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Surface Modification of a Perfluorinated Ionomer Using a Glow Discharge Deposition Method to Control Protein Adsorption

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Abstract

Nafion™ is the membrane material preferred for *in situ* glucose sensors. Unfortunately, surface properties of Nafion promote random protein adsorption and eventual foreign body encapsulation thus leading to loss of glucose signal over time. Here we detail surface modifications made by RF plasma deposition to Nafion with the intent to prevent random protein adsorption while providing enough functional sites (hydroxyl groups) to bind a biologically active peptide known to induce cellular adhesion (YRGDS). Nafion surfaces were modified by RF plasma polymerizing five different combinations of (1) tetraethylene glycol dimethyl ether (tetraglyme) and (2) 2-hydroxyethyl methacrylate (HEMA): pure tetraglyme, 2.5% HEMA/97.5% tetraglyme; 5% HEMA/95% tetraglyme, 10% HEMA/90% tetraglyme; and pure HEMA. Resultant surfaces were characterized by XPS (low and high resolution), dynamic contact angle, and atomic force microscopy. Protein adsorption and retention was determined and correlated to surface layer composition. The ability to bind a cell adhesion peptide was also determined and correlated well with surface layer composition.

1. Introduction

Increasing the lifetime of an implantable glucose biosensor would be quite advantageous for diabetic patients, decreasing their chances for related complications, such as blindness, kidney failure, heart disease, among others [1-3]. Implantable glucose biosensors are dependant on the transport of analytes to and from their surface. It is now well established that upon implantation, denatured and non-native protein structures facilitate the eventual formation of a fibrous capsule, responsible for limiting the utility of the biosensor in an *in vivo* environment [4]. To alter the typical biological response and limit the formation of a fibrous capsule, various approaches have been taken, the majority of which has revolved around creating “stealth” or “non-fouling” surface coatings, impervious to protein adsorption. Lopez and Ratner [5] developed a remarkably effective method of creating inert, “non-fouling” surfaces using the plasma deposition of ethylene glycol dimethyl ether, or “oligoglymes”, where monomers could be mono, di, tri, or tetra-glymes. The most effective at preventing random protein adsorption was found to be tetraglyme. Most recently this method has been used for decreasing fibrinogen adsorption and subsequent monocyte adhesion [6,7], both *in vitro* and *in vivo*. Interestingly, however, in the *in vivo* environment, there was an increase in monocytes adhesion to tetraglyme coated surfaces. Perhaps the most alarming phenomena observed was that after 4 weeks of implantation, the fibrous capsule around coated and uncoated implants was virtually the same,

even after proving that *in vitro*, the protein adsorption had been significantly decreased [7]. These findings indicate that a better approach may be to maintain a non-fouling substrata, while concomitantly immobilizing biological entities in order to control biological responses and elicit healing characteristics in the surrounding tissue.

The commercially available polymer Nafion™ (Dupont Chemical), has been used in various types of biosensors, including nitric oxide sensors [8-15] as well as glucose/oxygen sensors [16,17]. For a glucose biosensor, Nafion serves four purposes, namely: a) to reject large, negative molecules [18], b) to physically protect the glucose oxidase enzyme that drives the chemical reaction of the biosensor (acting as a physical barrier), c) to act as a glucose transport-limiting membrane, thereby reducing the oxygen requirement of the biosensor and d) to maintain some level of biocompatibility, especially after curing of the Nafion membrane [19].

This manuscript details the surface modifications done on the surface of Nafion. Our intent is to prevent the random adsorption of proteins at the surface and at the same time, provide enough functional sites (hydroxyl groups) to hold a biologically active peptide known to induce cellular adhesion (YRGDS). It is the goal of this work to engineer a glucose permeable membrane with a surface that cells actively interact with while preventing the foreign body response. Controlling cell behavior by reducing a fibrous capsule (composed mainly of collagen Type I) would increase the functional lifetime of an implanted glucose sensor. Tests on cell function as they relate to the synthesis of these new surfaces will be the subject of a subsequent manuscript in this series.

2. Materials and Methods

2.1 Preparation of surface coatings

Plasma polymerization, or polymerization initiated by a partially ionized gas generated by a radiofrequency glow discharge (RFGDD), is widely used for preparing coatings and insulating layers [20]. Its advantages have been described elsewhere [21].

Several variations of surface coatings were created using two base monomers individually or by combining the two at different concentrations. They were (1) tetraethylene glycol dimethyl ether (tetraglyme, Aldrich cat. no. 172405), known for its non-fouling characteristics [5] and (2) 2-hydroxyethyl methacrylate (HEMA, PolySciences Inc., ophthalmic grade) [22], known for its biocompatibility and hydroxyl group content. Combinations used were: pure tetraglyme, 2.5% HEMA with 97.5% tetraglyme; 5% HEMA with 95% tetraglyme, 10% HEMA with 90% tetraglyme and pure HEMA. Table 1 lists the different ratios of monomer feed, and subsequent labeling nomenclature.

Nafion disks were cut to shape (5×5 cm² or 6 mm diameter punch), cleaned, and placed on a rack. Monomer(s) were degassed by freezing-thawing cycles, and Nafion was etched in Argon plasma (40W, 0.175 mmHg) for 2 min. Monomer(s) were vaporized by heating to 105° C and RF maintained at 80 W for 1 min, 40 W for 30 sec, and 10 W for 17 min.

Either tetraethylene glycol dimethyl ether (tetraglyme, Aldrich cat. no. 172405), hydroxyethyl methacrylate (HEMA, PolySciences Inc., ophthalmic grade) or a mixture of both was placed in a custom made flask with a total volume of 20 mL. Monomer(s) were degassed by freezing-thawing three times with liquid nitrogen. Once inside the reactor, Nafion disks were etched briefly in an argon plasma (typically 40 W, 0.175 mmHg, 2 min) just prior to coating. Etching ensures the removal of atmospheric surface contaminants. Tetraglyme or tetraglyme mixed with HEMA was then vaporized by heating the monomer(s) to a 105-110°C and was then delivered to the reactor. At 350 mTorr, the RF plasma was maintained at 80 W for 1 min, was

then decreased to 40 W for 30 seconds, and was then maintained at 10W for 17 minutes, for a total running time of 18 minutes and 30 seconds. These conditions had been found to yield satisfactory non-fouling conditions on a similar substrate (Teflon) and were also found work for Nafion (see subsequent sections). After plasma deposition, the samples remained in the presence of the monomer for about 10 minutes to terminate reactive species on the film surface.

Nafion disks were also prepared with a pure HEMA surface using RF deposition. Nafion disks were cleaned and placed in the reactor as above. The HEMA monomer was then degassed three times using liquid nitrogen. Samples were then etched with argon plasma as specified above. HEMA was vaporized by heating the monomer to 60°C. Flow of vapor was regulated by a Teflon stopcock. At 350 mTorr, the RF plasma was maintained at 80 W for 1 min and kept at a pulsed plasma rate (in which the plasma is turned on and off every 100 ms) for 20 minutes. Samples were left in the reactor for 10 minutes to ensure the termination of reactive any species on the film surface.

2.2 XPS Analysis

X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis (ESCA), provides elemental characterization of a surface (with the exception of hydrogen and helium). The depth of a solid material sampled varies from the top 2 atomic layers to 15-20 layers. The area examined can be as large as 1 cm² or as small as 70 μm². XPS was used to analyze the newly synthesized modified Nafion surfaces for their elemental compositions using an SSX-100 surface analysis instrument (Surface Science Instruments or SSI) available at the National ESCA and Surface Analysis Center for Biomedical Problems (NESAC/BIO) at the University of Washington. The photoelectron take-off angle for the analysis was 55 deg, which corresponded to a sampling depth of 50 Å. Samples were mounted on an aluminum platform with double-sided tape. Charge compensation was provided by a 5eV flood gun and a fine nickel screen mounted 1-2mm above the samples. Survey scans (low resolution) were collected at three 1 mm spots per surface. High resolution C1s spectra were taken at the second spot per sample. The hydrocarbon peak for the XPS spectra acquired in this study was at a binding energy of 285.0 eV.

2.3 Investigation of hydroxyl content

To more clearly investigate the presence of hydroxyl groups at the surface of the tetraglyme, tetraglyme-HEMA and pure HEMA surfaces, a trifluoroacetic anhydride (TFAA, Aldrich Chemical) reaction was used. Figure 1.A depicts the chemical reaction, in which a fluorine group substitutes for any available hydroxyl group. Controls were necessary in order to determine the extent of the reaction. For a negative control, 20 ul of 2% (w/v) polystyrene (PS, Scientific Polymer Products, Inc.) in toluene was spin cast onto 12 mm glass coverslips at 4000 rpm for 20 seconds. For a positive control, a 2% (w/v) polyvinyl alcohol (PVA, Science Polymer Products, Inc.) solution was made in deionized water and was spin cast in a similar fashion. To properly dissolve the PVA, water was brought to 90°C for approximately 30 minutes using in a hot plate. This procedure has been described elsewhere [23, 24]. For the derivitization reaction to take place, samples were placed on a clean glass slide in a glass reactor tube. Placing the samples on a glass slide provided a reservoir for the insertion of 2 ml of TFAA into the larger glass reactor. Nitrogen was then streamed into the tube, before being capped by Teflon lined stopper. A hot plate was placed next to the glass reactor with an adjacent thermometer. Samples (chemical structure depicted in Figure 1.B) were heated to 50°C and allowed to react for 25 minutes. Samples were then carefully removed and placed in a tissue culture polystyrene dish, sealed and sent to be analyzed via XPS (as discussed above). Survey and high resolution C1s spectra were obtained to compare amount of fluorination.

2.4 Contact Angle Measurements

A common and inexpensive way of predicting material performance as it relates to its wettability is by measuring the surface energy via contact angles. After placing Nafion or modified Nafion surfaces on the stage of the goniometer, advancing and receding angles were obtained by carefully dropping a series of 7 μ l drops of water via an electric pump, always increasing the size of the drop. Five drops were dropped on one spot and contact angles taken after each one. Using the same system, 7 μ l of water were then aspirated back into the needle/tubing attached to the pump, and measuring the receding contact angle after each one (see Figure 3.5). The contact angle of polytetrafluoroethylene (Teflon) was measured as a control hydrophobic surface.

Critical surface tension (γ_c) was calculated from contact angle measurements by using the techniques developed by Fox and Zisman [25]. This technique uses several different liquid chemicals (with different surface tensions) to measure the contact angle. The chosen liquids and their respective surface tensions are as follows: Water (72.9); Formamide (58.4); Ethylene Glycol (47.99) (all units in dynes/cm). A 7- μ l drop of each liquid was dropped on the surfaces of Nafion and modified Nafion using a Rainin pipette. All measurements were done on a contact angle goniometer. (Courtesy of Dr. Allan Hoffman, University of Washington) and linear regression analysis was performed for data-fitting using Microsoft Excel.

2.5 Atomic Force Microscopy

In addition to surface chemistry and wettability of a material, surface topography (i.e. roughness) can also affect the type, quantity and conformation of an adsorbed protein layer [26]. For example, serum fibronectin has previously been shown to preferentially adsorb on rough, rather than smooth surfaces [27]. More recently, surface roughness has also been implicated with β 1 and β 3-integrin mediated cell adhesion [28], as well as having a direct relationship with collagen synthesis [29]. In order to investigate the surface roughness of the Nafion and modified Nafion surfaces, Atomic Force Microscopy (AFM) was used [30]. Surfaces were prepared as specified above, and examined by an AFM (Molecular Imaging PicoScan, Phoenix, AZ). Imaging was performed using acoustic AC (AAC) mode. Imaging in air was carried out with MikroMacsh (Wilsonville, OR) NSC35 ALBS tips. Three images were collected per scan: topography, phase, and amplitude. The topography images were further processed by second order line flattening and images were exported to Image J (NIH software).

2.6 Hydroxyl activation with Carbonyldiimidazole

Tetraglyme-HEMA surfaces were reacted with 1,1'-carbonyldiimidazole (CDI, Sigma) in anhydrous acetone. The process is depicted in Figure 2. Prior to the reaction, all glassware was dried in an oven, or gas flamed and samples were rinsed twice in acetone. This is because CDI can be easily hydrolyzed in solution, decomposing into two imidazole groups and CO₂. CDI (20mM) was then dissolved in acetone and added to samples (either Nafion modified disks or larger samples for cell culture). Samples were then placed in an oven at the temperature of 50°C for 2 hours. The concentration of CDI, time and temperature for reaction was previously determined as the optimal conditions for CDI reaction for polyHEMA substrates [24]. After 2 hrs, samples were removed from oven, and CDI solution was discarded. To remove the free imidazole molecule, samples were rinsed in acetone twice. Samples were immediately incubated with the YRDGS peptide, for immobilization onto CDI activated surfaces. The peptide was suspended at various concentrations in phosphate buffered saline (PBS) at a pH = 8.6, so that most or all amine groups on the surface of the peptide would be de-protonated. Peptide was allowed to react at 4 °C overnight.

2.7 Radiolabeling of proteins and peptide

In order to test the ability of the newly synthesized surfaces to repel proteins and/or attach YRGDS peptide, albumin, IgG, fibronectin, or the YRGDS peptide were radiolabeled with ^{125}I using the Iodobead™ method. The process is outlined elsewhere [31]. Briefly, peptide was reconstituted using PBS (Phosphate Buffered Saline) at pH= 7.2. First, Iodobeads were washed with 500 μl of reaction buffer per bead (PBS, pH 7.2). Beads were then dried on a Whatman filter paper. Next, one bead was placed in a 1.5 ml siliconized Eppendorf tube containing carrier free $\text{Na } ^{125}\text{I}$ diluted with reaction buffer and allowed to react for 5 minutes at room temperature. Next, the cold protein solution of known concentration (via BCA assay) was added to the bead mixture, sealed and allowed to react. The bead was separated from the solution after approximately 10 minutes (as this was previously determined to be an optimal time for reaction). The bead was then washed with a small quantity of buffer to recover any protein adhered to it. Unincorporated ^{125}I was removed from the iodinated protein using a hydrophobic column (Sep-Pak Cartridge, Waters, Inc.). First, the column was washed three times with 1 ml of 100 % ethanol. The column was then flushed with 10 ml of deionized water containing 0.1% trifluoroacetic acid, TFA, (Sigma). The newly iodinated samples were then loaded by first diluting them in 1.5 ml of deionized water containing 0.1% TFA. The loaded column was then rinsed twice with 2.5 ml of deionized water. Columns were finally eluted into siliconized tubes with 1.5 ml methanol, which recovered around 80% of the peptide. Methanol was then evaporated by placing the tube(s) in a concentrator/evaporator (Thermo Electron, Inc.) for approximately 4-8 hours or until peptide was dry. Radiolabeled peptide was then reconstituted in PBS and kept refrigerated at 4°C or frozen at -80°C, until needed and used within one-two weeks of iodination.

3. Results

3.1 XPS surface characterization

To characterize surfaces for their elemental composition, low resolution scans of unmodified and modified Nafion surfaces were done. Three scans were performed per surface. Figure 3 A-F shows unmodified Nafion, pure tetraglyme, 2.5% HEMA, 5% HEMA, 10% HEMA, and pure HEMA coated samples, respectively. Survey scans of Nafion exhibited fluorine, oxygen and sulfur (less than 2%), characteristic of its molecular structure. Low resolution scans from tetraglyme coated surfaces showed a PEO-like surface. Tetraglyme coated Nafion exhibited carbon and oxygen but no fluorine (Figure 3B). The same was true for subsequent mixed monomer surfaces.

From the low resolution scans, we obtain the elemental compositions of the surfaces, and list them Table 2. Each is the average of three scans per two samples $\pm$ standard deviation. All coatings exhibited an increase in the carbon and oxygen groups at the surface, and had a decrease in the fluorine groups, which are intrinsic in the Nafion structure. Based on their molecular structures, the theoretical C:O ratios for tetraglyme and HEMA are 2:1. The values reported here closely resemble these ratios, except for the surface coated with 5% HEMA.

High resolution scans of surfaces were taken at the C1s spectrum, to determine the types of carbon species of the surfaces. Figure 4 A-F shows the high resolution scans for unmodified Nafion and the various modified Nafion surfaces, in the same order as above. Unmodified Nafion contains peaks from fluorocarbon species in the range of 291-294 eV and showed no carbonyl groups (C=O) at 288 eV. Nafion coated with tetraglyme shows that the dominant carbon species was ether carbon (C-O) at 286.5 eV, with small amounts of hydrocarbon (C-H) at 285 eV and carbonyl groups (C=O) at 288 eV. Nafion surfaces modified with a 2.5% HEMA also exhibit similar percentages of the same species. Nafion surfaces modified with 5% HEMA exhibit an increase in the hydrocarbon content. Surfaces modified with 10% HEMA

exhibit very close ratios to that of tetraglyme coated surfaces, while HEMA coated surfaces follow the expected theoretical model of HEMA surfaces, previously estimated by XPS elsewhere [32], with an increase in hydrocarbons. Results are tabulated in Table 3.

3.2 Estimation of Interfacial Hydroxyl Levels

High resolution XPS scans can accurately detect the differences in the C1s carbon species as seen above. However, in order to more clearly investigate the presence of hydroxyl groups at the surface of the tetraglyme, tetraglyme-HEMA mixtures and pure HEMA surfaces, a trifluoroacetic anhydride (TFAA) derivatization reaction was utilized. A useful technique in determining the extent of hydroxyl groups on the derivatized surfaces is to obtain a Flourine to Carbon (F/C) ratio from the low resolution XPS survey scans. Chilkoti and Ratner have previously shown that TFAA vapor does not label alkenes, ethers, ketones, and aliphatic and aromatic esters. As discussed by the authors, TFAA displays seven-fold selectivity for hydroxyl groups as compared to acid groups [23]. These ratios are reported below in Figure 5. From this data, one can see that tetraglyme coated surfaces exhibited a very small F/C ratio, showing as expected, that hydroxyl groups were not present at this surface. Surprisingly, however, we found that as the concentration of HEMA (containing hydroxyl groups) increased in the monomer feed, hydroxyls were deposited on the surface at a corresponding lower ratio. Surfaces coated with pure HEMA exhibited a slightly higher ratio than theoretical ratio (0.5 vs. 0.38), most likely due to a thinner than desired coating. Positive (PVA) and negative (PS) controls also followed the theoretical values of 0.75 and 0, respectively.

3.3 Atomic Force Microscopy

Surface morphology and roughness profiles were obtained with AFM as rougher surfaces have previously been found to preferentially adsorb proteins as fibronectin, but not albumin [27, 33]. In addition, surface roughness has been implicated with $\beta 1$ and $\beta 3$ integrin binding [28] and cell differentiation [34,35]. To characterize changes in surface roughness, AFM images were taken of Nafion, tetraglyme coated Nafion and 10% HEMA coated Nafion. Images were processed using Image J (NIH Software), which provided a flattened topography image and a three-dimensional (3D) image. Figure 6 shows a sample of captured images at one of the sampling, namely, $6.72 \mu\text{m}^2$.

ImageJ was also used to generate several surface profile parameters (listed in detail in Appendix C), such as roughness averages (R_a), maximum profile valley depth (R_v), maximum profile peak height (R_p) and root mean square roughness (R_q , or RMS). RMS is the square root of the integral of the square of the roughness profile height over the evaluation length, and is expressed as follows:

$$RMS = \sqrt{\frac{1}{L} \int_0^L r^2(x) dx}$$

Table 4 lists the average RMS values obtained for the various Nafion surfaces.

3.4 Contact Angle Measurements

As expected, advancing angles were much lower on the tetraglyme coated surfaces, validating their hydrophilic nature. Surfaces modified with mixed monomers exhibited similar contact angles, and were less hydrophilic than tetraglyme coated surfaces but more hydrophilic than unmodified Nafion. Surfaces became less hydrophilic with a decrease in the content of HEMA at the monomer feed. Using Zisman plots of Nafion and tetraglyme, critical surface tension, γ_c , were found. Nafion exhibited a $\gamma_c = 5$ dyne/cm ($R^2 = 0.772$) and tetraglyme coated surfaces were found to have a $\gamma_c = 19$ dyne/cm ($R^2 = 0.999$) for tetraglyme coated surfaces,. Hence, tetraglyme treated Nafion membranes had an increase in their surface energy. This is consistent

with their increase in hydrophilicity, as was also seen by a plasma coating on polyurethanes [36]. In addition, this value is very close to the optimal range of minimum bioadhesion (20-30 dynes/cm), as has been previously found [37].

3.5 Protein Adsorption and peptide binding

Figure 7 shows the adsorption of albumin and fibronectin for the 200 µg/mL. The protein adsorption profile for unmodified Nafion surfaces correlated well with theoretical values previously calculated. Surfaces exhibiting ether carbons (tetraglyme) were the most effective at repelling proteins. Maximum protein adsorption was seen on surfaces containing an optimal amount of hydroxyl groups. Surfaces modified with 2.5% HEMA exhibited the most protein adsorption, followed by 5% HEMA surfaces. Surfaces modified with 10% HEMA exhibited a limited amount of protein adsorption, similar to ether carbon rich surfaces (non-fouling, *i.e.*, tetraglyme), and were chosen for subsequent cellular experiments.

The highest protein adsorption occurred on surfaces containing the highest amount of hydroxyl groups on the surface (from the 2.5% HEMA surfaces). This was initially surprising, as hydroxyl groups have been noted to be hydrophilic in nature and are generally correlated with low protein adsorption on such surfaces as hydroxyl terminated self assembled monolayers (SAMs). However, others have found that proteins such as fibrinogen and albumin can bind to hydroxylated surfaces. For example, the binding of human albumin is preferential to SAMs of hydroxylated surfaces, followed by methyl terminated, followed by fluorinated surfaces. This study is consistent with our finding of proteins adhering the most on surfaces rich in hydroxyl rather than fluorinated surfaces (Nafion). For example, 2.5% HEMA coated surfaces showed a much higher albumin adsorption profile, in comparison to unmodified Nafion surfaces. Figure 8 examines the correlation between protein adsorbed and hydroxyl groups (F/C ratios) for modified Nafion surfaces. When graphing theoretical values for HEMA coated surfaces, we observe that there is a direct relationship between hydroxyl content and protein adsorbed for both albumin and fibronectin. Upon an ANOVA analysis ($\alpha = 0.05$), we observe no statistical difference between protein adsorbed by 2.5% HEMA surfaces or HEMA coated surfaces, for either albumin or fibronectin. This signifies that as hydroxyl content increases, protein adsorbed increases as well.

3.6 Peptide modified Nafion

Modified Nafion surfaces exhibiting hydroxyl groups on their surfaces were modified with the YRGDS peptide via CDI chemistry. Radioactive studies were conducted to quantify the amount of peptide that could adsorb to the activated surfaces. As expected, more of the YRGDS peptide bound with increasing quantity of peptide concentration, and with increasing concentration of hydroxyl groups on the surface. Figure 10 shows that the highest amount of peptide was bound to pure HEMA coated surface, followed by 2.5% HEMA at the 200 µg/ml bulk concentration of YRGDS. At the 200 µg/ml bulk protein concentration, we also see that the least peptide was bound 10% HEMA coated surfaces and 5% HEMA modified surfaces.

4. Discussion

Specific changes to surface chemistry can render a biomaterial surfaces non-fouling to minimize random protein adsorption. In addition, sufficient functional sites can be deposited in order to bind biologically active entities, which can specifically attach cells and dictate cell behavior. Surfaces modified with 10% HEMA (90% tetraglyme) accomplished the goal of maintaining a non-fouling surface decorated with the YRGDS peptide and decreased the production of Type I collagen in an *in vitro* model (data not shown). Optimizing conditions for surface chemical groups, protein adsorbed, and peptide bound would theoretically dictate cell behavior such as the degree of cell spreading and the synthesis of extracellular matrix.

Controlling the synthesis of extracellular matrix components would in turn, modulate the thickness of a fibrotic capsule surrounding an implant. The modulation of an extracellular matrix component will be the subject of a subsequent manuscript.

Surface modification of Nafion was performed via the RF plasma deposition method previously described, with the goal of producing a surface able to both resist protein adhesion and support a bioactive ligand. Plasma deposited films resulted in a change in the surface roughness of the surface as was examined with AFM. Unmodified Nafion exhibited the roughest surface, followed by a 10% HEMA surface. The smoothest surface was obtained with a tetraglyme coating. Modified Nafion surfaces exhibited a decrease in fluorine (intrinsic to Nafion). For most surfaces, C:O ratios correlated well with theoretical values for both tetraglyme and HEMA. Surfaces modified with both 2.5% HEMA as well as 10% HEMA exhibited very little change from the tetraglyme structure in their high resolution C1s XPS. The 5% HEMA surface was slightly different, exhibiting a ratio of 2.7. Preserving ratios close to theoretical calculations indicates the preservation of the tetraglyme structure on the surface. Indeed, upon examination of the 5% HEMA surface with high resolution XPS, we note an increase in hydrocarbons with a decrease in ether carbons, which do not follow the structure of tetraglyme. We note however, the resemblance of the C1s species to HEMA coated surfaces. This was thought to indicate a shift in the structure to HEMA.

High resolution XPS data revealed similar percentages for carbon species among the various modified surfaces. Further characterization was conducted, however, in order to discern the exact nature of ether carbons found at the various surfaces, with the goal of semi-quantifying hydroxyl groups. The derivitization of the surfaces allows for more careful insight into the nature of ether carbon groups at the surface. Derivatized data showed a decrease in hydroxyl groups as the amount of HEMA was increased in the monomer feed. As previously noted, the plasma deposition method can yield surfaces that do not necessarily conform to the starting monomer(s) from which they are synthesized. It is important to keep in mind that there are many parameters that may be varied in the plasma deposition method (pressure, power, time, flow rate, etc.) that may, prove to be more effective in yielding a linear and direct relationship between monomer feed the outcome at the surface. The linking of peptide was dependent on the surface hydroxyl groups and we observed that an increase in hydroxyl groups correlated well with an increase in YRGDS attachment, with the hydroxyl groups being the most prominent on surfaces synthesized with 2.5% HEMA in the monomer feed.

Nafion surfaces coated with tetraglyme exhibited hydrophilic characteristics in comparison to untreated surfaces, and were more hydrophilic than surfaces coated with a 10%, 5% and 2.5% HEMA content in the monomer feed. Surfaces modified with 2.5%, 5% and 10% HEMA were found to have sequential decreasing amounts of hydroxyl groups on their surfaces.

Contact angle findings (data available) suggest that while hydroxyl groups are hydrophilic, they are not as hydrophilic as a purely tetraglyme coated surface, which exhibits no hydroxyl groups. In addition, for these surfaces, there is an inverse relationship (Figure 11) between the number of hydroxyl groups on a surface and their hydrophilicity, as has also been observed [38].

It was demonstrated by AFM, that the surface roughness of Nafion is decreased in both tetraglyme and 10% HEMA coated Nafion. The changes in surface topography seen in the new, coated Nafion may have contributed to its ability of repulsing proteins. Figure 12 below shows the correlation between protein adsorbed and surface roughness images obtained from 2.0 μm^2 AFM images. Rougher, unmodified Nafion surfaces (having a higher root mean square RMS value) adsorb more protein than smoother, tetraglyme and 10% HEMA modified surfaces. At the highest RMS value, fibronectin adsorbs to a higher degree than albumin.

Fibronectin has previously been found to preferentially adsorb to rougher, rather than smoother titanium surfaces.

In examining XPS data from modified Nafion surfaces, we saw that surfaces rich in ether carbons (such as tetraglyme) were the most successful at limiting protein adhesion. This phenomenon has been previously shown by Johnston, *et al.*, [39] where they successfully correlated protein resistance with ether carbon content.

Further examination is necessary however, to discern the nature of the C-O bonds (ether carbon vs. hydroxyls) at the surface for the successful immobilization of a biological peptide, such as YRGDS. This work revealed that the highest protein adsorption occurred on surfaces containing the highest amount of hydroxyl groups on the surface (from the 2.5% HEMA surfaces). This was initially surprising, as hydroxyl groups have been noted to be hydrophilic in nature and are generally correlated with low protein adsorption on such surfaces as hydroxyl terminated self assembled monolayers (SAMs) [22,40-42]. However, others have found that proteins such as fibrinogen and albumin can bind to hydroxylated surfaces. For example, Margel *et al.*, [38] found that the binding of human albumin is preferential to SAMs to hydroxylated surfaces, followed by methyl terminated, followed by fluorinated surfaces. This study is consistent with our finding of proteins adhering the most on surfaces rich in hydroxyl rather than fluorinated surfaces (Nafion). Figure 8 examines the correlation between protein adsorbed and hydroxyl groups (F/C ratios) for modified Nafion surfaces. From this figure we discern that there is an optimal amount of hydroxyl groups required to adsorb the most amount of protein. If the amount of hydroxyls surpasses a certain threshold however, the amount of protein will decrease. Figure 9 exhibits a similar relationship between protein adsorbed and the amount of hydrocarbons on the surface. A relationship could not be established between ether carbon rich surfaces, as no specific assay was carried out to identify their exact amount on the various surface.

5. Conclusions

Nafion™ is the membrane material preferred for *in situ* glucose sensors but unfortunately, Nafion promotes random protein adsorption and eventual foreign body encapsulation thus leading to the loss of glucose signal with time. Here, surface modifications were made to Nafion by RF plasma deposition with the intent to prevent random protein adsorption while promoting cell adhesion. Nafion surfaces were modified by RF plasma polymerizing five different combinations of (1) tetraethylene glycol dimethyl ether (tetraglyme) and (2) 2-hydroxyethyl methacrylate (HEMA). We were able to modify the Nafion base membrane surface to render it non-fouling. The tetraglyme/HEMA modifications also provided a controllable number of functional hydroxyl groups to which biomimetic molecules could be linked. We report here a flexible coating method to functionalize Nafion with a controllable amount of adhesion peptides necessary for mammalian cell attachment while negating random protein adsorption. Studies with fibroblast cells cultured on the various surfaces indicate the need for binding adhesion peptides to the non-fouling Nafion; these studies will be presented in a companion paper.

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Figure 1 A.

Trifluoroacetic anhydride reaction with hydroxyl containing surface

Figure 1 B.
Structure of samples to be analyzed by TFAA reaction

Figure 2.
Carbonyldiimidazole chemistry on modified Nafion surfaces

Figure 3. Sample survey scans of Nafion and modified surfaces. A) Unmodified Nafion. B) Tetraglyme Coated Nafion. C) 2.5% HEMA, 97.5% Tetraglyme. D) 5% HEMA, 95% tetraglyme. E) 10% HEMA, 90% tetraglyme. F) Pure HEMA

Figure 4. Sample high resolution C1s spectrum of Nafion and modified Nafion surfaces. A) Unmodified Nafion. B) Tetraglyme Coated Nafion. C) 2.5% HEMA, 97.5% Tetraglyme. D) 5% HEMA, 95% tetraglyme. E) 10% HEMA, 90% tetraglyme. F) Pure HEMA

Figure 5. Fluorine to Carbon Ratios for Derivatized Modified Nafion surfaces. Dashed lines show theoretical values for known sample profiles.

Figure 6.

Figure 7. Protein adsorbed on various Nafion surfaces after 24 hours of incubation at a 200 ug/ml bulk protein concentration. A) Albumin and B) Fibronectin. Solid bars represent Nafion samples after a PBS wash, and striped bars represent samples after an sodium dodecyl sulfate (SDS) wash.

Figure 8. Correlation between protein adsorbed and hydroxyl groups on modified Nafion surfaces. The symbol (◆) represents albumin, and (■) represents fibronectin.

Figure 9. Correlation between protein adsorbed and hydrocarbon groups on modified Nafion surfaces. The symbol (◆) represents albumin, and (■) represents fibronectin.

Figure 10.
YRGDS peptide linked to modified Nafion substrata.

Figure 11.
Correlation between contact angle and hydroxyl content

Figure 12. Correlation between proteins adsorbed (values after PBS rinse) and surface roughness as obtained from $2.0 \mu\text{m}^2$ AFM images. The symbol (◆) represents albumin, and (■) represents fibronectin.

Table 1

Surfaces treated with different monomer percentages

Percentage of Tetraglyme in feed	Percentage of HEMA in feed	Referred to as
100%	0%	Tetraglyme or pure tetraglyme
97.5%	2.5%	2.5% HEMA
95%	5%	5% HEMA
90%	10%	10% HEMA
0%	100%	HEMA or pure HEMA

Table 2

Elemental composition of Nafion and plasma deposited Nafion

Surface	%C	%O	%F	C:O Ratio
Nafion	24.07 ± 1.64	6.25 ± 0.26	42.88 ± 1.46	3.9:1
Nafion-tetraglyme	69.27 ± 0.04	30.55 ± 0.06	0.12 ± 0.04	2.3:1
Nafion - 2.5% HEMA	69.70 ± 0.38	30.22 ± 0.36	0.08 ± 0.02	2.3:1
Nafion - 5% HEMA	72.36 ± 0.02	27.03 ± 0.14	0.611 ± 0.15	2.7:1
Nafion - 10% HEMA	68.84 ± 0.47	31.12 ± 0.45	0.04 ± 0.03	2.2:1
Nafion - pure HEMA	69.76 ± 0.62	28.77 ± 0.55	1.46 ± 0.17	2.4:1

Table 3

C1s species for Nafion and modified Nafion substrata

Surface	%C-H (285 eV)	%C-OH, C-O-C (286.6 eV)	%C=O (289 eV)
Nafion	8.04 ± 0.22	4.62 ± 0.07	---
Nafion-tetraglyme	11.0 ± 3.3	85.8 ± 4.2	3.2 ± 0.88
Nafion - 2.5% HEM	23.3 ± 0.5	71.3 ± 1.1	5.5 ± 1.6
Nafion - 5% HEMA	53.3 ± 6.1	35.4 ± 6.4	11.3 ± 1.2
Nafion - 10% HEMA	13.7 ± 0.4	82.9 ± 0.7	3.3 ± 1.2
Nafion - pure HEMA	50.6 ± 0.06	33.9 ± 0.5	15.5 ± 0.4

Table 4

Root mean square roughness values obtained from average of 3 images per surface treatment. STD signifies standard deviation

Material	Root Mean Square Roughness (RMS) $\pm$ STD (6.72 μm^2)
Nafion	3.98 $\pm$ 0.89
Tetraglyme coated Nafion	8.32 $\pm$ 4.15
10% HEMA coated Nafion	4.63 $\pm$ 4.19