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Cold-plasma modification of oxide surfaces for covalent biomolecule attachment

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Abstract

While many processes have been developed to modify the surface of glass and other oxides for biomolecule attachment, they rely primarily upon wet chemistry and are costly and time-consuming. We describe a process that uses a cold plasma and a subsequent in vacuo vapor-phase reaction to terminate a variety of oxide surfaces with epoxide chemical groups. These epoxide groups can react with amine-containing biomolecules, such as proteins and modified oligonucleotides, to form strong covalent linkages between the biomolecules and the treated surface. The use of a plasma activation step followed by an in vacuo vapor-phase reaction allows for the precise control of surface functional groups, rather than the mixture of functionalities normally produced. By maintaining the samples under vacuum throughout the process, adsorption of contaminants is effectively eliminated. This process modifies a range of different oxide surfaces, is fast, consumes a minimal amount of reagents, and produces attachment densities for bound biomolecules that are comparable to or better than commercially available substrates.

Keywords: Microarrays; Cold plasmas; Surface chemistry

1. Introduction

Biological microarrays, composed of spots of different biomolecules attached to a substrate at known locations, are fabricated principally in two ways: synthesis directly on the substrate through photolithographic or chemical means (Fodor et al., 1991; Singh-Gasson et al., 1999; Hughes et al., 2001) or deposition of presynthesized or extracted molecules onto a chemically treated substrate that binds them (Schena et al., 1995). The latter way is required for those molecules that cannot currently be synthesized directly on a substrate, for example, many proteins (Templin et al., 2003) or carbohydrates (Wang et al., 2002).

Attachment to a substrate, either electrostatically or covalently, is critical to the production of microarrays using presynthesized or extracted biomolecules. Covalent attachment is preferred because attached molecules remain bound to the substrate after exposure to target solutions, multiple wash steps, and even repeated use of the microarray. Surface chemical functionalization processes have been developed to bind biomolecules covalently to a range of substrate materials, including glass (Conzone and Pantano, 2004), silicon (Strother et al., 2000), and diamond (Yang et al., 2002). Most of these processes rely on the use of wet chemistry, in many cases consuming significant volumes of rare, expensive, and/or environmentally unfriendly chemicals. Wet-chemical treatments may take anywhere from several hours to a day to produce a substrate ready for biomolecule attachment (Guo et al., 1994; Hermanson et al., 1992).

Cold plasmas can greatly reduce the complexity of preparing a chemically functionalized surface. A cold (non-equilibrium, near-room temperature) plasma is an ionized gas produced by applying an electric field to create a discharge in

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a vapor. An ionized gas permits the modification of even the most chemically resistant surfaces. We describe here the use of cold plasmas to modify the surfaces of oxides. Oxides are of particular interest as substrate materials for biomolecule attachment because of the wide use of glass slides in existing microarrays and the reflectivity in the visible spectrum of metals with native oxides, which can enhance the fluorescent signal from a microarray. Specifically, we create amine-reactive epoxide chemical functionalities on oxide surfaces.

Several prior efforts have been made to use cold plasmas for functionalizing surfaces. The use of cold plasmas of reactive vapors to create specific functionalities on surfaces has not produced exceptional results, primarily because the mixture of different chemical fragments and charge states found in a plasma formed from reactive vapors can produce a mixture of different chemical terminations on the surface (Denes et al., 1997). In one study, the attachment of different fragments on the surface was avoided by using the plasma only to activate the surface, which was then withdrawn from vacuum and exposed to a chemical bath to introduce the desired chemical functionalities (Yamada et al., 2000). This procedure, however, exposes the surface to atmosphere and the consequent contamination and deactivation of many of the active sites. The use of a chemical bath requires large quantities of chemicals and hinders the ability of the process to scale up. We resolve these issues by carrying out our entire process in vacuum, but using the cold plasma only to activate the surface and then using a vapor of the reactive species we want to attach on the surface.

2. Materials and methods

2.1. Plasma treatment

All of the steps in our treatment take place in a 15 in. diameter parallel-plate plasma reactor operated at a base pressure of 30 mTorr. The discharge is produced by a 30-kHz power supply. After each plasma treatment or vapor exposure, the chamber is again pumped down to 30 mTorr. The first step consists of a 2-min long oxygen plasma clean at 200 mTorr and 200 W, followed by an argon plasma exposure for the same time at the same conditions. Oxygen and argon plasmas are commonly used in semiconductor wafer cleaning for oxidizing and stripping photoresist and other adsorbed organics off silicon wafer surfaces (Vossen and Kern, 1978). By not allowing the plasma-cleaned substrates to see atmosphere until after treatment is completed, chemical functionalization will be unaffected by atmospheric contaminants. Existing wet-chemical procedures require baths in strong acids or bases and strong oxidizers (Halliwell and Cass, 2001) for cleaning and run the risk of surface contamination when the cleaned substrates are exposed to atmosphere between cleaning and treatment steps. FisherBrand Precleaned microscope slides were used as our glass substrates and no additional chemical cleaning was performed on our substrates prior to the plasma clean.

Following this surface preparation step, the substrates are exposed to a plasma of a 2:1 water/oxygen mixture. The purpose of this step is to terminate the surface with hydroxyl functional groups. The water/oxygen plasma is formed at a pressure of 200 mTorr and at 200 W for 2 min.

As the final reaction step, a 1-Torr vapor of epichlorohydrin (with no plasma) is introduced to the substrates and allowed to react for at least 30 min. Epichlorohydrin (C_3H_5ClO) is a relatively volatile liquid (vapor pressure of ~16 mmHg at 25 °C) composed of a single epoxide ring. A vapor is supplied to the chamber by opening a valve to a container of the liquid and allowing enough to evaporate to fill the chamber to a pressure of 1 Torr. As illustrated in Fig. 1, the epichlorohydrin epoxide ring reacts with the nucleophilic oxygen species introduced to the oxide surfaces, causing the ring to open and binding the epichlorohydrin to the surface (Merrill, 2004). The ease with which the chlorine group can leave, a unique property of epichlorohydrin, allows for the epoxide ring of epichlorohydrin to reform on the free end of

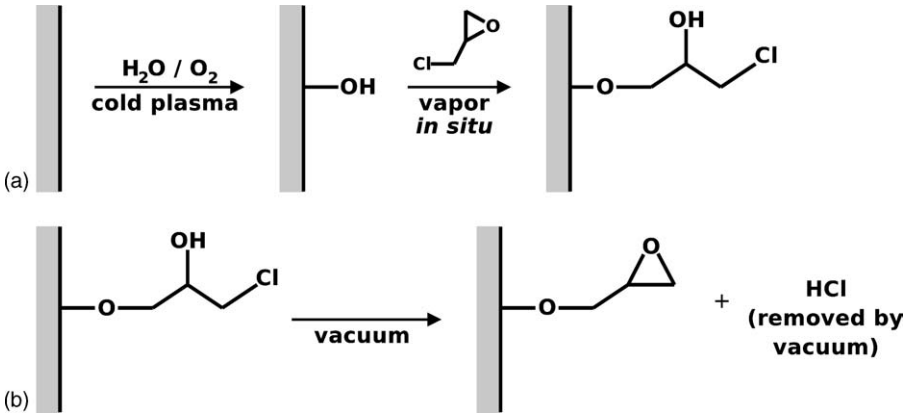

Fig. 1. Proposed reaction mechanism for the described treatment process. (a) First, the surface is terminated with hydroxyls by a water/oxygen plasma. The hydroxyls on the surface react with the epoxide ring of epichlorohydrin introduced as a vapor, covalently binding the epichlorohydrin to the surface. (b) Finally, exposure to vacuum removes HCl and allows the reforming of the epoxide on the free end of the epichlorohydrin, which epoxide-terminates the surface.

the bound molecule. In epoxy polymerisation, a strong base is normally used to draw off the HCl produced by the ring reformation (Chanda and Roy, 1998). In our process, we evacuate the epichlorohydrin and pump off the HCl in vacuum at room temperature for another 30 min. The epoxide-terminated substrates are then removed from the reactor and stored in dry conditions (<10% humidity), under which they retain their full activity for at least several months.

2.2. X-ray photoelectron spectroscopy

We used a Perkin-Elmer Physical Electronics 5400 small area system X-ray photoelectron spectroscopy (XPS) system with a Mg source at 15 kV and 300 W and a pass energy of 89.45 eV. In order to account for sample charging, we sputtered gold onto a portion of each sample and used the Au $4f_{7/2}$ peak as a reference. We compared a plasma-cleaned glass slide (exposed to ambient air) and a glass slide completely treated with our plasma process with a commercially available epoxide-terminated glass slide (TeleChem International Inc.).

2.3. Contact angles

We used a DataPhysics Contact Angle System OCA Plus 15 goniometer loaded with distilled water to measure sessile contact angles on treated, untreated, and commercially available substrates. Five locations were examined on each sample, with the average value being reported.

2.4. Atomic force microscopy

We use a digital instruments multimode atomic force microscope (AFM) in intermittent-contact mode to image the surfaces of untreated, treated, and commercially available substrates, as well as determine the root mean square (RMS) roughnesses of those surfaces.

2.5. Oligonucleotide deposition, immobilization, and detection

The 16-mer oligonucleotides were C6-amino-modified on their 5′ end and fluorescein-modified on their 3′ end. All oligos used in these studies were synthesized by the University of Wisconsin Biotechnology Center. These oligos were diluted to a 200 μM concentration in distilled water and deposited in 0.5 μL spots on the treated substrates. The amine-modified oligos were allowed to bind for 2 h in dry conditions, then a concentrated solution of 99% ethanolamine was spread evenly across the substrates underneath a coverslip and allowed to react for 30 min in order to block the remaining epoxide groups present on the surface. The substrates were rinsed with ethanol and distilled water and placed in a 2× SSPE buffer solution (20 mM NaH_2PO_4, 300 mM NaCl, 2 mM EDTA) for 1 h in order to remove non-specifically bound oligos. The substrates were then placed in a bath of distilled water for approximately 1 h. Before analyzing them in a Genomic Solutions GeneTAC UC4×4 microarray scanner, we spread a 1 M Tris–HCl buffer solution across the surface of the substrates to enhance the fluorescence of the fluorescein dye. We quantified fluorescence from the spots of bound oligonucleotides by finding the average fluorescence within an entire spot using the NIH ImageJ (Rasband, 1997–2004) analysis software and then averaging that value across 12 spots on a slide.

2.6. Oligonucleotide hybridization

Two different 31-mer sequences were immobilized on a glass slide treated with our process, 5′amino C6-T_{15}-GC TTA AGG AAG GTT CG-3′(sequence A) and 5′amino C6-T_{15}-GC TTA ACC ACC ATT CG-3′(sequence B), and hybridized with their complements, 5′(6-FAM)-CG AAC CTT CCT TAA GC-3′(complement A) and 5′(6-FAM)-CG AAT GGT GGT TAA GC-3′(complement B). We deposited a set of spots of sequence A along with a set of spots of sequence B, both on the same treated substrate, which had been subjected to the previously described binding, blocking, and washing procedure. For hybridization, the complements were diluted to a concentration of 10 μM in a buffer solution of 2× SSPE/0.2% SDS (20 mM NaH_2PO_4, 300 mM NaCl, 2 mM EDTA, 7 mM sodium dodecylsulfate). The fluorescently tagged complements were allowed to hybridize with the surface-bound oligos at room temperature for 1 h, then the substrates were washed for 15 min with 2× SSPE/0.2% SDS and dried. The substrates were scanned in the fashion described earlier.

3. Results and discussion

3.1. Surface characterization

We investigated the surface elemental composition of the treated substrates with XPS. High-resolution spectra of the C 1 s peak and an elemental analysis are shown in Fig. 2 and Table 1, respectively. Table 1 shows an increase in the relative amounts of Si and O in the glass surface at the expense of C after a plasma clean, indicating that adsorbed organics had been removed. The exposure to epichlorohydrin increases C on the surface and introduces a small amount of Cl, presumably from epichlorohydrin that has not yet reformed the epoxide ring during the 30 min vacuum HCl removal. In the substrate exposed to the full plasma treatment, the C–O component of the C 1s peak is greatly enhanced relative to the C–C component when compared with the substrate that was only plasma-cleaned. The C–O peak is also much larger, relative to the C–C peak, on our plasma-treated substrate than it is on the commercial epoxide-terminated substrate. A possible explanation is that the compound used to epoxide-terminate the commercial surfaces (such as the commonly used 3-glycidoxypropyltrimethoxysilane, $C_9H_2O_5Si$) has more C–C bonds within its structure than does epichloro-

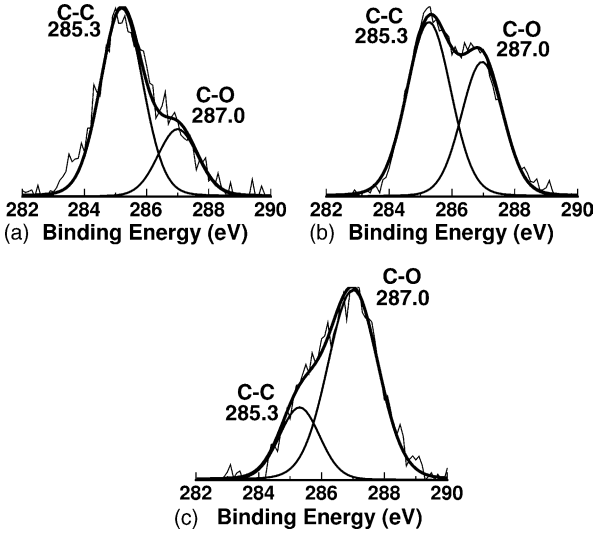

Fig. 2. High-resolution XPS scans of the C 1s peak for treated and untreated glass substrates from (a) an untreated glass slide; (b) a commercially available epoxide-terminated glass slide; and (c) an epoxide-terminated glass slide prepared using our process.

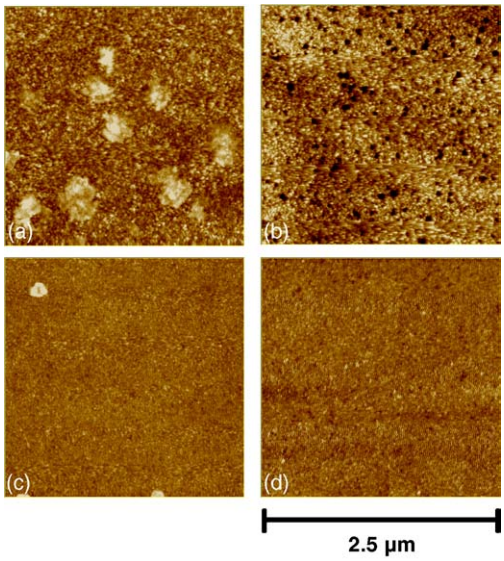

Fig. 3. Atomic force microscopy images of (a) an untreated glass slide; (b) a commercial epoxide-terminated glass slide; (c) a plasma-cleaned glass slide; and (d) a glass slide treated using our process. The height scale of each image is 10 nm. The surface roughness of the glass slide is reduced significantly after the plasma clean and storage in vacuum, presumably due to the removal of dust particles or other adsorbed contaminants. The cause of the observed roughness on the commercial epoxide-terminated slide is unknown.

hydrin. Adsorbed organic contaminants may also be present on the commercial slides, introduced either before or after their chemical treatment. It is also possible that our process introduces a higher density of epoxide groups, which contain two C–O bonds each.

Another measure of the nature of a surface can be obtained using contact angles. Sessile contact angles of distilled water droplets measured on a series of treated substrates are shown in Table 1. The plasma-cleaned substrates as well as the substrates that were plasma-treated, but not exposed to epichlorohydrin, had contact angles too low to detect, consistent with having a surface terminated by polar alcohol groups. After the surface is exposed to epichlorohydrin vapor, the contact angle becomes ∼67°. This contact angle is consistent with the termination of the surface by epoxide chemical groups, as these groups are moderately hydrophobic and contact angles in this range have previously been observed on other epoxide-terminated surfaces (Taylor et al., 2003). The higher contact angle relative to the commercial slides is consistent with the larger C–O peaks in the XPS data from our surfaces. Further evidence for the epoxide termination of these substrates comes from the fact that exposure of the fully treated substrates to ethanolamine, an amine-containing compound that reacts with epoxide groups to form polar alcohol-containing functionalities, reduces the surface contact angles below the level that can be measured.

In addition to performing XPS and contact angle studies, we used an AFM to observe the removal of contaminants from the surface of glass slides by looking at nanoscale surface roughness. Fig. 3 shows an untreated glass microscope slide, a commercial epoxide-terminated slide, a slide after the plasma-clean step, and a slide after the entire plasma-treatment process (including the epoxide termination). The RMS roughnesses of these substrates are 1.9, 2.4, 0.9, and 1.1 nm, respectively. The reduction in surface roughness shown in Fig. 3a relative to that in Fig. 3c is presumably due to the removal of adsorbed organics and dust particles. The roughness observed on the commercial slide does not appear to be caused by large particles present on the surface, but may be due to the morphology of the glass used.

3.2. Biomolecule binding

Epoxide chemical functionalities react with and covalently bind amine-containing biomolecules to a substrate. We tested

Table 1
XPS elemental analysis and sessile contact angles for a series of untreated, treated, and commercially available epoxide-terminated glass slides

Treatment	O (%)	Si (%)	C (%)	Cl (%)	Sessile contact angle (°)
Untreated	57	19	24	0	7.2 ± 3.5
Plasma-cleaned (exposed to atmosphere)	71	21	8	0	<2
Plasma-cleaned, water/oxygen plasma, no epichlorohydrin	75	16	9	0	<2
Plasma-cleaned, water/oxygen plasma, epichlorohydrin	70	17	10	3	66.5 ± 6.9
Commercial epoxide-terminated glass slide	58	26	16	0	46.1 ± 1.2

XPS data obtained from high-resolution scans of the C 1s, Si 2p, O 1s, and Cl $2p_3$ peaks.

the biomolecule-binding properties of our freshly prepared substrates and compared them to commercially available epoxide-terminated glass substrates fresh out of a vacuum-packed box (which may, of course, have been stored for some period of time). We find that spots on the glass slides treated using the cold-plasma process had on average 10% higher fluorescence than spots on the commercial slides, however, this increase was within the margin of error for the experiment (±20%). As controls, an untreated glass slide and a plasma-cleaned glass slide exposed to the same spotting and washing conditions as the treated substrates showed no fluorescence. A fluorescent image of spots deposited on a treated glass slide is shown in Fig. 4a.

Because the fluorescently tagged oligos are covalently bound to the surface, the intensity of fluorescence within spots of bound oligos should be an indication of the density of biomolecule binding on the treated substrates. Our results suggest that our plasma-based treatment process provides a number of attachment sites on the substrate surface at least comparable to that of the commercially available substrates, despite the fact that the plasma treatment has not been tuned for optimal binding site densities.

We tested our treatment process on several other oxide substrates, in addition to glass slides. Images of oligonucleotides chemically attached to fused silica, mirror-polished 304 stainless steel, and the native oxide on a silicon wafer are shown in Fig. 4b–d. The best performing of the various materials, functionalized mirror-polished stainless steel, has ∼10 times lower background fluorescence than glass slides plasma-functionalized at the same time and exhibits ∼2 times the fluorescent intensity in the spots of bound oligos (quantitated using the previously described procedure), possibly due to the reflective nature of the substrate. The same reduction in fluorescent background and increase in fluorescent signal is present when compared with commercial epoxide-terminated glass slides, suggesting that the functionalized mirror-polished stainless steel substrates may allow for more sensitive fluorescence-based assays than existing substrates.

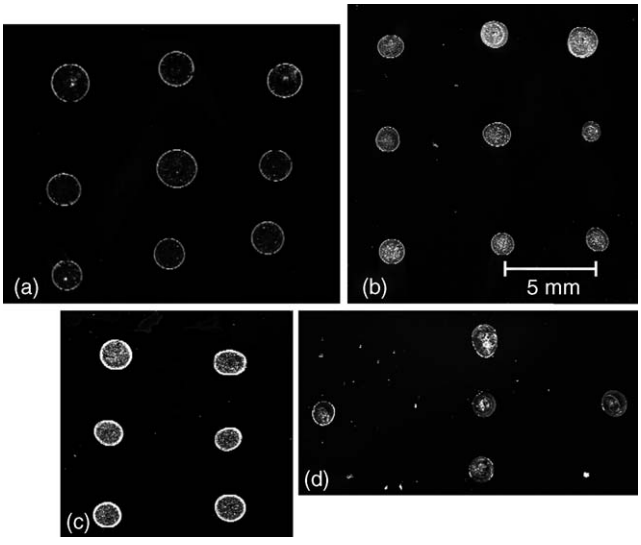

Fig. 4. Fluorescence scans of amine-modified, fluorescein-tagged oligonucleotides covalently bound on four different treated substrate materials. The four substrates are: (a) glass; (b) fused silica; (c) mirror-polished 304 stainless steel; and (d) native oxide on silicon. All spots were ∼0.3 μL in volume, deposited by a hand pipette. The fluorescent intensities from spots on each substrate were at least as strong as on the commercial epoxide-terminated glass slides. All images are to the same scale.

3.3. Oligonucleotide hybridization

To verify the biological activity of molecules covalently bound to our plasma treated epoxide-terminated surfaces, we attached single-stranded oligos and allowed them to hybridize with their fluorescently tagged complements. Fig. 5 shows an example of a glass slide on which spots of sequences A and B were bound, then hybridized first with complement A followed by complement B. The fluorescence in only spots of sequence A with exposure to complement A and subsequent fluorescence of spots of B with exposure to complement B demonstrates that oligonucleotides bound to our plasma treated slides are capable of sequence-specific hybridization.

3.4. Deposition of microscale spots

The high binding site density obtainable from our plasma functionalized substrates and increased fluorescent sensitivity from substrates such as stainless steel means that full advantage can be taken of recent technology that allows for the creation of much smaller, more uniform spots. One such device is called a fluid microplotter, which uses ultrasonics to deposit spots as small as 5 μm in diameter, consuming orders of magnitude less solution than current quill-pin-based spotters in the production of biological microarrays (Larson et al., 2004). In addition, at these smaller spot sizes, irregularities, such as the coffee-ring effect noticed in Figs. 4 and 5, are greatly reduced, thereby increasing the reliability of fluorescent-intensity measurements from the spots. Fig. 6a shows an image of a pattern of fluorescently tagged oligos deposited by the microplotter onto a glass slide treated with our process. The conditions for attachment are the same as those described previously. The spots in this image are 35 μm wide, with a 100 μm center-to-center spacing. Fluorescent intensity within the rectangular pattern

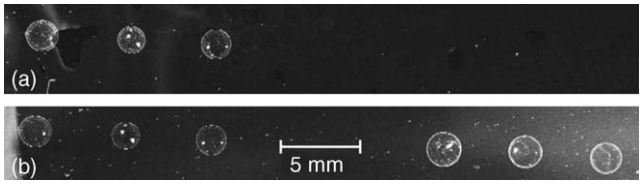

Fig. 5. Fluorescence scans of fluorescently tagged oligos hybridized to their surface-bound complements. A glass slide was treated with our process, then spotted with ∼0.3 μL spots of sequence A and B in two 3-spot lines, sequence A on the left, sequence B on the right. These slides were then hybridized with (a) complement A, followed by (b) complement B. All spots were ∼0.3 μL in volume, deposited by a hand pipette.

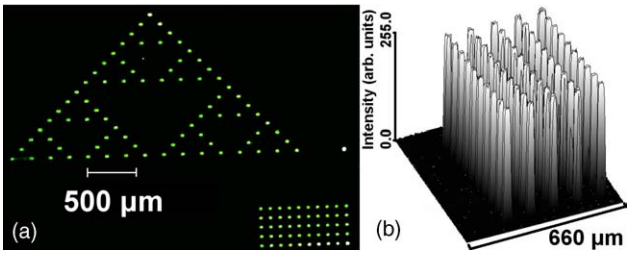

Fig. 6. Fluorescence scans of amine-modified, fluorescein-tagged oligos deposited by a fluid microplotter and bound to a glass slide functionalized with our process. The spots in these images are ∼35 μm in diameter, with a 100 μm center-to-center spacing. The two images are of (a) patterns deposited by a microplotter and (b) the fluorescent intensity of the spots in the rectangular spotted pattern, plotted in three dimensions. Even at this small a spot size, the bound oligonucleotides are easily detected over a minimal amount of substrate autofluorescence.

of Fig. 6a is plotted in Fig. 6b, demonstrating a large signal-to-background ratio with these small spots.

4. Conclusion

In summary, we have used cold-plasma activation and an in vacuo vapor-phase reaction to terminate a range of oxide-containing surfaces with epoxide chemical groups. This procedure is much more efficient than prior methods. Minimal amounts of reagents are consumed when compared with standard wet-chemical processes and much less processing time is involved, implying a much lower cost of fabrication for these epoxide-terminated slides. We have so far made no effort to optimize the process, with respect to the plasma clean parameters, the epichlorohydrin exposure time, or the pump-out time. Despite that, the performance of our epoxide-terminated slides equals or exceeds that of commercial slides. In particular, the remaining Cl seen in the XPS data suggests that improvements are possible in the binding-site density we can produce on the surface.

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