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Carbon Substrates: A Stable Foundation for Biomolecular Arrays

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Abstract

Since their advent in the early 1990s, microarray technologies have developed into a powerful and ubiquitous platform for biomolecular analysis. Microarrays consist of three major elements: the substrate upon which they are constructed, the chemistry employed to attach biomolecules, and the biomolecules themselves. Although glass substrates and silane-based attachment chemistries are used for the vast majority of current microarray platforms, these materials suffer from severe limitations in stability, due to hydrolysis of both the substrate material itself and of the silyl ether linkages employed for attachment. These limitations in stability compromise assay performance and render impossible many potential microarray applications. We describe here a suite of alternative carbon-based substrates and associated attachment chemistries for microarray fabrication. The substrates themselves, as well as the carbon-carbon bond-based attachment chemistries, offer greatly increased chemical stability, enabling a myriad of novel applications.

Keywords

amorphous carbon; diamond; microarray; monolayer; surface chemistry

1. INTRODUCTION

Biomolecular arrays—which for the purpose of this review, we define as planar substrates to which molecules of interest are immobilized in geographically distinct regions—allow for a few to millions of different molecules to be screened in parallel. The first high-density peptide arrays were developed in the early 1990s (1), subsequently extended to oligonucleotide arrays (2–4), and the concept has since expanded to include small molecules (5), carbohydrates (6, 7), proteins such as lectins or antibodies (7–9), and even cell lysates (10). The utility and performance of an array is critically dependent on both the substrate and the attachment chemistry linking the molecule of interest to the substrate.

DISCLOSURE STATEMENT

The authors note that they are inventors on patents and patent applications related to some of the material presented in this article, and there is thus potential for financial benefit derived from licensing royalties.

Although most arrays rely on glass substrates and some variation of a silane-based attachment chemistry, the stability of these chemistries can be problematic for applications involving high temperatures, extended incubation times, or exposure to organic solvents or extremes in pH (11–13). Since the first demonstration of oligonucleotides immobilized on a diamond substrate (14), carbon-based materials such as amorphous carbon, glassy carbon, or nanocrystalline diamond have been explored as alternatives to glass substrates, and many attachment chemistries have been developed. The primary advantage of these carbon-based substrates is their intrinsic stability, and the ability to attach biomolecules of interest to the substrate through highly stable carbon-carbon bonds. We review here this body of work, with a primary focus on oligonucleotide arrays, as they are the best-developed application area to date.

The Southern blot was one of the first examples of a DNA array, allowing the identification and quantification of biological DNAs that were enzymatically fragmented and size-separated in a gel, transferred to a nitrocellulose membrane, and then probed with radio-labeled complementary DNAs (15). Oligonucleotide arrays evolved from this gel-based method, and synthetic oligonucleotides attached to a glass substrate replaced the biological DNAs immobilized on nitrocellulose. The combination of several technologies has since driven the evolution of oligonucleotide arrays to their current state: arrays the size of a microscope slide containing hundreds of thousands (if not millions) of individual spots or features, each containing a different sequence of DNA. These technologies include (a) the ability to readily synthesize oligonucleotides with phosphoramidite-based chemistries; (b) the advent of instruments capable of fabricating arrays by either spotting minute volumes of a solution containing presynthesized oligonucleotides or synthesizing the oligonucleotides in a base-by-base manner directly on the surface; (c) analytical techniques such as confocal fluorescence imaging, which are capable of capturing high-resolution images of the arrays; (d) software, which can extract data from these images with sophisticated bioinformatics-based approaches; and last but not least, (e) the ever-increasing availability of high fidelity genome sequences to provide the information needed to design useful arrays for genomic analysis.

2. THE ANATOMY OF AN ARRAY

2.1. Overview

Arrays consist of three components: (a) the molecules of interest that will be arrayed; (b) the substrate on which the array will be fabricated; and (c) the surface chemistry used to modify the substrate, and attach the molecules of interest. Individual molecules are immobilized on the substrate by spotting techniques or by in situ combinatorial synthesis. Spotting techniques require the preparation of individual solutions of each molecule of interest, which in and of itself can represent a substantial challenge for a complex array with many features; however, this approach has the advantage that each of the molecules can be purified and characterized individually. These solutions are then spotted on the surface either manually or, more commonly, with robotic workstations.

Appropriate chemistries and instrumentation for solid-phase synthesis are needed for in situ array fabrication (1–3, 16–18). The fabrication of DNA arrays, for example, is based on

phosphoramidite chemistries and uses either acid- or photolabile protecting groups at the 5'-hydroxyl group of the deoxynucleoside phosphoramidites. Peptide arrays use standard solid-phase chemistries and amino acids with *t*-BOC, Fmoc, or photolabile protecting groups. It is not possible to purify these in situ synthesized molecules individually, and thus their fidelity is likely to be poorer than for spotted arrays. This approach has the tremendous advantage, however, of enabling the synthesis of hundreds of thousands of different molecules in parallel.

2.2. Surface Chemistries

There are many chemistries available to modify the surfaces of glass, metal, metal oxide, and carbon substrates; these chemistries can affect the wettability, charge, and reactivity of these surfaces. Introducing molecules that assemble on the surface to form ordered monolayers—the silanization of glass substrates or the formation of self-assembled monolayers (SAMs) of alkanethiols on noble metal substrates such as gold—is a convenient method to modify the surface of a substrate. The ease of forming SAMs on gold has afforded a means of prototyping chemistries for attaching biomolecules, preventing the nonspecific adsorption of proteins, studying the mechanisms of protein-surface interactions, and performing reactions directly on the modified surfaces(19).

Surface chemistry dictates how the molecules of interest are attached to the substrate; these interactions can be through nonspecific adsorption, electrostatic interactions, or the formation of covalent bonds. The covalent attachment of molecules to the substrate is desirable for applications that require prolonged incubation times, multiple washing steps, or exposure to stringent chemical environments, because it reduces the likelihood that the molecules of interest are removed unintentionally. The loss of noncovalently attached molecules from the surface is dependent on the strength of the interaction (or cumulative strength of many interactions) between the adsorbed molecule and surface, and can result in nonuniformity across an array and/or misinterpretation of the array data.

2.3. Covalent Attachment Strategies

Biomolecules can be covalently attached to the surface in a directed or randomized orientation. Proteins can be attached to a surface in a randomized fashion through formation of a covalent bond with a particular amino acid. A common strategy is to form an amide bond between *N*-hydroxysuccinimide (NHS) groups on a substrate and the lysines on the surface of a protein. Proteins can be oriented on a surface in a directed fashion with engineered affinity tags that are appended to the N or C terminus of an expressed protein. These affinity tags react with surface-bound molecules to form covalent bonds or strong noncovalent interactions. There are several strategies available for directed protein immobilization, such as the incorporation of a polyhistidine tail, a glutathione S-transferase domain, or a modified dehalogenase domain. These strategies have been reviewed in detail elsewhere (9, 20, 21).

Oligonucleotides are more amenable to directed orientation than proteins because of the ease in which suitable reactive groups (e.g., a primary amine, thiol, alkyne) can be incorporated in the sequence with modified phosphoramidites, which are widely available (22, 23). It is

common to incorporate these reactive groups at the 5' - or 3' - terminus of the oligonucleotide. As mentioned above, modified phosphoramidites have also been adapted for in situ synthesis of oligonucleotides directly onto a hydroxyl-terminated substrate (2).

2.4. Feature Size and Density

The number of features—geographic regions containing a single type of biomolecule—on an array determines the amount of information that can be obtained in a single experiment. In spotted arrays, the feature density is determined by both the volume of solutions of prepurified biomolecules spotted on the surface and the wettability of the surface. Robotically driven quill- or pin-based spotters deposit volumes of approximately 1 nL and typically yield arrays containing fewer than 1,000 features/cm²; piezoelectrically driven pipettes can deliver volumes as small as 3 pL and produce ~20-μm-diameter spots (24). High-density, in situ synthesized arrays (i.e., microarrays) can contain greater than 100,000 features/cm², and depending on the method of synthesis can contain oligonucleotides with lengths of up to 150 bases (3, 4, 25).

Two of the commercial methods for synthesizing microarrays combine photolithography and phosphoramidite chemistry. A single phosphoramidite with a photocleavable protecting group is coupled to a hydroxyl-terminated surface to yield an array that is compatible with photolithographic patterning (1–3). Illumination of the surface with UV light removes the protecting group from the phosphoramidites on the surface. A photoprotected nucleoside phosphoramidite is introduced to the surface and reacts with the newly exposed hydroxyl groups to form a phosphite bond, which is oxidized to form a more permanent and less labile phosphodiester bond. This illuminate-couple-and-oxidize process is repeated until the array is synthesized. Maskless array synthesis (3) uses a digital micromirror device to project virtual photomasks on the surface, and can prepare up to 786,000 features (14 μm × 14 μm each) in a single synthesis.

An alternative approach uses an inkjet-based technology to print nanoliter volumes of phosphoramidite solutions on the surface (4, 25). Here the nucleoside phosphoramidites include an acid-labile protecting group. High reaction efficiencies, presumably resulting from the small volumes of highly concentrated phosphoramidite, result in 99.5% coupling efficiency in these inkjet-printed arrays; in contrast to conventional phosphoramidite coupling efficiencies of approximately 98% (25). This improved reaction efficiency has afforded arrays with oligonucleotides up to 150 bases in length. Polymerase chain reaction (PCR) amplification, cloning, and sequencing of these long oligonucleotides showed that 47% (181 of the 384 analyzed) were the correct sequence (25).

2.5. Array Imaging

There are several analytical modalities capable of detecting and in some instances quantifying the reactions on the surface of an array. We focus here on two techniques that have been used extensively to evaluate arrays prepared on carbon substrates.

Confocal fluorescence imaging is widely employed to analyze microarrays because it has a limit of detection that is unsurpassed by most optical techniques. Fluorophores are introduced to oligonucleotide arrays through the hybridization of labeled complementary

molecules of DNA (cDNA) or RNA (cRNA); these labels can be fluorescent dyes or other moieties that are labeled with fluorescent molecules post-hybridization. In one workflow that utilizes post-hybridization labeling, a sample of biological RNAs are reverse-transcribed into cDNA molecules and then retranscribed into cRNAs with biotin-labeled nucleosides. After hybridization, the array is exposed to a solution of fluorescently labeled streptavidin, which binds to the biotin-labeled cRNAs. The fluorescence intensity of the hybridized features can be further amplified with a primary anti-streptavidin antibody and a fluorescently labeled secondary antibody (26). There are several studies that compare the effectiveness of different microarrays to quantify copy number variation (27,28); however, there is no study to our knowledge that directly evaluates the effectiveness of the individual workflows used to introduce labels to DNA or RNA samples prior to hybridization.

An alternative fluorescence-based readout incorporates a pair of fluorescent dyes (or a dye and a quencher) directly on the immobilized oligonucleotide. Increasing the physical distance between a fluorescent dye and a quencher causes an increase in the fluorescence intensity of the dye; the quenching mechanism is a result of fluorescence resonance energy transfer (FRET) (29). FRET-based readouts have been used to screen hybridization of biological samples to molecular beacons immobilized on the surface (i.e., an aptamer array) (30–33) and in assays that rely on the enzymatic cleavage of immobilized oligonucleotides (34, 35).

Surface plasmon resonance imaging (SPRi) eliminates the need to label molecules before exposing them to an array. Although fluorescence-based readouts are sensitive and fluorophores are easily incorporated into oligonucleotides, the selective labeling (and purification) of small molecules or proteins with fluorophores is not as straightforward. Reactive dye derivatives can be coupled to amines (e.g., NHS-activated dyes) or thiols (e.g., maleimide-activated dyes) on the surface of proteins. These reactions result in a heterogeneous population of proteins that contain differing numbers of dye molecules, which can be attached to the protein's surface in a variety of permutations. Many dye molecules are similar in molecular weight to small molecules, and labeling these small molecules can greatly alter their binding affinity.

SPR (surface plasmon resonance) and SPRi have proven powerful techniques not only to quantify the association of molecules in solution with molecules immobilized on a surface, but also to monitor these interactions in real time (36, 37). These techniques are less developed than fluorescence-based imaging technologies. Corn and colleagues (37, 38) have quantified the binding of DNA, RNA, and proteins to arrays of spotted oligonucleotides with SPRi. The sensitivity of SPRi is sufficient for routine monitoring of the hybridization of ~10-nM oligonucleotide solutions, and volumes as small as 10 μ L have been used in conjunction with microfluidic devices (corresponding to 1 fmol of DNA) (37). The sensitivity of this imaging technique has been further increased with post-hybridization reactions on the surface, such as rolling circle amplification of the captured oligonucleotides (increase in signal) (39), the selective incorporation of nanoparticles (an increase in signal) (40, 41), or the enzymatic removal of the captured or immobilized oligonucleotides from the surface (a decrease in signal) (42, 43).

3. CARBON SUBSTRATES: SURFACE CHEMISTRY

An early motivation for exploring carbon materials as an alternative substrate for fabricating biomolecule arrays resulted from an interest in bioelectronic devices, which incorporate the specificity of biomolecular interactions with the intrinsic properties of semiconductors. The susceptibility of native and modified silicon surfaces to oxidation upon prolonged exposure to atmospheric or aqueous environments (44–46) limits the utility of these materials. Carbon, unlike silicon and germanium, does not rapidly oxidize when exposed to atmospheric or aqueous conditions and thus offers a more stable substrate with tunable semiconductor properties (46, 47).

An early study comparing the stability of spotted arrays of presynthesized oligonucleotides attached to amine-terminated diamond, glass, glassy carbon, gold, and silicon substrates (Figure 1) showed that arrays prepared on diamond and glassy carbon retained greater than 95% of the surface-bound oligonucleotides after repeated use where the glass, gold, and silicon surfaces showed significant loss (14). The inability of the glass and gold substrates to withstand continued use is not surprising. The siloxane bonds formed when glass substrates are modified with silanes readily hydrolyze in the presence of weak acids or bases or when incubated in aqueous solutions for prolonged periods (11–14). The gold-sulfur bonds formed when alkanethiols self-assemble on a gold surface are also labile when stored under atmospheric or aqueous conditions for prolonged periods (48) or when exposed to mild oxidizers or UV light (14, 48–51).

The increased stability of modified carbon substrates increases the types of reactions, applications, and analyses that can be performed with biomolecule arrays. These early results demonstrated that functionalized carbon materials were a viable option for bioelectronic sensors and for the fabrication of biomolecular arrays and spurred research aimed at functionalizing carbon substrates, fabricating arrays on carbon substrates, and analyzing reactions occurring on their surfaces.

3.1. Carbon Substrates

Biomolecules have been attached to planar carbon substrates and carbon nanomaterials (52, 53). We focus here on three planar substrates: (a) glassy carbon plates, (b) nanocrystalline diamond films, and (c) amorphous carbon films. Each of these substrates can be prepared in the laboratory or are commercially available and have been used in oligonucleotide array fabrication.

Glassy carbon is a pyrolyzed polymeric material predominantly composed of sp^2 -hybridized carbon chains, which can be thought of as an entanglement of graphitic ribbons (54). Nanocrystalline diamond films are composed of a network of sp^3 -hybridized carbons and can be prepared on heated substrates through plasma-enhanced chemical vapor deposition of a mixture of methane and hydrogen gas (55); the crystallinity and smoothness of these diamond films depend on the synthesis parameters. Amorphous carbon films are a mixture of sp^2 - and sp^3 -hybridized carbons (56); the composition of this mixture is dependent on the deposition technique (e.g., ion beam deposition, chemical vapor deposition, plasma deposition, or magnetron sputtering) and the reaction conditions at the time of the

deposition. Magnetron sputtering of a graphite target is a room temperature process that produces thin films of amorphous carbon with a root mean square roughness of less than 0.1 nm (57). These sputtered films have been interfaced with electrode, microelectrode, and micromechanical devices (58), as well as metal films that are compatible with surface plasmon resonance spectroscopy (59, 60).

There are several protocols in the literature for preparing (and in the case of glassy carbon, polishing) the surface of these carbon substrates prior to functionalization. We focus here on hydrogen plasma treatment. Exposing the carbon surfaces to an inductively coupled hydrogen plasma (*a*) results in a substrate in which carbon dangling bonds at the surface are bonded to hydrogen atoms (61, 62) and (*b*) ensures substrates prepared at different times have a uniform composition because oxygen species on the surface are removed or reduced (59); this uniformity is important when performing mechanistic studies or comparing different surfaces chemistries. Finally, it (*c*) changes the electronic properties of the material (e.g., its work function or electron affinity) and determines its chemical reactivity (61–63).

3.2. Chemical Modification of Carbon Substrates

Several chemistries have been developed to modify the surface of hydrogen-terminated carbon substrates (62, 64–66). We highlight here three chemistries that result in the formation of a carbon-carbon bond between the surface and (*a*) an alkyl magnesium halide (67), (*b*) an aryldiazonium salt(68), or (*c*) an alkene-containing molecule (62).

Magnesium halide salts of alkanes, fluoroalkanes, and oligo(ethylene glycol)s were used to modify amorphous carbon substrates in a two-step process (Figure 2a) in which the surface was chlorinated in a solution containing PCl_5 , and then incubated in a solution containing an alkyl magnesium halide (67). These molecules change the wettability of the surface and can reduce the amount of nonspecific adsorption of protein to the surface (69). It is difficult to introduce functional groups for attaching biomolecules with this chemistry because these organometallic reagents are highly reactive. A similar two-step approach formed monolayers of alkanethiols on chlorine-terminated amorphous carbon or glassy carbon substrates (70, 71). Although the alkanethiols do not result in a carbon-carbon bond with the surface, they do provide a means of modifying both the wettability and chemical reactivity of the surface. The stability and durability of these thioether bonds formed with the surface have not been determined.

Aryldiazonium salts were used to modify planar carbon substrates (53, 72, 73) and carbon nanostructures (53). The structure of the diazonium cation determines if the attachment, which proceeds via an oxidation-reduction reaction that releases molecular nitrogen, is spontaneous or electrochemically driven. Oligonucleotides, proteins, and antibodies have been covalently attached to carbon surfaces modified with 4-nitrobenzenediazonium cations; once attached to the surface, the nitro group was electrochemically reduced to an amine (53, 74). Figure 2b outlines the process used to prepare an amine-terminated surface from a 4-nitrobenzenediazonium salt.

Carbon substrates are also readily modified with molecules containing terminal alkene groups (61–63, 75–77). In these reactions, a small volume of a neat alkene is placed on a

hydrogen-terminated surface, covered with a quartz cover slip, and illuminated with UV light. Detailed studies of this reaction on diamond and amorphous carbon substrates have shown that illuminating the surface with 254-nm light results in the ejection of electrons from the valence band of the material into the neighboring alkene liquid (61, 63, 77). The alkene group is believed to donate electron density to the newly formed holes in the carbon substrate, resulting in the formation of a carbon-carbon bond. Although ejection of an electron from the surface is needed to initiate the reaction, the efficiency of the reaction is dictated in part by the ability of the functional group opposite of the alkene to accept the ejected electrons into its lowest unoccupied molecular orbital (LUMO) (77). Figure 2c provides a detailed schematic of the proposed mechanism.

We focus the remainder of this review on the attachment of biomolecules to carbon surfaces modified with alkene-containing molecules because this chemistry (*a*) allows the surface to be photopatterned with more than one molecule. In one example, photomasks were used to pattern micron-sized features with different terminal functional groups (78). In another example, the surface was illuminated in the presence of two alkene-containing molecules with different terminal groups to yield a surface with different ratios of the two terminal groups (69). This chemistry also (*b*) relies on alkene-containing molecules, many of which are available commercially. Carbon substrates have been modified in this manner with molecules containing terminal alcohols, aldehydes, amides, carboxylic acids, ethers, esters, and halogens (11, 60, 62, 77, 79–81). Many of these functional groups are either directly compatible with biomolecule attachment or are compatible after an on-surface reaction. Finally, we focus on this chemistry because it (*c*) has been used to fabricate arrays of oligonucleotides, proteins, and small molecules on carbon substrates.

3.3. Characterization of Modified Carbon Substrates

The number of molecules composing a monolayer of an alkene molecule on a diamond substrate ($\sim 1.9 \times 10^{14}$ molecules/cm²) (61) is similar to the number of alkanethiol molecules in a SAM on a gold substrate ($\sim 4.5 \times 10^{14}$ molecules/cm²) (19, 82). There is not a single technique that can provide a complete picture of the bonding, overall structure, and dynamics of the low density of molecules attached to the surface. Combinations of optical, mass spectrometric, and electro-chemical measurements as well as indirect methods to probe the energy of the surface are needed to characterize these monolayers. The techniques used to characterize organic monolayers on semiconductor surfaces have been recently reviewed in detail elsewhere (46, 83–85).

4. CARBON SUBSTRATES: ARRAY FABRICATION AND ANALYSIS

4.1. Surface Chemistry

Amine-terminated amorphous carbon, diamond, and glassy carbon substrates have been used to immobilize both oligonucleotides and proteins (12, 14, 34, 62, 86–89). The carbon substrates were modified with trifluoroacetamide-protected 10-aminodec-1-ene (TFAAD, Scheme 1), which yields an amine-terminated surface after the acid- or base-catalyzed removal of the trifluoroacetate moiety. Oligonucleotides with terminal thiol groups were attached to these surfaces with a heterobifunctional linker containing an amine-reactive NHS

and a thiol-reactive maleimide (14, 87, 90). This immobilization chemistry has been well characterized spectroscopically and requires the oligonucleotides be in their reduced (thiol) form. Although this heterobifunctional linker is compatible with cysteine groups on the surface of a protein, a more common strategy is to form covalent bonds with surface-exposed lysines. Antibodies have been attached to amine-terminated diamond surfaces with a homobifunctional linker, glutaraldehyde (12, 88, 89).

Aldehyde- and carboxylic acid-terminated substrates further expand the available chemistries for immobilizing molecules on carbon substrates. An aldehyde-terminated surface reacts with amines reversibly through the formation of a Schiff base, or irreversibly with the aid of a mild reducing agent. The glutaraldehyde-terminated surface discussed above affords an amine-reactive surface, but requires several preparatory steps before biomolecules can be immobilized: the attachment of TFAAD molecules, removal of the TFA groups, reaction of the amine-terminated surface with glutaraldehyde and its subsequent reduction to a secondary amine.

An alternative approach, the attachment of 2-(10-undecen-1-yl)-1,3-dioxolane (Scheme 1), can yield an aldehyde-terminated surface after a single chemical deprotection (80). This two step-approach is required because the reaction conditions employed are not compatible with the direct attachment of aldehyde-containing molecules to the surface; the carbonyl group of photochemically excited aldehydes undergo a [2 + 2] cyclo-addition with alkenes (91). Illumination of amorphous carbon in the presence of 10-undecenal caused the neat alkene to polymerize on the surface, rendering it unusable (80). Amine-modified oligonucleotides were attached to amorphous carbon substrates modified with 2-(10-undecen-1-yl)-1,3-dioxolane (80).

Carboxylic acids also react with amines in the presence of an activating agent such as a carbodiimide. Converting a carboxylic acid to an acyl chloride increases its reactivity toward nucleophilic nitrogen, oxygen, or sulfur atoms, and eliminates the need for an activating agent. Carboxylic acids do not undergo noticeable side reactions when illuminated with UV light; thus, 10-undecenoic acid (Scheme 1) was attached directly to the surface without need of a protecting group (79,81). Acyl chlorides were formed by exposing the carboxylic-terminated surface to gaseous thionyl chloride in a dry desiccator; this gas-phase reaction was near complete as verified by elimination of the carbonyl stretch of the carboxylic acid ($1,712\text{ cm}^{-1}$) and the appearance of a new stretch at $1,801\text{ cm}^{-1}$, which was attributed to the carbonyl stretch of the acyl chloride. The newly formed acyl chloride groups were reacted with [4-(trifluoromethyl)phenyl]methanamine, [4-(trifluoromethyl)phenyl]methanol, or [4-(trifluoromethyl)phenyl]methanethiol to form the corresponding amide, ester, and thioester (79). This chemistry is summarized in Figure 3a. The formation of these species on the surface was confirmed by infrared spectroscopy (Figure 3b); the infrared assignments were verified with solution phase analogs whose structures were confirmed by NMR. Thiol- and amine-modified oligonucleotides were also immobilized on these surfaces (79).

Hydroxyl-terminated carbon substrates are compatible with phosphoramidite chemistry, and oligonucleotide arrays have been synthesized directly on amorphous carbon, diamond, and glassy carbon substrates by maskless array synthesis (11, 59, 60, 78, 92–94). Each of these

surfaces was first modified with 9-decene-1-ol. The carbon substrates withstand the chemical treatments common to in situ array synthesis: repeated exposure of the surface to organic solvents containing mild oxidizers, bases, and phosphoramidites. The average oligonucleotide density on these hydroxyl-terminated carbon surfaces is $3.6 (\pm 0.1) \times 10^{12}$ molecules/cm² (11, 59), slightly higher than the $2.1 (\pm 0.4) \times 10^{12}$ molecules/cm² collected from silanized glass substrates synthesized on the same instrument and with the same protocols.

Phosphoramidite chemistry is very versatile, and is easily modified to incorporate functional groups of interest. In one example, amorphous carbon substrates were modified with photo-protected thiol groups using 3-(2'-nitrobenzyl)thiopropyl phosphoramidite (92). Thiol-modified oligonucleotides were attached to this surface through the formation of a disulfide bond and then later cleaved with a reducing agent (dithiothreitol). Proteins were also attached to these thiol-terminated surfaces. In one example, the photopatterned surface was modified with a solution of *N*-biotinoyl-N₀-(6-maleimidohexanoyl)hydrazide or carbodiimide-activated biotin and then exposed to fluorescently labeled streptavidin (92).

4.2. Controlling Surface Density

The density of oligonucleotides immobilized on a surface not only affects the kinetics of hybridization when the surface is exposed to a solution containing the complementary sequence, but also affects the stability of the duplexes that are formed (95, 96). This surface density also affects the affinity of DNA-protein interactions (96) and can inhibit protein binding when the density is too high (97).

One means of reducing the density of biomolecules on the surface is to reduce the number of available reactive groups on the surface by forming a mixed monolayer. In a mixed monolayer, two (or more) molecules with similar structures but different terminal groups are combined in a defined ratio and then attached to the surface. To reduce the amount of nonspecifically adsorbed protein on an amine-terminated surface, varying ratios of TFAAD and an oligo(ethylene glycol) alkene were attached to a diamond surface, and then characterized with X-ray photoelectron (XP) spectroscopy (12, 69, 88). Increasing numbers of oligo(ethylene glycol)s on the surface decreased the amount of nonspecifically adsorbed protein—avidin, bovine serum albumin, casein, or fibrinogen—on the surface when compared to a TFAAD-only surface (69). The fractional composition of TFAAD in the neat alkene liquid did not, however, match the fractional composition of TFAAD on the surface. This disparity in the fractional composition of molecules in solution and on the surface is also common in mixed monolayers of alkanethiols formed on gold (19) and highlights that these relationships must be determined empirically.

The density of functional groups on the surface can also be controlled chemically without the need for mixed monolayers. In one example, a uniform layer of photoprotected phosphoramidites was attached to the surface of a hydroxyl-terminated amorphous carbon substrate. Varying fractions of the protecting groups were removed photolithographically by exposing different regions of the surface to UV light for different periods of time. The radiant energy dosage of the UV light (or the exposure time, measured in J/cm²) dictated the number of protecting groups removed. Coupling a phosphoramidite containing a protecting

group that was not photocleavable “capped” those reactive sites and reduced the total number of photoactive groups remaining in that region(78).

4.3. The Stability of Oligonucleotide Arrays on Carbon Substrates

The compatibility of a biomolecule array with a particular application is dictated by its stability: the ability to retain immobilized biomolecules after exposure to various reaction conditions or physical manipulations. In oligonucleotide arrays, for example, the stability can be determined by comparing the experimentally measured surface oligonucleotide density before and after exposure to a particular reaction condition.

Fluorescence images of the array are not adequate to quantify surface density because interactions with neighboring molecules and/or the surface itself can lead to quenching of the fluorescent dyes (34). Accordingly, wash-off experiments are employed for quantitation (98). In these procedures, which are summarized in Figure 1, fluorescently labeled oligonucleotides in solution are hybridized to complementary oligonucleotides on the surface, which is washed to remove nonspecifically adsorbed oligonucleotides and imaged. The array is then incubated in 8M urea, which disrupts the DNA duplexes and releases the hybridized strands into solution. This solution is collected and its fluorescence intensity is measured and compared to a series of fluorescence standards of known DNA concentration. The fluorescence image provides a measure of the area and uniformity of the DNA-containing features on the surface, and the wash-off experiment provides a measure of the total number of oligonucleotides hybridized to the array. These two measurements provide a straightforward means of determining the density of hybridizable sites on the surface.

A series of experiments comparing the stability of in situ synthesized oligonucleotide microarrays prepared by maskless array synthesis on glass and carbon substrates revealed that the carbon substrates lost less than 10% of the original number of immobilized oligonucleotides on the surface after 15 cycles of hybridization; glass substrates lost an average of 70% of the immobilized oligonucleotides (11, 59). Glassy carbon substrates were able to retain >95% of the oligonucleotides (*a*) after a 24-h incubation in a solution of phosphate-buffered saline at 60°C (11, 59), (*b*) after a 12-h incubation in a solution of 15% ammonium hydroxide (11), and (*c*) when cycled through the conditions needed for an on-chip PCR reaction (11, 59). In each of these experiments, the glass substrates retained less than 30% of their original fluorescence intensity.

The ability to reuse microarrays for multiple experiments not only decreases the costs associated with making or purchasing arrays [recently estimated at \$450 per array (99)], but also allows for direct comparison of biological replicates or other samples on the same array, eliminating potential artifacts due to array variability. Table 1 summarizes the hybridization density and stability of arrays prepared on carbon substrates; this information was obtained from papers in which both spotted and in situ synthesized oligonucleotide arrays were prepared and analyzed.

5. CARBON SUBSTRATES: APPLICATIONS

5.1. Scoring Single-Nucleotide Polymorphisms on Oligonucleotide Arrays on Diamond Substrates

In one example of a fluorescence-based readout, oligonucleotides containing a FRET pair were attached to a diamond substrate and used to detect the presence of single-nucleotide polymorphisms in a sample containing 100 attomoles of unamplified human genomic DNA (35). These polymorphisms were detected with the invasive cleavage reaction (34, 100): an enzymatic assay in which a structure-specific 5'-exonuclease cleaves a unique secondary structure formed between two adjacent oligonucleotides hybridized to a target sequence, upstream and downstream of the single nucleotide in question. The nucleotide at the 3'-end of the upstream oligonucleotide is designed to overlap at least one base in the duplex formed by the downstream oligonucleotide and the target strand. The unpaired region on the 5'-end of the downstream oligonucleotide can then be removed by the exonuclease. Complementarity between the downstream oligonucleotide and the target sequence at the position of overlap is required for efficient enzymatic cleavage, which is the basis for discrimination of single base changes. The downstream oligonucleotide was designed to include the cleavage site between the two dyes in the FRET pair and was immobilized on the surface. A comparison of the fluorescence intensity of the oligonucleotide FRET pairs before and after the reaction allowed the allele to be scored. Enzymatic cleavage of the downstream oligonucleotide releases the quencher moiety, and thus the fluorescence of that particular feature increased. Oligonucleotide arrays were prepared on amine-terminated gold, silicon, and diamond surfaces, and the scoring reaction was performed during a 10-h incubation at 58.5°C (35). Only the diamond substrate was able to retain the oligonucleotides on the surface and successfully score the sample of DNA. Both the silicon and gold substrates lost a significant number of the oligonucleotides (>25%) immobilized on the surface, compromising the ability to evaluate the assay results (35).

5.2. Label-Free Detection of DNA-DNA Interactions on Amorphous Carbon Substrates

In addition to monitoring hybridization with fluorescently labeled oligonucleotides, there are several examples of label-free detection of DNA-DNA interactions on carbon substrates. These include the gravimetric quantification of DNA binding to an oligonucleotide-modified amorphous carbon thin film that was deposited on a quartz-crystal microbalance (87), the detection and quantification of DNA binding to an oligonucleotide-modified amorphous carbon thin film deposited on a gold or silver thin film substrate with SPRi (discussed in detail in Section 5.4) (59, 60, 93), and the detection of DNA binding to an oligonucleotide-modified diamond substrate with electrical impedance spectroscopy (89, 101).

5.3. Arrays of Antibodies on Diamond Substrates to Detect the Presence of Bacteria

Although the majority of arrays prepared on carbon substrates have involved oligonucleotides, there are two recent examples of antibody-modified diamond substrates (12, 88). These surfaces, which were composed of mixed monolayers of TFAAD and 1-dodecene (12) or an oligo(ethylene glycol) alkene (88), were used to capture serotypes of *Escherichia coli* from a solution containing multiple species of bacteria. The antibodies—including anti-*E. coli* O157:H7, anti-*E. coli* K12, and anti-goat secondary antibodies—were

immobilized on aldehyde-terminated surfaces prepared by reacting deprotected TFAAD molecules with glutaraldehyde.

Anti-*E. coli*-O157:H7-modified diamond and glass substrates captured fluorescently labeled *E. coli*-O157:H7 from solution with similar efficiencies: 270 bacteria/mm² for the diamond substrates and 250 bacteria/mm² for the glass substrates (88). XP spectra of *E. coli* K12-modified substrates (recorded on the day of preparation and after a 14-day incubation in a buffered solution at 37°C) showed that the diamond surfaces were able to retain the antibodies, whereas greater than 50% of the antibodies had been lost from the glass substrate (88).

The capture efficiency of the immobilized anti-*E. coli* K12 antibodies on the diamond and glass substrates was also evaluated over a 28-day period. Each surface was exposed to a solution containing a mixture of fluorescently labeled *E. coli* and *L. monocytogenes* each day for a 28-day period, the number of captured bacteria counted, and the bacteria released from the surface before it was placed in buffer for continued incubation. The capture efficiency of the antibodies on each substrate was unchanged after a 7-day incubation at 4°C. The half-life of the antibody-modified glass substrates to prolonged incubation at 4°C was 27 days; the antibody-modified diamond surfaces showed no reduction in capture efficiency over the same period (12).

5.4. Label-Free Monitoring of RNA-DNA Interactions on In Situ Synthesized Arrays

Thin films of amorphous carbon were deposited on metal films to generate, aptly named carbon-on-metal substrates (59, 60). The amorphous carbon film provided the support needed for the in situ synthesis and subsequent usage of oligonucleotide microarrays. The metal underlayer retained its ability to interact with incident light to form surface plasmons. Oligonucleotide arrays on carbon-on-metal substrates have been used to monitor DNA-DNA, protein-DNA, and RNA-DNA interactions with both fluorescence confocal imaging and SPRi (60, 93).

In one application, the accessible sites of three different RNA molecules were determined by monitoring their hybridization to a DNA array with SPRi (93). RNA accessible sites—regions in a folded RNA molecule that are accessible to hybridization—are thought to play an important role in RNA interference. There are several software algorithms capable of computing the energy-minimized structures of an RNA molecule, but only a limited number of experimental techniques can assess RNA structure and sites accessible to binding (102, 103). In situ synthesized microarrays are ideal for these structure (or molecule)-specific applications because individualized arrays can be designed with the aid of computer software, allowing the user to control the number of features on an array and the sequence of oligonucleotides in those features. Spotted arrays, in contrast, would require the offline synthesis and purification of hundreds of oligonucleotides prior to spotting.

Tiling arrays of three different RNA molecules were fabricated: The arrays walked through the entire sequence of the RNA molecules one base at a time with a series of complementary oligonucleotides that were 6-, 8-, or 12-mers in length. These tiling arrays contained between 502 and 725 oligonucleotide features. In addition to sequence-specific tiling arrays,

a universal array containing 4,122 oligonucleotide features—all possible 6-mer combinations (4,96) and control features to ensure the fidelity of the array—was used to screen the accessible sites of the RNA molecules (93). The universal array is to our knowledge the highest density array used to date with SPRI.

The RNA accessible sites determined from the tiling and universal arrays were in agreement and showed that both targeted and nontargeted arrays can probe RNA structure. The lowest energy structures of the RNA molecules predicted from computer-aided algorithms did not agree with the accessible sites determined by hybridization. These differences may not reflect errors in either calculation or experiment, but rather may reflect that the lowest energy configuration of an RNA molecule cannot capture the dynamics of RNA structure and binding.

Images of the arrays obtained from an SPRI experiment (Figure 4) not only eliminated the need for labeling the RNA with fluorophores, but also provided qualitative (identifying the accessible sites) and quantitative (as the intensity of the SPR signal is directly related to the number of RNA molecules which hybridize to a feature, and thus provides insight into the affinity/stability of these interactions) information about RNA binding.

5.5. On-Array Transcription and Assembly of Full-Length RNAs from In Situ Synthesized Arrays

In a separate study, the ability to readily design and synthesize a microarray of arbitrary size, feature number, or oligonucleotide sequence was used not to detect oligonucleotide hybridization (or protein binding) but rather to synthesize the full-length RNA molecules needed to express a green fluorescent protein (ZsGreen I) (94). To prepare the RNA molecules, the 696 nucleotide (nt) cDNA sequence was divided into 18 segments ranging in length from 18 to 58 nt and synthesized as individual features on an amorphous carbon substrate; there were also 17 splint sequences, which when transcribed were complementary to portions of two neighboring segments and whose function was to stitch all of the segments into the full-length RNA by hybridization (Figure 5a). Each oligonucleotide feature contained the promoter site for T7 RNA polymerase (Figure 5b).

To initiate transcription, oligonucleotides complementary to the promoter site of T7 polymerase were hybridized to the array in the presence of T7 RNA polymerase and the components needed for in vitro transcription. The RNA products were assembled directly after transcription by the addition of RNA 5'-pyrophosphohydrolase (to convert the 5'-triphosphate to a monophosphate) and T7 RNA ligase 2. The resulting RNA was amplified with reverse transcription PCR with the appropriate restriction sites such that the DNA product could be ligated into plasmids compatible with cell-free eukaryotic protein expression; translation of the on-chip synthesized RNA (which yielded 44% of the total number of full-length RNAs with correct sequences) yielded fluorescent protein. Figure 5 summarizes the transcription, assembly of the transcripts into a full-length mRNA, and production of the translated protein.

The stability of oligonucleotide arrays on carbon substrates, combined with the ability to readily assemble and translate full-length RNAs, could allow for the generation of “transcription chips” that could be stored for prolonged periods and used multiple times.

6. SUMMARY AND FUTURE CHALLENGES

Carbon substrates, the surface chemistry used to modify the substrate, and the oligonucleotide arrays fabricated on these substrates through spotting or in situ synthesis outperform arrays prepared on glass, gold, or silicon equivalents. Their high stability provides a platform to expand the types of chemistries, reactions, analyses, and postprocessing steps associated with array-based screens. In the case of oligonucleotide arrays, this stability makes possible new analytical approaches that extend beyond the current methods that rely solely on hybridization. Amorphous carbon films have been used to prepare carbon-on-metal substrates and applied to quartz crystal microbalances, and thereby make possible label-free array analyses.

6.1. Substrate Availability and Surface Chemistry

Despite their stability, carbon substrates have not been widely adopted for biomolecule array fabrication. Amorphous carbon, diamond, and glassy carbon substrates are commercially available, but require the end user to modify the surface prior to usage. The carbon-carbon bonds formed between the substrate and an alkene functional group are very stable and are compatible with photolithographic patterning. The majority of work with this alkene attachment chemistry has utilized hydrogen-terminated substrates. The mechanism of the carbon-carbon bond formation is still an active area of investigation, and terminating the surface with hydrogen serves as a means of ensuring the surfaces are chemically uniform. Hydrogen termination of diamond substrates increases the favorability of reaction with the alkene (61, 62) but is not required with certain alkenes. In our own work (M.R. Lockett & L.M. Smith, unpublished data), we have also shown that alkenes attach to the surface of amorphous carbon films in the absence of hydrogen termination, and mechanistic studies have also shown that alkenes react more readily with oxidized amorphous carbon substrates than with hydrogen-terminated substrates (62, 63).

Continued work on elucidating the alkene-surface reaction mechanism and the development of alternative chemistries to modify these materials will likely result in methods to modify the surface chemistry of carbon substrates that are akin to the widely adopted procedures used to silanize glass or to prepare SAMs on gold.

6.2. High-Density Array Examples

The examples of oligonucleotide microarrays presented here are demonstrations of the types of reaction conditions and analytical modalities compatible with carbon substrates. Glass substrates are the preferred substrate for commercially produced microarrays, but are not compatible with label-free methods such as SPRi and require the use of fluorescent dyes. The RNA-accessibility work on amorphous carbon substrates discussed above would have been compromised by a need for the incorporation of fluorescent tags, due to the possibility that they could perturb RNA secondary structure.

The ability of microarrays prepared on carbon substrates to retain surface-immobilized oligonucleotides after prolonged incubations at high temperatures, in harsh solvent environments, or when repeatedly used, suggests that these arrays can be used for more than just the hybridization-based readouts currently employed in the research community. Combinatorial synthesis of biomolecules other than oligonucleotides is a topic of growing interest, and perhaps these stable substrates could help usher in the advent of in situ synthesized RNA, oligosaccharide, or small-molecule arrays.

6.3. Continued Development

An important question when developing any new technology is “where do we go from here?”. The work presented here shows some promising applications of arrays on carbon substrates—e.g., antibody capture arrays for diagnostic purposes, oligonucleotide arrays (transcription chips) for the expression of proteins on demand with in vitro transcription and translation systems, microarrays compatible with SPRi—and highlights that an improvement in surface (and surface chemistry) stability has paved the way for several new application areas previously unavailable with silanized glass substrates. The unprecedented chemical stability offered by carbon substrates and the associated carbon-carbon bond-based attachment chemistries offer a myriad of new possibilities for biomolecular analysis, to be developed over the coming years.

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Figure 1.

(a) Schematic of the steps taken to determine the stability of oligonucleotide arrays in which thiol-modified oligonucleotides were covalently immobilized on amine-terminated diamond, glass, glassy carbon, gold, and silicon substrates (b). Fluorescence intensities obtained from fluorescence confocal images of each array after exposure to a solution of fluorescently labeled complements (i.e., hybridized array). Each surface underwent 30 complete cycles of hybridization and dehybridization. UNCD, ultrananocrystalline diamond. Reprinted with permission from Reference 14. Copyright 2002, Nature Publishing Group.

Figure 2.

Modification of carbon substrates with (a) a two-step reaction in which the surface is chlorinated and then reacted with an organometallic reagent, (b) an aryldiazonium salt, or (c) an alkene-containing molecule with a UV light-mediated reaction.

Figure 3.

(a) Schematic of the gas-phase reaction used to convert terminal carboxylic acid groups on a carbon substrate to an acyl chloride group. (b) Representative spectra obtained from infrared reflection-absorption spectroscopic measurements of an acyl chloride surface after reacting with [4-(trifluoromethyl)phenyl] methanol (*red*), [4-(trifluoromethyl)phenyl]methanethiol (*brown*), and [4-(trifluoromethyl)phenyl] methanamine (*blue*). Reprinted with permission from Reference 79. Copyright 2009, American Chemical Society.

Figure 4.

(a) SPRi difference image of a DNA tiling array designed to step through the nucleotide sequence of the premiR-155 RNA molecule. This difference image was obtained by subtracting an image of the array after hybridization (after exposure to a 1- μ M solution of premiR-155) from an image of the array prior to introducing the premiR-155-containing solution. The brightness of an individual feature increases with increasing numbers of hybridized premiR-155 molecules; gray regions contain low numbers of DNA-RNA duplexes, and white regions contain several DNA-RNA duplexes. (b) Histogram of the hybridization data obtained from the SPRi difference image. The y-axis corresponds to the average change in brightness (or reflectivity, delta %R, and the x-axis corresponds to the location of the surface-bound oligonucleotide probe on the premiR-155 molecule. (c) The lowest energy structure of premiR-155 predicted by Mfold (104, 105). The 6-mer accessible sites determined from the hybridization experiment are indicated with the red line. Reprinted with permission from Reference 93. Copyright 2009, American Chemical Society.

Figure 5.

Illustration of the RNA-mediated gene assembly process. (a) A gene target is divided into segment (*red*) and splint (*blue*) sequences, which are then (b) synthesized on an amorphous carbon surface. Each sequence contains the complement of the T7 RNA polymerase (RNAP) promoter sequence (*yellow*) at the 3'-terminus. (c) Hybridization of an oligonucleotide matching the T7 promoter sequence produces the duplex needed for RNA polymerase binding (and subsequent transcription) to occur. (d) RNA segments and splints have their terminal triphosphate units trimmed to monophosphates with pyrophosphohydrolase, the RNA strands assemble through RNA-RNA hybridization, and the nicks are sealed to yield the desired full-length RNA. (e) Detailed view of the surface-bound oligonucleotides and the

T7 RNA polymerase complementary oligonucleotides. Reprinted with permission from Reference 94. Copyright 2012, Angewandte Chemie International Edition.

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Scheme 1.

Alkene molecules used to modify carbon substrates for the immobilization of biomolecules.

Table 1

Oligonucleotide density and hybridization cycle stability on carbon substrates

Substrate	Surface chemistry ^a	Modified oligonucleotide	Oligonucleotide density (10 ¹² molecules/cm ²)	Number of hybridization cycles	Oligonucleotide loss (%)	Reference
Spotted Arrays						
Amorphous	aldehyde	DNA-NH ₂	4.4 (±0.1)	NA	NA ^b	80
Amorphous	amine	DNA-SH	2.3 (±0.5)	NA	NA	79
Amorphous	acyl chloride	DNA-NH ₂	2.0 (±0.2)	NA	NA	79
Amorphous	acyl chloride	DNA-SH	1.1 (±0.2)	NA	NA	79
Amorphous	thiol	DNA-SH	1.0 (±0.1)	NA	NA	92
Diamond	amine	DNA-NH ₂	3.1 (±0.7)	30	<5	14,86
Glassy carbon	amine	DNA-NH ₂	NA	30	<5	14
In Situ Synthesized						
Amorphous	hydroxyl	phosphoramidite	7.4 (±0.4)	15	<5	59
Diamond	hydroxyl	phosphoramidite	4.4 (±0.1)	20	<5	11
Glassy carbon	hydroxyl	phosphoramidite	2.4 (±0.1)	20	9	11

^aCompounds and respective molecules: aldehyde, 2-(10-undecen-1-yl)-1,3-dioxolane; amine, 10-aminodec-1-ene (TFAAD); acyl chloride, 10-undecenoic acid; thiol, 3-(2'-nitrobenzyl)thiopropyl phosphoramidite coupled to a 9-decene-1-ol-modified surface; hydroxyl, 9-decene-1-ol; phosphoramidite, (2'-nitrophenyl)propoxycarbonyl-protected nucleoside phosphoramidites.

^bAbbreviations: NA, no data available; TFAAD, trifluoroacetamide-protected 10-aminodec-1-ene.