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Protein Resistance of Surfaces Modified with Oligo(Ethylene Glycol) Aryl Diazonium Derivatives

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Anti-fouling surfaces are of great importance for reducing background in biosensor signals. Oligo(ethylene glycol) (OEG) moieties are commonly used to confer protein resistance on gold, silicon and carbon surfaces. Herein, we report the modification of surfaces using electrochemical deposition of OEG aryl diazonium salts. Using electrochemical and contact angle measurements, the ligand packing density is found to be loose, which supports the findings of the fluorescent protein labelling that aryl diazonium OEGs confer resistance to nonspecific adsorption of proteins albeit lower than alkane thiol-terminated OEGs. In addition to protein resistance, aryl diazonium attachment chemistry results in stable modification. In common with OEG species on gold electrodes, OEGs with distal hydroxyl moieties do confer superior protein resistance to those with a distal methoxy group. This is especially the case for longer derivatives where superior coiling of the OEG chains is possible.

1. Introduction

Often amperometric biosensors are required to operate in complex biological media such as blood or food. Part of the challenge such devices face, is that the matrix to be analysed contains many protein species that may foul the electrode and many other electroactive species that may give interfering current signals. The challenge here is that most amperometric biosensors require small diffusing redox active species to be able to move between the biological recognition molecule and the electrode. Hence they are open and accessible to species in solution. We[1] and others[2] have addressed this issue using a modified electrode where electroactive interferences and proteins are hindered from accessing the electrode interface using oligo(ethylene oxide) molecules (OEGs). In such interfaces, as the OEG molecules will passivate the electrode, to achieve electrochemical transduction of the resultant biosensor, molecular wires are used. This strategy has been employed with considerable success for DNA biosensors[2], enzymatic systems[1a] and immunosensors[1b-d]. For example, we have employed our immunosensor that uses this interface to detect the antibiotic enrofloxacin in undiluted milk and achieved analytical results as good, if not better, as those achieved with a standard LC-MS/MS technique[1d]. We have since expanded the applications of this immunosensor to the analysis of proteins in serum[3].

The effectiveness of the OEG components in these interfaces is crucial to the final device performance. The ability of OEG molecules to limit nonspecific adsorption of proteins to surfaces when they are coupled to a gold surface by alkanethiol chemistry is well studied. Grunze *et al.*[4] has previously investigated OEGs and other oligoethers on both gold and silver surfaces and has concluded three general guidelines as to what is required for SAM's to have good protein resistance[5]. Firstly, the hydrophilicity of the termination is important. The more hydrophilic the distal end is, the better the protein resistance[5-6]. Secondly, the hydrophilicity of the internal units is important; the less hydrophilic the internal structure is, the less protein resistant the surface is[5,7]. Thirdly the lateral packing density is also of importance[5,8].

We[9] and others[10] have been exploring the use of aryl diazonium salt chemistry for modifying electrode surfaces primarily because such chemistry gives a very stable modification layer. On these substrates very little is known regarding the ability of the OEG molecules to resist nonspecific adsorption of proteins. The only studies to explore this class of molecules derived from aryl diazonium salts are on carbon electrodes by Downard *et al.*[11] and furthermore Liu and Gooding[1a] suggest these molecules may not be as effective as the alkanethiols on gold. The effectiveness of OEG layers has been shown to vary considerably on different substrates. For example on silver the density of OEG molecules was considerably higher with a decrease in the efficacy at hindering nonspecific protein adsorption[5,12]. Similarly, on silicon the efficacy is also not as good as on gold but in the case of silicon this is attributed to a lower density of OEG groups than on gold[13].

From these previous studies it has been shown that OEG packing and water solvation are important factors in conferring protein resistance. Protein resistance via OEGs has been seen on all surfaces, however only the difference of chain length and distal ends has been thoroughly explored on gold and silicon.

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The purpose of this paper is to determine the effectiveness of aryl diazonium OEGs for the production of protein resistant layers on thin pyrolysed photoresist carbon films (PPF) and gold surfaces. We explore the effect of changing the distal end and chain length of the aryl diazonium OEGs by monitoring the efficacy of these molecules at resisting nonspecific adsorption of proteins. The aryl diazonium OEG-modified surfaces are compared to self-assembled monolayers of thiolated OEGs on gold surfaces by measuring the amount of protein on the surface directly with a fluorescence microscope[11].

2. Results and Discussion

2.1 Investigating the packing of aryl diazonium OEGs on PPF and gold

In order to explore the effects of changing chain lengths and distal end groups on protein resistance of surfaces, molecules based on tri(ethylene glycol), hexa(ethylene glycol), decane, and methyl-terminated versions thereof were prepared. A total of eight aryl diazonium and thiolated OEGs were synthesised for the purposes of this study (Scheme 1). Details of the syntheses can be found in the Supplementary Information.

By observing the ease of surface modification, the electrochemical properties of the formed layer, and by making contact angle measurements, it is possible to say something about the packing density and therefore the relative protein resistance of aryl diazonium OEGs.

2.1.1 Deposition of aryl diazonium OEGs

The reduction of the OEG aryl diazonium salts was conducted by cycling the potential between 0.2 V to -1.5 V for PPF surfaces and 0 V to -1 V for gold surfaces. The smaller potential range employed on gold is because of the reduction of water on the gold surfaces beyond -1 V. The cyclic voltammograms of the reductive adsorption of all compounds on carbon and gold surfaces are shown in the Supplementary Information. In the process of reductively adsorbing the organic layer the electrode is passivated. The passivation of the surface by the attachment of the OEG aryl diazonium salts to the surface typically took more redox cycles than has been observed with smaller and simpler aryl diazonium salts. That is, 5 to 20 cycles were required to achieve a good passivating layer with OEG aryl diazoniums compared with 2 cycles for 4-carboxyphenyl diazonium salt[14]. Similar passivation behaviour was observed with *in situ* attachment of aryl diazonium OEGs. The greater difficulty in passivating the electrodes could be linked to the conformation of the OEG. At first it is loosely packed with no structure and could partially block the surface from further modification until more molecules are attached to the surface to give a more structured conformation. As observed by Whitesides and co-workers[12] that for loosely packed OEG_3OMe on gold surfaces, the OEG tail takes on an amorphous like structure. As the surface becomes more densely packed it takes up a helical structure, and as it becomes more densely packed, a planar *trans* formation is taken. It was also discussed by Downard *et al.* that the layers formed by the reduction of aryl diazonium salts are loosely packed.[11] For subsequent studies of the different molecules, to allow consistent comparison with the different modifying molecules, a well passivated surfaces were used such that the ability of the surface chemistry to provide protein resistance was investigated.

The electrochemical reduction of the aryl diazonium salts gave more well-defined voltammetric peaks than on the PPF surfaces than those observed of the gold surfaces (Figures S1 and S2). It has been observed previously by Gooding and co-workers that aryl diazonium salts behave differently towards gold surfaces than carbon surfaces.[15] For small aryl diazonium salts the reduction peak tends to be more well defined on gold than carbon surfaces, but for larger aryl diazonium salts the behaviour on gold surfaces in comparison to carbon surfaces can vary. This variation in the behaviour of the electrochemical reduction large aryl diazonium salts towards gold surfaces and carbon surfaces is noted by the broadness in the voltammetric reductions peaks on the gold surfaces (Figure S2).

2.1.2 Electrochemical Passivation of Modified Surfaces

To evaluate the modification of surfaces with aryl diazonium salts a comparison of the electrochemistry at the underlying PPF electrode before and after modification was performed with 1.0 mM $Fe(CN)_6^{3-/4-}$ in the form of potassium ferricyanide. The results of these experiments are shown in the Supplementary Information (Figures S3 and S4). It was observed that the aryl diazonium salt derived layers passivated the electrodes towards $Fe(CN)_6^{3-/4-}$ and that all three OEGs tend to passivate the surface to near the same extent (Fig. S3).

The aryl diazonium salt derived layers on gold are observed to behave in a similar manner to those on PPF. The passivation of $Fe(CN)_6^{3-/4-}$ (Fig. S4) can be seen. It is also noted that both the decane and perhaps OEG_6OH aryl diazonium modified layers give complete passivation towards $Fe(CN)_6^{3-/4-}$ (Fig. S4) whilst both OEG_3 aryl diazonium modified layers give similar passivation results.

2.1.3 Contact Angles

To examine the hydrophobicity and hydrophilicity of the modified surfaces contact angles on the surface with water were measured and compared with literature values (Table 1). From literature it was observed that by increasing the number of ethylene glycol units the contact angle decreases slightly, and changing the distal group from hydroxy to methoxy increases the contact angle.

The contact angles of the aryl diazonium derived PPF surface have lower contact angles for that of the OEG derivatives to those of both the bare PPF and the decane aryl diazonium derived surface. A similar trend was observed for the aryl diazonium salt derived layers on gold surfaces.

The contact angles of the OEG aryl diazonium derived layers on the PPF were larger in comparison to those on gold suggesting that the packing of the OEGs on PPF is less dense than on the gold surfaces. This difference in contact angles from PPF to gold, and the suggested better packing of the aryl diazonium derived layers on gold than PPF is not reflected in any difference in the protein resistance, as will be discussed later.

Comparing the contact angle results with those observed from deposition of aryl diazonium OEGs and electrochemical passivation of the modified surfaces, a consistent trend indicating a loosely packed molecular layer can be discerned. The large number of cycles required in the surface preparation can be linked to an extended conformation of OEGs hindering the attachment of further molecules. The assessment of electrochemical passivation indicated that, in the presence of redox-active species, only decane presented a completely blocked surface. Finally, the contact angle measurements showed a deviation from the trend observed on densely-packed thiol layers on gold where attachment of hydrophobic distal groups increases the contact angle. By contrast, in the loosely-packed aryl diazonium OEG layers, conformation upon attachment to the surface was more important, with a hydrophobic distal group not significantly increasing the contact angle. Hence, compared with a tightly-packed surface layer, a lower protein resistance would be expected for the aryl diazonium-formed layers.

2.2 Resistance to Non-Specific Protein Adsorption on Aryl Diazonium OEG-Modified Surfaces

For the purposes of this study, the ability of a modified surface to resist non-specific protein adsorption is defined as the reduction in adsorbed fluorescently-labelled BSA (fluorescein-BSA, or FITC-BSA) on OEG modified surface as compared to a decane-modified surface. This ability to reduce the amount of protein can be referred to as protein resistance.

Downard *et al.* have previously used a fluorescent microscope to look directly at modified surfaces to measure the amount of protein on the surface qualitatively against bare surfaces[11,16]. The fluorescence microscope method allows a qualitative comparison of the different OEG aryl diazonium derived surfaces by the comparison of the modified areas of PPF to the surrounding bare PPF surface. By using large area electrochemical cells the PPF surfaces could be prepared that had a bare outer edge and a modified inner section as illustrated in Scheme 2. This allowed for internal calibration of the samples.

Images were taken of both the modified and unmodified areas (Figure 1). The fluorescence of each surface was characterised by measuring the mean grey scale of each image. For each surface the mean grey scale of the modified area was compared to either the bare surface, as carried out for PPF surfaces, or the decane modified surface, as for the gold surfaces (as the bare gold surface was found to quench the fluorescence of the FITC).

The aryl diazonium derived surfaces on PPF are compared to both the OEG aryl diazonium derived gold modified surfaces and the thiol derived gold surfaces. The use of the OEG thiol derived surfaces, as a comparison, will allow benchmarking of the performance of the aryl diazonium salts to a well understood OEG modified surface that is effective at reducing nonspecific adsorption of proteins[12].

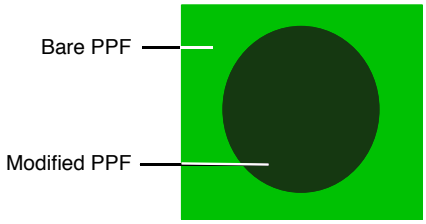
It can be seen from the results shown in Figure 2, that alkane thiol terminated ethylene glycols gave the biggest reduction in protein resistance of around 90% in comparison to decane modified surfaces, as expected from the work of Harder *et al.*[12]. Aryl diazonium derived ethylene glycol derivatives on PPF showed an increase in protein resistance in comparison to those on gold, with a reduction of between 50-70%. It was seen from Boecking *et al.*[13a] that OEG_3OMe modified silicon surface gave a 70-80% reduction of protein in comparison to octadecyl Si-C layers. The observed difference in protein resistance may be due to difference in packing density between OEG layers on PPF and OEG layers on silicon.

The changes in the distal end and amount of ethylene glycol units had little effect on protein resistance for OEG aryl diazonium derived modification layers on PPF. As Grunze *et al.*[5] showed that both these factors should have an effect on protein resistance. Therefore, the low molecular packing density of the aryl diazonium salt derived layers must be dominant factor in determining the protein resistance on these PPF surfaces. The effect of the change of the distal end of OEG-aryl-diazonium-modified-gold surfaces shows a decrease in the non-specific adsorbed protein from methoxy to hydroxyl (Figure 2). The variation of protein adsorption between alkane (decane) modified surfaces was also investigated. It can be seen that little difference can be observed between the thiol-derived-gold surfaces and the aryl-diazonium-derived-PPF surfaces in the intensity of fluorescence measured. The aryl diazonium derived gold surfaces though, were twice the intensity of measured fluorescence in comparison to both the aryl diazonium derived PPF surface and the thiol derived gold surfaces (see Figure S5). This difference in intensities suggests that the aryl diazonium derived gold surfaces absorb greater amounts of protein in general, but that the degree of protein resistance can still be modulated by modifying the surface with aryl diazonium OEGs.

Some recent work of aryl diazonium OEGs grafted to a carbon surface has been conducted by Liu *et al.* [1a,17] and Downard and co-workers[11,16]. Liu showed that a tri(ethylene glycol) aryl diazonium with a methoxy on the distal end gave a reduction in protein adsorption in comparison to a bare glassy carbon[1a]. Whilst Downard and coworkers investigated the difference in protein adsorption of the same OEG aryl diazonium salt as well as various other PEG molecules attached to glassy carbon surfaces through electrochemical reduction.

Downard and co-workers also studied a diamine PEG and an OEG with a propyl amine distal end[11,16]. From this latter study it was noticed that the aryl diazonium OEG did not have good protein resistance and showed more protein on the modified surface than that of the bare glassy carbon. This was also thought to be due to the loose packing of the aryl diazonium salt derived layers, and is consistent with previous studies showing that nitroazobenzene films have a greater than 50 % free volume[11].

Scheme 1. Oligo (ethylene glycol) derivatives used for determination of protein resistance on PPF and gold surfaces. Proximal end: **1-5)** diazonium salts; **6-8)** thiols. Distal portion: **1,7)** OEG_3OH; **2,8)** OEG_6OH; **3)** OEG_3OMe; **4,6)** Decane; **5)** OEG_6OMe.



Scheme 2. Illustration showing a PPF sample exposed to BSA-FITC with a bare PPF outer edge and a modified inner circle.

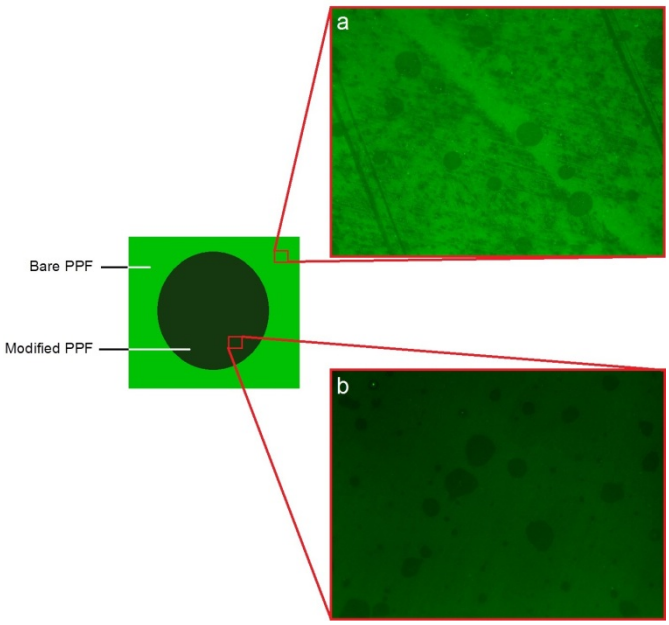


Figure 1. Resistance to protein adsorption on modified PPF surface. Insets show images taken with a fluorescence microscope with a 60×60 oil lens of a) bare and b) OEG_3Me modified regions of a PPF surface. The fluorescence intensity is taken to be indicative of the amount of adsorbed FITC-BSA.

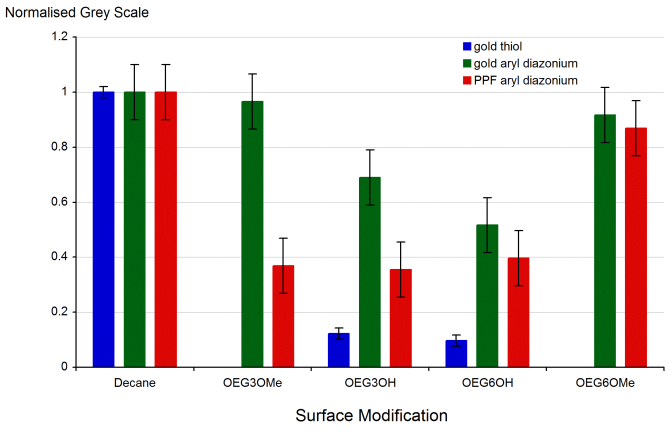


Figure 2. Ratio of mean grey scale of OEG aryl diazonium derived surfaces on gold and PPF and OEG thiol derived gold surfaces in comparison to decane aryl diazonium derived PPF and gold surfaces plus dodecane thiol derived gold surfaces. By comparing to decane modified surfaces and indication as to the resistance of the surface to non-specific protein adsorption is gained. As were recorded from the measurement of the fluorescence of BSA-FITC absorbed onto the surface *via* a fluorescence microscope. Error bars indicate the standard deviation.

Table 1. Comparison of contact angles of aryl diazonium derived layers on gold and PPF surfaces, and thiol derived surfaces. Literature values were also noted for comparison. Where ± is the standard deviation of three measurements.

Surface group	Si [a]	Gold (thiol)[b]	Gold (thiol)	Gold (aryl diazonium)	PPF
Bare				82.2 ± 1.5 °	90.8 °
Decane			105.4 ± 2.5°	84.8 °	76.6 ± 6.0 °
OEG_3Me	57-59 °	65 °		51.3 ± 4.5 °	51.9 ± 3.1 °
OEG_3OH		34 °	30.0 ± 4.0 °	57.0 ± 3.3 °	68.5 ± 4.5 °
OEG_6Me	49 °	67 °		37.5 ± 20.5°	17.0 ± 4.8 °
OEG_6OH		32 °	45.8 ± 9.4 °	54.8 ± 2.9 °	69.4 ± 7.6 °

[a] Literature values[13]. [b] Literature values[5].

3. Conclusions

From this study it can be concluded that the use of OEG-aryl-diazonium-derived layers on both gold and PPF surfaces were able to reduce the amount of protein adsorbed onto the surface when compared to alkane-aryl-diazonium-derived layers. The protein resistance of the OEG aryl diazonium derived PPF surface is consistent with previous studies[12-13,16,18] showing that loosely packed surfaces produce lower protein resistance. As seen by the difficulty of deposition of the aryl diazonium layer, the incomplete electrochemical passivation, and the similarity of the contact angles, it is concluded that the packing of the OEG aryl diazonium derived modification layers on PPF are loosely packed.

The use of a fluorescent microscope to measure qualitatively the amount of protein on the surface is useful for PPF surfaces where the fabrication method only allows for modification on one side of a chip. Using FITC-labelled BSA, it was possible to compare the protein resistance between surfaces modified with different aryl diazonium OEGs.

Experimental Section

Chemicals, Solvents and Materials: The following chemicals, solvents and materials were purchased, and used without modification or purification unless stated otherwise, tri(ethylene glycol), tri(ethylene glycol) monomethyl ether, hexa(ethylene glycol), 1-fluoro-4-nitrobenzene (99% purity), tetrabutylammonium hydrogen sulphate (98% purity), sodium bicarbonate, nitrosonium tetrafluoroborate, tetrahydrofuran (THF), sodium hydride (70% purity), and iodomethane were purchased from Aldrich (Sydney, Australia), 1-decanol was purchased from Sigma (Sydney, Australia). Sodium hydroxide, ethyl acetate, magnesium sulphate, hydrochloric acid, ethanol, acetonitrile, methanol, ammonia solution, and diethyl ether were purchased from Ajax Chemical Co. (Sydney, Australia). The 10% palladium on carbon was purchased from Lancaster Synthesis (MA, USA). Silica gel (grade 9385, 230-400 mesh, Merck) was used for column chromatography and silica gel on aluminium (60 F254, Merck) for thin layer chromatography (TLC).

Potassium ferricyanide (99% purity) was purchased from Aldrich (Sydney, Australia). Fluorescein-labelled BSA (FITC-BSA) was purchased from Sigma (Sydney, Australia).

All the aqueous solutions were prepared in Milli-Q water (18 MΩ cm, Millipore, Sydney, Australia).

Instrumentation: ^{1}H NMR spectra were obtained on a Bruker DPX300F (300 MHz) spectrometer. ^{1}H chemical shifts (δ) are reported in parts per million (PPM) downfield from TMS.

^{13}C NMR spectra were obtained on a Bruker DPX300F (75.6 MHz) spectrometer. ^{13}C chemical shifts (δ) are reported in parts per million (PPM) downfield from TMS.

Electrochemical measurements were performed with an Autolab PGSTAT 12 potentiostat (Eco Chemie, Netherlands) interfaced to a personal computer. Data acquisition was performed using GPES and Nova software. Cyclic voltammetry and square wave voltammetry experiments were carried out with a conventional three electrode system, comprising a bare or modified working electrode, a platinum flag counter electrode and a Ag|AgCl 3.0 M NaCl reference electrode (from Bioanalytical Systems Inc., Lafayette, IN, USA). All potentials are reported *versus* this reference at 25°C unless otherwise stated.

General Synthesis of Aryl Diazonium Derivatives of Ethylene Glycol: All aryl diazonium salts used were custom synthesised. The same method for the synthesis of the aryl diazonium salts was carried out for all OEG derivatives. The previous method for the synthesis of OEG_3OMe[1a,17] has been modified, with the first two steps of tosylation of the OEG and subsequent addition of a nitrobenzene replaced with a single step of addition of the nitrobenzene by reacting with 1-fluoro-4-nitrobenzene. Details of individual syntheses for each aryl diazonium salt are given in the Supplementary Information.

Setup for Electrochemical Modification of Surfaces:

Aryl Diazonium OEG attachment to PPF: For the assessment of aryl diazonium salts on carbon electrodes, 1.4 x 1.4 cm plates of pyrolised photoresist (PPF) were used, fabricated as described previously[19].

An Ag|AgCl 3.0 M NaCl reference electrode and a platinum wire counter electrode were placed in the top of a custom-made Teflon electrochemical cell[20]. The PPF was sandwiched in the base of the electrochemical cell which gives a controlled area of PPF by allowing only a disc with a 1 cm diameter to be exposed to the appropriate aryl diazonium salt. The rest of the surface was left unmodified such that each sample has its own control surface.

Each surface modification solution was made separately for each surface due to the large surface area required. Acetonitrile was degassed before making the solution to reduce volume loss. Tetrabutyl ammonium tetrafluoroborate (17 mg) was used as the electrolyte and 10 - 20 mg of OEG was used (see Table S1, Supplementary Information). The two dry ingredients were placed in a small glass sample vial and degassed acetonitrile (1 mL) was added and the solution was added to the Teflon cell. A platinum counter electrode and a Ag|AgCl reference electrode was used and the cell was cycled from 0.2 V to 1.2 V several times depending on the OEG aryl diazonium salt used (see Table S1, Supplementary Information).

Water contact angles of the modified surfaces were measured using Ramé-Hart standard contact angle goniometer and data acquisition collected on a personal computer. The angles were processed with *dropimage*.

Aryl diazonium and alkanethiol attachment to gold: For gold surfaces, a 1 cm by 0.5 cm gold foil pieces were used as the surface substrates. The foil pieces were cleaned by leaving them in a solution of piranha (sulphuric acid and hydrogen peroxide with a 2:1 ratio respectively) overnight. The surfaces were then rinsed with copious amounts of Milli-Q water followed by ethanol. When modified with aryl diazonium salts, the same modification procedure as for the PPF surfaces was employed. When the gold surfaces were modified with alkanethiols the cleaned gold surfaces were soaked for 1 hr in redistilled ethanol to remove oxides. The foil pieces were then placed in a solution of the appropriate alkanethiol in ethanol (1 mM, 3 mL) for 18 hrs at room temperature before being removed and rinsed with ethanol. The alkanethiols used were decanethiol, triethylene glycol mono-11-mercaptoundecyl ether and hexa(ethylene glycol)mono-11-mercaptoundecyl ether.

Electrochemistry of Modified Surfaces: Each surface was tested before and after modification with 10 mM ferricyanide ($Fe(CN)_6^{3-/4-}$) in 1.0 M KCl. All solutions were degassed with dry nitrogen for 15 minutes prior to use. For each redox probe, three cycles at 100 mV s^{-1} were run employing CV techniques. Scan ranges were -0.6 V to 1.0 V for ferricyanide, unless otherwise stated.

Non-Specific Protein Adsorption Measurements: For each OEG aryl diazonium derivative, nine PPF surfaces were made and four gold surfaces. The PPF surfaces were modified in the middle of the wafer leaving an unmodified section for comparison on the outside. Gold foil pieces were completely modified on both sides leaving no unmodified section for comparison. Exposure to protein was carried out in a dimly lit laboratory to prevent the BSA-FITC from fading. Modified surfaces were exposed to 1.5 mL of BSA-FITC (1 mg/mL) in PBS solution for 1 hour in plastic Eppendorf tubes. The reactions were protected from light by covering with foil. The BSA-FITC solution was filtered through a 0.22 μm sized filter before exposure to the modified surface to remove large clumping of the protein before it is exposed. After an hour of exposure, the solution was then removed. A solution of PBS was subsequently added to the tubes containing and the surfaces were soaked for 7 min. The PBS solution was removed and replaced with Milli-Q water, twice, to remove any salts from the surface. The surfaces were gently dried with nitrogen and mounted surface side down onto glass microscope slide covers that were cleaned via sonication in ethanol and rinsing with ethanol and dried under nitrogen. The surfaces were fixed to the glass slide coverslip with mowiol, an antifade fixative. The samples were left over night in the dark to set.

The amount of BSA-FITC adsorbed onto the surface was recorded by measuring the intensity of fluorescence with a Leica DM IL inverted microscope with transmitted light LED back illumination and an EL6000 Fluoro system. The images were taken with a ProgRes CFscan CCD

camera attached to the Leica DM IL inverted microscope. The filter set GFP was used for blue excitation and the objective 63x NA1.25 oil was used to investigate the surfaces. The images were exposed for 100 ms. For the PPF surfaces six images were captured on the non-modified outer areas of the PPF and six images taken from the modified inner area. By taking images on the same surface of modified and bare surfaces exposed the BSA-FITC allows an internal standard for the adsorption of protein. The amount of fluorescence was then processed using the image processing program, *ImageJ*. For the analysis of the images the average amount of grey scale was measured for each image of the intensity of fluorescence on the surface and divided by the area measured. The fluorescence intensity could then be normalised for each set of samples by dividing the measured intensity by the bare surface value to get a grey scale ratio. The control surface was modified with decane, and a final normalisation against the decane ratio was performed to allow for comparisons between the PPF and gold surfaces.

For the gold, six images were taken anywhere on the gold surfaces, as the entire surface was modified and the intensity was standardised against a surface modified with the alkane, either the aryl diazonium decane derivative and the dodecane thiol derivative which was measured on the same day.

Controls were run of each OEG aryl diazonium derivative on both PPF and gold surfaces. For each OEG aryl diazonium salt three PPF surfaces were modified and three gold surfaces were modified, as per the premade diazonium aryl diazonium salt attachment as discussed above. The modified surfaces were exposed to a solution of PBS for 1 hour in plastic tubes. The solution was then removed and another solution of PBS was added and the surfaces were soaked for 7 min. The PBS solution was then removed and replaced with Milli-Q water, this was repeated to remove any salts from the surface. The surfaces were gently dried with nitrogen and mounted surface side down onto glass microscope slide covers with mowiol. The glass microscope slide covers were cleaned via sonication in ethanol and rinsing with ethanol before being used. The samples were then left over night in the dark to set. The fluorescence of each surface were measured on the fluorescence microscope in the same manner as the modified surfaces exposed to protein.

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