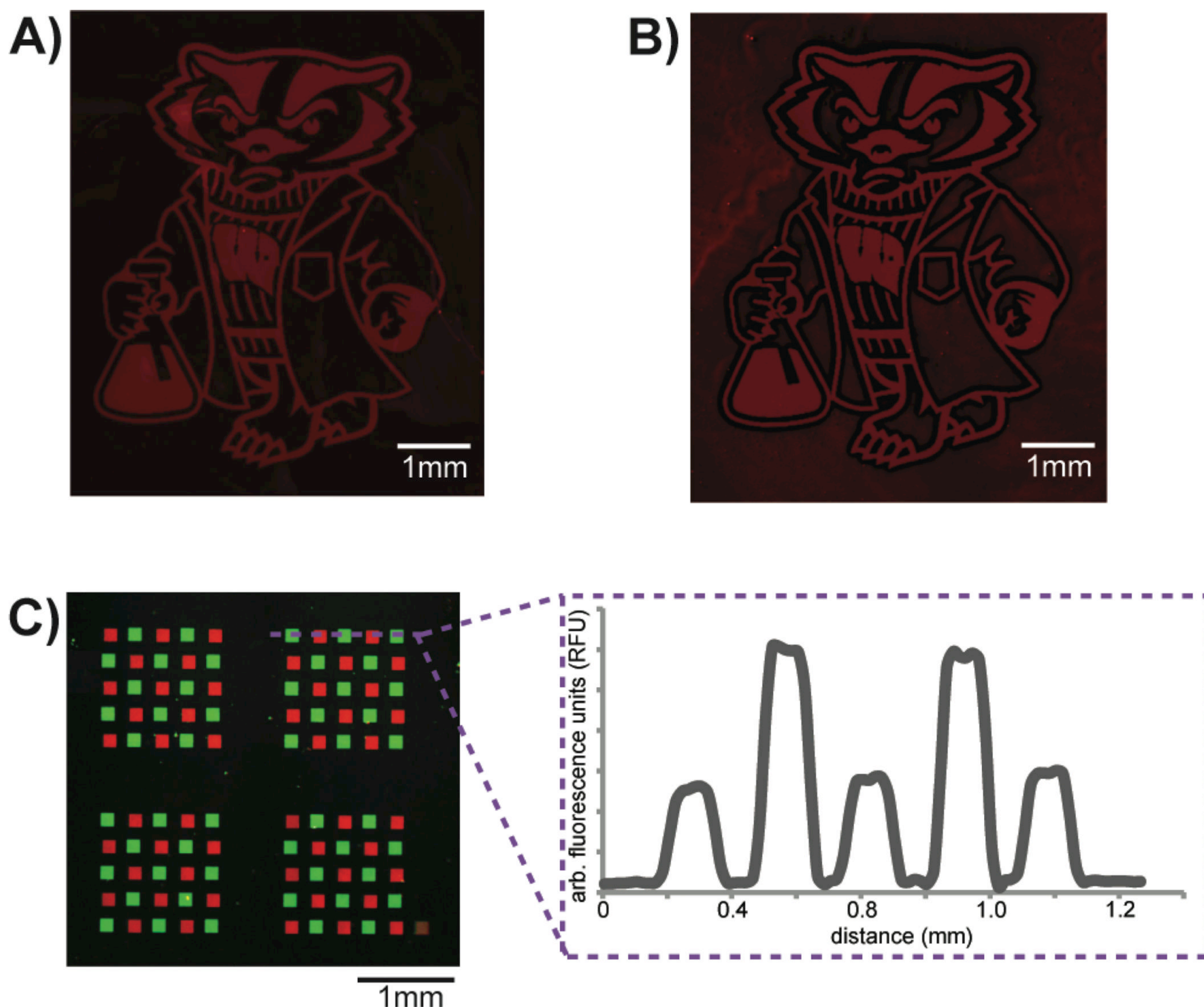

Figure 2.

(A, B) Images of two larger-scale arrays of oligonucleotides, fabricated in the shape of Bucky Badger, composed of the Probe 2 oligonucleotide sequence and hybridized with Cy3-labeled Complement 2 (red) prior to imaging (note: images best viewed in color). The image in (A) is of an array fabricated on a glucamine-functionalized PEI/PVDMA film; the image in (B) is of an array fabricated on a 4-amino-1-butanol-functionalized PEI/PVDMA film. (C) Representative image of oligonucleotide arrays of Probes 1 and 2 arranged in a checkered pattern (individual array features are 144 × 144 μm) synthesized on a glucamine-functionalized PEI/PVDMA film fabricated on the surface of a glass substrate. The arrays were hybridized with fluorescein-labeled Complement 1 (green) and Cy3-labeled Complement 2 (red) prior to imaging. A line intensity profile for one row of features of the array is also shown. The intensity values of each feature are the summation of the fluorescein (green) and Cy3 (red) fluorescence intensities.

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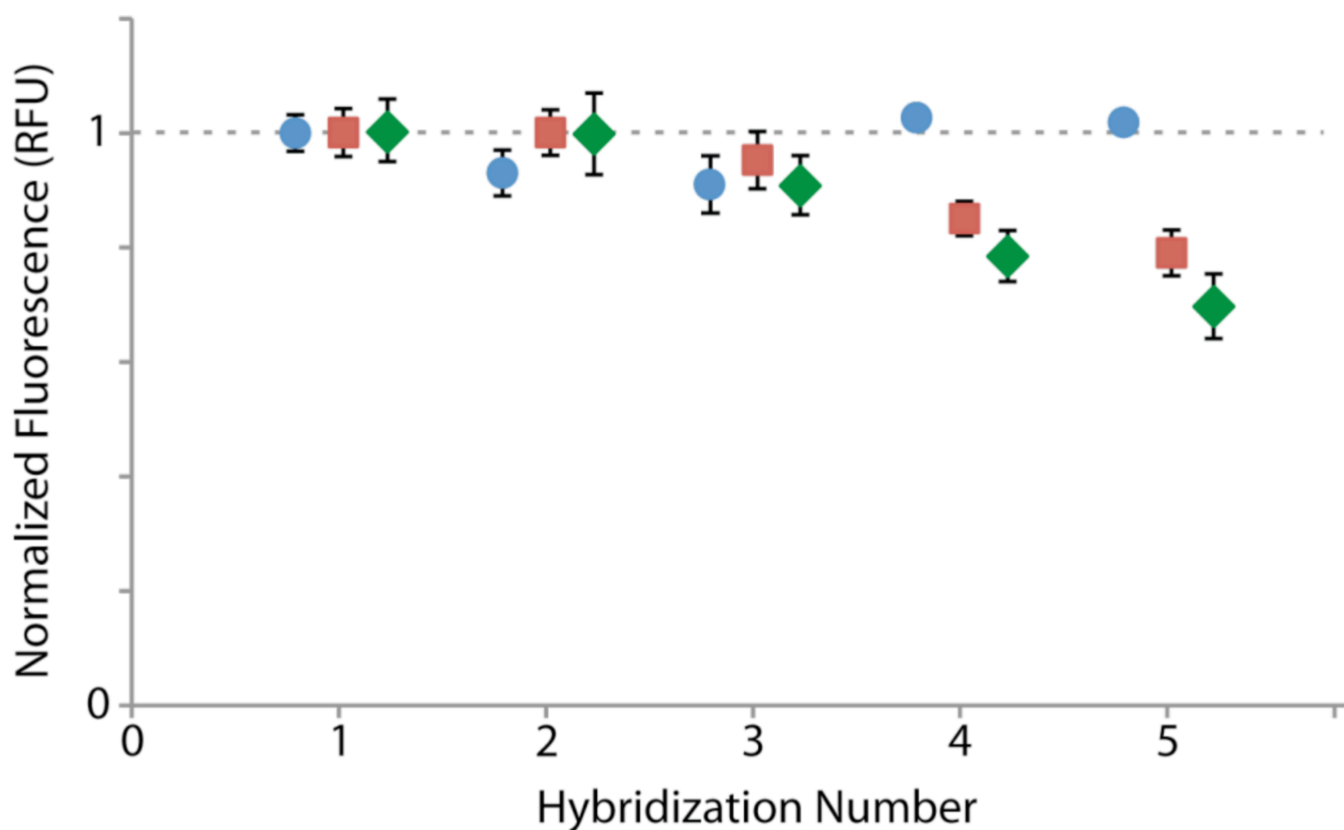

Figure 3.
The stability of each oligonucleotide array was determined by monitoring the fluorescence intensity of the hybridized features as a function of hybridization cycle. Each array was hybridized with fluorescently labeled complement (Cy3 fluorescence data shown here), a fluorescence image was obtained, and then dehybridization was effected using an 8 M urea solution. The fluorescence values presented are normalized to the initial fluorescence intensity upon the first hybridization. The fluorescence intensity as a function of hybridization cycle for an array synthesized on (●) a silanized glass substrate, (■) a glucamine-treated PEI/PVDMA film fabricated on a glass substrate, and (♦) a glucamine-treated PEI/PVDMA film fabricated on a thin sheet of PET.

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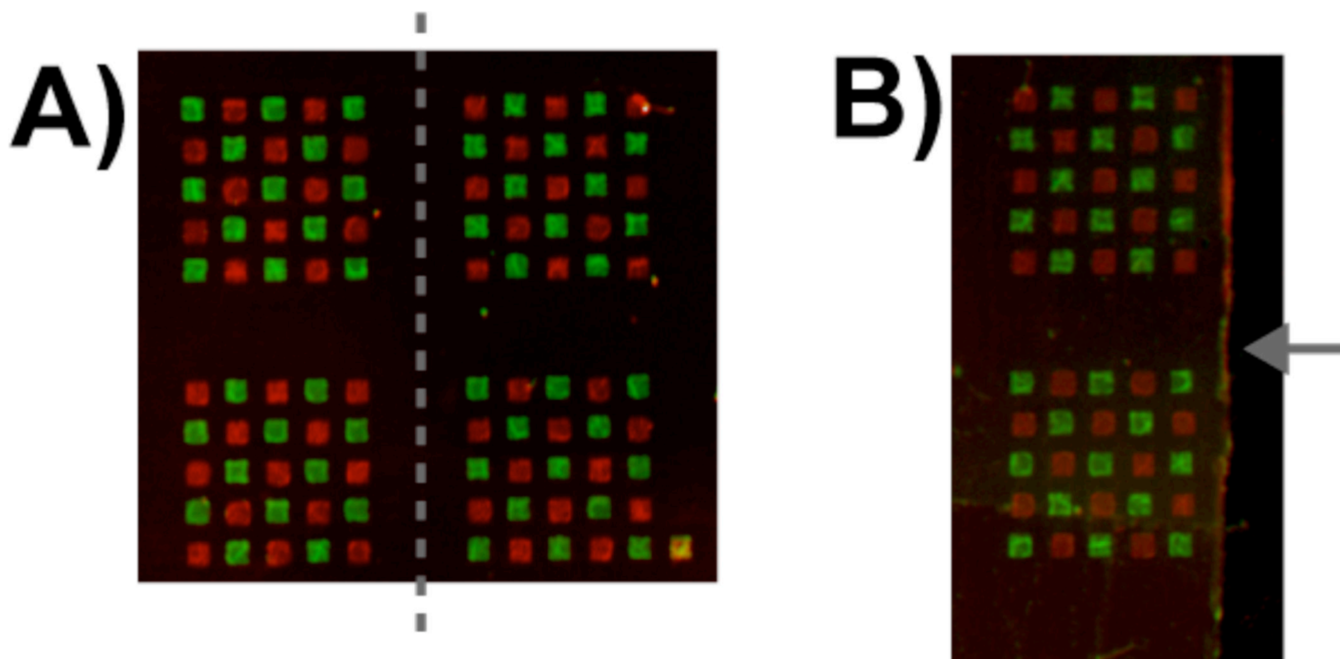

Figure 4.
(A) Representative image of an oligonucleotide array synthesized on a PET sheet coated with a glucamine-functionalized PEI/PVDMA film (hybridized with fluorescein-labeled Complement 1 (green) and Cy3-labeled Complement 2 (red) prior to imaging). The dashed line indicates the region where the array was subsequently cut in half using a razor blade to produce the smaller substrate fragment shown in (B). (B) This array fragment cut from the substrate shown in (A) was dehybridized in 8 M urea and then rehybridized prior to fluorescence imaging (the gray arrow indicates the edge where the larger array was cut).

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Table 1

Structures of oligonucleotide sequences used in this study.[a]

	Sequence (5'→3')
Probe 1	CCACTGTTGCAAAGTTATT $(T)_{10}$--|
Probe 2	CGCTTCTGTATCTATATTCATCA $(T)_{10}$--|
Complement 1	AATAACTTTGCAACAGTGG - (F)
	AATAACTTTGCAACAGTGG - (3)
Complement 2	TGATGAATATAGATACAGAAGCG - (F)
	TGATGAATATAGATACAGAAGCG - (3)

[a] Probe oligonucleotides were *in situ* synthesized directly onto hydroxyl-functionalized substrates (in a 3' → 5' direction) using NPPOC-protected phosphoramidite bases and Maskless Array Synthetic methods. Each probe oligonucleotide was separated from the surface by 10 thymidine (T) residues. Complementary oligonucleotides were synthesized using standard phosphoramidite chemistry. A fluorescein (F) or Cy3 (3) dye moiety was attached to the 3' end of the complement oligonucleotides.

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Table 2

Characterization of oligonucleotide arrays fabricated on glass substrates.[a]

	***fluorescence intensity* (RFU)**		***signal-to-noise***		***hybe density***
	FAM	**Cy3**	**FAM**	**Cy3**	**($\times 10^{12}$oligos/cm^2)**
Silanized Glass	38688.6 ± 432.4	35523.4 ± 1468.3	134	159	1.45 ± 0.39
Film-Coated Glass					
Aminobutanol-treated	20371.6 ± 1971.5	23071.6 ± 2761.4	94	81	1.24 ± 0.72
Glucamine-treated	23382.5 ± 1196.5	28164.9 ± 2073.7	118	106	1.18 ± 0.54
Film-Coated PET					
Glucamine-treated	15330.9 ± 1818.4	28405.6 ± 2918.5	68	59	1.09 ± 0.42

[a]Oligonucleotide arrays were synthesized on glass substrates (silanized glass or glass coated with PEI/PVDMA films treated with 4-amino-1-butanol or D-glucamine). Oligonucleotide arrays, composed of a checkered pattern of Probe 1 and Probe 2, were synthesized on each substrate. The average fluorescence signal and average signal-to-noise values were determined after hybridization with fluorescently labeled Complement 1 and 2. The values reported are for n = 3 arrays. The average fluorescence intensity signal was determined by integrating the fluorescence intensity of each feature on the array. The average background fluorescence intensity, which was used to calculate the signal-to-noise ratio, was determined by integrating the fluorescence intensity of areas on the array that did not contain the oligonucleotide features. The average signal-to-noise ratio was calculated using Eqn 1. The average hybridization densities were obtained by collecting the hybridized complements in a known volume of 8 M urea and comparing the fluorescence intensity to a calibration curve. Uncertainties shown correspond to the calculated standard error of the mean.