Poly(NaSS) Functionalization Modulates the Conformation of Fibronectin and Collagen Type I To Enhance Osteoblastic Cell Attachment onto TiAl4V

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Poly(NaSS) Functionalization Modulates the Conformation of Fibronectin and Collagen Type I To Enhance Osteoblastic Cell Attachment onto Ti6Al4V

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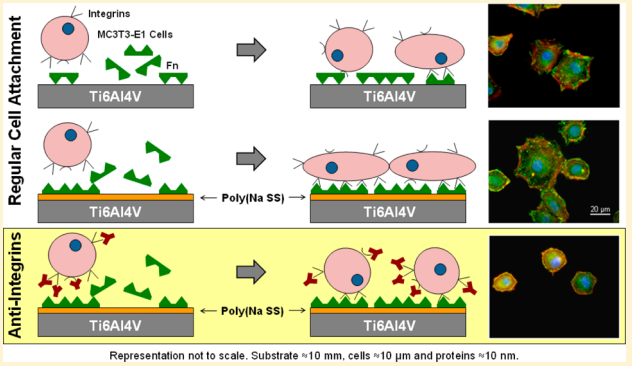
ABSTRACT: Functionalization of surfaces with poly(sodium styrenesulfonate) (poly(NaSS)) has recently been found to enhance osteointegration of implantable materials. Radical polymerization of poly(NaSS) on titanium (Ti)-based substrates has been used to improve their long-term performance by preventing fibrosis and consequently implant loosening. However, the influence of the sulfonate groups on the early cell behavior and the associated molecular phenomena remains to be understood. In this work, we used quartz crystal microbalance with dissipation (QCM-D) to elucidate the role of poly(NaSS) in enhancing osteoblastic cell attachment. This was measured by following the cell attachment using the MC3T3-E1 cell line, on fetal bovine serum (FBS) preadsorbed surfaces and on substrates adsorbed with a series of relevant proteins, bovine serum albumin (BSA), fibronectin (Fn), and collagen type I (Col I). Comparison of the performance of poly(NaSS) with other clinically important substrates such as Ti alloy Ti6Al4V, gold, and poly(desamino-tyrosyl-tyrosine ethyl ester carbonate) (poly(DTEc)) indicates poly(NaSS) to be a superior substrate for MC3T3-E1 cells attachment. This attachment was found to be integrin mediated in the presence of Fn and Col I. Antibodies specific to the RGD peptide and the N- and C-terminal HB-binding domains reacted more intensively with Fn adsorbed on poly(NaSS). Fn adapts a conformation favorable to RGD mediated cell attachment when adsorbed onto poly(NaSS).



1. INTRODUCTION

Protein adsorption onto a biomaterial surface is a complex phenomenon that occurs soon after the biomaterial is exposed to the biological environment. The events include the transport of the protein (diffusion and convection) from surrounding body fluids or serum-containing media into the interfacial region, the adsorption of the protein to the surface, and the subsequent protein relaxation to optimize protein−surface and protein−cell interactions.[1] The proteins that reach the surface provide a network of adhesive ligands for the attachment of cells and mediate the host response.[2−4] The orientation,[5] conformation,[6] and packing density[7] of the proteins determine how the bioactive sites are presented to integrins. Integrins are the only ones among the many classes of cell-adhesion receptors that mediate cell-extracellular matrix (ECM) adhesion and are thus responsible for the initial cell attachment.[8] Adhesion of cells to ECM proteins generates signals that regulate cell survival, cycle progression, and phenotype expression.[9,10]

Integrins consist of 18 α-subunits and 8 β-subunits and play important roles in signal transduction and in the actin cytoskeleton organization of different cell types.[11] In osteoblasts, its early binding to implantable materials has been shown to be strongly associated with two integrins: the $\alpha_5\beta_1$, primarily a fibronectin (Fn) receptor,[12] and the $\alpha_2\beta_1$, a collagen type I (Col I) receptor.[13] Fn is an adhesive protein with a main integrin-recognition site, the arginine-glycine-aspartate (RGD) amino acid sequence. The bonding of $\alpha_5\beta_1$ to the RGD peptide has been shown to promote cell attachment.[14] RGD is found in a number of ligands which interact with integrins including Col I.[13] The adsorption of these two proteins on implanted substrates enhances cell activity (attachment, growth, and proliferation). Because the structure and conformation of the proteins are determined by the surface characteristics of the substrate, the cells behavior is in essence indirectly influenced by the substrate.

Titanium (Ti) and its alloys are preferred in orthopedic applications when the implant material is in direct contact with

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bone. These materials possess excellent corrosion resistance, low toxicity, "acceptable" compatibility with the living tissue, and good mechanical properties, namely high tensile strength and durability, high ductility, and low density.[10,15,16] One drawback, however, is the possible aseptic loosening due to inadequate tissue response (i.e., fibrous tissue formation and/or infection) and integration of the implant.[17] One of the approaches to address these problems is to chemically modify the surfaces of the implant, such as by grafting of bioactive polymers onto Ti substrates to create biomimetic surfaces.

Many polymers bearing chemical functionalities such as carboxylate or sulfonate groups capable of modulating the biological response have been tested.[18] Polymers bearing ionic sulfonate groups have been shown to stimulate osteoblastic differentiation in addition to inhibiting bacterial adhesion.[19] Poly(sodium styrenesulfonate) (poly(NaSS)) has been successfully grafted onto model polymeric surfaces, poly(ethylene terephthalate), with promising *in vivo* results.[20] Poly(NaSS) grafted onto polymeric substrates has been shown to induce specific protein adsorption patterns that favors early cellular response.[2] Poly(NaSS) is stable in physiological environments and is not susceptible to enzymatic degradation, overcoming the limitations of pre-existing strategies of incorporation and/or release of bone-promoting proteins (BMPs,[21] collagen[22]) and antibacterial drugs (gentamycin, etc.).[23] Still, the effect of the poly(NaSS) grafted onto Ti-based materials on the proteins adsorption behavior and consequent cell attachment is not completely understood.

Quartz crystal microbalance with dissipation (QCM-D) is a fast and accurate technique that monitors frequency and energy dissipation response of the freely oscillating sensor and is frequently used in the study of complex biomolecular systems.[24] In earlier studies, Marxer et al. followed the viscoelastic properties and adsorption levels of Fn and bovine serum albumin (BSA) on Ti and gold substrates.[25] Ni et al. deposited Col I and decylbisphosphonate (DBP) layer by layer onto Ti surfaces and found enhanced osteoblastic proliferation and differentiation in the presence of Col I.[26] Molino et al. demonstrated that the surface topography does not influence the Fn adsorption but induces a two-phase adsorption on BSA that starts with the arrival and initial adsorption of the protein molecules and is followed by a postadsorption rearrangement of its conformation to a more dehydrated and compact conformation.[27] Tagaya et al. followed the effect of interfacial proteins, BSA, Fn, and Col I, on the osteoblastic cells adhesion to hydroxyapatite nanocrystals and concluded that Fn and Col I exercise a bigger influence on the cells morphology, expanding their cytoplasm, than BSA.[28]

In this paper we present the result of our study of the influence of three important proteins in bone regeneration, BSA, Fn and Col I, on the attachment of MC3T3-E1 osteoblastic cells onto Ti6Al4V physisorbed with poly(NaSS). The results are compared with those from other clinically relevant substrates, Ti6Al4V, gold, and poly(desamino-tyrosyl-tyrosine ethyl ester carbonate) (poly(DTEc)). Poly(DTEc) has been found to be ideally suited for the fabrication of scaffolds for bone regeneration[29] as indicated by the cancellous bone fracture fixation studies in rabbits[30] and its unique osteocompatibility in canine models.[31] Antibodies against specific receptors in the cellular membrane were used to understand the protein−cell interactions, and the conformation of Fn was explored by following the interaction of its active sites with the cells.

2. MATERIALS AND METHODS

2.1. Materials. *2.1.1. Substrates Preparation.* Four substrates were selected to study protein adsorption and cell attachment. Gold-coated QCM-D sensors (5 MHz) with and without a 50 nm thick vapor deposited Ti6Al4V layer were purchased from Q-Sense AB (Goetenberg, Sweden). The fundamental resonance frequency of the crystals was 5 MHz. Some of the Ti6Al4V-coated sensors were used as received (substrate 1), and some were coated with poly(NaSS) (substrate 2) by physisorption over 15 h from a 15% (w/v) aqueous solution. Some gold-coated sensors were used as received (substrate 3), and some were spin-coated with 1% (w/v) poly(DTEc) in tetrahydrofuran (OmniSolv) (substrate 4).

Before a QCM-D measurement, the sensors were sonicated in 99% ethanol (10 min, Sigma) and twice in Milli-Q ultrapure water (10 min each, Millipore), followed by drying in N_2 and UV ozone sterilization, again for 10 min.

2.1.2. Protein Solutions. Bovine serum albumin (BSA, Sigma), human fibronectin (Fn, Sigma), and collagen type I (Col I, Sigma) were used at different concentrations, mimicking their proportion (Fn/BSA) in the human plasma. BSA was used at 4000 μg/mL in phosphate buffered saline solution (PBS, Sigma), Fn at 20 μg/mL in PBS, and Col I at 10 μg/mL in acetate buffer (0.1 M, pH 5.6).

2.2. Methods. *2.2.1. Comparative Cell Attachment to Ti6Al4V and Poly(NaSS)-Coated Substrates. Cell Expansion.* MC3T3-E1 cells, mouse calvaria-derived osteoblast-like cell line (American Type Culture Collection), were used in this study. Before the QCM-D tests, cells were expanded in Minimum Essential Eagle Medium-Alpha (MEM-α, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C in an atmosphere of 5% CO_2.

Cell Attachment: FBS Preadsorption. In the first set of tests (absence of single protein adsorption), a baseline was established with PBS, and afterward culture medium supplemented with 10% FBS proteins (complete medium, CM) was injected and left in contact with the surfaces until saturation. MC3T3-E1 cells (5×10^4 cells/mL) were introduced into the module and the attachment was followed for 2 h (summary of the sequence: PBS−CM−cells in CM).

Cell Attachment: BSA or Fn or Col I Preadsorption. In a second set of experiments, cell attachment was followed in the presence of BSA, Fn, and Col I. In these experiments, a baseline was first established (PBS), and then the protein solution (BSA or Fn or Col I) was passed over the sensors for 1 h. Nonspecific binding sites were blocked by passing 1% (w/v) BSA in PBS for 30 min. This was immediately followed by the introduction of serum containing medium (CM), which was left until saturation was reached. In the end, cells were injected and left in contact with the substrates for 2 h. PBS was used to remove unattached protein molecules between injections (summary of the sequence: PBS−BSA or Fn or Col I−PBS−1% BSA−PBS−CM−cells in CM).

The frequency dissipation data were collected in a static (0 μL/min) mode with the solution left undisturbed on the surface of the sensors during the experiment.

2.2.2. Protein Adsorption. The adsorption of BSA, Fn, and Col I on the different substrates was carried out at 37 °C. Each of the proteins was introduced into a QCM-D module at a rate of 25 μL/min. The flow was maintained until the saturation point of each protein was reached. A baseline was obtained with PBS, and the same solution was used to remove unattached protein after saturation. The influence of the acetate buffer solution was less than 5 Hz and was therefore small enough to be neglected during the analysis.

2.2.3. Antibody Interference Studies with Integrin-Dependent Cell Attachment. Substrates coated with proteins, Fn and Col I, were used to determine the role of the integrins on the attachment of osteoblastic cells. For Fn, antibodies against the integrins $\alpha_5\beta_1$ were used (Millipore, anti-integrin α_5 MABT18 and anti-integrin β_1 CBL1348, both reactive to mouse), and for Col I, anti-integrins $\alpha_2\beta_1$ were selected (Millipore, anti-integrin α_2 CBL1345 and anti-integrin β_1 CBL1348, both reactive to mouse). All antibodies were used at 100 μg/mL. These integrins are of particular importance to the osteoblastic attachment in the presence of the respective proteins. In each experiment, equal amounts of cells and antibodies were combined

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in one solution. Three solutions were prepared for use on Fn preadsorbed substrates: cells + anti-integrin α_5; cells + anti-integrin β_1; and cells + anti-integrins $\alpha_5\beta_1$. The same was done for Col I preadsorbed substrates. The sequence of solutions and the contact periods applied were the same as in section 2.2.1.

The influence of integrins on the cells attachment was also assessed using fluorescent microscopy (ZEISS Axiolab, Germany). Cells were cultured for 2 h at 37 °C on Fn preadsorbed Ti6Al4V and poly(NaSS) physisorbed sensors with and without anti-integrins (same combinations as before). The medium was first removed, the surfaces washed with PBS, and the cells fixated with 4% formaldehyde (Sigma) in PBS for 30 min at 4 °C. The sensors were then washed twice with a 4 mg/mL BSA/PBS solution, permeabilized with 0.1% of Triton X100 (Sigma) in PBS, and immersed for 30 min in a 3% PBS/BSA solution under agitation. Antivinculin (Sigma) diluted in 1% PBS/BSA (1/200 v/v) was added to each sample and incubated for 1 h at 37 °C. Before adding each dye reagent, the samples were washed two times with 0.05% Tween 20 (Sigma) in PBS. The subsequent staining procedure was conducted protected from light to prevent antibody inactivation. The IgG antibody (rabbit antimouse, Molecular Probes) diluted in 1% PBS/BSA (1/200 v/v) was left in contact with the surfaces for 30 min at room temperature (RT). Then, the alexafluor 488 phalloidin (1/40 v/v in 1% PBS/BSA, FluoProbes) was added and kept for 1 h at RT. Finally, 20 μg/mL of DAPI (Sigma) dissolved in water were added and left to coat the surfaces for 10 min at RT. In the end, the samples were washed twice with dH_2O and stored at 4 °C. This staining procedure was applied to highlight the focal adhesion points (antivinculin, green), actin fibers (phalloidin, red), and nucleus (DAPI, blue) of the cells in order to have a better perception of their morphology. Photographs were taken using a digital camera (Olympus Camedia C-5050). The cells area was evaluated using the Image Pro Plus 5.0 software.

2.2.4. Effect of Poly(NaSS) Coating on the Fn Orientation at Ti Alloy Substrates. The conformation of Fn adsorbed onto Ti6Al4V/poly(NaSS) substrates was inferred through the expression of heparin (HB) and RGD binding sites. For this purpose, the amount of antibodies bound to each of these sites was monitored using QCM-D. Three different antibodies (Millipore, all reactive with human fibronectin and used at 100 μg/mL) against each of the N-terminal (MAB1936) and C-terminal (MAB1935) HB domains and the RGD peptide (MAB1934) were used. The binding of antibodies to these and all the other binding sites on Fn was assessed using a polyclonal antibody (Millipore, AB1945). In all the experiments, antibodies were left in contact with Fn-adsorbed substrates until saturation was reached (25 μL/min).

2.3. QCM-D Analysis. QCM measurements were carried out on a Q-Sense E4 instrument (Q-Sense AB). A peristaltic pump (Ismatec, IDEX Health & Science GmbH, Wertheim, Germany) at constant flow rate of 25 μL/min (nominal) was used during protein tests.

In analyzing QCM-D data, when the surface coatings and adsorbed protein are rigid and laterally homogeneous, the dissipation change is negligible compared to the frequency change, the hydrated surface mass can be calculated using the Sauerbrey equation $\Delta f = -C\Delta m$, with the mass sensitivity of the crystal C equal to 17.7 ng/(cm^2 s).[32] The increase in dissipation was less than 1×10^{-6} per 20 Hz drop in frequency for BSA and Fn, and hence the Sauerbrey equation was used to estimate the adsorbed protein mass with hydration. Data from the 3rd to the 11th overtones were used. Although the dissipation was larger with collagen, to keep the analysis consistent, we used Sauerbrey even in this instance, thus underestimating its adsorbed mass.

2.4. Statistical Analysis. All experiments were conducted in triplicate. Numerical data were reported as mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the posthoc Bonferroni test, using the GraphPad Prism 5.0 software. Significance was defined as having $p < 0.05$.

3. RESULTS AND DISCUSSION

The presence of the poly(NaSS) polymer on the physisorbed sensors was confirmed by X-ray photoelectron spectrometry (XPS, K-Alpha XPS Instrument, Thermo Scientific). Four substrates were selected for this study and organized according to their wettability, from more hydrophilic to more hydrophobic as indicated by their contact angles: Ti6Al4V (30.9 ± 2.7°), poly(NaSS)-coated sensors (44.9 ± 2.5°), gold (67.2 ± 4.8°), and poly(DTEc) (77.3 ± 3.6°).

3.1. Comparison of Cell Attachment to Ti6Al4V and Poly(NaSS). The attachment of MC3T3-E1 cells onto a poly(NaSS) coating is compared with those onto uncoated Ti6Al4V, gold, and poly(DTEc) surfaces in Figure 1A. These

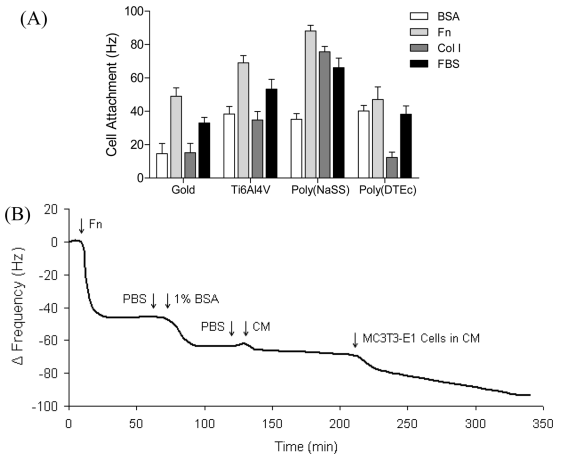

Figure 1. (A) MC3T3-E1 cells attachment (2 h, 37 °C) onto Ti6Al4V, Ti6Al4V physisorbed poly(NaSS), gold, and poly(DTEc) sensors preadsorbed with FBS, BSA, Fn, and Col I, under static conditions. (B) Pattern of frequency shift during cell attachment tests in static conditions. Though the image represents the cell attachment on gold sensors preadsorbed with Fn, all sensors behaved similarly with the three proteins (CM = complete medium or MEM-α supplemented with 10% FBS).

experiments were carried out in the presence of 10% FBS supplemented medium (MEM-α) (control) and with preadsorbed protein BSA, Fn, and Col I. Here, the use of FBS containing medium during cell attachment had minimal effect in the outcome of the experiments, since the FBS proteins were blocked by 1% BSA solution as demonstrated in Figure 1B.

The FBS columns in Figure 1A show that under the conditions of the experiment the cell attachment is better on poly(NaSS) coating than on uncoated Ti6Al4V and the other substrates. This result is in agreement with previous studies in which poly(NaSS) was found to promote the attachment of osteoblastic cells onto Ti-based substrates.[19] Most interestingly, the attachment of the cells onto poly(NaSS)-coated substrates remains high even when Fn and Col I are preadsorbed onto the substrate prior to cell exposure, while BSA inhibits the cell attachment. Interestingly, Col I preadsorbed surfaces displayed the lowest cell attachment rates with the exception of poly(NaSS)-coated sensors. Moreover, Fn increased the cell attachment for all substrates. This is expected, since Fn is known to promote cell attachment because of the role of its RGD sequence in the integrin-mediated recognition processes.[33] Furthermore, the RGD sequence is found in Col I as well, but not in BSA.[13] This finding was further investigated by studying the interplay of protein adsorption and the potentially RGD/integrin-dependent mechanism.

3.2. Protein Adsorption. To better understand the cell attachment results, the adsorption of BSA, Fn, and Col I onto

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physisorbed poly(NaSS), uncoated Ti6Al4V, gold, and poly-(DTEc) substrates was investigated. The hydrated surface masses of protein specific to the various substrates, calculated from QCM-D experiments, are compared in Figure 2. It should

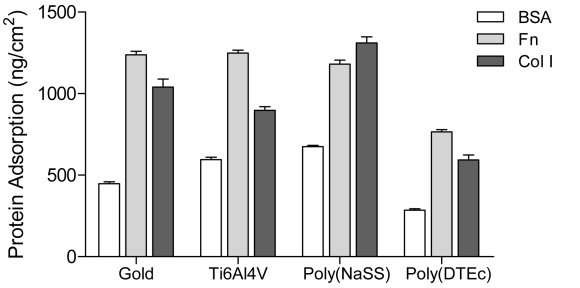

Figure 2. Adsorption of BSA, Fn, and Col I onto Ti6Al4V, Ti6Al4V physisorbed poly(NaSS), gold, and poly(DTEc) at 37 °C and 25 μL/min flow, until saturation.

be noted that unlike surface plasmon resonance and ellipsometry, QCM-D is sensitive to the hydration state of the adsorbed layer. For the majority of the cases, the poly(NaSS) coating shows the highest amounts of adsorbed protein. The only exception was Fn; however, no statistical significance between surfaces was detected. This is expected since poly(NaSS) with its sulfonate (SO_3^-) pendant chains is a polyanion, and the electrostatic interaction is a main driver for protein adsorption.[34] On the other hand, adsorption to uncharged surfaces, such as gold and poly(DTEc), is dependent on weaker forces such as hydrophobic contributions or London dispersion forces. Changes in protein conformation or even denaturation along with loss of functional activity may occur when hydrophobic amino acid side chains are exposed to the interface.[35,36] The presence of poly(NaSS) may reduce these effects due to its hydrophilic character which makes proteins less susceptible to structural changes and less tightly bound to the surface, so their original conformation can be preserved.[3,37] Moreover, previous studies have shown that poly(NaSS) grafted substrates are able to adsorb proteins until saturation. It was demonstrated, for instance, that the saturation level of BSA was 3 times greater on chemically grafted surfaces than on ungrafted surfaces.[38]

Poly(DTEc) sensors show some interesting results. Even though adsorption was small with each of the three proteins investigated, cell attachment was high.[29,39] It could be because although electrostatic interactions are responsible for protein adsorption, on poly(DTEc) surfaces there could be a different class of specific binding interactions that promote cell attachment. It is possible that despite the low concentration of the adsorbed proteins on poly(DTEc), the conformation/orientation of the adsorbed protein, on this hydrophobic surface is favorable for cell attachment.

3.3. Antibody Interference with Integrin-Dependent Cell Attachment. Studies were carried out to see how antibodies interfere with the cell attachment that is initiated by the interaction between the integrins on the cells membrane and the binding domains/sequences of the adsorbed proteins. Specifically, we wanted to understand how poly(NaSS) coating effects Fn and Col I adsorption and then cell attachment. Anti α_5 and anti β_1 were associated with cells to block interactions with Fn preadsorbed substrates, while anti α_2 and anti β_1 anti-integrins were used for the Col I. These are heterodimers of

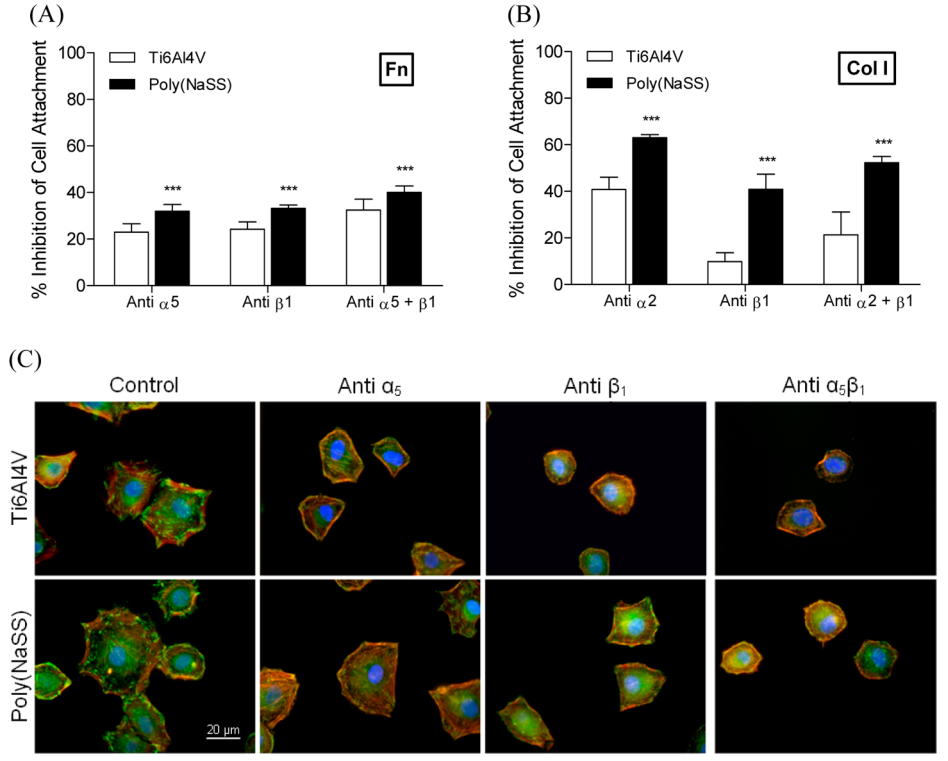

Figure 3. Percentage of cell attachment inhibition on Ti6Al4V and poly(NaSS) physisorbed sensors, preadsorbed with (A) Fn and (B) Col I, by the presence of anti-integrins (2 h at 37 °C and 0 μL/min). (C) Morphological characteristics of cells cultured for 2 h on Fn preadsorbed substrates, in the absence (control) and presence of anti-integrins. Significant differences between surfaces are indicated by an asterisk (*$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$).

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two noncovalently associated transmembrane glycoproteins α and β and are recognized preferentially by the Fn and Col I integrin binding domains in the presence of osteoblastic cells.[12,13] Figures 3A and 3B show the extent to which the attachment of MC3T3-E1 cells on the Fn and Col I preadsorbed surfaces, respectively, are inhibited by the presence of these antibodies. In addition to their superior cell adhesivity, the poly(NaSS) physisorbed surfaces showed higher osteoblastic attachment inhibition than the bare Ti6Al4V, in the experiments with antibodies. While the inhibition was only 40% with Fn, the inhibition with Col I, in some cases, was greater than 60%. One possible explanation for this observation is that poly(NaSS) changes the exposure of the specific protein domains to the cells, and this might lead to more integrin-mediated interactions. The main binding regions responsible for cell attachment in Fn are the HB domains, the synergy peptide PHRSN, which stabilizes the RGD−integrin interactions and preserves its specificity, and the central adhesive peptide RGD;[14,40] and those in Col I are the HB domains, the RGD peptide and the von Willebrand factor A-like domain (A-domain) also known as inserted domain (I-domain).[41] At these sites, the interactions between cells and material are mediated by integrins. Biomaterials surfaces are known to induce changes in the proteins conformation during adsorption by means of intermolecular forces. van der Waals forces, Lewis acid−base forces, electrostatic forces, and hydrophobic/hydrophilic interactions are some of the many intermolecular events that affect the intrinsic structural stability of proteins.[42,43] In poly(NaSS), the SO_3^- pendant chains (polyanion) interact with Fn and Col I by means of electrostatic and hydrophilic interactions.[44] It has been shown that as a result of these interactions, regardless of their original structural arrangement, proteins unfold to a more stable conformation, thereby increasing the exposure of important active binding regions.[45]

Another interesting aspect from the results is the importance of each subunit α and β to the final cell attachment. By itself, each anti-integrin may interact with other heterodimers. For instance, the anti-integrin β_1 can be recognized by more than one α subunit, i.e., $\alpha_3\beta_1$, $\alpha_4\beta_1$, and $\alpha_v\beta_1$, therefore explaining the increased inhibition in cell attachment registered in Figure 3A,B (first two columns). Still, in combination the reaction with the respective heterodimer, $\alpha_5\beta_1$ in Fn and $\alpha_2\beta_1$ in Col I, is predominant.[12,13]

In addition to mediating cell attachment, the receptors α and β also play an important role in the actin cytoskeleton organization. To assess this influence, images of individual cells stained with phalloidin (actin fibers) were taken after 2 h of culture on Fn preadsorbed Ti6Al4V and poly(NaSS) physisorbed sensors in both the absence and presence of the antibodies (Figure 3C). In the absence of the antibodies (control), the focal adhesions (fluorescent green dots) are abundant. Integrins are the major transmembrane components present in focal adhesions. These specialized contact points provide a structural link to the actin cytoskeleton allowing the spread of the cytoplasm in all directions, developing multiple points of interaction.[46] The amount of focal adhesions detected (Figure 3C) on the Ti6Al4V was 1/6 of that on the physisorbed sensors. These results demonstrated the influence of poly(NaSS) on the interactions between MC3T3-E1 and Fn.

The use of antibodies induced a 20−50% reduction of the cells cytoplasm, size and extensions, and number of focal adhesions. However, it is remarkable to see that even when the antibodies block the $\alpha_5\beta_1$ there are still many focal adhesions detected on poly(NaSS) physisorbed sensors. We suspect that poly(NaSS) allows more than one type of integrin-mediated interactions between osteoblastic cells and the Fn binding regions, despite the clear preference for the $\alpha_5\beta_1$ combination. The $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_3$ are possible alternatives known to support osteoblastic cells attachment in the presence of Fn.[47] Similar observations are expected on Col I preadsorbed substrates.

3.4. Effect of Poly(NaSS) Coating on the Fn Orientation at Ti Alloy Substrates. While Figure 2 shows that amount of Fn adsorbed is about the same on gold, Ti6Al4V, and poly(NaSS), Figure 1 shows that the cell attachment is the highest with poly(NaSS). The hypothesis is that the orientation or the confirmation of Fn, not just the amount adsorbed, plays a role in cell attachment. This hypothesis that poly(NaSS) coating affects on the orientation/conformation of Fn adsorbed compared to uncoated Ti6Al4V was investigated using specific antibodies recognizing HB domains (N- and C-terminal) and the RGD sequence (Figure 4A). The data shown in Figure 4B were obtained to

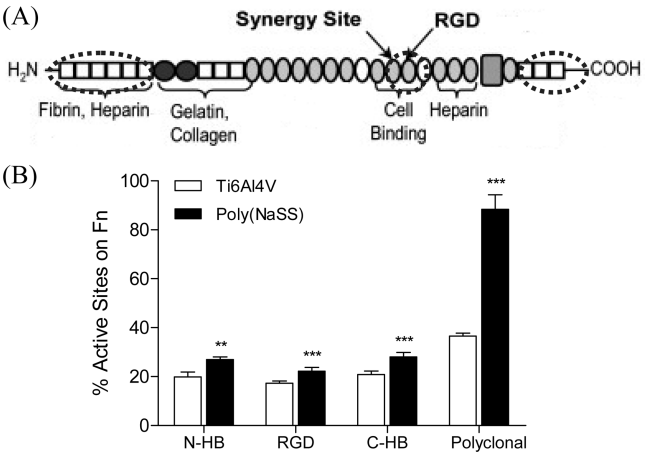

Figure 4. (A) Schematic structure of a Fn fragment, with identification of binding domains of interest [adapted from ref 41]. (B) Percentage of active sites, namely RGD peptide and heparin domains N-terminal (N-HB) and C-terminal (C-HB), exhibited by Fn preadsorbed on Ti6Al4V sensors with and without poly(NaSS) (25 μL/min). Significant differences between surfaces are indicated by an asterisk (**$p < 0.001$ and ***$p < 0.0001$).

demonstrate the effect of poly(NaSS) on the Fn orientation at the interface. The results show that the exposure of the three sites was enhanced by the presence of the polymer, confirming that the orientation of the protein, and most likely its conformation, is different on poly(NaSS) relative to uncoated Ti6Al4V. The conformation and orientation of a protein adsorbed on a surface have a significant effect on the binding of cells because they determine the exposure of the cell-binding sites. It has been shown that both the RGD and the HB domains are directly involved with the attachment of osteoblastic cells to Fn.[48] These observations were also confirmed by the use of a polyclonal antibody against the entire Fn molecule in which the exposure of the Fn active sites was found to be 2 times higher on poly(NaSS) physisorbed surfaces than on uncoated Ti6Al4V. Latz et al. reached similar conclusions with poly(methyl methacrylate) (PMMA) substrates grafted with poly(NaSS). They found enhanced expression of the C-terminal HB domain, and even by the

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entire molecule, and they attributed this to the conformational changes in Fn induced by the sulfonate groups. They also found increased cellular attachment and adhesion strength stimulated by integrin activation at the RGD and HB binding sites.[2] These results validate our findings and support our conclusions on the integrin mediated cell attachment, thus demonstrating the influence of the poly(NaSS) on both protein adsorption and cell attachment.

4. CONCLUSIONS

The effect of preadsorption of BSA, Fn, and Col I on uncoated and poly(NaSS) physisorbed Ti6Al4V, gold, and poly(DTEc) sensors on osteoblast-like cells attachment was investigated using the QCM-D technique. Cell attachment was found to depend on the substrate and adsorbed protein. Poly(NaSS) exhibited the highest cell adhesivity, particularly in the presence of Fn and Col I. Integrin-dependent attachment was observed between the MC3T3-E1 cells and the Fn/Col I preadsorbed poly(NaSS) sensors. Our preliminary findings also reveal that poly(NaSS) exerts a large influence over the Fn protein conformation that results in more active binding sites (RGD and HB domains) to be exposed to cells.

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Notes

The authors declare no competing financial interest.

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