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Reduction of 3T3 fibroblast adhesion on SS316L by methyl-terminated SAMs

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ABSTRACT

Inhibiting the non-specific adhesion of cells and proteins to biomaterials such as stents, catheters and guide wires is an important interfacial issue that needs to be addressed in order to reduce surface-related implant complications. Medical grade stainless steel 316L was used as a model system to address this issue. To alter the interfacial property of the implant, self-assembled monolayers of long chain phosphonic acids with $-CH_3$, –COOH, and –OH tail groups were formed on the native oxide surface of medical grade stainless steel 316 L. The effect of varying the tail groups on 3T3 fibroblast adhesion was investigated. The methyl-terminated phosphonic acid significantly prevented cell adhesion however presentation of hydrophilic tail groups at the interface did not significantly reduce cell adhesion when compared to the control stainless steel 316L.

1. Introduction

Medical grade stainless steel 316L (SS316L) has been widely used as a biomaterial due to its corrosion resistance and very good mechanical properties [1]. It is employed in many medical devices such as guide wires, orthopedic implants, and most commonly in the manufacture of vascular stents. Stents are used after angioplasty, a surgical method to clear the narrowing of the arteries, to prevent the re-closure of the artery. Though this process is very successful, some complications arise due to thrombosis and neointima formation on the stents because, like most artificial materials, stainless steel 316L also provides a good surface for non-specific protein and cell adhesion. Therefore, surface chemistry that prevents protein and cell adhesion to the substrates and minimizes the host–implant inflammatory responses [2,3] is still needed. Though the monolayer formation and cell adhesion studies on model systems like gold and silicon is well established, these studies on more practical biomaterial alloys like stainless steel is necessary. We present a significant step towards mitigating non-specific cell adhesion by the formation of monolayers with functionalized tail groups using SS316L substrates as the direct model system.

Self-assembled monolayers (SAMs) have developed as a commonly employed method of altering the interfacial properties of materials for their potential applications in the field of adhesion, corrosion inhibition, nano-lubricants and nano-scale devices [4–8]. Two of the most significant advantages of SAMs over other methods of surface coating are the ease in engineering interfacial structures at the molecular level and the ability to tailor the surface properties by functionalizing the tail group of the SAMs. Many tail groups have been employed to render surfaces inert to protein and non-specific cellular adhesion. While oligo-ethylene glycol [9–11] has been the standard by which inert surfaces are measured, other groups such as mannitol [12], maltose [13], taurin [14], and tertiary amine oxides [15] have rendered gold surfaces inert. This effect may be due to their ability to order surrounding water molecules, excluding them from the surface and rendering the surface inert to protein adsorption. However, the molecular basis for the resistance of these surfaces has been debated, due to conflicting data in the literature. For example, Krishnan et al. proposed that more proteins adhere on hydrophobic surfaces due to the hydrophobic effect by which proteins are expelled from the aqueous solution in order to increase hydrogen bonding among water molecules at the expense of less favorable water–protein interactions. Expelled proteins readily displace water from the hydrophobic surface region and become adsorbed [16]. While, Cooper et al., found that 3T3 fibroblast and primary human osteoblast attachment and spreading on methyl-terminated thiols on gold were poor when compared to carboxylic acid terminated SAMs. They also found that the chain length affected the cell attachment only in the case of methyl-terminated thiols and not for hydroxyl and carboxylic acid terminated SAMs [17–19]. Similar results were found using osteoblast cell lines where the focal contact and cell growth was highest for carboxylic acid terminated SAMs and least on methyl-terminated thiols on gold [18]. There have been several works on silicon, using silane-based self-assembly, which specify that hydrophobic substrates resist protein and cell adhesion more when compared to controls and hydrophilic surfaces [19–23]. Due to many interdependent, complex interactions between cells and organically modified surfaces, the phenomenon of controlling

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cell adhesion to surfaces has been difficult. A survey of literature suggests that formation of an inert surface utilizing self-assembled monolayers is a combination of many parameters including substrate wettability [24,25], tail functionality, lateral packing density [24], conformational flexibility of the molecule [11,26], structure of water on the surface [27–30] and kosmotropicity [14].

Although there is a large body of work performed on model substrates such as gold and silicon, these substrates cannot be employed in biomedical applications due to their poor mechanical properties, and standard thiol chemistry has not been successfully employed on the native oxide surface of SS316L. Additionally, experiments, which utilized gold-coated and uncoated stainless steel stents in patients with coronary artery disease, showed an increased risk of restenosis after placement of gold-coated stents in patients vs. uncoated stents [31]. It is noteworthy that although SS316L is widely used as a biomaterial, very few papers of SAMs on stainless steel have been reported in literature [32–36]. Therefore, this paper is the first report of mouse 3T3 fibroblast cell attachment studies on covalently bound, mechanically stable, ordered monolayers of phosphonic acids with varying tail groups on the native oxide surface of SS316L substrates. The tail groups include hydrophobic and hydrophilic moieties.

2. Experimental section

2.1. Self-assembled monolayer formation

2.1.1. Materials and methods

Stainless steel 316L foils (0.5 mm, 99.99% pure, wt.%: Fe 66%, Cr 19%, Ni 10%, Mn 3% and Mo 2%) were obtained from Goodfellow Inc. 12-carboxydodecanephosphonic acid [37] and 11-hydroxyundecanephosphonic acid [38–40] were synthesized in the lab using literature procedures. Tetrahydrofuran (THF), methanol, triethyl phosphite (98%), hydrobromic acid (48%), 11-bromoundecene (98%), chlorotrimethylsilane (97%) and sodium iodide (ACS reagent grade) were obtained from Aldrich Chemical Co. and were used without further purification. Hydrogen peroxide (30%) and sodium hydroxide were obtained from Fisher Scientific and used without further purification. Anhydrous dichloromethane (99.9%) was obtained from Acros and used without further purification.

2.1.2. Substrate preparation and monolayer formation

Stainless steel 316L (SS316L) substrates were cut into 1 cm × 1 cm coupons and polished with 220, 400 and 600 grit silicon carbide paper followed by polishing with Buehler Ecomet 4 mechanical polisher using 600, 800, 1200 grit silicon carbide paper and then a 1 μm diamond suspension. The polished samples were cleaned by ultrasonication in methanol (15 min) followed by immersion in boiling methanol for 10 min to remove traces or residues of organics and metallic dust. The cleaned substrates were stored in an oven at 120 °C overnight under atmospheric pressure and gasses. SEM data on the substrates shows that the metals are evenly distributed throughout the SS316L oxide before and after heating process with no phase separations or surface segregation. As previously reported in our publication [41], XPS analysis of the substrates was performed using a Phoibos 150 hemispherical energy analyzer (SPECS) and a monochromatized Al (1486.6 eV) source. Compositional results for the SS316L reference substrate were in reasonable agreement with the nominal SS316L bulk elemental composition (Fe 66.01%, Cr 19.19 %, Ni 9.17 %, Mn 3.22% and Mo 2.42%.) Deconvolution of Fe2p, Cr2p, C1s and O1s peaks was in good agreement with the stoichiometry of surface oxides ((Fe_2O_3 (18%) and Cr_2O_3 (8%)). These data indicate that our cleaning and heating procedures do not change the native oxide composition of the surface.

The cleaned room temperature substrates were dipped in a 1 mM solution of octadecylphosphonic acid (CH_3–P) in dry THF and excess solution was removed by evacuation (0.1 Torr). For SAMs of 12-hydroxydodecanephosphonic acid (OH–P) or 12-carboxydodecanephosphonic acid (COOH–P), the SS316L coupons were dipped in a 1 mM solution of the acid in dry THF for 2–3 min and the sample were stored in an oven at 120 °C overnight before further analysis. All samples were rinsed and sonicated in THF to remove any weakly adsorbed material or multi-layers.

2.1.3. Analysis techniques

SAMs were analyzed using a Nexus 470 FT-IR, Diffuse Reflectance Infrared Fourier Transform spectroscopy (DRIFT; Thermo Electron Corporation). Static contact angles were measured using VCA Optima Goniometer to determine the wettability of the surface using the procedure described by Bain [42]. PicoSPM Atomic Force Microscope (Molecular Imaging) operating in non-contact mode using a silicon nitride cantilever with a resonance frequency of 160–170 kHz and a typical spring constant of 40 N/m was used to confirm the uniformity of the film on the surface.

3. Cell experiments

3.1. Materials and methods

3T3 Swiss Albino mouse embryo fibroblast cells were obtained from ATCC (CCL-92). Dulbecco's modified Eagle medium (DMEM) was obtained from Invitrogen Corp. Trypsin neutralizing solution (TNS) and trypsin/EDTA were obtained from Cambrex Bio Sciences (New Zealand). Bovine calf serum, trypsin dissociation reagent 0.05% (1×), and penicillin/streptavidin solution (1%) were obtained from Fisher Scientific Co. FITC-conjugated vinculin were obtained from Sigma Chemical Co. The viability/cytotoxicity kit (L-3224) was obtained from Molecular Probes.

3.2. Cell culture and seeding procedure

The cells were maintained in culture in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum and 1% penicillin/streptavidin antibiotic and incubated at 37 °C and 5% CO_2. They were passaged every 3 days and discarded after 15 passages. Cells were removed by trypsinization, washed once with DMEM and resuspended in culture medium. The modified SS316L substrates were placed in a 24 well plate and sterilized with 70% ethanol for 10 min and then rinsed with sterile distilled water 3 times. The sterilized substrates were left in the hood to dry completely.

The suspended cells were counted using a haemocytometer after staining with trypan blue then seeded at the density of 10,000 cells/well. After incubating the substrates with cells in humidified air with 5% CO_2 for 24 h at 37 °C, the samples were rinsed with PBS three times and analyzed by live/dead assay.

3.3. Live/dead assay

A viability/cytotoxicity kit (Molecular Probes) was used to provide a simultaneous determination of the presence of live and dead cells on the surface. Live cells fluoresce green and dead cells fluoresce red when viewed under the fluorescent microscope.

After washing the incubated wells containing the cells with PBS to remove the non adherent cells, 0.5 ml of the live/dead stain was added to each well and the well plate was covered with aluminum foil and placed in the incubator for 30 min. Three samples of each type of modification were used for every set and four such sets were performed. Each sample was mounted on the microscope slide and viewed under fluorescence Nikon Eclipse microscope at 10× magnification. Five different spots on each sample were observed and counted for cells and the average value used for calculation of significance. Viability calculations were performed on each sample by counting the number of live cells divided by total number of cells.