A PRECISION TECHNOLOGY FOR CONTROLLING PROTEIN ADSORPTION AND CELL ADHESION IN BIOMEMS

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ABSTRACT

A surface coating technique is investigated to enhance device biocompatibility by eliminating bio-fouling, the strong but non-specific affinity of proteins and cells to attach to surfaces. This coating is a conformal, thin poly(ethylene glycol)-like film deposited in a glow discharge of tetraglyme. Substrates with different chemistries are successfully modified, and exhibit ultralow protein adsorption and cell attachment with the coating. This "stealth" or "non-fouling" coating can also be faithfully patterned using standard photolithography processes. The interaction of proteins and cells with patterned surfaces is limited only to the protein-adhesive domains, thus creating heterogeneous patterns of proteins and cell cultures on the surface. The potential benefits of our technique to applications such as cell-based assays and micro-electrodes are discussed.

INTRODUCTION

Biocompatibility is essential to successful biomedical devices. Low material toxicity is an important criterion. Bio-fouling is another important measure of biocompatibility [**1**] because of the strong affinity of proteins to physically adsorb to synthetic surfaces.[**2**] More importantly, the adsorbed protein layer can further mediate additional biological responses, like cell attachment and activation, and can create unpredicted perturbations to device operation. For example, analytical applications, such as lab-on-a-chip, are reduced to one-use systems because of a possible cross contamination from one run to the next. In addition, rapid analysis in complex bodily fluids is difficult due to background drift and deteriorated signal quality.[**3**,**4**] Biocompatibility issues are even more challenging in *in vivo* settings, such as a subcutaneously implanted glucose sensor. In the *in vivo* case, a successful integration of the device into the host environment must be achieved without encapsulation.[**5**]

Despite the clear motivation to eliminate any adhesion between proteins and surfaces, in some cases the ability to enable protein adhesion to certain restricted regions on the device surface is very desirable. Such spatially controlled heterogeneous surface can be advantageous in the operation of some bio-microelectromechanical systems (bioMEMS). Cell-based micro assays are one example. In these assays, miniaturized cell cultures are to be maintained only on pre-selected sensors while additional sensors remain free of cells to provide a background signal.[**6**] Such devices require two different surface chemistries on the assay surface, with good alignment to the sensors.

Because non-specific protein adsorption and cell attachments are surface reactions, surface modification is one technique to control bio-fouling.[**7**] A promising approach is based on poly (ethylene glycol), or PEG. PEG is a neutral, hydrophilic hydrogel that effectively forms hydrogen bonds with water.[**8**] This structured water layer is thought to contribute to the protein resistance of PEG. Other factors proposed to contribute to the PEG's protein resistance include a steric exclusion mechanism involving surface mobility and the dynamics of PEG chains.[**9**] PEG is also non-toxic and FDA-approved for use in biotechnology and consumer applications. Because PEG lacks the mechanical integrity and strength, it is used as a coating rather than as a structural material. Various methods have been adopted to modify surfaces with PEG. The coating process determines the surface coverage. To a large extent, the success of PEG surface modification is a function of surface coverage of ethylene oxide moieties.[**10**] Therefore, the choice of coating technique can have a direct impact on the coating performance.

Here we present a surface coating technique to achieve precisely patterned surfaces consisting of protein adhesive and non-adhesive micro-domains, and to enhance the biocompatibility of electrochemical sensors. In our study we use glow discharge plasma polymerization of tetraglyme (CH_3-O-(CH_2-CH_2-O)$_4$-CH_3). Plasma polymerization is a gas-phase, thin-film deposition process. This process is capable of producing thin solid PEG-like coatings that offer superb surface coverage and protein resistance.[**11**] Previous studies demonstrated the protein resistance of plasma polymerized tetraglyme (pp4G) coatings.[**11,12,13**] Coated surface plasmon resonance biosensors were resistant to protein adsorption in protein solutions.[**12**,**13**]

The focus of our current study is to examine the compatibility between the pp4G coating and photolithography and micro-machining processes in order to allow adaptation of this non-fouling technology as part of the MEMS toolbox. Using environmental scanning electron microscopy (ESEM) we verified the pattern definition. Electron Spectroscopy for Chemical Analysis (ESCA), a surface sensitive analytical technique, was utilized to analyze the coating surface chemistry after the photoresist lift-off. We found that the patterning process did not affect the pp4G coating. Protein adsorption and cell culture experiments demonstrated the spatial control of these interactions on patterned pp4G surfaces. Finally, to verify the applicability of pp4G to electrochemical sensors for neural recording, the ionic conductivity of coated and bare microelectrode arrays was measured.

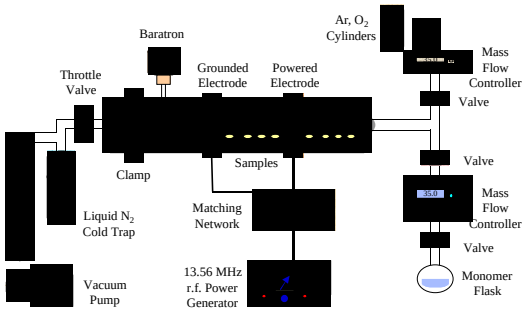
EXPERIMENTAL PROCEDURES

Sample Preparation. We used conducting silicon wafers as substrates for the pp4G coatings. The wafers were sputtered with silicon nitride or oxidized for electrical passivation. Nitride is better suited for our experiments because it has better corrosion resistance in aqueous environments than oxide.[**14**]

To achieve the pp4G patterning, photoresist (AZ 1512) was deposited, and then exposed and developed in order to create openings in the resist for the later pp4G lift-off. After plasma oxygen descum etch the wafers were introduced to the plasma reactor for the pp4G deposition.

Plasma Polymerization of Tetraglyme. The apparatus for plasma polymerization is a home-built system as shown in Figure

1. The electrodes are 1 inch wide copper bands wrapped around the glass chamber (100 mm diameter and ca. 700 mm long). The electrodes are connected to a radio frequency (13.56 MHz) power



supply and a manual matching network.

Figure 1. *A diagram of the plasma polymerization reactor.*

After briefly cleaning the substrates in an argon plasma, tetraglyme vapor was introduced to the chamber at a flow rate of 1.3 sccm. The plasma power was first maintained at 80 W for 30 seconds at 350 mT to deposit an adhesion-promoting layer. The power was then reduced to deposit the soft, functional PEG-like top coating. It was maintained at 15 W for one minute, then at 10 W for another 30 seconds for a total plasma-on time of 2 minutes. Additional details of the monomer preparation and the deposition process will be discussed elsewhere.[**15**]

Integrating the lift-off and the plasma deposition processes was rather straightforward. However, special attention had to be directed to the location of the wafer in the plasma chamber. We placed the wafers in low monomer condensation regions to prevent tetraglyme vapor from dissolving the photoresist. After the plasma deposition, the wafers were rinsed in acetone (in ultrasonic bath) to remove the photo-resist (lift-off).

ESCA. ESCA analyses were performed on a Surface Science Instruments S-Prove ESCA instrument with an aluminum $K\alpha_{1,2}$ monochromatic X-ray source and a hemispherical energy analyzer. An X-ray sampling spot with a 150-µm diameter is used. Typical pressures in the analysis chamber during spectral acquisition were 10^{-9} Torr. Spectra were collected with the analyzer at 55^{o} with respect to the surface normal of the sample, resulting in a data collection depth of 50-80 Å. Analyses were performed on pp4G patterned on a substrate with metal and thermal oxide regions.

Protein Adsorption. Bovine serum albumin (BSA) was labeled with fluorescent tags (Oregon Green, Molecular Probes). The adsorption experiment was carried out at room temperature in citrated phosphate buffer solution at pH 7.4. A protein concentration of 50 µg/mL was used. After 1.5 hours, the surface was rinsed repeatedly and thoroughly in buffer.

Cell Culture. Bovine aortic endothelial cells (BAECs) were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum, 1% 100mM MEM sodium pyruvate solution, 1% 10mM MEM non-essential amino acids solution, 100 units/mL penicillin and 100 µg/mL streptomycin. BAECs were cultured on 75 cm^2 tissue culture flasks inside a humidified incubator at 37 ^{o}C with 5% CO_2 and 95% air.

Cell Patterning and Staining. Patterned pp4G surfaces (ca. 15 mm by 15 mm) were sterilized in 70% ethanol overnight and washed with Dulbecco's phosphate buffer (dPBS) for two or more times prior to use. BAECs in the ninth passage were used to make a cell suspension of 5.5 10^5 ± 1.4 10^5 cells/mL with a 97% cell viability. The cell concentration and viability were determined using trypsin blue stain and the hemocytometer. Two mL of the cell suspension was added to each 35-mm dish for a cell seeding density of 1.1 10^5 cells/cm^2. The cells were cultured in a 37 ^{o}C incubator for 4 days. For fluorescence staining, cell cultures were first rinsed with dPBS, fixed in 3.7% formaldehyde solution, and blocked with 1% BSA. The cell cytoskeleton was labeled with rhodamine phalloidin (Molecular Probes), and the cell nucleus was labeled with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, Molecular Probes).

RESULTS AND DISCUSSION

We begin our discussion with a visual examination of the pp4G coating after lift-off. In Figure 2, we show an ESEM image of patterned pp4G coating on silicon nitride substrate with predefined chrome-gold (CrAu) features. The advantages of our process are readily seen: The coating has good adhesion to the silicon oxide substrate and to the metal, it is reliably patterned and can be very easily incorporated onto complex structures.

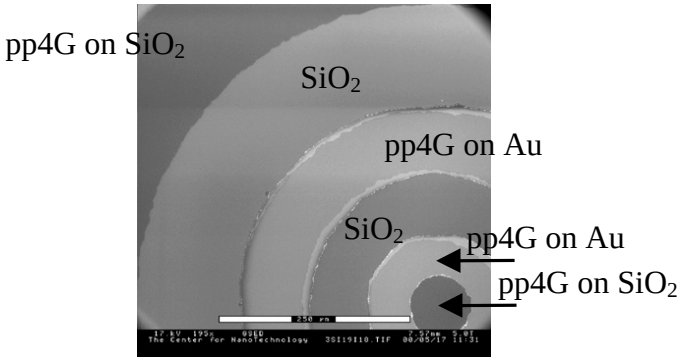


Figure 2*. ESEM image of pp4G features after lift-off. The pp4G structures are aligned to predefined CrAu structures. Similar results were obtained for structures as small as 10 µm. While current results were produced with low-cost printed masks (features > 10µm), we expect minimum feature size and alignment precision for these protein-resistant coatings to be limited only by the lithographic resolution.*

Surface Chemistry. The next step in characterizing the patterned pp4G coating is to study the effect of patterning and substrate materials on its chemistry. The surface chemistry of pp4G on SiO_2 and on metal layers was examined using ESCA. Figure 3a shows a survey scan of a patterned coating on thermal oxide. The carbon-to-oxygen ratio of the coating is 2.2, similar to the ratio previously observed for non-patterned pp4G, and it is also comparable to the C-to-O ratio of PEG, which is 2. Also detected is a trace amount of residual lead (< 0.1%) on the SiO_2 surface from the electrochemical deposition of electrodes. Very similar composition was observed on pp4G coatings on metal electrodes after the patterning process. A C-to-O ratio of 2.1 (± 0.1, n=2) and more than 0.1% of lead (0.2 ± < 0.1%) was observed in this case.

Figure 3b shows the carbon 1s high-resolution scan of a patterned pp4G on SiO_2. Three peaks of 1.30 eV peak width were used in the peak fitting process. The dominant carbon functional group is the ether carbon (C-O) group at 286.6 eV (80.3%). There is a small peak at 285.0 eV from the C-C crosslinks, and another peak at 287.8 eV from the higher oxidized carbons. Again, similar chemistry was observed on the C1s spectra of both non-patterned pp4G and patterned pp4G on electrode surface as well. Based on

the ESCA analysis, it is concluded that the coating retained its PEG-like characteristics after the resist lift-off process. It was also observed that the coating has similar adhesion to different materials present on the same substrate.

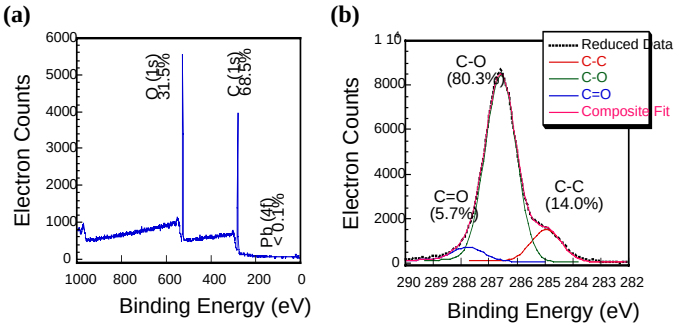


Figure 3. *ESCA data of a pp4G coating after the photoresist lift-off process: (a) survey scan and (b) carbon 1s high-resolution scan from a patterned pp4G coating on thermal oxide.*

Protein Adsorption. The positive results of the ESCA analysis, shown in Figure 3, are verified by the results of protein adsorption tests. Figure 4 shows a fluorescence microscopy image of adsorbed fluorescently labeled BSA on a patterned surface.

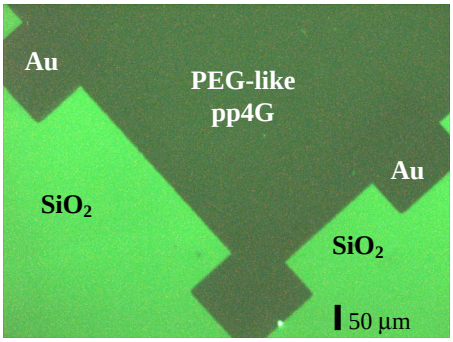


Figure 4. *A fluorescence microscope image from a fluorescently labeled protein adsorption experiment on a patterned pp4G and SiO_2 surface. Adsorbed proteins covered exposed SiO_2 areas while pp4G coated domains resisted protein adsorption.*

Here, an array of Au squares (200 by 200 µm) was first deposited on an oxidized silicon wafer, then pp4G was deposited and patterned. The coating was measured to be approximately 20 nm from a stylus stepper (Alpha-Step). It is readily seen, in Figure 4, that proteins adsorbed non-specifically only to exposed SiO_2 areas (bright areas) whereas the pp4G coated regions appear dark in the absence of adsorbed proteins. Note that the Au grids appear dark as well because of the well-known quenching interaction between fluorophores and metal surfaces [**16**].

Cell Patterning. The data presented above demonstrate the remarkable properties of the pp4G as a micro-machined non-fouling material. As such, the patterned pp4G coatings appear to be an excellent choice for building micro-scale cell cultures. For the cell culture experiment we used a substrate with silicon oxide micro-domains on a pp4G background. After 4 days of culture, adherent cells were fixed and stained. The cell cytoskeleton, *f*-actin, stained with rhodamine phalloidin is false-colored red (appears bright). DNA inside the cell nucleus, stained with DAPI, is false-colored blue (appears dark). Cells were found to adhere only to the oxide domains and not the pp4G background. Figure 4 shows the microscopy image of a "W"-shaped cell culture on the patterned substrate.

The "W"-shaped silicon oxide domain is cell-adhesive because of the adsorbed serum proteins from the culture medium as cells attached to adsorbed protein layers. The non-fouling background prevented cell adhesion by blocking protein adsorption. BAECs, which typically prefer to form a monolayer in culture, started to attach to each other due to a limited number of adhesive sites on patterned surfaces. Cell multilayers and cellular aggregates were observed as a result. However, BAECs did not attach or migrate onto the non-fouling pp4G background. The cell pattern was observed for more than two weeks in culture until the cells died off, likely due to a lack of nutrients from overcrowding in the multilayer. Particularly, the cells attached to the surface were affected more severely because of the reduced nutrient transport to the bottom of the multilayered structure.

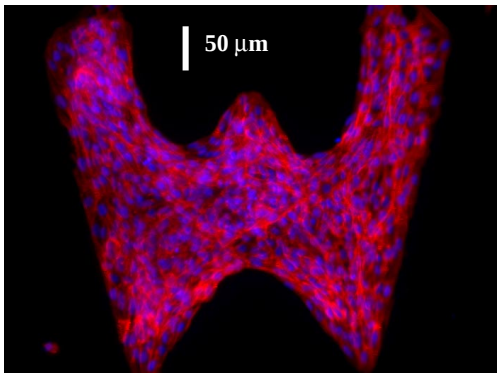


Figure 5. *A cell pattern achieved by culturing BAECs on a "W"-shaped silicon oxide micro-domain in a pp4G background (200x). Despite the multilayer formation of these cells on silicon nitride, they remained within the domain boundary.*

Electrode Coatings. The well-hydrated nature of the pp4G coating and its thickness suggest its potential as an inert protection layer for electrochemical electrodes. Good ionic conductivity is particularly important for the construction of reliable microelectrodes for neural recording.[**17**] We verify the conductivity properties of pp4G by comparing the ionic conductivity of electroplated electrodes with and without pp4G coatings. The pp4G coating was integrated into the following process: A CrAu seed layer for an electroplating step was sputtered on a nitride layer. Photoresist was then patterned and the wafers were electroplated with platinum and then rinsed in acetone to remove the photoresist. A second layer of photoresist was applied and patterned and the seed layer was etched in gold etchant. A third lithography process defined the pp4G patterns.

Our devices consist of two sets of six square electrodes with different sizes (250 to 2250 µm side lengths). One set is coated (Figure 6a) while the second is bare. To demonstrate the effectiveness of the coating we show images of the electrodes with and without pp4G (Figures 6b and 6c, respectively) after 24 hours in a solution with bovine aortic endothelial cells. In Figure 6b the entire surface is covered with adherent cells, albeit not yet a confluent monolayer after one day. While in Figure 6c, due to the protection provided by the pp4G coating, cells do not attach to the electrodes.

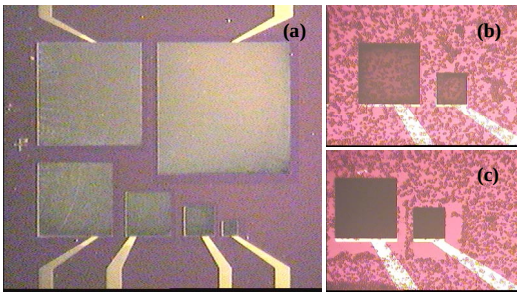


Figure 6. *(a) Electroplated platinum electrodes (dark squares) with pp4G coating on silicon nitride surface. The two smallest electrodes ((b) bare and (c) coated) after 24 hours in a medium with bovine aortic endothelial cells.*

AC resistivity for bare and pp4G coated electrodes was found to be close (i.e. at 10 Hz the resistivity is $7 \cdot 10^9$ and $5 \cdot 10^9$ $\Omega\mu m^2$ for bare and coated electrodes respectively) in cell-containing media. This suggests the ability of the pp4G to protect the electrodes from bio-fouling while maintaining the electrode conductivity properties. Further *in vivo* experimental study with coated and bare electrodes will clarify the significance of the coating to various issues such as tissue irritation, signal to noise ratio, signal stability, and signal distortion.

CONCLUSIONS

In summary, key features of this non-fouling technology include excellent coating quality, good biocompatibility, and ease of incorporation into MEMS fabrication. Because pp4G is patterned by a photoresist lift-off process, high alignment precision and good spatial resolution are attainable. This enabling technique, thus, offers precise spatial control of protein adsorption and cell attachment with good alignment to underlying features. Such precision can be a great advantage in engineering the surface chemistry of bioMEMS, biosensors, and cell-based assays to better interface devices with the biological environment. In addition to being a non-fouling material, pp4G is also ionically conductive. Thus, it can be applied to control bio-fouling on micro-electrodes without affecting the electrode conductivity properties.

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