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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 $-\text{CH}_3$, $-\text{COOH}$, $-\text{OH}$ tail groups were formed on the native oxide surface of medical grade stainless steel 316L. 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.

Keywords

Self assembled monolayers; phosphonic acid; Stainless Steel; cell adhesion; hydrophobic; methyl-terminated

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.

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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 was 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 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.

Experimental Section

Self-Assembled Monolayer Formation

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.

Substrate preparation and monolayer formation—Stainless steel 316L (SS316L) substrates were cut into 1cm × 1cm 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 ultra-sonication in methanol (15 min) followed by immersion in boiling methanol for 10 mins 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 were 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 were in good agreement with the stoichiometry of surface oxides ((Fe₂O₃ (18%) and Cr₂O₃ (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 1mM solution of octadecylphosphonic acid (CH₃-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 1mM 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.

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.

Cell Experiments

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% (1X), 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.

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% CO₂. 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 mins 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% CO₂ for 24 hours at 37 °C, the samples were rinsed with PBS three times and analyzed by live/dead assay.

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 minutes. 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 10X 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.

Vinculin localization—FITC-conjugated vinculin (Sigma Chemical Co.) was used to observe the focal adhesions on control SS316L, CH₃-P modified modified substrates using 3T3 fibroblast cells. 3T3 fibroblast cells incubated for 24 hrs on the substrates were washed with PBS twice and fixed with 3.7% paraformaldehyde at room temperature for 10 minutes and then permeabilised with 0.1% triton X in PBS for 3–5 minutes. The stock solution of FITC-conjugated vinculin was diluted 1:50 using PBS and about 0.5 ml of the working solution was added to each well. The well plate was incubated at 37°C for 45 minutes. The samples were then rinsed with PBS twice before viewing by fluorescence Nikon Eclipse microscope with a FITC filter at 40X magnification. The modified substrates were observed for spreading and focal contacts.

Statistical methods—One-way ANOVA (analysis of variance) was used to determine the statistical difference between the means of data sets for cell experiments with the modified substrates and multiple comparisons of the sample means was performed using a Bonferroni post hoc test using confidence level of 0.05.

Results and Discussion

Monolayers were formed on the native oxide of SS316L utilizing octadecylphosphonic acid (CH₃-P), 12-hydroxydodecanephosphonic acid (OH-P) and 12-carboxydodecanephosphonic acid (COOH-P) using a simple solution deposition method. The method of formation of the monolayers of methyl terminated phosphonic acid has been reported previously. [41,43]DRIFT infrared spectroscopy, AFM, MALDI-TOF MS,[44] and contact angle goniometry were performed to ensure monolayer formation, uniformity and stability (Table 1). Results of all characterization techniques were consistent with ordered, stable, uniform monolayer formation for all of the molecules. The monolayers contain order in the alkyl chain, according to the infrared spectroscopy results, because it has been observed that an

‘ordered’ film is characterized by alkyl chains in an all-trans configuration that are all tilted from the normal to the surface at the same angle which results in $\nu_{\text{CH}_2 \text{ asym}} \leq 2918 \text{ cm}^{-1}$. [45–48] All monolayers were found to be stable in protic and aprotic solvents,[43] as well as tissue culture conditions.

The hydrophilic tail functional groups are available for further interfacial molecular interactions with other organics and biomolecules as evidenced by infrared spectral data and contact angle data. For example, Figure 1 shows the OH and CH₂ region of a sonicated sample of 11-hydroxyundecanephosphonic acid deposited on SS316L. It can be seen in the spectra that $\nu_{\text{CH}_2 \text{ asym}} = 2916 \text{ cm}^{-1}$ indicating an ordered monolayer and $\nu_{\text{OH}} = 3738 \text{ cm}^{-1}$ indicating the presence of free hydroxyl group.

Contact angle measurements were consistent with the presentation of the functional groups at the interface. Methyl-terminated, densely packed, well-ordered SAMs predominantly expose methyl groups at the surface, decreasing the surface wettability and increasing the contact angle compared to control SS316L surface.[49] Hydrophilic tail groups exposed at the interface increase the surface wettability and decrease the contact angle value versus the control. The contact angle values for the –OH, –COOH terminated stainless steel surfaces were consistent with the exposure of hydrophilic groups at the interface as shown in Table 1. Therefore, contact angle values along with the results from IR confirm the availability of the tail terminus at the interface for further interaction with biomolecules and other organics. These functional groups presented may change the interaction between the surface and the cells.

In order to determine the stability of the interfacial systems developed in this work under physiological conditions and their viability for use in biomedical implants, cell attachment studies using fibroblast cells were conducted on four sets of independent experiments with three samples each of all functionalized SAMs of phosphonic acid head groups. The substrates were modified with –CH₃, –COOH, –OH terminated phosphonic acids. Cells were seeded at a density of 10,000 cells/well in DMEM medium and incubated for 24 hrs. The samples were then rinsed with PBS three times and analyzed for live and dead cells using the viability/cytotoxicity kit. The attachment of live and dead cells to the modified SS316L substrates was examined using fluorescence microscopy to evaluate the number and shape of the cells on each surface. From the images, we observed that the number of live cells attached was highest for the control (Figure 2A). Additionally, the cells looked uniformly and densely well spread on the control samples. The number of cells on CH₃-P (Figure 2C) was the least in number. The cells on these SAMs were rounded and small, indicating a poor interaction with the surface, exhibiting the potential the CH₃-P SAMs have in abating extensive surface fouling. The cells attached to OH-P (Figure 2B) and COOH-P were similar to those found on control stainless steel 316L substrates.

Overall, the numbers of dead cells (red) were very few when counted and compared to the live ones. The viability on the control SS316L substrate and all the modified substrates were greater than 88% as shown in Table 2. This indicates that the viability is high and the modified surfaces are non-toxic to the cells.

Statistical analysis of the number of cells attached to the surfaces was performed by one-way ANOVA followed by Bonferroni post hoc test. The bar graph in Figure 3 shows the normalized number of cells on each of the modified substrates along with their statistical significance of difference when compared to control SS316L. Independent experiments were normalized to the control set at 100 % to enable comparison between different experiments.

There was a dramatic and statistically significant difference between the number of cells attached to the control and CH₃-P ($p < 0.001$). The CH₃-P surfaces reduced cellular

attachment by 77% (standard error = 6%) versus control. The cells attached to OH-P, COOH-P were not significantly different from the control. Overall, the methyl-terminated phosphonic acid surfaces mitigated the non-specific attachment of cells the best.

The success of the methyl terminated SAMs is analogous to a few studies that have shown that hydrophobic methyl terminated thiols on gold and silanes on silicon induce minimal cell adhesion and spreading of fibroblast cells.[50] These results diverge from most studies of thiols on gold,[11,16,51–53] which show that methyl groups are poor at resisting non specific adhesion of cells while hydrophilic tail group derivations provide the most effective monolayer for reducing non-specific cellular adhesion. The underlying reason for the very sparse attachment of cells to methyl terminated SAMs on SS316L is unclear. It may be due to the combination of the hydrophobic nature of the tail functionality, the long alkyl chain and the tightly bound phosphonic acid head group which provide a closely-packed barrier to cell adhesion on the rough SS316L surface.

Since the morphology of the cells is as important as the number of live cells attached to a substrate[54,55], the quality and stability of adhesion of the cells on the surface was investigated by performing cell experiments with the control and modified surfaces and staining the cells with anti-vinculin. Vinculin is a membrane-cytoskeletal protein associated with focal adhesion and adherens junctions, which are complexes that nucleate actin filaments and crosslinkers between the external medium, plasma membrane, and actin cytoskeleton.[56] Lack of vinculin demonstrates a decrease in spreading of cells, accompanied by reduced stress fiber formation, formation of fewer focal adhesion points. [50] Thus, vinculin plays an important role in controlling the shape and attachment of cells based on its ability to modulate focal adhesion structure and function.

Three sets of experiments were performed on control, CH₃-P using FITC-conjugated vinculin to study the focal adhesion formation. The focal adhesion formation visualized in the control surfaces indicated that the cells were well spread and attached to the surface, most likely through the deposition of fibronectin onto the surface by the cells followed by mediated attachment and spreading.[51,57,58] In addition to supporting minimum cell adhesion on the CH₃ terminated phosphonic acid (Figure 2), CH₃ surfaces also did not support cell spreading and focal contact formation as evidenced by the smaller cells which are less spread out and without focal contacts as seen in Figure 4.

Conclusion

The surface wetting properties of SS316L can be controlled through the formation of functionalized self-assembled monolayers on the native oxide surface of SS316L. These properties in turn modulate the interaction between cells and the surface. The hydrophobic surface (CH₃-P) most effectively mitigated non-specific cell adhesion, reducing it by 77% when compared to the control. In addition to supporting minimum cell adhesion, the CH₃-P surfaces also did not support cell spreading and focal contact formation. The ability of the methyl terminated phosphonic acid surface to minimize non-specific cell adhesion is a significant and important step to the controlled functionalization of metal oxide biomaterials.

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Figure 1.
DRIFT spectra for 11-hydroxyundecanephosphonic acid modified SS316L.

Figure 2.
A sample of live/dead images using 10X magnification of A) Control B) OH-P C) CH₃-P
Magnification 10X objective.

Figure 3. Normalized number of 3T3 cells on each modified SS316L substrate after 24 hours. a = $p < 0.001$ compared to the control SS316L, b = $p > 0.05$ compared to control SS316L

Figure 4.
FITC conjugated anti-vinculin stained 3T3 fibroblasts on A. Control B. CH₃-P modified substrate at 40X magnification.

Table 1

CH₂ _{assym} values by IR indicating ordered films and contact angle measurements for all modifications on SS316L.

Modification	CH ₂ _{assym} values (cm ⁻¹)	Contact angle values	Standard deviation
Control SS316L	-	53.6°	3.0°
CH ₃ -P	2912	108°	3.0°
OH-P	2914	59.8°	5.2°
COOH-P	2915	52.3°	1.8°

Table 2

Summary of results obtained from the viability/cytotoxicity experiments. The percentage viability was calculated by dividing the number of live cells by the total number of cells.

Modification	Normalized Number of Live Cells	Percentage Viability
Control	100	97
CH ₃ -P	23	92
OH-P	66	88
COOH-P	67	93