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The method of surface PEGylation influences leukocyte adhesion and activation

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Abstract The influence of different surface modifications with poly(ethyleneglycol) (PEG) layers on the adsorption of fibrinogen and the adhesion and activation of macrophage-like human leukocytes was investigated. Poly(ethylene terephthalate) (PET) was modified using pulsed AC plasma polymerization with two types of starting monomers to generate: 1) a reactive acid surface using maleic anhydride (MAH) as monomer, and 2) a PEG-like surface using diethyleneglycol methyl vinyl ether (DEGVE) as monomer. The MAH surface was used as a reactive platform to graft linear chains of non-fouling mPEG via an intermediate layer of poly(ethyleneimine) (PEI) under lower critical solution temperature (LCST) conditions of the mPEG. The DEGVE monomer is used to create PEG-like layers by use of low power plasma conditions. The ability of the surfaces to resist protein adsorption was investigated quantitatively using ^{125}I-radiolabeled human fibrinogen, and the conformation of the adsorbed protein was tested using an anti-fibrinogen monoclonal antibody in an enzyme-linked immunosorbent assay. The results showed that PEGylated surfaces adsorbed significantly less (up to 90% less) fibrinogen, and that unfolding of adsorbed fibrinogen was more pronounced on the linear mPEG layers than on the PEG-like plasma polymer surfaces. Adhesion of in-vitro differentiated macrophage-like U937 cells was reduced on both the PEG-like plasma polymer surfaces and the linear mPEG layers compared to the unmodified PET surface, but cells adhering to the PEG-like plasma polymer surfaces secreted less tumor necrosis factor-α (TNF- α) than cells adhering to the linear mPEG layers. In conclusion, the method for preparing non-fouling surfaces for long-term implanted devices influence surface-induced cellular responses of the host.

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1. Introduction

All biomaterial surfaces developed so far adsorb proteins immediately after implantation. The adsorbed protein layer is the first step in a series of processes that direct cellular interactions with biomaterials and ultimately cause implant failure. Recognition of the protein layer by monocytes and macrophages is a pivotal step in the organization and development of the foreign-body inflammatory response. Adherent monocytes/macrophages may fuse to form foreign-body giant cells (FBGCs), even in *in-vitro* assays [1, 2]. Monocytes and macrophages have been shown to be involved in both the healing of tissue around implants and in the inflammatory processes that lead to implant failure [3]. Therefore, controlling adhesion and activation of inflammatory cells to the biomaterial surface is desirable for modulating the biocompatibility of long-term implanted materials. Monocyte and macrophage adhesion is mediated by complex interactions between the material surface and adsorbed proteins [4]. The cells adhere to the protein adlayer using cell-membrane-bound receptors [5]. Activated macrophages can further release signaling factors to modulate the function of other cell types involved in the inflammatory response [6]. The cytokines interleukin-1α (IL-1α), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are the most

important pro-inflammatory signals produced by these cells [7, 8].

Both chemical [9] and topographic [10] modifications of biomaterial surfaces have shown promise at controlling protein adsorption and subsequent adhesion of monocytes/macrophages. Brodbeck *et al*. reported that hydrophilic and anionic surfaces limit macrophage adhesion and fusion [11]. Furthermore, it was shown that hydrophilic surfaces inhibit cytokine secretion by adherent cells, possibly resulting in a reduced inflammatory reaction [12]. These surfaces are hypothesized to reduce protein adsorption and therefore lack the specific ligands for the cell-surface receptors that promote cell adhesion, survival and fusion. On the other hand, cationic surfaces adsorb more protein and thus contain abundant ligands to promote inflammatory responses [13]. Therefore, hydrophilic surfaces that adsorb minimal amounts of protein are considered useful for minimizing the inflammatory reaction to biomaterials. Polyethyleneglycol (PEG) has been extensively investigated for biomedical applications, and immobilization of PEG on surfaces is known to decrease protein adsorption [14]. Many models have been proposed to explain the mechanisms involved, but steric stabilization and excluded-volume are the most commonly accepted [15, 16]. It has been reported that monocytes and macrophages show reduced adhesion to PEG-containing materials [17, 18]. Shen *et al*. produced a PEG-like coating by plasma deposition of tetraethylene glycol dimethyl ether and showed that monocyte adhesion to modified surfaces was significantly lower than adhesion to an unmodified surface [19].

Several studies have demonstrated that adsorbed serum proteins mediate monocyte/macrophage adhesion to and survival on biomaterial surfaces [20, 21]. The presence of fibrinogen (Fg) in the layer of adsorbed proteins attracts more inflammatory cells than when Fg is absent [22]. In a model of acute inflammation where polyethylene terephthalate (PET) disks with adsorbed Fg were implanted into the peritoneum of mice, analysis of explants showed that both neutrophils and monocytes/macrophages adhered to the adsorbed Fg [23]. Adhesion of leukocytes to adsorbed Fg is mediated by the β_2-integrin Mac-1 [24–26] and intercellular adhesion molecule-1 [27], whereas Fg-induced activation of these cells is mediated primarily by toll-like receptor-4 [28]. In agreement with this, we recently reported that proteins recognized only by β_2-integrins can serve as adhesion substrates, but do not result in activation of the adherent leukocytes [29]. Even if Fg is adsorbed in relatively small amounts, low fouling surfaces may differ in the way they cause unfolding of the Fg molecule, and thus in which leukocyte receptors that recognize the adsorbed Fg.

In the present study, our aim was to compare the ability of two types of PEGylated surfaces to adsorb Fg and subsequently serve as substrates for adhesion of in-vitro differentiated, human macrophage-like cells. One of the surfaces was prepared by pulsed AC plasma polymerization of diethylene glycol divinyl ether (DEGVE), and the other was prepared by the grafting of linear PEG chains to an amino-functionalized maleic anhydride plasma polymer surface at the lower critical solution temperature (LCST) of the PEG. The surface characterization (XPS, ATR-FTIR and ToF-SIMS) of these surfaces has recently been described [30]. Fibrinogen adsorption is quantified using ^{125}I-radiolabelling and the reactivity of the fibrinogen is determined by enzyme linked immunosorbent assay (ELISA). Finally, a human macrophage (dU937 cell-line) adhesion and proinflammatory responses (TNF-α secretion) was determined.

2. Materials and methods

2.1. Polymer and chemicals

PET film (Mylar, 150 μm thickness) was purchased from Trafoma A/S (Denmark) and cut in 13-mm diameter discs using a punch. Discs were cleaned by Soxhlet extraction with hexane/ethanol for 24 h. Tissue-culture grade polystyrene (TCPS) was obtained from Nunc (Denmark). (1–Ethyl–3-(3-dimethylaminopropyl)carbodiimide-hydrochloride (EDC), N-hydroxysuccinimide (NHS), 1, 2-(methylenedioxy) benzene (MDOB), sodium cyanoborohydride and 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were from Fluka (Germany). Phosphate-buffered saline (PBS), polyethylenimine (PEI, MW 25000), diethylene glycol vinyl ether (DEGVE), maleic anhydride (MAH), bovine serum albumin (BSA), human Fg and monoclonal mouse anti-human Fg (IgG2a) were from Sigma-Aldrich (Germany). Tween 20 and HRP-conjugated rabbit anti-mouse secondary antibody were obtained from Pierce (France). Methoxy-terminated PEG-aldehyde (mPEG, MW 5000) was obtained from Nektar (USA). ^{125}I-labelled Fg (^{125}I-Fg) was purchased from Amersham Bioscience (Germany). Citrate-phosphate-buffered saline (cPBS) contained 10 mM citric acid, 10 mM Na_2HPO_4 and 120 mM NaCl and pH 7.4 was adjusted with 0.1 M NaOH. ABTS buffer contained 40 mM citric acid, 3.25 mM sodium perborate tetrahydrate and 60 mM disodium hydrogen phosphate.

2.2. Chemical functionalisation of PET

The PET samples (13 mm disks) were modified by pulsed AC plasma polymerization in a custom-built plasma unit. The plasma system used to deposit thin films to the PET substrate has been described previously [30]. To prepare the first plasma polymer surface, argon plasma activated PET discs were modified with co-monomers of MAH and MDOB, introduced via separate lines, to produce a reactive maleic

anhydride coating on the PET surface (PET-MAH). Deposition of MAH and MDOB was carried out at a plasma current of 15 mA (resulting in a plasma power of 6 W) for 10 min. The maleic anhydride groups on the PET-MAH modified substrate were further hydrolysed in 0.1 M HCl for 3 h in order to produce reactive carboxyl groups (PET-COOH). The PET-COOH surface was further modified by immobilisation of PEI using the EDC/NHS method. Here, the carboxyl groups are activated with 0.1 M EDC/0.1 M NHS in water for 20 min at room temperature. After rinsing with water, the samples were incubated with 3 mg/ml PEI in carbonate buffer, pH 8.4 for 1 h in order to produce an amino-functionalised surface (PET-PEI). Subsequently, mPEG was grafted onto the aminated surface by reductive amination, using $NaCNBH_3$ (PET-PEG). The grafting was performed under marginal solvation conditions (lower critical solution temperature, LCST) [31]. The mPEG (1 mg/ml) was dissolved in 0.1 M PBS buffer at pH 7.4 containing 0.6 M K_2SO_4. $NaCHBH_3$ (3 mg/ml) was added prior to the immersion of the aminated PET substrates. The reaction was carried out at 60°C for 6 h.

The second set of PEG surfaces was prepared by the direct plasma polymerization of diethylene glycol vinyl ether (DEGVE) on the PET disks (PET-DEGVE). The deposition time was 30 minutes and the plasma current was varied 10, 5 and 2.5 mA resulting, in an effective plasma power of 3.6, 1.4 and 0.6 W respectively. This resulted in the generation of surfaces with a varying degree of monomer fragmentation in the gas phase and thus surfaces close to that expected for a conventionally polymerized PEG at low power.

2.3. Protein adsorption

2.3.1. Adsorption of ^{125}I-Fg

The unmodified and modified PET disks were immersed in a 200 μg/ml Fg solution in cPBS buffer pH 7.4 for 1 h. ^{125}I-Fg was added to adjust a specific activity of at least 2000 cpm/μg protein. The unlabelled-labelled ratio was 20:1. After rinsing 4 times with cPBS the radioactivity of the discs were measured in a Canbera 20 Gamma counter (Canbera, USA), and the amount of adsorbed Fg was calculated (ng/cm^2). The background counts were 35 cpm in the I-125 window.

2.3.2. ELISA

ELISA was performed after adsorption of Fg (0.5 mL; 200 μg/mL in cPBS) to 13 mm diameter TCPS disks (reference material) and the modified PET disks at 37°C for 1 h in 24-well plates. The antibody, monoclonal anti-human fibrinogen (clone FG-21) from Sigma-Aldrich (Germany), is specific for human fibrinogen and recognises an epitope sensitive to protein reduction. Primary antibody was added in a dilution of 1:5000 in blocking buffer (1% (w/v) BSA in PBS) for 1 h, followed by addition of the HRP-conjugated rabbit anti-mouse secondary antibody in a dilution of 1:5000 in blocking buffer. After 2 h, the ABTS enzymatic reaction was performed for 20 min in ABTS buffer. Finally, the optical densities were measured at 405 nm using a VICTOR-3 1420 multilabel counter (Perkin Elmer, Finland). Between each step, the samples were rinsed three times with wash buffer containing 1% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS.

2.4. Cell experiments

The experimental setup for the in-vitro cell experiments is shown in Fig. 1.

2.4.1. Cell culture and differentiation

The human promonocytic cell line U937 was obtained from the American Type Culture Collection (USA) and grown in suspension (0.2–1.0 $\times$ 10^6/ml) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4.5 g/l glucose, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1.0 mM sodium pyruvate and 2.0 mM ultraglutamin (all from

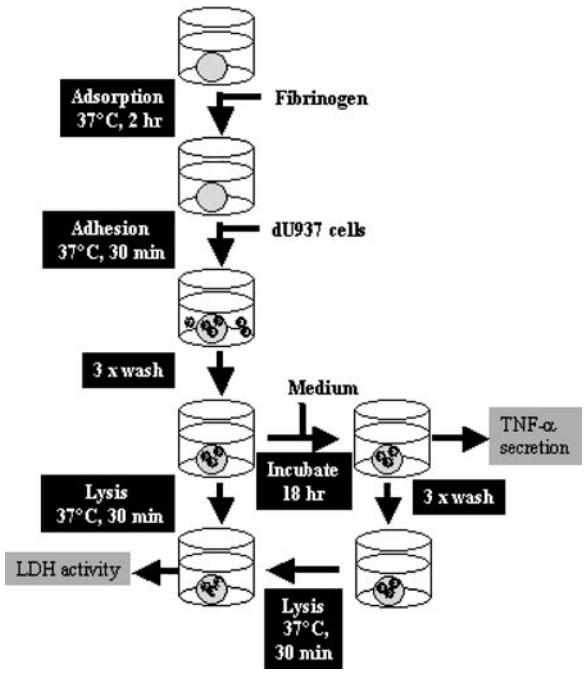

Fig. 1 Experimental setup for the adhesion and activation experiments. Disks were incubated in DPBS or in DPBS containing 200 μg/ml fibrinogen. After rinsing twice, dU937 cells were added and allowed to adhere to the disks for 30 min. Non-bound cells were removed by washing three times with HBSS. The disks were transferred to new wells containing lysis buffer. After lysis, adherent cells were determined by measuring the lactate dehydrogenase (LDH) activity. For the determination of TNF-α secretion, disks with adhered and washed cells were incubated for 18 h at 37°C in fresh medium. TNF-α concentration in the supernatant was measured by a standard sandwich ELISA.

BioWhittaker, Belgium). U937 cells were differentiated into macrophage-like cells (dU937) by incubation (1.2×10^6/ml) for 20 h with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), resulting in adhesion of cells to the TCPS flasks (Nunc). The adherent cells were then grown without PMA for 48 h (change of medium after 24 h) and dissociated using a non-enzymatic cell dissociation solution (C-5789; Sigma-Aldrich). Dissociated cells were centrifuged (500 *g*, 5 min), resuspended in RPMI 1640 with 10% heat-inactivated FBS or complete growth medium, as indicated, and used in assays as described below. U937 was expanded and differentiated at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.4.2. Cell adhesion

Disks were placed in 24 wells plates, hydrated in Dulbecco's phosphate-buffered saline (DPBS) (BioWhittaker) over night, followed by incubation with or without Fg (1.0 ml; 200 μg/ml) for 2 h at 37°C. The disks were then rinsed twice in DPBS, and dU937 cells were added (800 μl; 0.1×10^6 cells/ml). After 30 min at 37°C in a humidified atmosphere of 5% CO_2 in air, the disks were washed three times with 800 μl HBSS (Sigma-Aldrich), and transferred to new wells containing 600 μl lysis buffer (1% Triton X-100 in DPBS). The disks were incubated for 30 min at 37°C, and cell lysates were harvested. The number of adherent cells was determined by measuring the lactate dehydrogenase (LDH) activity of these lysates using a cytotoxicity detection kit (Roche, Germany) according to the manufacturer's instructions. Briefly, 100 μl lysate and 100 μl catalyst and dye reagent mixture were mixed, and incubated in the dark at 25°C for 30 min. The reaction was stopped by adding 50 μl 1.0 M HCl, and the absorbance at 490 nm was measured in a microplate reader (model ELx808; Bio-Tek Instruments, USA). The number of cells in each well was calculated using the A490 values from samples and standards containing LDH from a known number of cells.

2.4.3. TNF-α secretion

Coating of disks with Fg and adhesion of dU937 cells to disks was performed as described above. After adhesion of cells to disks and washing three times with 800 μl HBSS, disks with adherent cells were given 1.0 ml of complete media and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 18 h of incubation, 150 μl of supernatant was harvested and transferred to U-bottomed polypropylene microwells. The supernatants were centrifuged (500 *g*, 10 min), and 120 μl secondary supernatants were harvested. TNF-α concentration in the supernatants was measured by sandwich ELISA using the DuoSet development kit (R&D Systems, UK) according to the manufacturer's instructions. Briefly, capture antibody was adsorbed (25°C, 18 h) to Maxisorp microwells (Nunc). Wells were washed, blocked and incubated with 100 μl of sample or standard (with a known concentration of TNF-α) for 2 h at room temperature. Bound TNF-α was then detected by subsequent incubations with biotinylated detection antibody and horseradish-peroxidase conjugated streptavidin. SureBlue TMB peroxidase substrate and TMB stop buffer (Kirkegaard & Perry Laboratories, USA) was used for quantification of bound horseradish peroxidase. Absorbance at 450 nm was measured in the ELx808 microplate reader, and the TNF-α concentration was calculated using the A450 values from samples and dilutions of standards. TNF-α activities were related to the number of cells on the disks. The number of cells was determined using the LDH assay described above.

3. Results and discussion

3.1. Surface modification and characterisation

The modified surfaces used in this study have previously been extensively characterised with XPS, ToF-SIMS and ATR-FTIR [30]. In order to provide a background for the rationale behind the surface modification and the surface analysis, the results are briefly discussed here. Two approaches were followed to create two types of PEG surfaces:

1) Grafting of linear mPEG chains (Mw 5000) to a hydrolyzed anhydride plasma polymer surface via an intermediated linker layer of PEI. PEI was chosen to aminate the acid surface because creating covalent bonds using water-soluble carbodiimide chemistry is a well-established method and to provide a high density of reactive amino groups best suited for the reductive amination step for the final immobilization of the mPEG molecules. The mPEG was grafted under lower critical solutions temperature (LCST) or 'cloud point' conditions. In combination with the high density of reactive sites on the PEI layer, these conditions are necessary to achieve maximum graft density of the mPEG chains, and thus maximize the steric repulsion capabilities of the PEG layer [31]. When PEG is grafted in a good solvent (i.e. at room temperature), the larger hydration volume of the mPEG chains excludes neighboring chains from reactive sites on the surface during the grafting process, and the result is a surface with low graft density. The ATR-FTIR results showed distinct bands associated with the acid groups, e.g., a predominate C=O stretching band at 1730 cm^{-1}, demonstrating creation of the PET-COOH surface. The results of the subsequent modification steps were more clearly seen with XPS and ToF-SIMS, since the layers were < 10 nm thick. The XPS showed high nitrogen content detected on the PET-PEI surface and a high ether (C-O) component (characteristic of mPEG) in the high-resolution carbon 1s spectra for the mPEG

surface. The ToF-SIMS results confirmed these observations by providing mass spectra that were distinctive fingerprints of both the PEI and mPEG layers.

2) Direct creation of a PEG-like surface by pulsed AC plasma polymerization of diethyleneglycol vinyl ether (PET-DEGVE). In the pulsed AC mode, it is possible to vary the plasma 'on and off' times by variation of the pressure in the chamber, and directly vary the current input to the electrodes. As a consequence, the power density can be decreased to levels where minimal fragmentation occurs, which results in plasma-initiated radical polymerization. The result is a surface that resembles PEG. In this study, the power density was varied in order to change the extent of fragmentation and create surfaces that differed in their degree of PEG-like nature. Both ATR-FTIR and XPS results showed increases in the retention of the ether component expected for PEG as the power density was decreased. ToF-SIMS analysis confirmed that the most PEG-like PET-DEGVE surface (2.5 mA) had a characteristic PEG surface mass spectrum [30].

3.2. Fibrinogen adsorption

Adsorbed Fg has been shown to be one of the key proteins in stimulating inflammatory responses to biomaterials [32]. In order to investigate the influence that the amount of adsorbed Fg has upon macrophage adhesion and activation, adsorbed Fg was quantified using ^{125}I-Fg in single protein adsorption experiments. The amount of Fg adsorbed to each of the surfaces is shown in Table 1.

For the unmodified PET control surface, the adsorbed amount of Fg was equivalent to that expected for a disordered monolayer ($\sim$900 ng/cm^2). Surprisingly, twice as much Fg adsorbed to the PET-COOH surface compared to the unmodified PET, which is equivalent to two monolayers of Fg. Both Fg (isoelectrical point pI 5.5) and the carboxyl groups of the coating have a net negative charge at physiological pH 7.4. Thus, one would expect an equivalent amount of Fg adsorption, or a small reduction in Fg adsorption, due to charge repulsion effects between Fg and the carboxyl groups of the PET-COOH surface. Although it should be stressed that the pI of a large multidomain protein like Fg represents an average value over the entire surface of the molecule, and it will undergo structural rearrangements during adsorption to overcome charge repulsion. We speculate that the high level of Fg adsorption is the result of Fg diffusion into the plasma polymer layer. Recent colloid-probe AFM measurements with a Fg coated tip have shown that a strong adhesive force occurs between Fg and the PET-COOH surface when MDOB is used as co-monomer during plasma deposition, compared to the PET control [33]. On the other hand, a much weaker adhesive force is observed between the Fg-coated colloid and the PET-COOH surface when MAH is polymerised as a single monomer. This suggests that the plasma co-polymer of MAH/MDOB most likely produces a gel-like structure capable of binding high levels of protein.

Table 1 ^{125}I-Fg adsorption to the surface modified PET surfaces

Sample	adsorbed Fg [ng/cm^2]
PET	881 ± 112
PET-COOH	2037 ± 39
PET-PEI	860 ± 131
PET-PEG	206 ± 14
PET+DEGVE (10 mA,)	218 ± 39
PET+DEGVE (5 mA)	178 ± 14
PET+DEGVE (2.5 mA)	92 ± 13

After immobilisation of the PEI to the PET-COOH (PET-PEI), a monolayer coverage of Fg is re-established. It is likely that the positively charged amino groups of the PEI surface facilitate electrostatic interactions with the negatively charged Fg molecules. However, because the PEI molecules contain ethylene units along the backbone, hydrophobic interactions are also likely to play a role in the adsorption process. After immobilization of a high graft density of mPEG chains (PET-PEG) to the PET-PEI surface, Fg adsorption was reduced to 206 ng/cm^2 ($\sim$80% reduction). This demonstrates that the mPEG layer provides a significant steric barrier against Fg adsorption; however, the level of adsorption is NOT zero. Even with a high degree of grafting measured it is difficult to fully explain why the PEG layer fails to fully prevent Fg adsorption without being speculative. However, it is likely that the final mPEG layer is not optimal in terms of the graft density, possibly because of the PEI linker layer not presenting a sufficient number of amino groups to facilitate the formation of a high graft density PEG layer. Additionally, the surface chemistry can only be measured in the dry state, and the surface may have significant defects after hydration to account for the low level of Fg adsorption. Measurement of such surface properties still remains a challenge to modern surface science.

As previously shown, XPS and TOF-SIMS spectra of the DEGVE plasma polymer layers indicated a chemical composition characteristic of the native DEGVE structure [30]. In addition, the DEGVE films deposited at different powers showed differences in surface chemistry, in which higher retention of monomer structure and a more PEG-like structure in deposited films was obtained by lower plasma power. Fg adsorption to the DEGVE surfaces (Table 1) was significantly reduced when compared to untreated PET surfaces, and increased with plasma power. Adsorption was the lowest on DEGVE surfaces made using a plasma current of 2.5 mA ($\sim$90% reduced as compared to untreated PET) increased at 5 mA ($\sim$80%) and further increased at 10 mA ($\sim$75%). Therefore, the plasma power affects the chemistry of the deposited films, which again affects the protein

Table 2 XPS N/C ratio after Fg adsorption to the PET and PET-DEGVE surfaces.

Sample	N/C ratio
PET	0.197
PET+DEGVE (10 mA)	0.027
PET+DEGVE (5 mA)	0.024
PET+DEGVE (2.5 mA)	0.015

The absolute error of the XPS measurement is 5–10%. Results are an average of three measurements.

repellent properties of the surface. XPS analysis was also performed on the same surfaces after exposure to Fg. Since PET or DEGVE plasma polymer surfaces do not contain nitrogen, the XPS signal can be used to determine the extent of protein adsorption, where the content of nitrogen in the amino acids of the protein backbone is measured. Table 2 shows the XPS N/C ratio for the DEGVE surfaces after Fg adsorption.

As expected, the unmodified PET surfaces adsorbed high amounts of Fg. The N/C ratio of 0.197, which is close to that of pure protein, suggests that the layer is almost 10 nm thick. For the DEGVE films, the N/C ratio decreased with decreasing deposition power and reached a value of 0.015 on the PET-DEGVE 2.5 mA surface, which is in accordance with the results obtained with ^{125}I-Fg.

To further investigate the influence of plasma modification on Fg adsorption, ELISA was performed on disk surfaces with pre-adsorbed Fg. ELISA has been shown to be a powerful tool for assessing protein adsorption phenomena and has been extensively applied in understanding of the parameters that influence such adsorption [34]. It is an immunological method based on the key-lock principle formed between the protein as an antigen and a highly specific antibody. Furthermore, ELISA can provide information on the conformation of a protein, if the primary antibody reacts with an epitope that is sensitive to the unfolding of the protein. The main disadvantage of this method is that the results are related to a reference material. Radiolabeling and ELISA protein adsorption studies are complementary in the evaluation of the relationship between the total amount of adsorbed protein and the protein conformation on the surface. The ELISA results for Fg adsorption to the unmodified and modified PET surfaces is shown in Fig. 2 as optical density relative to reference material TCPS. Optical density of TCPS was treated as 100%.

The level of primary antibody reactivity on PET with adsorbed Fg was determined to be 40% of the reactivity obtained with TCPS with adsorbed Fg. This slightly increased to 46% on the PET-COOH surface and further increased to 60% on the PET-PEI surface. These results are not in agreement with ^{125}I-Fg adsorption experiments where the PET-COOH surface showed twice as much adsorbed Fg

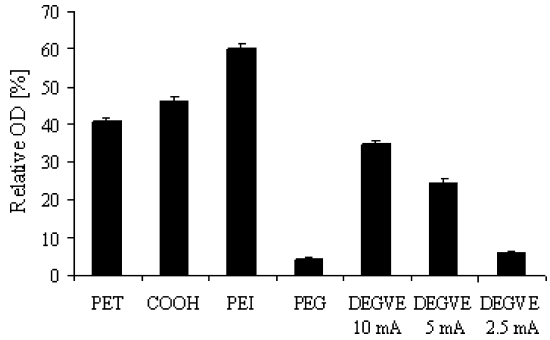

Fig. 2 ELISA determination of Fg adsorption to unmodified and modified PET surfaces. Disks were incubated in cPBS containing 200 μg/ml Fg for 1 h. Adsorption of Fg was measured by a standard sandwich ELISA, using a primary antibody recognizing an Fg epitope sensitive to reduction. Optical density is given relative to TCPS control surface (100%). Results are shown as mean ± SD (n=4).

amount than PET and PET-PEI surfaces. Apparently not all adsorbed molecules are accessible to the primary antibody, suggesting a partial unfolding of the protein. Modified PET surfaces terminated with grafted PEG chains and treated with Fg showed very low primary antibody reactivity, whereas DEGVE-terminated PET surfaces with less or the same amount of Fg adsorbed (Table 1) showed significantly higher primary antibody reactivity, suggesting that the adsorbed Fg molecules were more denatured on the mPEG surface than on the DEGVE surface. The antibody reactivity to the Fg-treated DEGVE surfaces depended on the plasma power used in preparing the DEGVE surfaces, and was highest on the DEGVE film deposited at the highest power (equivalent to 10 mA) and decreased as the surfaces became more PEG-like at the low applied plasma power (equivalent to 2.5 mA). When measuring total bound Fg a similar pattern was seen (Table 1).

3.3. Adhesion and activation of macrophage-like cells

Fg has been shown to mediate proinflammatory responses to implant surfaces mainly by recognition of a small peptide that is situated in the Fg γ chain (residues 192–202) [35]. Additionally, it has been demonstrated that Fg modulates secretion of pro-inflammatory IL-1α, (TNF-α) and anti-inflamatory interleukin-10 (IL–10) cytokines [36]. Thus, Fg plays an important role during the sequential stages of inflammation and tissue response at implant surfaces. In order to determine the relative importance of Fg in mediating adhesion of macrophage-like cells on modified PET surfaces, experiments were performed in the absence or presence of preadsorbed Fg (Fig. 3).

In the absence of preabsorbed Fg, the number of adherent cells to PET, PET-COOH and PET-PEI were similar (Fig. 3a). Further modification with mPEG (PET-PEG) clearly reduced the number of adhering cells. A clear reduction in cell adhesion was also achieved on PET surfaces that were plasma

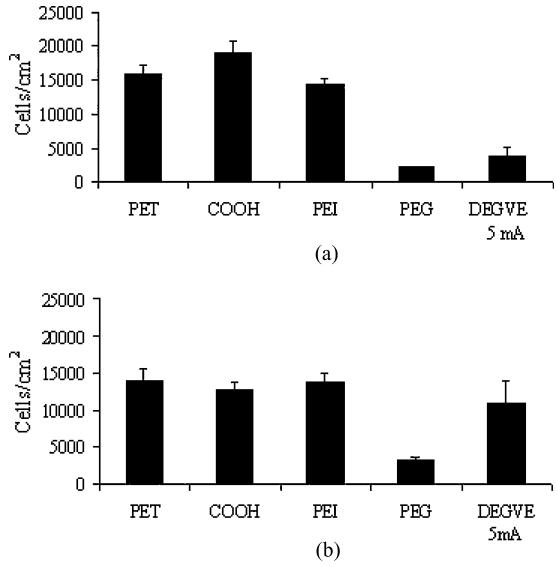

Fig. 3 Adhesion of macrophage-like dU937 cells to PET modified surfaces in the absence (a) and presence (b) of preadsorbed Fg. Disks were incubated in DPBS or in DPBS containing 200 μg/ml Fg. After rinsing twice, dU937 cells were added and allowed to adhere to the disks for 30 min. Non-bound cells were removed by washing three times with HBSS. The disks were transferred to new wells containing lysis buffer. After lysis, adherent cells were determined by measuring the lactate dehydrogenase (LDH) activity. Bars represent results from a representative experiment shown as mean ± SD (n=4).

modified with DEGVE using a current of 5 mA (the PEG-like surface). Preadsorption of Fg had almost no effect on macrophage-like cells adhesion to PET, PET-COOH and the PET-PEI surfaces. Whereas preadsorption of Fg to PEG surfaces only increased the number of adhering macrophage-like cells slightly, a more clear increase in adhesion of these cells was observed for the PEG-like DEGVE surface (Fig. 3b).

Comparing the data in Table 1 and 2 with the adherence of cells to the disks, indicate that there is a more clear correlations between the amount of Fg adsorbed to the disks that is still recognized by the conformational specific antibody and the adherent cells, than there is between total amount of Fg adsorbed to the disks and adherent cells. Further experiments are needed using antibodies directed towards macrophage-receptor ligands in Fg to fully explain the relationship between cell behavior and protein adsorption. Settling of macrophages and adsorption of serum proteins to the surfaces may occur simultaneously.

We are currently investigating protein adsorption to these surfaces from serum using surface-MALDI-ToF, in an effort to identify other proteins than Fg responsible for macrophage adhesion and activation. Figure 4 shows an example of a surface-MALDI experiment recorded from a PET with adsorbed Fg exposed to 10% FBS. Clearly there are a number of proteins detected, as determined from the unique ions in the spectrum, which coadsorb with Fg despite the latter existing as a monolayer. E.g, albumin is detected at 67kDa, and the peak at 45 kDa is most likely α-antichromtryspin. The low mass range (inset) shows peaks most likely assignable to apolipoproteins and fragments of proteins. The nature of this adlayer is dependent on the surface to which the Fg adsorbs [37].

The degree of retention of monomer structure in the DEGVE coatings was shown to influence protein adsorption, and experiments were performed to investigate the effect of subtle differences in surface chemistry on macrophage-like cell adhesion and secretion of TNF-α. The results were compared to the PET surface with grafted linear PEG chains and the results are shown in Fig. 5.

Whereas the absolute number of adherent cells in Fig. 4 is generally higher than in Fig. 3, the relative numbers show

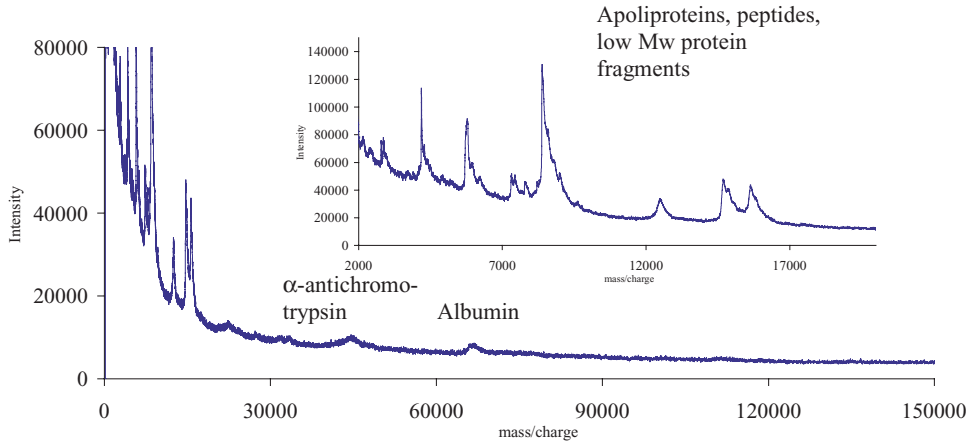

Fig. 4 Surface-MALDI spectrum of the PET-Fg surface after exposure to 10% FBS (the insert is the low mass range). After FBS exposure the surface is rinsed three times with PBS and twice with water. The surface is then allowed to air dry followed by application of the matrix solution (0.5 μL of sinapinic acid in a 1:1 solution of water and acetonitrile). After solvent evaporation and matrix crystal formation the PET substrate is adhered to the MALDI sample plat using double-side tape. The analysis is performed using a Buker Reflex IV instrument in the linear detector mode. Desorption is aided by a 3ns pulsed N_2 UV laser.

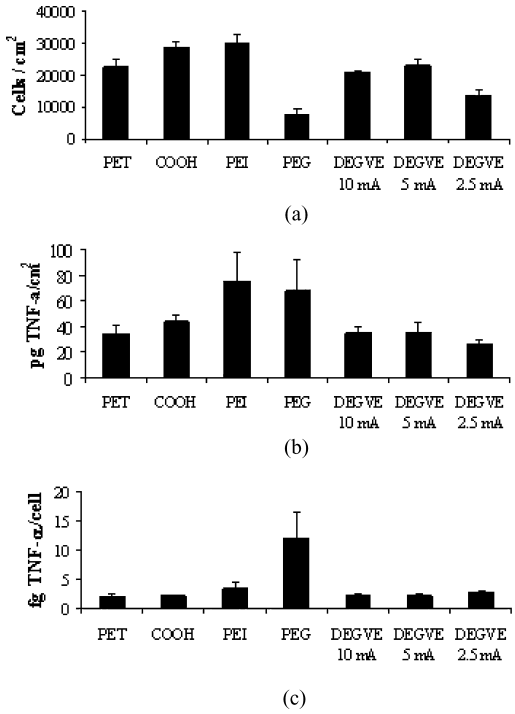

Fig. 5 Macrophage-like cell responses upon exposure to modified PET surfaces pretreated with Fg: (a) cell adhesion, (b) TNF-α secretion per disk area, and (c) TNF-α secretion per adherent cell. Disks were incubated in DPBS or in DPBS containing 200 μg/ml Fg. After rinsing twice, dU937 cells were added and allowed to adhere to the disks for 30 min. Non-bound cells were removed by washing three times with HBSS. The disks were transferred to new wells containing lysis buffer. After lysis, adherent cells were determined by measuring the lactate dehydrogenase (LDH) activity. For the determination of TNF-α secretion, disks with adhered and washed cells were incubation for 18 hr at 37°C in fresh medium. TNF-α concentration in the supernatant was measured by a standard sandwich ELISA. Bars represent results from a representative experiment shown as mean $\pm$ SD (n = 4). Note that the data presented in (c) is calculated using the data given in (a) and (b).

the same tendency. This difference is probably a combined effect of the manual counting of cells for the standard curve in the LDH assay, as well as an effect of the differences in the day to day physically properties of differentiated U937 cells.

In the presence of preadsorbed Fg, no significant difference in the number of adherent cells was seen when using plasma deposition currents of 10 or 5 mA (Fig. 5a). A reduction in the number of adherent cells was seen only at a very low deposition power (equivalent to 2.5 mA). These results show that adhesion of dU937 cells depend on the DEGVE deposition power and indicate that the degree of retention of monomer structure of DEGVE indeed has an effect on the ability of the cells to adhere. Interestingly, the linear mPEG showed slightly lower levels of attachment consistent with the ELISA results.

TNF-α secretion from dU937 during the first 18 h of adhesion to Fg-pretreated disks was determined (Fig. 5b). In the presence of preadsorbed Fg, there were no clear differences in the amount of TNF-α secreted from the macrophage-like cells, except for the PET-PEI and PET-PEG modified surfaces, which showed higher TNF-α secretion. Both the mPEG and DEGVE treated surfaces are chemically similar and bind fewer cells than untreated PET surfaces, however, there is a much higher TNF-α secretion from macrophage-like cells adhering to the PEG surface compared to the DEGVE coated surfaces (Fig. 5c). There was no difference in TNF-α secretion between DEGVE surfaces deposited by different plasma power.

The linear mPEG surface (PET-PEG) is relatively efficient at reducing adhesion of macrophage-like cells, but those cells that do attach are in a more activated and pro-inflammatory state. Thus either the surface by itself or other proteins present seems to be important for the activation state of the adherent cells. This implied that in an implant situation such low-fouling mPEG surfaces would only provide a temporary barrier against adverse responses, and suggest that a surface that totally rejects macrophage adhesion is more desirable. An alternative is to find surfaces that do bind cells to some degree, but which at the same time fails to activate them.

4. Conclusion

In this study we have investigated the influence of different types of surface PEGylation, using PET as substrate, on both Fg adsorption, and the adhesion and activation of macrophage-like cells. Linear grafted mPEG and plasma polymer DEGVE coatings showed a reduced adsorption of Fg and reduced adherence of macrophage-like cells. After Fg preadsorption the number of adherent cells increased only slightly on the mPEG surfaces, but on the PEG-like DEGVE plasma polymer surfaces the difference is greater compared to no Fg adsorption. Furthermore, we show that the extent of cell activation (secretion of TNF-α) is greater for those cells adhering to the linear mPEG surfaces compared to the plasma polymer DEGVE surfaces. Furthermore, we demonstrate, in preliminary surface-MALDI experiments, that serum components, even after pre-adsorption of Fg, adsorb to all surfaces, most likely influencing the cell behaviour. The combined experimental approach provides novel insights into the biocompatibility of different surfaces aimed at being protein and cell resistant.

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