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Proteins and cells on PEG immobilized silicon surfaces

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Abstract

Silicon surfaces were modified by covalent attachment of a self-assembled (SA) polyethylene glycol (PEG) film. Adsorption of albumin, fibrinogen, and IgG to PEG immobilized silicon surfaces was studied by ellipsometry to evaluate the non-fouling and non-immunogenic properties of the surfaces. The adhesion and proliferation of human fibroblast and Hela cells onto the modified surfaces were investigated to examine their tissue biocompatibility. Coated PEG chains showed the effective depression of both plasma protein adsorption and cell attachment to the modified surfaces. The mechanisms accounting for the reduction of protein adsorption and cell adhesion on modified surfaces were discussed.

Keywords: Silicon; PEG immobilization; Protein adsorption; Cellular interaction; Ellipsometry

1. Introduction

Microfabrication technology, a new and powerful tool in the manufacturing of various types of silicon-based biomedical microdevices (BioMEMS), is making a considerable impact in recent biotechnological research. Among the biomedical microdevices (BioMEMS) are biosensors [1, 2], silicon-based, microfabricated devices for electrophoretic separation [3], and their integration into DNA analysis systems [4]. Significant emphasis has also been placed on silicon bioseparation nanofilters [5] and controlled cell culture environments. It is noted that control of pore geometries in the range of tens of nanometers is required for specific applications of both the microfabricated filter and cell culture technologies [6, 7]. In such applications, the control of biofouling associated with protein adsorption is a critical performance parameter.

A further example of the necessity of accurate control over non-specific protein adsorption is provided by the technology of microfabricated biocapsules for cell xenotransplantation [8]. The objective of this technology, directed at the therapy of diabetes and other pathologies, is to isolate cell grafts from the host immune system, while preserving the viability of the transplanted hormone-secreting cells. Tight control over protein adsorption is needed in this context, not only to ensure long-term patency of the pores in the immunoisolation membrane, but also to minimize fibrotic response. Cell adhesion and subsequent proliferation is dictated by initial protein adsorption. A decrease in protein adsorption usually leads to a decrease in cellular attachment. The activation of collagen specific receptors upon cell adhesion to an implant surface can lead to collagen deposition and subsequent walling of the capsule implant by deposition of a fibrous layer.

Whatever the application, microfabricated devices in use may come into contact either with blood, tissues, or both. Like all blood-contacting materials, the use of microdevices is therefore limited by surface-induced thrombosis initiated by the adsorption of plasma proteins, followed by the adhesion of cells [9–11]. Upon implantation in tissues, devices are encapsulated by fibrous tissue. Severe inflammation or excessive fibrosis

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may cause tissue necrosis, granulomas or tumorgenesis [12, 13]. Thus, it is of considerable interest to improve the biocompatibility of silicon microdevices and develop silicon surfaces with reduced protein and cell adherence for the prevention of fouling and fibrotic responses upon implantation.

Significant control over protein adsorption and cell adhesion has been achieved in many biomaterials by the surface immobilization of polyethylene oxide (PEO) or polyethylene glycol (PEG), a water-soluble, nontoxic, and non-immunogenic polymer film [14–16]. The science and technology of PEO/PEG immobilization on polymeric surfaces have recorded major advances in the past decade [17–20]. Several techniques, including physical adsorption, graft polymerization, and chemical coupling have been applied to immobilize PEO/PEG to substrates with hydroxylated groups. PEO and its block copolymers have been coated on glass surfaces through physical adsorption [21], and polymerization by plasma and photo-induced coupling has been successfully introduced to graft PEO onto glass [22, 23].

The PEO coatings deposited by the cited methods have proven quite successful in depressing protein adsorption and cell attachment. However, the thickness of these coatings may limit their viability in applications where nanometer-scale dimensional requirements must be enforced, in the the field of BioMEMS and otherwise. Stark et al. [24] grafted diepoxide PEG to amine derivatized silicon surfaces. Kishida et al. [25] coupled PEG with a terminal carboxyl group to form an ester linkage with the hydroxyl groups on cellulose surface. Of concern in these technologies are the complexity and time requirements of immobilization protocols.

In a recent work by the authors, a simple chemical coupling technique was introduced to modify silicon surfaces by coating a self-assembled (SA) PEG multilayer [26]. In the present communication, the performance of the SA PEG coatings is addressed, by investigating their interactions with plasma proteins and cells. Albumin, fibrinogen and IgG were chosen as model proteins in this study, the latter two in view of the fact that they control thrombogenicity and immunogenicity. For the cell–surface interaction studies, fibroblast and adherent HeLa cells were selected in view of their anchorage-dependent properties and role in the fibrotic response [27, 28]. Their attachment and growth were studied by a cell culture method to evaluate the tissue compatibility of the modified surfaces.

2. Materials and methods

2.1. Materials

Bovine serum albumin (lyophilized, Fraction V) and fibrinogen (lyophilized, Type IV, 95% clottable) were purchased from Sigma (St. Louis, MO). Human gammaglobulin (50% solution) was donated by American Red Cross. Lyophilized proteins were stored in a dessicator at 4°C. Proteins in a concentrated form at 1% (w/w) in PBS were stored in 1 ml aliquots at −20°C. Protein solutions were either freshly prepared by dissolving different amounts of lyophilized proteins in phosphate-buffered saline (PBS) solutions or were obtained by quick thawing the aliquots at 37°C. The same buffer was used throughout an experiment for the rinsing steps to dilute the protein solutions.

PEG ($M = 600$), silicon tetrachloride (99.9%, 100 ml), benzene (anhydrous, 1 L), and triethylamine (NEt_3) were purchased from Aldrich (Milwaukee, WI). All the reagents were used without further purification. Air-sensitive reagents from Aldrich are packaged in special bottles for storing and dispensing quantities of those reagents without exposure to atmospheric moisture or oxygen.

Phosphorus-doped silicon wafers with 100 orientation were purchased from Wafernet. The wafers were coated with 2 μm photoresist on the polished side and cut into 8×12 mm slides using a disco saw. The photoresist was stripped out by PRS 2000 solution at 90°C for 20 min and thereafter care was taken not to touch the highly polished side of slides in all handling steps. The silicon slides were cleaned before use by immersion in a piranha solution (5:1 of v/v sulfuric acid : peroxide) for 10 min at 120°C, followed by rinse in running deionized water until the water resistivity reached 12 mΩ.

2.2. Surface modification

PEG was immobilized on silicon by the functionalization of a PEG precursor by the formation of $SiCl_3$ groups at its chain ends, followed by reaction of surfaces with compounds of the form PEG-$OSiCl_3$. The silanols formed after hydrolysis of the compound condense with silanols on silicon surfaces, leaving the surface modified with the remaining PEG moieties.

PEG-$OSiCl_3$ was synthesized by reacting PEG with silicon tetrachloride with a reagent NEt_3 as a catalyst. The formation of the dominant product PEG-$OSiCl_3$ was achieved by the use of excess amount of PEG reactant and short reaction time. Since the reaction involved air-sensitive reagents, it was carried out in a glass apparatus and prevented from exposure to the atmosphere.

PEG was dissolved in 80 ml anhydrous benzene to make 1% concentration of solution. NEt_3 (80 mg, 0.78 mmol) was added dropwise to the solution at 25°C. After stirring for one hour, the reaction mixture was followed by the addition of silicon tetrachloride (24 mg, 0.14 mmol) and stirred for another 5 min at room temperature. The major products of the reaction were unreacted PEG, and PEG-$OSiCl_3$, NEt_3HCl which precipitates in benzene and dissolves in ethanol. The reaction mixture was filtered through a sintered glass funnel. The

filtrate was directly employed to silanize silicon substrates without further purification since the excess of the non-reacted PEG is expected to have no harmful effects on the silanization process. The PEG moiety provided a terminal hydroxy group that could be utilized for the formation of another PEG layer. The thickness of the PEG film was controlled by the reaction time of the silanization process.

2.3. Protein adsorption

Prior to the adsorption, both modified and unmodified silicon samples were removed from their storage, rinsed with deionized water and ethanol, respectively, and blown dry using nitrogen [29, 30]. The individual samples were transferred into wells of a standard 24-well tissue culture dish (14 mm across internal diameter); 1000 μl of desired concentration of the protein was added with a pipette. Adsorption was allowed to proceed in an incubator for 1 h at 37°C. Upon completion of an adsorption experiment, the samples were washed 3 times with deionized water for the removal of non-adsorbed protein and salts in PBS. The samples were then dried with nitrogen and the thickness of the adsorbed protein was measured by an ellipsometer immediately.

2.4. Air/solid ellipsometry

An automated Gaertner ellipsometer (Model L116A, Gaertner Scientific Corp, Chicago, IL) was employed with an incident angle of 70° and a wavelength of 6328 Å. The refractive indices and thicknesses of PEG and protein films were determined using a computer program accompanying the ellipsometer. Five ellipsometer measurements at different locations of each sample were conducted and the average was then calculated. The thicknesses of protein layers were corrected for background PEG film and oxide layer by measuring untreated surfaces and subtracting the relative values [31]. The amount of absorbed protein in ng cm^{-2} was estimated from thickness data using the method by Stenberg and Nygren [32],

$$m = d_p \rho, \tag{1}$$

where ρ is the density of the adsorbed protein and d_p is the thickness of the protein layer. The thickness of the protein film was obtained by the equation

$$d_p = (n_{ox}^2 - 1) n_p^2 d_{ox} / n_{ox}^2 (n_p^2 - 1) \tag{2}$$

where d_{ox} is the thickness of oxide film; n_p and n_{ox} are the refractive indices of the protein and oxide film, respectively. Substituting the values $\rho = 1.37$ g cm^{-3} and $n_p = 1.55$ [32] and $n_{ox} = 1.46$ [33], it follows that

$$m = 12 d_{ox} \tag{3}$$

with m in ng cm^{-3} and d_{ox} in Å.

2.5. Contact angle measurement

Contact angles with water were measured in air by the sessile drop method using a contact angle goniometer (Rame-Hart, Inc.). Readings were made after the angles were observed to be stable with time. Five readings were made at different spots on each sample and averaged.

2.6. Attachment and proliferation of cells

Cell adhesion properties of PEG coated and uncoated silicon surfaces were evaluated in vitro by cultivating two cell lines, a finite cell line of human lung fibroblast (IMR90) and a continuous cell line of human epithelial cells (Adherent HeLa), on these surfaces. Both cell lines are anchorage dependent.

2.6.1. Cell culture and number determination

Human fibroblasts were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan UT) in a humidified incubator with 5% CO_2. The fibroblasts were removed from tissue culture flasks by incubating in 0.05% trypsin/0.6 mM EDTA for five minutes. Hela cells in DMEM + 10% FBS were harvested from a T-flask. Cell suspensions were counted in a particle counter (Coulter Co.) equipped with a 100 μm diameter orifice. Cells were then reseeded on modified and unmodified silicon surfaces for either cell attachment or cell growth monitoring.

2.6.2. Cell attachment assay

The number of cells attached to modified and unmodified silicon substrates was determined at the time intervals of 30, 120, 240 and 300 min. For both cell lines, approximately 3×10^5 cells in 0.5 ml culture medium containing 10% FBS were pipetted into each well of 24-well tissue culture plates containing the modified and unmodified silicon substrates. After incubation, the sample substrates were transferred to empty culture plate wells and rinsed twice with 1 ml PBS to remove unattached cells. Substrates were then incubated with 0.2 ml 0.05% trypsin–EDTA to remove attached cells. Trypsin was subsequently neutralized with 0.3 ml DMEM + 10% FBS. Cells detached from the substrates were counted twice with a particle counter and also a hemacytometer.

2.6.3. Cell proliferation assay

The measurement of change in number of adherent cells on the substrates was determined at 1, 2 and 4 days. Cell suspensions of 3×10^5 in 0.5 ml of medium with 10% FBS were cultured in 24 well tissue culture plates containing the substrates in a humidified incubator for up to 4 days. Substrates were transferred into new dishes and culture medium was replaced with fresh medium every

24 h so that only the change in number of attached cells would be monitored. Cells on the substrates were detached by trypsinization and counted as described above.

3. Results and discussion

3.1. Protein adsorption

The thickness of the PEG coating for modified silicon samples was 45 ± 5 Å by ellipsometry. The PEG film was stable in water and ethanol for the studied period of time. The contact angles of unmodified and PEG modified silicon surfaces were less than 10° and in the range of 15–20°, respectively. The hydrophilic character of the PEG coating is associated with its ability to tetrahedrally coordinate water molecules about the ether group through hydrogen bonding to the ether oxygen (H-bond acceptor) [10]. Unmodified surfaces may have smaller contact angles than the PEG modified surfaces, possibly in relation with O^- groups.

The time dependence of albumin, fibrinogen, and IgG adsorption on unmodified and modified silicon surfaces is shown in Fig. 1. Reported are mean values of three measurements with standard deviations. The concentrations used correspond to (pseudo-) plateaus in the respective adsorption isotherms. The adsorption of the plasma proteins increased with time, presumably in association with the formation of a multilayer or the occurrence of structural rearrangement of the adsorbed proteins [34, 35]. For both unmodified and PEG modified surfaces, protein adsorption increases rapidly at the beginning and then slowly with time. However, as shown in the figure, precoating silicon surfaces with PEG led to a marked reduction of protein adsorption. It is known that protein molecules are adsorbed on substrates through hydrophobic, hydrogen, and ionic interactions [36]. As indicated previously, both unmodified and modified surfaces are hydrophilic. This suggests that protein adsorption on both surfaces is dominated by interactions other than hydrophobic. Apart from hydroxylated groups, the unmodified silicon surface, unlike the modified surface, possesses O^- groups which allow a direct interaction between protein and surface through ionic and hydrogen bonding. For the modified silicon surface, a non-ionic water-soluble PEG multilayer tethered on silicon has a potential to suppress the direct contact of proteins with the substrate surface and shield the charged groups from the surface. This gives rise to a decrease in the ionic interaction and hydrogen bonding between the PEG films and substrate. The steric force exerted by the immobilized PEG film may also play an important role in hindering protein adsorption on silicon surfaces [37–39].

The adsorbed amount of IgG on silicon is close to that of fibrinogen, and greater than that of albumin. The

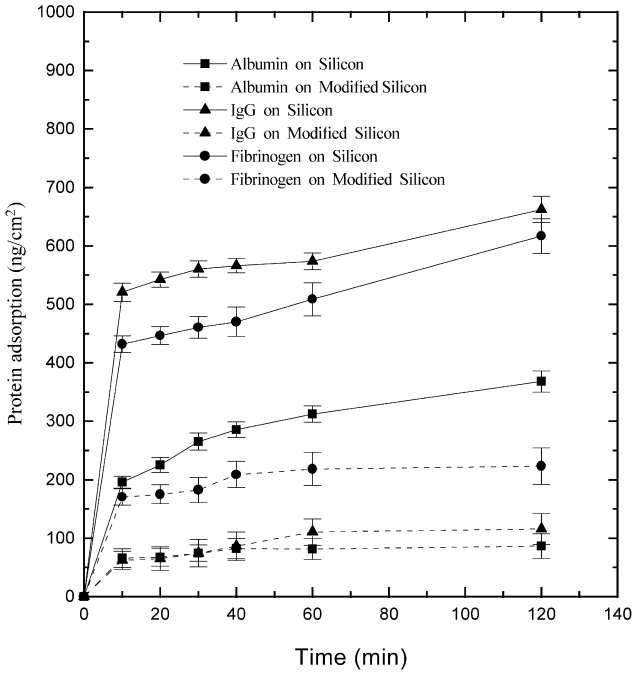

Fig. 1. Adsorption of albumin, fibrinogen and IgG onto silicon and PEG modified silicon surfaces under pH = 7.2 and 2 mg ml^{-1} of protein concentration. Reported are average values of experiments performed in triplicate with standard deviations.

molecular sizes of IgG, fibrinogen, and albumin are $45 \times 45 \times 235$, $60 \times 60 \times 450$ and $40 \times 40 \times 140$ Å, respectively [40]. The adsorbed mass correlated reasonably well with protein molecular size. All proteins showed a certain amount of adsorption on PEG modified surfaces. This suggests the possibilities of interactions between protein and PEG chains or between penetrating proteins and substrate chains. The amount of IgG adsorbed on the PEG modified surface is a little larger than that of albumin, possibly in association with the difference in molecular size between BSA and IgG. It is of interest to note that IgG and fibrinogen show different adsorption behavior towards the PEG modified surfaces. The adsorbed amount of fibrinogen on the modified surfaces is much more pronounced than that of IgG, while both adsorbed on the unmodified silicon surfaces with similar amounts. This may be due to the high surface activity of fibrinogen at solid–liquid interfaces as compared to other proteins [34, 41]. Fibrinogen is likely to have a stronger affinity with the adsorbing surface, and a greater steric force is therefore required to prevent its adsorption as compared to IgG. This may be further compounded by the difference in configuration for the proteins adsorbed on the PEG layer.

Adsorption on modified surfaces reached a maximum after approximately 60 min for all three of the proteins under investigations. By contrast, adsorbed amounts on unmodified surfaces increased monotonically in the time

interval considered. The reduction of albumin, fibrinogen, and IgG adsorption on PEG modified surface at 120 min were about 75, 80 and 65%, respectively.

In closing, it is noted that ellipsometry has been extensively employed in investigations of biological interactions in air/solid interfaces [29, 40, 42]. The calculation of the surface concentration requires estimation of average refractive index and density of adsorbed protein. However, any difference between the real and assumed values of the adsorbed protein results in a systematic error but does not change the relative values and the conclusions. A comparison of ellipsometric and radiotracer experimental values of adsorbed proteins onto silicon surfaces demonstrated a reasonable agreement between the two techniques [43].

3.2. Cellular interaction

Figure 2 shows the adhesion of fibroblast and HeLa cells to PEG treated and untreated silicon substrates with respect to time. Data points are mean values of three measurements with standard deviations. Cell adhesion and a subsequent increase in the number of attached cells on both surfaces increased with time. However, significantly less initial cell adhesion was observed on PEG-coated surfaces for both cell types. These observations are consistent with the results of other investigations involving cellular interaction with PEG modified polymeric surfaces [44, 45]. It is speculated that a decrease in the adsorption of cell adhesive proteins causes a reduction in the number of adherent cells. Furthermore, the steric repulsion exerted by the PEG films together with the lack of ionic interaction between the approaching cells and PEG chains is likely to play an important role in the reduction of cell adhesion to the surface. PEG molecules in culture medium can exist in a number of spatial configurations and PEG chains can also occupy a certain volume because of the ease in rotation about single bonds constituting the polymer backbone [45, 46]. Therefore, the adhesion of large number of cells is thermodynamically unfavorable as a result of a loss in entropy due to the reduction in the number of PEG chain spatial configurations.

Figure 3a shows the change in number of adherent fibroblasts onto differently treated surfaces, determined after 1, 2 and 4 days, due to adherent cell proliferation, cell detachment and/or cell death. As seen in this graph, precoating with PEG chains caused significant reduction in the number of adherent fibroblasts over time. The number of cells on the silicon control samples increased with time and cell confluence was achieved in 48 h. However, the rate of change in cell number on the PEG-modified surfaces decreased with time and cell confluence had not been attained by 96 h. The decreased rate of confluence can be attributed to the low initial cell attachment and cell density on the modified surface. In

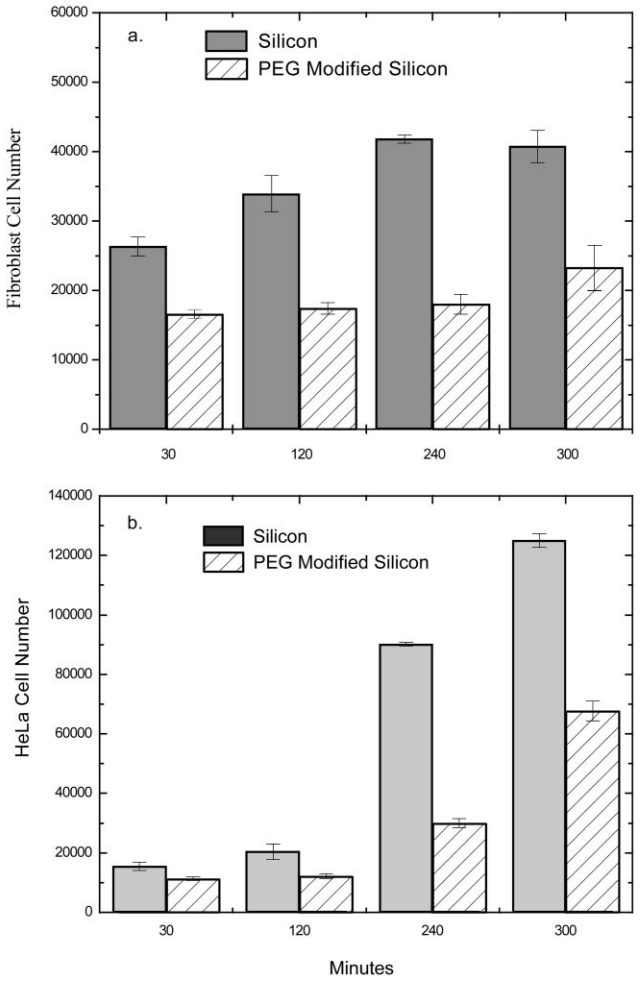

Fig. 2. Adhesion of fibroblast (a) and HeLa (b) cells onto silicon and PEG modified silicon surfaces. Data points are mean values of experiments performed in triplicate with standard deviations.

Fig. 3b, the growth of HeLa cells on unmodified and modified silicon surfaces is shown for different times after seeding at a density of 10^4 cells cm^{-2}. The number of adherent HeLa cells on both surfaces also increased with time, although the final cell counts on modified surfaces were significantly reduced. The fact that there was some cellular adhesion on both substrates agrees with findings by Drumheller and Hubbel [47] which showed that polymer networks with low molecular weight PEGs (4000, 8000, 10 000 g mol^{-1}) supported confluent monolayers but with reduced confluency kinetics. The details of the interactions of cells with surfaces remain poorly understood and debatable. However, it is known that PEG chains deter or hinder the adhesion of cells to the silicon substrate and therefore can cause subsequent low cell proliferation [48]. As revealed in Fig. 3, both cell lines exhibited monolayer growth, but fibroblasts had a slower growth rate compared to HeLa cells, which is consistent with the results of Wise et al. [49].

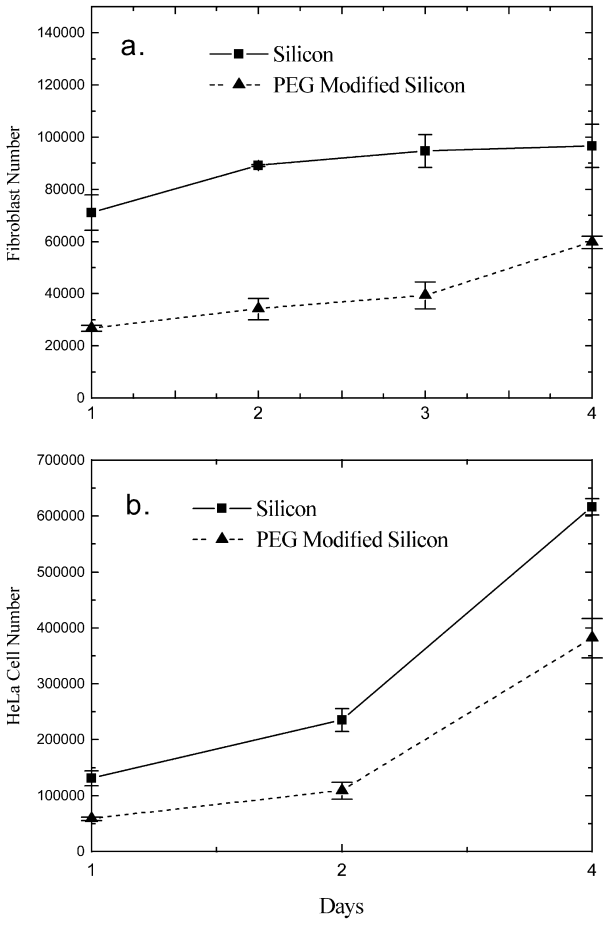

Fig. 3. Proliferation of fibroblast (a) and HeLa (b) cells on silicon and PEG modified silicon surfaces. Data points are mean values of experiments performed in triplicate with standard deviations.

The reductions in the adhesion and proliferation of fibroblast and HeLa cells increased with time and maximum values were about 55–62%, with the exception of the slight decrease of the reductions at the last data point for each experiment. The reason for the observation is not clear at this time. However, it may be due to the ability of some adherent cells to reorganize adsorbed serum proteins to maximize cell interactions [50]. The density of specific adsorbed serum proteins on surfaces, specifically fibronectin and vitronectin, has been shown to be critical in promoting cell adhesion [51]. Since, our surfaces did support some protein adsorption, this density of adsorbed cell adhesive proteins from the serum-containing culture media may have been at a sufficient level to support cell adhesion and subsequent proliferation. Additionally, Norde and Lyklema [52] reported that at low PEG surface coverage, polymer and/or protein bridging may lead to cell adhesion whereas at higher coverage, steric repulsion tends to prevent overall cell attachment. Thus, since our surfaces support low protein adsorption, such low-density polymer coverage could, in fact, support some cell adherence, although significantly less quantities than untreated surfaces.

It is interesting to note that the rates of change of cell number were similar despite significantly lower initial cellular adhesion on the PEG modified surfaces. This seems to indicate that PEG modified surfaces do not adversely affect cell biochemical functionality and growth characteristics, but rather physically hinder cell approach and attachment to the surface. The similar rates of change of cell numbers on both surfaces suggest that cell detachment or death does not occur and that the adherent cells are growing normally on the modified substrate.

4. Concluding remarks

Silicon surfaces were modified by covalently attaching a PEG film to reduce protein adsorption and cell adherence. In an encouraging development for silicon-based plasma-fractionation membranes, the reductions of albumin, IgG, and fibrinogen adsorption onto PEG treated silicon surfaces as compared to untreated silicon surfaces were 76, 82, and 64%, respectively. Maximum reductions of adhesion and proliferation of fibroblast and HeLa cells were in the range of 50–62%. While these results seem encouraging, full-scale in vivo investigation on the thrombogenicity, immunogenicity, and fibrotic response will be required, in order to determine the viability of PEG-coated silicon microcapsule implants.

In closing, it is noted that the surface modification characterized in this study is very general in nature and has potential for use in several diverse fields. The hydroxyl groups of the introduced PEG chains may provide the opportunity for further modification by chemically attaching a variety of functional groups. These modifications can be carried out to tailor silicon-based biomaterials to meet the specific needs of different biomedical applications.

Acknowledgements

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