Published in final edited form as:

J Biomed Mater Res A. 2011 January ; 96(1): 150–161. doi:10.1002/jbm.a.32954.

Complement activation on poly(ethylene oxide)-like RFGD-deposited surfaces

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Abstract

Non-specific protein adsorption, particularly fibrinogen (Fg), is thought to be an initiating step in the foreign body response (FBR) to biomaterials by promoting phagocyte attachment. In previous studies, we therefore prepared radio frequency glow discharge (RFGD) polyethylene oxide (PEO)-like tetraglyme coatings ($\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_4\text{CH}_3$) adsorbing less than 10 ng/cm^2 Fg and showed that they had the expected low monocyte adhesion *in vitro*. However, when these were implanted *in vivo*, many adherent inflammatory cells and a fibrous capsule were found, suggesting the role of alternative proteins, such as activated complement proteins, in the FBR to these materials. We therefore investigated complement interactions with the tetraglyme surfaces. First, because of its well known role in complement C3 activation, we measured the hydroxyl group (-OH) content of tetraglyme, but found it to be very low. Second, we measured C3 adsorption to tetraglyme from plasma. Low amounts of C3 adsorbed on tetraglyme, though it displayed higher binding strength than the control surfaces. Finally, complement activation was determined by measuring C3a and SC5b-9 levels in serum after incubating with tetraglyme, as well as other surfaces that served as positive and negative controls, namely poly(vinyl alcohol) hydrogels, Silastic sheeting, and poly(ethylene glycol) self-assembled monolayers with different end groups. Despite displaying low hydroxyl group concentration, relatively high C3a and SC5b-9 levels were found in serum exposed to tetraglyme, similar to the values due to our positive control, PVA. Our results support the conclusion that complement activation by tetraglyme is a possible mechanism involved in the FBR to these biomaterials.

Keywords

Complement activation; foreign body response; poly(ethylene oxide); protein adsorption; biocompatibility

Introduction

Radiofrequency glow discharge plasma (RFGD) generated poly(ethylene oxide)-like tetraglyme surfaces developed in our lab have been shown to adsorb extremely low levels (usually $<10 \text{ ng/cm}^2$) of proteins from dilute solutions [1]. This ability to largely resist protein adsorption, however, is reduced as radiofrequency (RF) deposition power is increased, thus giving us the ability to control the degree of protein adsorption resistance of our materials by controlling our tetraglyme films' ethercarbon content [2]. Because fibrinogen (Fg) has been identified as playing an important role in monocyte recruitment during the foreign body response (FBR) [3,4], surfaces displaying extremely low levels of protein adsorption, such as ours, are expected to have a reduced FBR as compared to highly fouling surfaces. Yet, in our previous studies [1,2], we found that there was a high number of inflammatory cells adherent on plasma polymerized tetraglyme after 24 hour

subcutaneous implantation *in vivo*, even though the same type of surface had exhibited a high degree of resistance to protein adsorption and cell adhesion *in vitro*. Furthermore, after 28 days of implantation, a fibrous capsule was formed around the materials, with no statistically significant difference in the thickness of the capsule compared to control surfaces. Thus, while trying to better understand the *in vivo* inflammatory response to tetraglyme surfaces, we became interested in the role of complement proteins and the potential for complement activation by our surfaces.

Complement is one of the self/non-self immune recognition and surveillance systems in higher animals. The complement system is made up of approximately 30 proteins, which participate in a complex cascade of biochemical transformations that is one of the body's principle means for relatively rapid attack of "non-self" species in the body. Complement activation occurs via three different pathways: the classical pathway, the alternative pathway, and the mannose-binding lectin pathway [5]. Although the triggers and the initial steps in the activation of each of these pathways differ, they all converge at the formation of C3 convertase.

It is generally accepted that biomaterial-induced complement activation is initiated by free hydroxyl (-OH) or amine (-NH₂) groups on the material's surface, as they promote the formation and the covalent binding of the cleaved form of C3, C3b, on the material's surface, a process also leading to the release of C3a as a normally soluble activation product [6]. Furthermore, activating materials also facilitate the binding of Factor B to C3b, forming the C3 convertase, which catalyzes the cleavage of more C3 [7], thus amplifying the response. However, the adsorption of C3 onto material surfaces does not always lead to the activation of the complement system. For instance, C3 can adsorb onto biomaterial surfaces through electrostatic or hydrophobic interactions leading to surface bound but uncleaved C3 [6]. In addition, C3b adsorption onto non-activating surfaces facilitates its binding to Factor H and Factor I, which quickly leads to C3b inactivation [8].

C3 cleavage and degradation products are of great importance in inflammation. C3a is an anaphylatoxin known to activate and recruit phagocytes, while iC3b, a degradation fragment of C3b, is an opsonin [9] known to be a ligand for a monocyte adhesion receptor, known as CR3, Mac-1, or CD11b/CD18 [10,11]. In addition, C3b is involved in mediating monocyte and macrophage phagocytosis and cell adherence [12], as well as contributing to the formation of C5 convertase, which causes the cleavage of C5 into the potent anaphylatoxin C5a and C5b. The presence of C5b initiates the formation of the membrane attack complex (MAC, C5b-9), the common terminal complex for all pathways of complement activation [13].

A number of hydroxyl-rich biomaterials, such as cellulose dialysis membranes [14], hydroxyethyl methacrylate-ethyl methacrylate (HEMA-EMA) copolymers [15], and poly(vinyl alcohol) (PVA) hydrogels [16], have been found to activate the complement system upon contact with blood and blood components. A study by Tang et al supported the idea that hydroxyl-rich surfaces cause complement activation to a greater degree than do amine and carboxyl-rich surfaces, as hydroxyl-rich thiols on gold showed a higher degree of complement activation *in vitro* in C3b and SC5b-9 levels and a corresponding higher number of adherent neutrophils and macrophages than other thiol functionalities [17]. Furthermore, the number of adherent inflammatory cells to these surfaces was greatly impaired when a complement-depleted mouse model was used [17]. Hirata et al [18] further tested OH- and CH₃-SAMs for C3b immobilization and Factor Bb release into the liquid phase. The OH-SAMs were found to induce a significantly greater degree of complement activation via the alternative pathway, and complement activation by OH-SAMs was found to increase with increasing surface -OH group density [19].

The role of alternative pathway complement activation by PEO and PEO-like coatings is an area that has received little study. The effect of end groups of the PEOs on complement activation was reported recently. PEG-acrylate films were found to be more complement activating than sulfonated PEG-acrylate films [20], which was attributed to the exposed hydroxyl group on the PEG-acrylate films. Both intact and cleaved C3 adsorb on PEO-thiols on gold surfaces [21] after incubation with plasma. Nanocapsules coated with PEG were found to be less complement activating than MePEG coated nanocapsules, a difference attributed to the conformation of the PEG chain and its ability to prevent protein adsorption from occurring [22]. OH terminated PEG coated surfaces activate the complement system to a larger degree than CH₃ terminated PEG coated surfaces, although the degree of complement activation by CH₃ terminated PEG coatings was found to increase with storage time, due to oxidation of the coatings over time [23].

The role of complement proteins in the FBR has been largely overlooked and is not well understood, particularly for ultra low protein adsorption surfaces like tetraglymes, despite the evidence that complement proteins and activation fragments are known to adsorb onto various surfaces and can directly impact inflammatory cells. Thus, the research presented in this and in a forthcoming publication was designed to provide a more complete picture of the role of complement proteins in the FBR. Although we have evaluated complement-mediated monocyte adhesion as well as protein adsorption and complement activation with respect to our PEO-like tetraglyme surfaces, only the complement protein adsorption and activation data are presented here. First, we measured complement factor 3 (C3) adsorption and retention on tetraglyme surfaces generated using different RF deposition powers (10W and 80W). Because there was some adsorption, we also needed to know if it was accompanied by activation of the adsorbed proteins. That led us to investigate whether our surfaces were inducing complement activation by measuring two activation by-products, C3a and inactivated membrane attack complex (MAC), SC5b-9, in normal human serum after incubation with tetraglyme surfaces and a variety of other surfaces used as controls, including poly(vinyl alcohol) hydrogels (PVA), Silastic sheeting, and self-assembled monolayers (SAMs) including two oligo(ethylene glycol) SAMs. Although PVA, Silastic, and the SAMs included in this study were not included in our lab's previous *in vivo* studies, we know that all of these materials elicit the foreign body response [24,25,26]. In fact, PVA sponges are often used to model acute and chronic inflammatory states, including the foreign body reaction [26]. Monocyte adhesion data presented elsewhere [27] has shown that C3 removal from serum or plasma decreased adhesion greatly and that C3 restoration to the depleted serum or plasma greatly increased adhesion. Those results support the findings of the current study in which we shown complement proteins are activated by tetraglyme, in that both studies show that complement protein interactions, namely adsorption and activation, play an important and previously overlooked role in the FBR to tetraglyme.

Materials and Methods

The fluorinated ethylene propylene (FEP, (CF(CF₃)-CF₂(CF₂-CF₂)_n)_m) film used as the substrate for our PEO-like films was a gift from Du Pont de Nemours & Co. (Circleville, OH). The film was 0.125 mm thick, and was cut into 7.9 mm diameter disks using a die punch. Once cut, samples were cleaned by two successive sonications in methylene chloride, acetone, and methanol, for 10 minutes each. FEP disks were left to dry in a laminar flow hood, and stored at room temperature in a clean polystyrene Petri dish, wrapped in Parafilm, until ready to use. Tetraethylene glycol dimethyl ether (tetraglyme, CH₃O(CH₂CH₂O)₄CH₃) monomer was purchased from Sigma-Aldrich (St. Louis, MO).

The 50,000-kDa poly(vinyl alcohol) polymer (PVA, [-CH₂CHOH-]_n) was a gift from Kuaray Specialties Europe (KSE, Frankfurt, Germany). Enough PVA was dissolved in

sterile-filtered distilled water to make a 10% (w/v) solution. Once fully dissolved, the solution was poured into 35 mm polystyrene Petri dishes and allowed to dry in a laminar flow hood, a process that usually required about 1 week. The resulting film was re-hydrated using ultra-pure water for a minimum of 24 hours prior to use. The PVA hydrogel sheet was then cut into 8-mm diameter disks using a sterile biopsy punch (Picu Punch) and placed in fresh ultra-pure water until ready to use. The resulting hydrogel disks were approximately 0.5 mm in thickness.

Silastic sheeting with a thickness of 0.5 mm was a gift from Dow Corning Corporation (Midland, MI), and was cut into 7.9 mm-diameter disks using a die punch. Samples were rinsed with 18M Ω deionized water once, and then sonicated twice in a 0.1% Ivory Flakes soap solution for 10 minutes. The Ivory soap cleaning protocol is recommended by the manufacturer of medical grade silicone. Previous studies by members of our lab found no residual soap detectable by electron spectroscopy for chemical analysis (ESCA) (unpublished observations). Next, the samples were sonicated four times in fresh distilled water for 10 minutes. The samples were allowed to dry in a laminar flow hood, placed in a clean Petri dish, sealed with Parafilm, and stored at room temperature until ready to use.

Buffers and reagents

Citrate phosphate buffered saline containing 3 mM NaN₃ and 10 mM NaI (CPBSzI) was used at a pH of 7.4 for radiolabeled protein adsorption studies. Phosphate buffered saline (PBS) was purchased from Sigma-Aldrich (St. Louis, MO). Heparinized, EDTA, and plain vacutainers were purchased from Becton Dickinson (Franklin Lakes, NJ). Purified human fibrinogen (>95% purity and >95% clottable) was purchased from Enzyme Research Laboratories (South Bend, IN). Purified Complement C3 was purchased from Sigma-Aldrich (St. Louis, MO), EMD Biosciences (San Diego, CA), and RDI Division of Fitzgerald Industries International (Concord MA). Absolute ethanol was purchased from Aaper (Shelbyville, KY).

Sample preparation

Radio-frequency glow discharge (RFGD) plasma deposition of tetraglyme—A capacitively coupled radio-frequency glow discharge plasma system, previously described in detail in the literature [28], was used to generate our tetraglyme coatings. Briefly, the system consists of a vacuum pump, a mass flow controller to determine the flow rate of monomer and gas, a monomer reservoir, copper electrodes, a cylindrical reaction chamber for sample preparation, and a 13.56 MHz radio-frequency power source.

FEP substrates were suspended from a glass frame using fishing line to ensure that both sides of the disks were coated at once. Glass cover slips were placed on a specially designed sample rack that allowed the cover slips to stand vertically on their edge, exposing both sides to the plasma. Both of these set-ups eliminated the need for two runs to coat both sides of the substrate and the resulting damage on the side coated first, which has been observed previously by the first author (unpublished observations) and others in our group [29].

Samples were loaded 1 to 3 inches upstream of the RF coil of the plasma reactor, and treated with a 3-minute argon plasma generated using 40W and 350 mtorr. This step etches away surface impurities and also introduces free radicals at the surface that can react with subsequent gaseous compounds. The Ar was completely evacuated from the reactor chamber and tetraglyme monomer vapor was introduced. Tetraglyme was then deposited as a polymeric film by subjecting substrates immersed in its vapor to an initial 80W 1-minute exposure immediately followed by either (a) a 10-minute 80W exposure, (b) a 20-minute 10W exposure, or (c) a 20-minute 20W exposure, all at 350 mtorr. Samples were then

placed in a clean polystyrene Petri dish, sealed with parafilm, and stored at room temperature until ready for use.

Preparation of PEG self-assembled monolayers (SAMs) on gold—Glass cover slips (8 mm, ProSci Corp) were cleaned with detergents, water, and several solvents. First, the samples were subjected to ultrasonication in a 1/64 dilution of Isopanasol (C.R. Callen Corp, Seattle, WA) solution for 10 minutes before rinsing with copious amounts of 18M Ω deionized water (Millipore, Billerica, MA). The samples were then sonicated once for 10 minutes each in water, acetone, and methanol. The cover slips were dried under argon (Airgas) and stored in Petri dishes sealed with parafilm.

The cleaned glass cover slips were sputter coated with gold using a MRC 822 system equipped with an argon gas matrix. A 150 Å layer of Ti/W was first sputtered onto the glass to promote adhesion of gold on to the substrate. Then, a 1000 Å gold layer (99.99% purity) was deposited on the surfaces. Deposition was at a pressure lower than 3.0×10^{-6} Torr and at a rate of 100 Å/min for Ti/W and 300 Å/min for gold. Double-sided samples were manually flipped and resputtered using an identical protocol. All steps were conducted at room temperature in a clean room environment. Substrates not immediately used were stored in clean Parafilm-sealed Petri dishes under argon.

Self-assembled monolayers with either (1-mercapto-11-undecyl) tetra (ethylene glycol) (designated “PEG”-SAM, $\text{HS}(\text{CH}_2)_{11}\text{O}(\text{CH}_2\text{CH}_2\text{O})_4\text{H}$) or methoxy-capped tetra (ethylene glycol) (“MePEG”-SAM, $\text{HS}(\text{CH}_2)_{11}\text{O}(\text{CH}_2\text{CH}_2\text{O})_4\text{CH}_3$) (both acquired from Asemblon Inc, Redmond, WA) were prepared by submerging gold substrates in 1 mM solutions in absolute ethanol for 1 hour. Undecanethiol SAMs ($\text{HS}(\text{CH}_2)_{11}\text{H}$, (Asemblon Inc, Redmond, WA) were generated by submerging gold sputtered substrates in a 1 mM solution in absolute ethanol for 24 hours. After assembly, the samples were rinsed with copious amounts of ethanol, sonicated in ethanol for 2 minutes, rinsed in ethanol a second time, and then blown dry and stored under argon until analysis. The two different adsorption times used were based on previous in-depth XPS and FTIR studies on the adsorption times needed to produce the least Gauche defects while minimizing the amount of unbound and oxidized sulfur. Due to the differences in interchain forces of the different molecules, different adsorption times were found to yield optimized results for each solution (data not shown).

Surface characterization using ESCA—All surfaces were characterized using an SSX-100 spectrometer (Surface Science Instruments, Mountain View, CA) equipped with a monochromatic Al $K\alpha_{1,2}$ x-ray source. The samples were analyzed at a 55° take-off angle, resulting in data reflecting the composition of the topmost 50–80 Å of the surface. Compositional survey and detailed scans (S_{2p}) were acquired using an analyzer pass energy of 150 eV and an x-ray spot size of 1000 μm . High-resolution spectra were acquired at a pass energy of 50 eV and resolved into individual Gaussian peaks using a least-squares fitting routine in the Service Physics ESCAVB Graphics Viewer.

Three replicates of tetraglyme films deposited at 10, 20, or 80W were analyzed, with three spots collected for each replicate. At each spot, a composition survey scan (0–1000 eV) and a high-resolution C_{1s} scan were collected. In the case of the SAMs, three composition survey scans (0–1000 eV) were collected, along with a corresponding detailed S_{2p} scan. High-resolution (Au_{4f} , S_{2p} , and C_{1s}) scans were also collected. All binding energies (BE) were referenced to the lowest resolved C_{1s} peak BE, corresponding to the hydrocarbon peak (CH_x) at 285.0 eV. Composition data are reported as the average of the collected spots for all samples of one type, and presented as atomic percentage.

Hydroxyl derivatization with Trifluoroacetic Anhydride (TFAA)—Tetraglyme coatings on FEP disks and glass cover slips were used. Spin coated polystyrene (PS, Scientific Polymer Products, Inc., Ontario, NY) samples were used as negative controls, and were made by spin coating 10 μ l of 2% (w/v) PS in toluene on 8-mm diameter glass cover slips for 20 seconds at 4000 rpm. Poly(vinyl alcohol) (PVA, KSE, Frankfurt, Germany) coatings were used as positive controls, and were made by spin coating 10 μ l of 2% (w/v) PVA in purified H₂O under the same conditions as PS coatings. The samples were allowed to dry for one hour before they were flipped and recoated on the uncoated side. Samples were allowed to dry for an additional hour, placed in Petri dishes, and sealed with parafilm until use.

The TFAA derivatization procedure has been described in the literature [30]. Briefly, samples were placed on a glass slide, and the slide was then placed inside a large glass test tube. Next, 2 mL of TFAA was injected into the tube in the area directly below the slide, making sure not to touch the samples with the liquid TFAA. The test tube was evacuated and then flushed briefly with nitrogen and capped with a Teflon-lined cap. The reaction was allowed to proceed for 8 hours at 25°C, and the materials were analyzed using ESCA, keeping untreated controls and reacted samples separate to eliminate contamination risk. Survey and high-resolution C_{1s} spectra were collected, and the fluorination degree and the C to F ratio were used to determine the number of surface hydroxyl groups, as each hydroxyl group present will react with TFAA marking the reaction with three fluorine atoms [30,31].

Complement C3 radiolabeling—Shortly before use, two Iodobeads® (Pierce Biotechnology, Rockford, IL) were washed with 1 mL PBS, and dried on filter paper to remove loosely bound particles. Iodobeads® were added to 0.25 mL PBS, and 1mCi Na¹²⁵I (Amersham) was added to the beads and allowed to react for 5 minutes at room temperature. Next, 0.5 ml of a 0.2 mg/ml C3 solution in PBS was added to the Iodobeads®, and allowed to react for 15 minutes at room temperature. Unbound ¹²⁵I was separated from the labeled protein by two passes through Econo-Pac® 10DG desalting chromatography columns (BioRad, Hercules, CA). Radiolabeled complement C3 was stored at -80°C, and used within four weeks. ¹²⁵I incorporation rates of approximately 40% were achieved.

C3 adsorption—FEP and tetraglyme coated FEP prepared at 10 or 80W were placed in 1.5 ml polystyrene sample cups, and soaked in 0.3 ml degassed CPBSzI for 1 hour at 37°C, ensuring that the disk was completely submerged. At the end of the soaking period, the buffer was replaced with 100% normal human serum with enough added ¹²⁵I-C3 to reach a specific activity of 40 cpm/ng. Samples were incubated for two hours at 37°C. A 2-hour adsorption time was chosen based on many previous studies in our lab showing that steady state adsorption is achieved in 1–2 hours [32,33]. At the end of the adsorption time, samples were rinsed free of bulk protein with CPBSzI by dip rinsing three times. The amount of surface bound ¹²⁵I was measured using the Cobra II gamma counter. Finally, the amount of adsorbed protein (C3_{ads}, ng/cm²) was calculated using the amount of surface bound ¹²⁵I, the specific activities of the protein solutions used for the adsorption, and the surface area of both sides of the sample.

After measuring the amount of adsorbed C3 as described above, samples were placed in a 2% (w/v) sodium dodecyl sulfate (SDS, Sigma-Aldrich, St. Louis, MO) in PBS solution for 24 hours to determine the amount of irreversibly bound C3 on 10W, FEP, and 80W samples. A 24-hour soaking period was chosen to allow for full desorption of loosely adsorbed protein. After the 24 hours, samples were removed from the soaking solution, rinsed with CPBSzI, and placed in fresh gamma counter tubes. The surface bound (C3_{irr}) and eluted ¹²⁵C3 (C3_{SDS}) in the 2% SDS solution were counted for 1 minute using the Cobra II gamma counter. The adsorption retention of C3 on the tested surfaces, defined as the

percentage of initially adsorbed C3 retained on the surface after soaking for 24 hours in the 2% SDS solution, was then calculated as follows:

$$\text{Adsorption Retention} = 100 \times (C3_{ads} - C3_{irr}) / C3_{ads}$$

Corrections for radioactive decay and variation in counter efficiency and background were made.

Preparation of EDTA human blood plasma—Human blood plasma was prepared from healthy donors' blood collected via venipuncture into K₃ (EDTA) vacutainers (BD Biosciences) by trained phlebotomists at the University of Washington Medical Center, under a protocol approved by UW Human Subjects Committee. The collected blood was then gathered into a 50 mL conical tube (Falcon) and centrifuged for 20 minutes at 314g at 4°C. The platelet rich plasma layer was carefully collected and moved to a polycarbonate centrifuge tube. The plasma was then centrifuged for 30 minutes at 31,400g at 4°C to remove platelets in the protein solution. The topmost plasma layer was collected, aliquoted, and frozen at -80°C or promptly used. EDTA plasma from multiple donors (N = 3 donors) was pooled before use.

Preparation of human blood serum—Human blood serum was prepared from healthy donors' blood collected into glass vacutainers without anticoagulants by trained phlebotomists at the University of Washington Medical Center, under a protocol approved by UW Human Subjects Committee. Blood was allowed to coagulate for 1 hour at room temperature, and for an additional hour at 4°C. Serum was then collected into a 50 mL conical tube (Falcon) using a 5 mL serological pipette, exercising care not to disturb the blood clot, and centrifuged at 896g. The serum was then filtered using a 0.22 µm pore size filter (Millipore, #SCGP00525), and either used immediately or promptly frozen at -80°C. Serum from multiple donors (N = 3 donors) was pooled before use, unless otherwise noted.

Enzyme Linked Immunosorbent Assay (ELISA) for complement activation—Commercially available ELISA kits (Quidel, San Diego, CA) were used to determine the degree of formation of the anaphylatoxin C3a and the soluble, inactive form of the membrane attack complex (MAC), SC5b-9, as a result of complement activation. C3a is formed during the initiation stage of the alternative and classical activation pathways of the complement system, and is short-lived, degrading to C3a-desArg shortly after being generated. Thus, measuring the amount of C3a-desArg (referred to as C3a for ease) is an appropriate method for measuring the degree of complement activation during the initial stage of complement activation. Measuring the amount of SC5b-9, also known as the terminal complement complex, generated as a result of biomaterial contact with serum was also done as an additional way to determine complement activation by biomaterials.

C3a formation assay—The following protocol was adapted from the manufacturer's recommendations. Four biomaterials were included in the C3a formation assay: FEP, 10W and 80W tetraglyme coatings, and hydroxyl terminated PEG-SAMs. FEP was included as a negative control, while PEG-SAMs were included as positive controls. Untreated polystyrene (PS) was also included as a negative control.

Replicates of each of the four biomaterials included in the study were placed in 48-well plates and were washed by three 30 minute soaks in ultra-pure water, followed by three 30 minute soaks in sterile 1X PBS (pH = 7.2). Three replicates of each sample type were assayed. After the last soak, samples were moved to a new 48-well tissue culture plate, and 200 µL of pooled human serum added to each well, ensuring that the disk was completely

submerged in the protein solution. Because Ca^{2+} and Mg^{2+} are necessary for complement activation, EDTA plasma was used as a negative control. Samples were incubated at 37°C for 60 minutes. After incubation, EDTA was added to the samples incubated with serum at a final concentration of 10 mM, and the plate was placed on ice.

Incubated serum samples were diluted 1:22,500, while EDTA plasma samples were diluted 1:1000 prior to adding 100 μl of each to a prepared microassay plate coated with monoclonal antibody specific to C3a. After incubating for 60 minutes at 25°C , the microassay plate strips were rinsed, and 100 μl of the horseradish peroxidase conjugated antibody that detects C3a antigens was added. The plates were incubated for an additional 60 minutes at 25°C . The wells were rinsed once more, and 100 μl of the chromogenic substrate was added and allowed to incubate for another 15 minutes at 25°C before 100 μl of the stop solution was added to each well. All of the steps were carefully timed to ensure accurate results. Upon completion of the assay and within 1 hour of adding the stop solution to the samples, absorbance readings were taken at 450 nm with a reference wavelength at 650 nm ($A_{450-650}$ value) using a microplate reader (Molecular Devices Corporation, Union City, CA). The standards provided by Quidel were used to generate a calibration curve, which allowed us to determine the concentration of C3a-desArg present in serum and EDTA plasma as a result of complement activation due to contact with the biomaterials tested ($\mu\text{g}/\text{ml}$).

SC5b-9 formation assay—The following protocol was adapted from published protocols [8,31] as well as from the manufacturer's recommendations. A variety of biomaterials were included in the assay: FEP, 10W, 20W, and 80W tetraglyme coatings, undecanethiol self-assembled monolayers (SAMs) on gold, hydroxyl terminated PEG-SAMs, methoxy terminated MePEG-SAMs, Silastic, and PVA. Silastic was included as a negative control, while PVA was included as a positive control. The PEG SAMs were included as PEG controls.

Three replicates of each of the biomaterials included in the study for each of the three sera tested were placed in 48-well plates and washed by three 30 minute soaks in ultra-pure water, followed by three 30 minute soaks in sterile 1X PBS ($\text{pH} = 7.2$). Because all samples included in the study were treated in the same manner, possible complement activation as a result of endotoxin contamination in the water would be observed throughout all the samples tested. In addition, the SC5b-9 values detected for our control PVA and Silastic samples are similar to those previously published [8], giving us good evidence that endotoxin contamination was not a problem. Thus, differences in complement activation between the samples can be attributed to biomaterial-induced or alternative complement activation. After the last soak, samples were moved to a new 48-well tissue culture plate, and 200 μL of human serum was added to each well, taking care to maintain the sample submerged in the serum. Samples were incubated at 37°C for 90 minutes. After incubation, EDTA was added to each well at a final concentration of 10 mM, and the plate was placed on ice.

Shortly before beginning the assay, the prepared microassay plate strips coated with monoclonal antibody specific to SC5b-9 were hydrated by soaking with the wash buffer provided by the kit manufacturer for 30 seconds. This was repeated twice. Wells were emptied, and residual buffer was removed by firmly tapping on an absorbent surface. Incubated serum samples were diluted 1:150 prior to adding 100 μl to a hydrated, prepared microassay plate well. After incubating for 60 minutes at 25°C , the microassay plate strips were rinsed, and 50 μl of the horseradish peroxidase conjugated antibody that detects SC5b-9 antigens was added. The plates were incubated for an additional 30 minutes at 25°C . The wells were rinsed once more, and 100 μl of the chromogenic substrate whose turnover is catalyzed by HRP was added and allowed to incubate for another 15 minutes at 25°C before

the 100 μ l of the stop solution provided by Quidel was added to each well. All of the steps were carefully timed to ensure accurate results. Upon completion of the assay, an absorbance reading was taken at 450 nm (A_{450} value) within 30 minutes of adding the stop solution to the samples, using a microplate reader. The standards included in the Quidel kit were used to generate a calibration curve, which allowed us to determine the concentration of soluble SC5b-9 in the serum (μ g/ml).

Statistical analysis—To determine statistical significance, the data were analyzed using one-way ANOVA, and the means were compared using Bonferroni's post-hoc correction with an $\alpha = 0.05$.

Results

Surface characterization

RFGD tetraglyme films—The elemental and high-resolution ESCA C_{1s} composition for 10W, 20W, and 80W tetraglymes are presented in Table 1. The C/O ratio was found to be the lowest for 10W tetraglyme coatings (2.2) consistent with its PEO-like surface chemistry. The 20W tetraglyme coatings had a slightly higher C/O ratio, 2.6, due to a higher carbon content than that observed on 10W tetraglyme films. Finally, the 80W tetraglyme coatings displayed the lowest amount of oxygen and the highest amount of carbon on the surface, resulting in an extremely high C/O ratio of 11.1. Three peaks were resolved from the high-resolution C_{1s} spectra, corresponding to C–C/C–H (285 eV), C–O (286.6 eV), and C=O (288 eV). The films generated at the lower deposition powers had a surface chemistry most like PEO, with the ether carbon peak dominating the spectra. Consistent with the elemental analysis shown on Table 1, 10W tetraglyme had the highest ether carbon content, $82 \pm 1\%$. As has been shown previously, ether carbon content was found to decrease with increasing deposition power, with 20W tetraglyme peaks having an ether carbon content of $69 \pm 1\%$, and 80W samples having an ether carbon content of $7 \pm 2\%$.

No fluorine from the FEP substrate was detected in any of the samples, consistent with a uniform, pinhole free coating. ESCA is highly sensitive to fluorine, which has a high photoemission cross-section, approximately four times higher than carbon: for equal amounts of fluorine and carbon, fluorine will have a signal that is four times more intense. Thus, we can be confident that the lack of fluorine in our elemental composition indicates that the coatings are at least 100Å thick at all points. These findings were corroborated by the lack of substrate-related CF_2 and CF_3 peaks in the high-resolution C_{1s} ESCA spectra collected.

PEG self-assembled monolayers on gold—All elements expected from the thiols (ie O_{1s} , C_{1s} , and S_{2p}) and the substrate (Au_{4f}), were detected with the XPS compositional survey scans of the PEG and MePEG SAMs (Table 2). No unexpected elements were detected. A summary of the corrected surface elemental composition for these two SAMs is presented in Table 2, including the (C+O)/Au and C/O ratio. The elemental compositions of the PEG and MePEG monolayers generated are in good agreement with the expected values based on thiol stoichiometry. As the thickness of well organized PEG layers in a homogenous PEG SAM interfere with the emission of gold electrons from the substrate, the (C+O)/Au ratio is useful in estimating the expected thickness for a uniform, well organized PEG film [32,33]. The theoretical values presented in Table 2, which correspond with the expected values for alkanethiol SAMs of a similar thickness as those generated, were found to be less than one standard deviation from the experimental measurements.

The S_{2p} high-resolution spectra of the PEG SAMs exhibited the expected doublet (data not shown) [34]. A strong S $2p_{3/2,1/2}$ doublet at 161.9 eV ($S_{2p_{3/2}}$), indicative of a strongly

bonded thiolate anchor, and a smaller amount (<20%) of unbound alkanethiol and dialkyl disulfides at a $S\ 2p_{3/2}$ of ~164 eV are visible. The unbound sulfur is common for highly hydrophilic SAMs and is still suggestive of a well ordered monolayer [35,36]. The high-resolution S_{2p} scan of the MePEG shows only bound sulfur indicated by a single doublet at 164 eV. The C_{1s} spectra of both PEG types are dominated by two peaks centered at ~285.0 eV and 286.8 eV representative of the C-C/C-H and C-O bonds, respectively.

Hydroxyl derivatization with Trifluoroacetic Anhydride (TFAA)—Because hydroxyl (-OH) groups on a biomaterial's surface have been found to be involved in the activation of the complement system [16], we tried to detect hydroxyl groups in tetraglyme films. Hydroxyl and ether carbon peaks in ESCA overlap, thus derivatization of hydroxyl groups using trifluoroacetic anhydride (TFAA, $(CF_3CO)_2O$) was used for quantitative analysis of the percentage of hydroxyl groups present on our materials. We found that both 10W and 80W samples had a very low hydroxyl content on their surfaces, about $2.4 \pm 0.4\%$ and $2.7 \pm 0.3\%$, respectively (Table 3). Negative and positive controls, PS and PVA, respectively, were included to ensure that the gas phase reaction proceeded to completion. The values for each of these controls matched expected theoretical values, further supporting the significance of our results.

C3 adsorption—Adsorption of C3 from 100% normal human serum was measured on 10W and 80W tetraglyme coatings and FEP films (Figure 1). FEP samples displayed the greatest amount of adsorbed C3, $37 \pm 10\text{ ng/cm}^2$, while 80W tetraglyme samples had the second highest amount of adsorbed C3, $21 \pm 5\text{ ng/cm}^2$ and 10W tetraglyme samples had the lowest amount of adsorbed C3, $14 \pm 2\text{ ng/cm}^2$. The amount of irreversibly bound C3 was measured on these surfaces after soaking them in 2% SDS buffer for 24 hours at room temperature (Figure 1.a). The highest amount of irreversibly adsorbed C3 was observed on 10W tetraglyme samples ($4 \pm 0.2\text{ ng/cm}^2$), with the amount of irreversibly bound C3 decreasing in the order of 10W tetraglyme > 80W tetraglyme > FEP, though the difference between 10W and 80W samples was small. These results suggest a stronger protein-surface interaction observed on the hydrophilic PEO-like 10W surfaces than the other two surfaces. In fact, adsorption strength, defined as the percentage of initially adsorbed protein retained on the surface after a 24-hour SDS elution, shows that a greater percentage of C3 was retained on the 10W tetraglyme surface than on the 80W or FEP surfaces (Figure 1.b).

C3a formation assay—Figure 2.a shows the amount of C3a detected in 100% normal human serum after incubating for 60 minutes with five different materials: untreated polystyrene (PS) control, FEP, 10W and 80W tetraglyme films, and PEG-SAMs. Special care was taken in the handling of the serum in this study to minimize complement activation, placing it on ice before the start of the assay and immediately after the end of the assay. The levels of C3a present in 100% serum incubated in untreated PS plates was found to be significantly lower than that observed from 10W and PEG-SAMs, but not FEP or 80W tetraglyme. FEP and 80W tetraglyme coatings were found to be low complement activators, while 10W tetraglyme coatings were found to be highly complement activating and PEG-SAMs only moderately complement activating. As indicated by the asterisks in Figure 2, the levels of C3a detected in serum with all biomaterial tested were significantly less than for the 10W tetraglyme film, meaning that 10W tetraglyme films were indeed the strongest complement activator tested. The levels of C3a detected in serum decreased in the order of 10W tetraglyme > PEG-OH SAMs > 80W tetraglyme $\geq$ FEP > PS.

EDTA plasma was used as a control in this assay (Figure 2.b), as EDTA is a strong metal ion chelator known to inhibit both the classical and the alternative complement activation pathways [37]. As shown in Figure 2.b, C3a formation was indeed much lower in EDTA

plasma than in serum. No statistically significant difference was observed in the levels of C3a generated for any of the materials tested using EDTA plasma.

The results of the C3 formation assay reported here were reproduced two separate times using different fresh tetraglyme batches as well as serum and EDTA plasma pools.

SC5b-9 formation assay—The amount of soluble SC5b-9 (inactivated membrane attack complex, MAC) generated in 100% normal human serum as a result of complement activation by a variety of biomaterials was also measured using a commercially available ELISA kit (Figure 3). A hydroxyl-rich positive control, PVA, was included, as well as Silastic, a negative control containing no hydroxyl or amine groups. Fluorocarbon-rich FEP, the substrate used for our tetraglyme coatings, was included, as were the three tetraglyme coatings generated using 10, 20, and 80W deposition powers, displaying different ether carbon contents. In addition, PEG, MePEG, and undecanethiol self-assembled monolayers (SAMs) on gold were included as PEG controls. The PEG-SAMs contain a hydroxyl group terminus, while the MePEG-SAMs contain a methoxy terminus, similar to tetraglyme. The undecanethiol SAMs were included as a control for the PEG SAMs, and are composed of methoxy-terminated $(\text{CH}_2)_{11}$ chains.

The greatest amount of SC5b-9 was generated in serum incubated with poly(vinyl alcohol) (PVA) hydrogels, our positive control, about 10.2 ± 1.4 $\mu\text{g/ml}$. As summarized by the asterisks in Figure 3, we then tested to see if any of the average SC5b-9 values for other materials tested were statistically different than the average for PVA. The two tetraglyme coatings with high ether carbon generated using a 10W and 20W deposition powers, had SC5b-9 levels around 6.4 ± 0.8 $\mu\text{g/ml}$ and 6.2 ± 1.1 $\mu\text{g/ml}$, respectively. While these average values are lower than that generated by PVA, the difference in averages is not statistically significant, suggesting that 10W and 20W tetraglymes are, within the probability value for our statistical testing, as potent complement activators as PVA. Furthermore, the SC5b-9 levels for 10W and 20W tetraglyme were almost two times higher than that observed for Silastic, our negative control, at 3.4 ± 1.9 $\mu\text{g/ml}$, FEP, at 3.7 ± 0.8 $\mu\text{g/ml}$, and hydrocarbon rich 80W tetraglyme films, at 3.3 ± 1.3 $\mu\text{g/ml}$, all significantly lower than PVA. The hydroxyl terminated PEG-SAMs tested had SC5b-9 levels comparable to those seen for 10W and 20W tetraglyme coatings, 6.6 ± 1.3 $\mu\text{g/ml}$, while the methoxy terminated MePEG-SAMs appear to be less complement activating, with SC5b-9 values of approximately 4.7 ± 1.6 $\mu\text{g/ml}$. The hydrocarbon-rich undecanethiol SAMs were included as controls for the two PEG-SAMs, and were found to generate the lowest amount of SC5b-9 in serum after incubation, about 1.8 ± 0.7 $\mu\text{g/ml}$, lower than the negative control included.

The results of the SC5b-9 formation assay reported here were reproduced two separate times using different fresh tetraglyme batches as well as serum from three different donors each time.

Discussion

Because the complement system is known to be involved in inflammatory responses and is known to directly affect monocyte adhesion and activation, this study focused on assessing potential complement activation by PEO-like tetraglyme coatings using three approaches.

First, because we knew that biomaterial-induced complement activation has been linked to surface amine ($-\text{NH}_2$) and hydroxyl ($-\text{OH}$) groups [17], the hydroxyl group content on the ether carbon-rich tetraglyme films was measured. The hydroxyl and ether carbon binding energies in ESCA overlap, so TFAA derivatization was used to determine the presence of hydroxyl groups on the tetraglyme surface. No delamination or film thinning was expected

as a result of the vapor-phase reaction. Nevertheless, tetraglyme coatings deposited on glass were included as controls, to ensure that no artifacts were introduced due to the fluorine containing substrate. As expected, tetraglyme surfaces contained only a small amount of surface hydroxyl groups, about 2% for ether carbon-rich 10W tetraglyme coatings generated on both FEP and on glass cover slips and about 3% for hydrocarbon-rich 80W tetraglyme coatings (Table 3). The positive and negative controls matched the theoretical values of 0% and 50% for polystyrene (PS) and polyvinyl alcohol (PVA), providing evidence that the TFAA derivatization reaction progressed to completion and confirming the accuracy of the hydroxyl values measured for our tetraglyme surfaces.

The second approach we used to assess potential complement activation was to measure C3 adsorption to our surfaces. Although the low hydroxyl group content on tetraglyme coatings suggested that biomaterial induced complement activation should not occur, other researchers found that a small amount of the complement protein C3 and its activation fragments readily adsorb onto PEO [21]. Thus, C3 adsorption from 100% normal human serum was measured (Figure 1.a). Tetraglyme coatings generated using a 10W deposition power adsorbed less C3 than 80W tetraglyme films, which in turn adsorbed less C3 than FEP films. Although the adsorption of C3 to 10W tetraglyme was relatively low, approximately 20 ng/cm², we next investigated the strength of the protein-surface interaction. A weak interaction between C3 and the tetraglyme surface was expected, as covalent bond formation is characteristic of nucleophile-containing surfaces, which are known to induce complement activation.

To investigate the strength of the protein-surface interaction between adsorbed C3 and tetraglyme surfaces, the amount of irreversibly bound C3 on the surfaces was determined by measuring the amount of eluted ¹²⁵I-radiolabeled C3 from each of the materials after soaking in 2% SDS for 24 hours [38,39](Figure 1.b). Both 80W and FEP samples had a lower amount of retained C3 than did 10W samples, though the differences were not statistically significant. However, the percentage of originally adsorbed C3 retained on each of these materials after elution with SDS, known as adsorption strength, was found to be higher on 10W tetraglyme films than on either 80W or FEP samples, suggesting a tighter interaction between the adsorbed C3 and the 10W tetraglyme coating. The differences in adsorption strength between 10W samples and FEP were statistically significant, while the differences between 10W and 80W samples were not. Because there is a lower amount of C3 adsorbed on the 10W materials, these results might simply be reflecting the ability of the protein to make multiple contact spots on the surface, thus forming a tighter, more stable bond. However, the higher adsorption strength observed on our PEO-like tetraglyme films can also be a sign of surface-complement interaction, which has been previously shown to promote the generation of C3 convertase [5]. C3 cleavage resulting from potential complement activation was not studied as a part of this elution study. Nevertheless, the obtained results are the opposite of what might be expected of low-fouling coatings, suggesting the C3 adsorbed on tetraglyme coatings is held covalently, as might be the case if the protein is chemically bonded to the surface via hydroxyl group interaction (but, measured –OH content is very low).

Although adsorption of C3 on a biomaterial's surface is not always an indication of complement activation, its adsorption and high retention on tetraglyme even after SDS elution raised some concern. Thus, our final approach to assess potential complement activation by tetraglyme was to conduct two complement activation assays, which measured the activation markers C3a and SC5b-9. The formation of C3 convertase is the first step in the activation of the complement system, resulting in the cleavage of C3 into the anaphylatoxin C3a and the opsonin C3b, both of which are of biological importance for their effect on inflammatory cells. Because C3b is often adsorbed onto activating surfaces, C3a-

desArg, a degraded inactive and more stable form of C3a, is typically used as the target for detection in the fluid phase as a valuable marker of the progress of the initial stage of complement activation. The inactive form of the membrane attack complex (MAC), SC5b-9, was also probed in this study, as the cleavage of C5 during complement activation results in the generation of the potent anaphylatoxin C5a. Although C3a-desArg and SC5b-9 are common to all activation pathways, they are used here to measure material induced complement activation, as tetraglyme samples in this study were used immediately after being generated in the RFGD plasma reactor, believed to eliminate bound endotoxin. Endotoxin testing conducted on RFGD samples has confirmed this.

Thus, we used a commercially available ELISA kit to probe for C3a-desArg, referred to as C3a for convenience, in 100% serum incubated with biomaterial disks for 60 minutes (Figure 2.a), using EDTA plasma as a control (Figure 2.b). The largest amount of C3a was detected in serum incubated with 10W tetraglyme films, with the hydroxyl PEG-SAMs generating the second largest amount of C3a. FEP and 80W tetraglyme films, on the other hand, had the lowest amount of C3a generated, and were thus deemed the lowest complement activating materials tested. We tested all materials for statistically significant differences against the highest complement activator, 10W tetraglyme, and found that all materials generated a significantly lower amount of C3a than 10W tetraglyme, as indicated by asterisks (Figure 2.a), supporting our conclusion that 10W tetraglyme is indeed a strong complement activator. Furthermore, complement activation results were consistent with the C3 elution studies, where FEP and 80W samples retained a lower percentage of the initially adsorbed C3 than did 10W samples. Thus, C3 retention strength may be a more appropriate measure of complement activation potential by a biomaterial than total amount of adsorbed C3.

When the levels of the inactive membrane attack complex (MAC), SC5b-9, generated in serum after contact with biomaterials was measured (Figure 3), the 10W and 20W tetraglyme samples were found to be moderately complement activating. These materials generated higher SC5b-9 levels than the negative control, Silastic, and a lower amount than the positive control, PVA. Tetraglyme samples generated using an 80W deposition power, on the other hand, were found to be non-activating, as approximately the same amount of SC5b-9 was generated as that observed with Silastic. These values were approximately 1.5 times larger than that observed from our untreated polystyrene control. Despite the variations in the surface chemistries of 10W and 20W tetraglyme samples, no difference was observed in the amount of SC5b-9 generated by each of these materials, suggesting a common surface characteristic of high ether carbon coatings that may be involved in complement activation. Furthermore, the low levels of SC5b-9 observed with the low ether carbon, high hydrocarbon 80W tetraglyme films support this. However, the specific surface functionality responsible for this complement activation phenomenon is unclear at this time. Previous studies have examined in detail the chemistry of these low power tetraglyme films and no obvious candidate chemical group as the complement trigger has been found[40].

Like 80W tetraglyme coatings, FEP substrates were also found to be less complement activating than 10W tetraglyme, as was determined using the C3a assay. PEG and MePEG SAMs, with hydroxyl and methoxy end groups, respectively, displayed slightly different degrees of complement activation. The hydroxyl terminated SAMs generated SC5b-9 levels similar to the 10W and 20W tetraglyme coatings, while the methoxy terminated SAMs were less complement activating, with lower SC5b-9 levels. Undecanethiol controls were found to be the least complement activating surfaces, suggesting that the levels of SC5b-9 generated by PEG- and MePEG-SAMs stem from the PEG and terminal group exclusively, and are not a result of substrate exposure. Further study will be necessary in order to understand why hydroxyl-terminated PEG-SAMs generate similar amounts of SC5b-9 than low power

tetraglyme coatings, which contain a very low surface hydroxyl concentration. We again tested all our materials for statistically significant differences against our greatest complement activator, our positive control, PVA. Silastic, FEP, MePEG-SAMs, undecanethiol SAMs, PS, and 80W tetraglyme samples all generated significantly lower amounts of SC5b-9 than PVA. However, 10W and 20W tetraglymes and PEG-SAMs all generated similar levels of SC5b-9, which were not significantly lower than the levels generated by PVA, supporting our conclusion that these materials are strong complement activators.

The results obtained in the studies presented here, particularly the detection of the activation products C3a and SC5b-9 in serum that has been incubated with high ethercarbon tetraglyme films, raise many questions that still need to be answered. First, it is currently unclear which surface characteristic of these tetraglyme films is responsible for complement activation, as low hydroxyl levels have been measured on these surfaces. Recent research [41] suggests that adsorbed proteins on a biomaterial's surface, such as IgG, might initiate complement activation via the classical pathway, generating C3b molecules that can then adsorb on a biomaterial's surface, triggering the alternative pathway amplification loop. Though we did not test this hypothesis, C4 depleted sera could be used to incubate materials prior to activation assays, thus excluding possible classical and mannose-binding lectin pathway activation.

Secondly, the formation of the measured activation products arises from the cleavage of molecules whose fragments are known to be potent anaphylatoxins. By studying the levels of SC5b-9 produced, we can estimate the relative amount of C5a generated, which is of interest as a potent anaphylatoxin known to activate leukocytes. Similarly, C3a measurement is a useful monitor of C3 fragment levels, such as C3b and iC3b. C3b is an opsonin involved in mediating phagocytosis and cell adhesion by monocytes and macrophages, while iC3b, a degradation product of C3b, is known to be a ligand for the monocyte $\alpha_M\beta_2$ integrin.

Although monocyte interactions with these surfaces were not probed as part of this study, the implications of complement activation with regards to monocyte adhesion cannot be ignored. Monocytes are the primary cell type of interest for their role in the FBR, and studies aimed at better understanding the FBR to biomaterials need to consider the relationship between the mechanism being investigated and its effect on inflammatory cells. Our studies on the adhesion of primary human monocytes, presented in a forthcoming publication [40], support the importance of complement proteins, particularly C3 and its fragments, in monocyte adhesion. Specifically, the absence of C3 in a variety of protein solutions led to a drop in the number of adherent monocytes to tetraglyme, which could be restored upon replenishment of C3 to the protein solution [40].

Finally, we became interested in the interaction between the complement system and tetraglyme coatings as we tried to understand the classic FBR observed upon implantation of tetraglyme coatings despite the low protein adsorption and monocyte adhesion *in vitro*. Because samples for these *in vitro* complement studies were used immediately after being generated in the RFGD plasma reactor and thus were sterile at that time, no further sterilization procedure was implemented, unlike our *in vivo* samples, which were soaked in ethanol prior to implantation. Nevertheless, endotoxin tests conducted on both ethanol soaked polymerized tetraglyme coatings [1] and tetraglyme coatings fresh from the RFGD plasma reactor (data not shown) confirmed lack of endotoxin contamination, so we felt justified in not soaking our polymerized tetraglyme films in ethanol prior to conducting these studies. Since various complement proteins are known to recruit and activate inflammatory cells, we carefully designed these studies to shed some light in the area of complement activation by low-adsorbing synthetic surfaces. The findings presented here and

in our forthcoming publication [40] indicate an important but unexpected interaction between complement proteins and PEO-containing surfaces that may have implications in the FBR to these materials. However, despite using a well-controlled *in vitro* model for our studies, care must be taken when extrapolating from our results, obtained using a simplified environment with static serum, to explain the phenomena observed *in vivo*, where a much more complex system is involved. Nevertheless, our findings suggest an important, yet previously overlooked, role of complement proteins and their activation, and which requires closer study.

Conclusion

Complement C3 adsorption to 10W tetraglyme coatings from 100% serum was found to be relatively low, approximately 20 ng/cm², and was lower than that observed on 80W and FEP films. Because complement activation by biomaterials hinges on the covalent binding of C3 to nucleophiles on the surface, we probed the hydroxyl content of tetraglyme surfaces, and found it to be low, about $2.4 \pm 0.4\%$ for 10W and $2.7 \pm 0.3\%$ for 80W tetraglymes, respectively. To gain a better understanding of the protein-surface interaction, we tested the retention strength of C3 on these surfaces using SDS elution. Despite adsorbing the lowest amount of C3 of all the surfaces, 10W tetraglyme coatings retained the greatest percentage of originally adsorbed C3, suggesting a strong or covalent interaction between adsorbed C3 and these surfaces. Relatively high levels of C3a and SC5b-9 formation were found in serum incubated with 10W tetraglyme, probably due to alternative pathway complement activation. These findings suggest that complement activation and/or adsorption may have consequences upon introducing tetraglyme materials into the body, and may be a contributor to the FBR to a tetraglyme coated surface.

Acknowledgments

We gratefully acknowledge financial support from the NSF through UWEB EEC9529161 and the NIH through the Engineered Biomaterials Training Grant (T32 GM065098). We thank NESAC/BIO (supported by NIH 5P41 EB002027 from the National Center for Research Resources) for support of the surface analysis experiments.

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Figure 1.

a) Radiolabeled pure C3 was adsorbed to 10W and 80W tetraglyme coatings, as well as FEP films, from 100% normal human serum for 2 hours, and eluted with 2% SDS for 24 hours. Data are displayed as mean $\pm$ SEM; $n = 3$. Asterisks denote the statistically significant differences in the amount of eluted C3 from 10W tetraglyme as compared to FEP ($\alpha = 0.05$). b) Protein adsorption strength of C3 from 100% serum was defined as the percentage of adsorbed protein that was retained on the materials' surface after soaking in 2% SDS for 24 hours, and was measured by ^{125}I adsorption. Data are displayed as mean $\pm$ SEM; $n = 4$. Asterisks denote the statistically significant differences in the adsorption strength of C3 on 10W tetraglyme and FEP ($\alpha = 0.05$).

Figure 2.

C3a (µg/ml) generated in (a) 100% pooled human serum (N = 3 donors) and (b) 100% pooled EDTA plasma (N = 3 donors) as a result of biomaterial induced complement activation. Data are displayed as mean $\pm$ SEM; n = 3. Asterisks denote statistically significant differences in amount of C3a (µg/ml) generated by the various materials tested as compared to 10W tetraglyme ($\alpha = 0.05$). All samples tested were found to be lower complement activators than 10W tetraglyme.

Figure 3.

Biomaterial induced complement activation was evaluated by measuring the amount of soluble SC5b-9 (in µg/ml) present in 100% serum after incubating for 90 minutes at 37°C with different biomaterials. Data are displayed as mean ± SEM; n = 9 per material (n = 3 per donor). Asterisks denote statistically significant differences in the amount of SC5b-9 (µg/ml) generated in serum by various biomaterials as compared to PVA (positive control; alpha = 0.05). There was no statistically significant difference in the average amount of SC5b-9 generated by 10W and 20W tetraglyme and PEG-SAMs as compared to PVA, indicating they are strong complement activators.

Table 1

Elemental composition and high resolution carbon functional group composition (C_{1s}) of 10W, 20W, and 80W RFGD tetraglyme coatings analyzed using XPS. Data are displayed as mean $\pm$ SEM; n = 3.

Sample	O _{1s}	C _{1s}	C/O	C-C/C-H	C-O	C=O
10W	31 $\pm$ 0.3	69 $\pm$ 0.3	2.2	14 $\pm$ 1	82 $\pm$ 1	5 $\pm$ 0.3
20W	28 $\pm$ 0.3	72 $\pm$ 0.2	2.6	26 $\pm$ 1	69 $\pm$ 1	5 $\pm$ 0.3
80W	8 $\pm$ 0.1	92 $\pm$ 0.2	11.1	88 $\pm$ 1	7 $\pm$ 2	5 $\pm$ 3

Table 2

Elemental composition data and surface coverage of the SAMs on gold as determined by XPS at a takeoff angle of 55°. ^a

Sample	Atomic Percent				Atomic Percent w/o Gold				C/O	(C+O)/Au
	Au 4f	C 1s	O 1s	S 2p	C 1s	O 1s	S 2p			
PEG	17.3	60.1	20.2	2.4	72.7	24.4	2.9	3.0	4.64	
Theoretical	...	...	...	...	76.0	20.0	4.0	3.8	4.61	
MePEG	17.5	60.2	20.2	2.1	74.4	23.1	2.4	3.2	4.58	
Theoretical	...	...	...	...	76.9	19.2	3.8	4.0	4.84	

^a All standard error of the means <2%

Table 3

Hydroxyl group concentration as determined by ESCA analysis of TFAA derivatized surfaces. Data are displayed as mean $\pm$ SEM, with n = 3.

Sample	F/C	(-OH)%
Spin-cast PVA	0	51.5 $\pm$ 0.5
TFAA derivatized spin-cast PVA	0.74 $\pm$ 0.0	49.8 $\pm$ 0.3
Theoretical (Derivatized)	0.75	
Spin-cast PS	0	0
TFAA derivatized spin-cast PS	0	0
10W Tetraglyme coatings on FEP	0.03 $\pm$ 0.01	2.1 $\pm$ 0.01
10W Tetraglyme on glass cover slips	0.03 $\pm$ 0.0	2.4 $\pm$ 0.01
80W Tetraglyme coatings	0.03 $\pm$ 0.0	2.7 $\pm$ 0.01