Abstract: Activation of the complement system is one way in which the human body reacts to foreign materials that come in contact with blood. Poly(ethylene oxide) (PEO) has been used quite frequently to modify biomaterial surfaces to prevent protein adsorption and cell adhesion. Despite extensive use of PEO, however, PEO-induced complement activation has not been examined before. We examined the complement activation by PEO chains grafted to glass surfaces. PEO was grafted to trichlorovinylsilane-treated glass (TCVS-glass) by γ-irradiation using PEO homopolymer, Pluronic® F108 (PF108), and PEO-polybutadiene-PEO triblock copolymer (COP5000). Complement activation was assessed by measuring the plasma C3a level. Of the three polymers grafted (PEO, PF108, and COP5000), only PF108 showed significant increases in complement activation over controls. Complement C3a production on PF108-grafted glass was linearly dependent on surface concentration of grafted PF108. The C3a concentration increased from 46 ng/mL to 316 ng/mL as the surface PF108 concentration increased from 0 – 0.25 mg/cm². Kinetics of C3a generation by PF108-grafted surfaces show that 60% of the steady state C3a concentration was generated during the first hour of plasma exposure. When the same PF108-grafted glass surface was repeatedly exposed to fresh plasma, the amount of C3a generated decreased by 70% after the first exposure. This supports the “single-hit” mechanism in complement activation. PEO homopolymer did not activate complement in bulk solution, and, thus, it appears that C3a complement activation by PF108-grafted surfaces is due to the presence of poly(propylene oxide) units. Grafting of PEO using PEO-containing block copolymers requires examination of complement activating properties of the non-PEO segment. © 1999 John Wiley & Sons, Inc. J Biomed Mater Res (Appl Biomater) 48: 640–647, 1999

Keywords: complement activation; poly (ethylene oxide) grafting; glass surfaces; Pluronic surfactants; single-hit mechanism; gamma-irradiation; plasma

INTRODUCTION

The reaction of the human body to implantable materials is diverse and complex. Protein adsorption, cellular adhesion, and complement activation are manifestations of a myriad of reactions of the human body toward foreign materials. When a biomaterial encounters blood, protein adsorption is the first step in a series of events leading to thrombosis and failure of the biomaterial. In addition, the body’s recognition of implanted foreign materials begins with activation of the complement system. The complement system is a host defense system comprising more than 20 plasma proteins. The complement system can be activated by various substances recognized as foreign to the body. Two pathways of activation of the complement system exist: the classical and the alternative pathways. Both pathways are activated by immunoglobulin-based stimulants (immunologic) and direct (non-immunologic) stimulants, such as polysaccharides, lipid A, and trypsin-like enzymes. Lipopolysaccharides contain a complex polysaccharide component to which lipid A is attached. Lipopolysaccharides derived from gram negative bacteria are potent activators of the complement system and are used widely as a positive control in complement activation assays. When the complement system is activated, a cascade of chemical reactions takes place leading to inflammation.

The relationship between surface-induced complement activation and thrombosis is not clear, but there have been claims that complement activation promotes thrombosis by enhancing cellular adhesion, platelet aggregation, and platelet activation. Complement activation is also suspected of causing reduction of platelets in the circulation, due possibly to its enhancement of platelet aggregation. Complement activating surfaces attract neutrophils and leukocytes that release chemicals such as histamine that promote platelet adhesion and aggregation. Platelet aggregation and consumption are observed in parallel with complement activation in platelet concentrates stored following standard blood-banking procedures. In addition, aggregation and adhesion
of platelets onto poly(methyl methacrylate) (PMMA) and nylon-6,6 surfaces were found to be significantly lower when exposed to decomplemented blood compared to the same material exposed to normal blood.9 Dog experiments with vascular grafts modified with various polymers showed that those surfaces causing more complement activation were also more thrombogenic, and that infusion of a decomplementing compound resulted in marked decreases in thrombogenesis.8

Although many investigators have examined complement activation using a wide variety of biomaterials,8,9,13–18 systematic studies of complement activation by poly(ethylene oxide) (PEO)-grafted surfaces have not been reported. PEO grafting is known to prevent protein adsorption and platelet adhesion in vitro.19–22 Ex vivo studies, however, have shown that PEO grafting may not render blood contacting biomaterials nonthrombogenic.20,23 Thrombus formation on PEO grafted biomaterials in ex vivo and in vivo experiments is commonly observed.23,24 Since PEO-grafting has been used as one of the major approaches for making surfaces biocompatible,25–28 it is important to examine the complement activating potential of PEO-grafted surfaces. This may provide new information necessary to understand differences in results between in vitro and ex vivo (or in vivo) studies.

**MATERIALS AND METHODS**

**PEO Grafting**

Glass coverslips (#1 thickness, Bellco Glass Inc., Vineland, NJ) were cleaned by soaking in a 50:50 mixture of chromic acid and sulfuric acid overnight and washed extensively with running distilled, deionized water (DDW). They were dried in a 60°C oven. Clean glass coverslips were coated with trichlorovinylsilane (Aldrich, Milwaukee, WI) following a procedure used previously in our laboratory.29 Briefly, clean glass coverslips were immersed in a 5% solution of trichlorovinylsilane (TCVS) in chloroform (analytical grade, Mallinkrodt, Paris, KY) for 3 h. Trichlorovinylsilane-treated glass (TCVS-glass) coverslips were rinsed with chloroform and ethanol in sequence twice followed by a copious amount of DDW before drying and curing at 60°C. TCVS-glass coverslips were stored covered at room temperature until use.

PEO was grafted to the TCVS-glass surfaces using Pluronic® F108 (PF108) (BASF, Parsippany, NJ), PEO-polybutadiene-PEO triblock copolymer (COP5000), and PEO homopolymer (PEO2000) [molecular weight = 2,000 (1,900 – 2,200), >95% purity, Fluka Chemical Co., Ronkonkoma, NY] (Table I). COP5000 with PEO chains of molecular weight of 5,000 was synthesized in our laboratory. PEO-grafting and surface characterization are described in our previous publications.19,25,28–33

Solutions of PF108, COP5000, and PEO2000 were prepared in DDW at concentrations ranging from 0.01–10 mg/mL. TCVS-glass coverslips were exposed to these polymer solutions in disposable glass culture tubes (16 mm × 100 mm, Pyrex®, Corning Inc., Corning, NY). Care was taken to ensure that both faces and edges of coverslips were exposed to the grafting polymer solutions. After 1 h of adsorption, the polymer-adsorbed coverslips were exposed to γ-radiation from a 60Co source in the presence of DDW (grafting condition A) or in the presence of bulk aqueous polymer solution (grafting condition B) for a total radiation dose of 0.8 Mrad. After γ irradiation, glass coverslips were soaked in 1% SDS solution overnight and rinsed thoroughly with running DDW for 3 min to remove noncovalently attached polymer molecules. Our previous study showed that the attached PEO remained on the surface after treatment with SDS and extensive rinsing.28–30 The PEO-grafted glass coverslips were dried at 60°C overnight and used for complement activation experiments within two days.

**Surface Density of Grafted PEO Chains**

PF108 was radio-labeled using a method described previously.32 Briefly, the terminal hydroxyl groups of PEO chains on PF108 were reacted with a 5-fold molar excess of p-methoxyphenylchloroformate (MPC, Aldrich, Milwaukee, WI) in acetonitrile for 48 h at room temperature. The ester product (MP-PF108) was precipitated in cold anhydrous ether (analytical grade, Mallinkrodt, Paris, KY), filtered, and washed with 500 mL fresh ether twice to remove unreacted MPC. The modified polymer was then radio-labeled with 125I using the Iodo-Gen reagent (Pierce Co., Rockford, IL). Radio-labeled polymer was separated from free 125I by gel filtration using Sephadex® G-10 (exclusion limit of 700 Daltons, Pharmacia, Piscataway, NJ). The radio-labeled polymer was diluted with DDW to prepare adsorption solutions with concentrations ranging from 0.01–10 mg/mL. TCVS-glass capsillaries were injected with the polymer solutions and adsorption was allowed to continue for 1 h at room temperature. They were then rinsed with DDW to remove excess polymer and the surface-bound radioactivity was determined with a γ-scintillation counter (Gamma 5500B®, Beckman, Arlington Heights, IL). The raw γ counts were divided by polymer solution specific activity and sample surface area to yield adsorption data in μg/cm².

**Complement Activation**

Blood was drawn from three healthy human donors into a chilled potassium-EDTA (0.07 mL, 0.34 M K3-EDTA in a 13 mm × 100 mm tube) Vacutainer (Becton Dickinson, Franklin Lakes, NJ). The raw counts were divided by polymer solution specific activity and sample surface area to yield adsorption data in μg/cm².
Lakes, NJ) by a certified technician. Blood was not pooled, and blood from each donor was used in a separate experiment. The blood was transferred immediately into chilled 15 mL polypropylene centrifuge tubes (Corning, Corning, NY) and centrifuged at 2,000 g for 15 min at 4°C in a fixed-angle rotor centrifuge (JA21, Beckman, Arlington Heights, IL). The separated plasma was used immediately within 1 h.

A lipopolysaccharide (LPS) from E. coli, (Serotype 0111: B4, Sigma, St. Louis, MO), a potent complement activator, was used as a positive control. The effect of concentration of LPS on the extent of complement activation was examined. LPS was mixed with plasma in a sterile tissue culture polystyrene multiwell plate (Falcon, #3047, Becton Dickinson, Franklin Lakes, NJ) to give final concentrations of LPS in plasma ranging from 1–52 mg/mL. The multiwell plate was incubated at room temperature for 1 h. Plasma samples were removed and examined for complement activation by measuring the C3a concentration using a commercial human complement C3a des Arg125 I assay system (Amersham Life Sciences, Arlington Heights, IL). A concentration of LPS that gave an optimum degree of complement activation was chosen and used as a positive control.

PEO-grafted glass coverslips (circular, #1 thickness, 1.2 cm diameter) were hydrated with normal saline (Sigma, St. Louis, MO) for 1 h in a sterile multiwell plate prior to exposure to plasma. One milliliter of plasma was placed into each well. Samples used were glass coverslips grafted with PF108, COP5000, or PEO 2000. Glass and TCVS-glass coverslips were used as controls. The multiwell plate was incubated at room temperature for 1 h. Plasma samples (0.45 mL) were taken and assayed for C3a levels.

**Kinetics of Complement Activation**

Kinetics of complement activation by PEO-grafted surfaces were examined using glass grafted with PF108. A PF108 solution (concentration of 10 mg/mL in DDW) was used for grafting TCVS-glass coverslips (#1 thickness, 9 mm × 22 mm). PF108-adsorbed glass coverslips were gamma-irradiated in the presence of PF108 solution (total radiation dose of 0.8 Mrad), washed with 1% sodium dodecyl sulfate (SDS) and rinsed with DDW before drying at 60°C overnight. The surface PEO chain concentration was determined by 125I-labeled experiments to be 0.25 μg/cm². PF108-grafted surfaces were exposed to plasma and incubated at room temperature. Plasma samples (0.45 mL) were taken at timed intervals up to 48 h post-exposure and assayed immediately for C3a.

**Effect of Repeated Exposure to Fresh Plasma**

Glass coverslips (#1 thickness, 9 mm × 35 mm), grafted with PF108 at a surface PEO concentration of 0.25 μg/cm², was hydrated with normal saline (0.9% NaCl solution) for 1 h. Normal saline was replaced with plasma and incubated for 1 h at room temperature. Plasma-exposed coverslips were rinsed briefly with normal saline and placed in fresh plasma every hour. Plasma samples were taken for analysis every hour up to 4 h.

**Complement Activation by Solution-Phase Polymers**

Solutions of PF108, COP5000, and PEO2000 were prepared in concentrations twice the desired final concentrations. Final polymer concentrations after mixing with fresh plasma ranged from 0.7–714 μM. One milliliter of each PEO solution was placed in a well in a multiwell plate and mixed with 1 mL of fresh plasma. The multiwell plate was incubated at room temperature for 1 h. Plasma samples (0.45 mL) were taken and assayed for C3a.

**RESULTS**

**Complement Activation by PEO-Grafted Glass Surfaces**

The amount of C3a generated by the added lipopolysaccharide (LPS) in human plasma was linearly dependent on the concentration of LPS added over the range of 1–51.8 mg/mL (Fig. 1). The amount of C3a generated in control plasma alone was subtracted from each value to distinguish the influence of LPS on C3a generation (i.e., complement activation).

Figure 2 shows complement activation detected on control glass surfaces and surfaces grafted with PEO, PF108, and COP5000 under two different grafting conditions. Background C3a level in the plasma incubation control (Control I in Fig. 2) was 337.1 ± 45.3 ng/mL. The background level of plasma C3a, as determined for a total of 57 normal human
male and female, was $104 \pm 6\text{ ng/mL}$ with a range of $47$–$211\text{ ng/mL}$. The value for our incubation control is higher than this reported endogenous C3a level in normal human plasma. This may be due to C3a activation by the polystyrene multiwell plate during plasma incubation. Treatment of glass with TCVS and exposure to $\gamma$-radiation did not change complement C3a activation significantly. TCVS-glass (Control II in Fig. 2) generated a C3a concentration of $384.7 \pm 189.4\text{ ng/mL}$, while TCVS-glass subjected to $\gamma$-irradiation in the presence of DDW (Control III of Fig. 2) resulted in C3a concentration of $321.1 \pm 109.0\text{ ng/mL}$. There were no statistical differences among these values. Glass surfaces grafted with PEO homopolymer activated complement to the similar extent as these controls. PEO homopolymer-grafted surfaces gave C3a concentrations of $496.6 \pm 206.8$ and $423.1 \pm 135.6\text{ ng/mL}$ when grafted under conditions A and B, respectively. The C3a concentrations generated by COP5000-grafted glass were $591.7 \pm 2.7$ and $524.1 \pm 92.8\text{ ng/mL}$ for grafting conditions A and B, respectively. Glass surfaces grafted with PF108 under Condition A gave a C3a level of $468.5 \pm 84.7\text{ ng/mL}$, whereas surfaces grafted under Condition B generated $733.3 \pm 101.5\text{ ng/mL}$ of C3a. These differences in amounts of C3a generated between the two grafting conditions were not statistically significant ($p > 0.1$) when PEO2000 and COP5000 were used for grafting. However, PF108 grafting resulted in statistically significant differences ($p < 0.1$) in the amount of C3a generated depending on the grafting condition. Grafting condition B, in which samples were exposed to $\gamma$-radiation in the presence of polymers in bulk solution, usually results in higher surface concentration of grafted PEO chains. The significant differences in complement activation observed with PF108-grafted surfaces indicate that the surface concentration of the grafted PF108 may affect the overall complement activation in certain cases. For this reason, the effect of the surface concentration of the grafted PF108 was examined in more detail.

**Effect of the Surface PEO Concentration**

Effect of the surface concentration of PF108 on complement activation is shown in Figure 3. Surface concentration of PF108 was varied from $3.9$–$35.1\text{ pmol/cm}^2$. The plasma background C3a concentration was subtracted from the total value. Complement C3a activation linearly increases with the increase in surface PF108 concentration. The C3a concentration changed from $104$–$375\text{ ng/mL}$ as the PF108 surface concentration increased from $0$–$0.25\mu\text{g/cm}^2$. It is apparent that the higher the surface PF108 concentration, the more activating the surface toward C3a.

**Kinetics of C3a Generation**

C3a generation by PF108-grafted surfaces was monitored for up to $48\text{ h}$ of exposure to plasma. Glass coverslips with a surface PF108 concentration of $0.25\mu\text{g/cm}^2$ were used as the test surface. Figure 4 shows the kinetics of C3a generation by the PF108-grafted surface (● in Fig. 4). A rapid increase in C3a concentration was observed within $1\text{ h}$ of exposure.

![Figure 3. Concentration of C3a generated as a function of the surface concentration of grafted PF108. The background level C3a in plasma was subtracted to distinguish the effect of PF108 chains on C3a generation. TCVS-glass exposed to $\gamma$-irradiation was used as a control. $n = 3$, mean ± S.D.](image-url)
followed by a slow increase reaching a plateau after 24 h. During this same time period, increases in the concentration of C3a in the plasma incubation control were also observed (△ in Fig. 4). To separate the effect of PF108 on complement activation, the amount of C3a generated by the control plasma sample was subtracted from the original readings of C3a concentration from the test material. Complement C3a activation by the PF108-grafted surface only (○ in Fig. 4) remained constant for the first 24 h. Thus, it appears that the surface grafted PF108 has only limited capacity to induce complement activation.

Repeated Exposure

To gain information on surface-grafted PF108 effects on complement activation, the same PF108-grafted surface was exposed to fresh plasma repeatedly. Plasma was replaced with fresh plasma every hour up to 4 h. Figure 5 shows that the PF108-grafted surface decreases C3a formation substantially upon repeated exposure to fresh plasma. To separate the effects of the surface-grafted PF108, C3a generated by the control plasma (△ in Fig. 5) was subtracted from the total value (● in Fig. 5). The resulting net C3a level (○ in Fig. 5) generated by the first exposure to plasma was 591 ng/mL, and this value decreased to 162.6 ng/mL at the second exposure. Only a minimal C3a effect was observed by surface-grafted PF108 at the fourth plasma exposure.

Complement Activation by Free Polymer Molecules in Plasma

Since PEO-grafted surfaces were found to be complement activating, we investigated the ability of polymer molecules dissolved in bulk aqueous solution to activate complement C3a as well. Solutions of PEO2000, COP5000, and PF108 in concentrations ranging from 1.4 – 1428.6 μM were tested for complement activation. To extract the effect of the dissolved polymer molecules, the C3a value for the incubation control was subtracted from the C3a value obtained for each polymer concentration. As shown in Figure 6, C3a concentration levels in plasma increased as bulk concentrations of PF108 and COP5000 increased. The C3a level generated by the dis-
solved PEO2000, however, remained relatively constant near 150 ng/mL even though the PEO2000 concentration increased to 714 μM. The difference in complement activation by PEO homopolymer (PEO2000) and PEO-containing block copolymers (PF108 and COP5000) suggests that polymer chemistry in segments other than the PEO block is responsible for observed complement C3a activation.

**DISCUSSION**

Blood compatibility of a biomaterial depends on the nature of interactions between blood components and the biomaterial surface. It is known that grafting hydrophilic molecules such as albumin, PEO, and heparin on biomaterial surfaces reduces protein adsorption and platelet adhesion.1,33 Hydrophilic materials such as cellulose dialysis membranes, poly(hydroxyethyl methacrylate) (polyHEMA), and Sephadex® are known to activate the complement system via the alternative pathway. The alternative pathway of complement activation begins with the covalent attachment of C3b, a complement system protein, to surfaces bearing nucleophiles such as amino and hydroxyl groups.16,35 Surface nucleophiles react with a labile thioester group on the C3b molecule resulting in a covalent attachment. Once bound to the surface, the C3b molecule interacts with other complement system proteins to form the C3 convertase, which augments C3 cleavage and the subsequent deposition of more C3b molecules on the activating surface. Grafting of PEO or PEO-containing polymers onto biomaterial surfaces introduces hydroxyl groups. PEO hydroxyl groups are, therefore, likely to be involved in complement activation. The flexible PEO chain extends from the surface into bulk aqueous solution making the hydroxyl groups accessible for interaction with complement proteins. As shown in Figure 3, higher surface concentrations of grafted PF108 result in increased complement activation. Increased concentrations of hydroxyl groups on the surface may be a contributing factor.

Generation of C3a by a complement activating surface in contact with plasma follows a “single-hit” mechanism as suggested for poly(HEMA) by Payne et al.15 In this mechanism, an activating surface causes a burst in the production of C3a the first time it comes in contact with plasma. Subsequent exposures of the same surface to fresh plasma generate only a small quantity of C3a. This single-hit mechanism is consistent with the observation that hemodialysis membranes become less and less complement activating with repeated use.36 We also observed that complement activation by the PF108-grafted glass follows the same “single-hit” mechanism as other complement activating surfaces. Figure 5 shows that the PF108-grafted glass, upon exposure to fresh plasma, causes an initial burst of C3a production followed by a decline in C3a generation on subsequent fresh plasma exposures. We also examined the kinetics of complement activation by PEO-grafted glass. The rate of C3a generation by the PEO-grafted surface was the highest during the first hour of exposure (Fig. 4). Maximum complement activation was observed only after the first 1 h of exposure to plasma, and this also support the “single-hit” burst mechanism of C3a generation by the PEO-grafted surfaces.

The degree of complement activation by PEO-grafted surfaces has indeed been shown to be lower compared to the lipopolysaccharide positive control. However, to truly assess the significance of complement activation by PEO-grafted surfaces, it would be proper to compare them with other polymeric materials with a history of complement activation such as Cuprophane® and nylon oxygenator membranes.15 Complement activation by these materials is suspected of causing complications such as pulmonary dysfunction,37 hypoxemia,38 and reduction of circulating platelets in the blood.12 Table II shows amounts of C3a generated by PEO-grafted surfaces used in our study, as well as by nylon oxygenator membrane, Cuprophane®, and zymosan found in the literature.15 Glass surfaces grafted with PEO homopolymer generated the lowest amount of C3a (19 ng/cm²), whereas COP5000- and PF108-grafted glass surfaces generated 41.5 ng/cm² and 45 ng/cm² of C3a in our assay, respectively. The C3a level generated by Cuprophane and nylon were reported to be 63 ng/cm² and 70 ng/cm², respectively. Differences in C3a generation between the PEO homopolymer and PEO-containing block copolymers (PF108 and COP5000) indicates that factors other than hydroxyl groups may also play a role in complement activation. It is not clear at this point which groups of the hydrophobic segments of block copolymers are responsible for complement activation. It is clear, however, that PEO-containing block copolymers having shorter hydrophobic segment elicit less complement activation.

**TABLE II. Complement Activation by PEO-Grafted Surfaces Compared to Other Surfaces Known to Activate Complement**

<table>
<thead>
<tr>
<th>Surface</th>
<th>cm²/0.5 mL Plasma</th>
<th>ng C3a/cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO2000-grafted glass</td>
<td>2.26</td>
<td>19</td>
</tr>
<tr>
<td>COP5000-grafted glass</td>
<td>2.26</td>
<td>41.5</td>
</tr>
<tr>
<td>PF108-grafted glass</td>
<td>2.26</td>
<td>45</td>
</tr>
<tr>
<td>Cuprophane®</td>
<td>5</td>
<td>48</td>
</tr>
<tr>
<td>Nylon oxygenator®</td>
<td>5</td>
<td>63</td>
</tr>
<tr>
<td>Zymosan®</td>
<td>250</td>
<td>48</td>
</tr>
</tbody>
</table>

* From Ref. 15.
that mediate the adhesion and activation of platelets on complement activating surfaces. Activation of the complement system is known to mediate chemotactic, adhesive, and phagocytic responses of polymorphonuclear leukocytes to foreign substances. Leukocyte adhesion to complement activating surfaces is caused by the interactions of complement fragments bound to the activating surface with receptors on the leukocyte membrane. Surface-bound leukocytes release chemicals such as platelet-activating and tissue factors. Activation of platelets by the complement-activating factor and the initiation of coagulation by the tissue factor may play a critical role in the process of surface-induced thrombosis.

We have also shown that PEO chains grafted onto biomaterial surfaces enhance, albeit modestly, complement activation. This activation of the complement system, however modest it may be, may be enough to initiate platelet adhesion and subsequent thrombosis on biomaterial surfaces used in long-term therapy. When PEO-containing block copolymers are used for surface modification of biomaterials, complement activation can be significantly higher due to the activation by non-PEO segments of the copolymers. Thus, examination of the complement-activating properties of PEO-containing block copolymers may be necessary.

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