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Original Article

Cell Spreading and Morphology Variations as a Result of Protein Adsorption and Bioactive Coating on Ti6Al4V Surfaces

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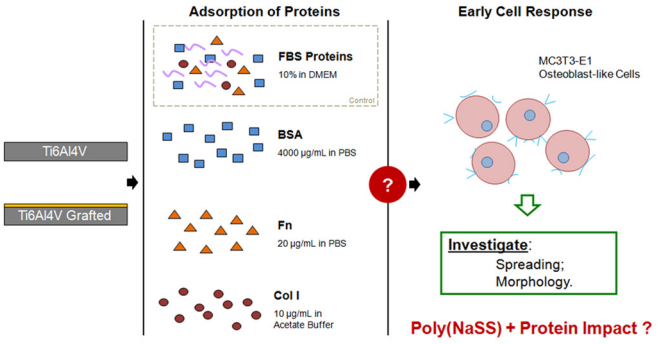
Graphical abstract



Abstract

Objectives: This study investigates the role of poly(sodium styrene sulfonate) (poly(NaSS)) chemically grafted onto Ti6Al4V surfaces on the early spreading and morphology of osteoblastic cells (MC3T3-E1). Moreover, it uncovers important information about the influence of different plasma proteins in the early instances of cell–surface interaction, namely spreading, confluence and focal adhesions formation.

Methods: Fetal bovine serum (FBS), albumin (BSA), fibronectin (Fn) and collagen type I (Col I) proteins were pre-adsorbed onto ungrafted (bare Ti6Al4V, control) and poly(NaSS) grafted Ti6Al4V surfaces. MC3T3-E1 cells were cultured from 30 min to 4 h and their spreading, morphology, confluence and number of focal adhesions was evaluated.

Results: Cell spreading was intensified in the presence of the sulfonate groups even after 30 min of interaction, regardless the proteins at the interface. Fn and Col I, as adhesive proteins, influenced the most the cell expansion and its morphology, revealing their impact on cell attachment very early. At 4 h, this influence was still extremely important particularly in the presence of poly(NaSS) and was highlighted by an increased number of focal adhesions and enhanced cell area. Despite its early influence, BSA represented the smallest improvement in cell spreading and confluence between the four treatments.

Conclusion: Chemically grafting titanium surfaces with poly(NaSS) instigates significantly the cell early response by promoting a rich protein interface.

Keywords: Poly(NaSS) grafting; Ti6Al4V; Fibronectin; Collagen type I; Spreading; Morphology

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1. Introduction

Cell–surface interactions are dependent on the initial adsorption of proteins. Once an implantable material enters in contact with the living system, rapid adsorption of plasma proteins occurs on the surface. These proteins provide an adhesion network for the attachment of cells and mediate all the interactions that follow [1–4].

Protein adsorption is a complex and competition-based process very difficult to predict. It depends not only on the nature of the proteins in the mixture but also on the physicochemical properties of the surface. Generally, proteins seek active sites on the surface and compete with each other for those sites, until they finally adsorb [5,6]. Their conformation, type and amount are influenced by the properties of the outermost layer of the implantable material and, according to that, trigger an appropriate cell response through linkage with the integrin receptors located on the cell surface. This is the basis for the long-term compatibility of a biomaterial and its successful implantation [7].

Because of the limitations of bare titanium (Ti) and its alloys during or after implantation, e.g. ion release and aseptic loosening [8], surface modification techniques have been employed to modify the physical and chemical properties of the surface of these metals. One of the most common strategies is to immobilize calcium and phosphate onto to surface to induce hydroxyapatite precipitation in biological environments [9]. Another consists in the functionalization of the surface with proteins or peptides, like the RGD sequence [10]. More recently, grafting of bioactive polymers bearing anionic functions, like sulfonate (SO_3^-) groups, has proven to be most useful to modulate the biological response [11]. This is a recent approach in which randomly distributed chemical groups from "model" polymers, such poly(sodium styrene sulfonate) (poly(NaSS)), are chemically grafted by radical polymerization onto polymeric or metal surfaces to induce specific interactions with selective adsorbed adhesive proteins [12–14]. Indeed, the negative ionic groups distributed along the polymer macromolecular chains can generate new active sites for protein association. The grafting of poly(NaSS) onto Ti6Al4V surfaces has been successfully accomplished by our laboratory [13,15,16], producing a highly stable coating that both prevents bacterial adhesion and promotes osteointegration [17]. More recently, it was shown that poly(NaSS) alters the conformation of important adhesive proteins, such as fibronectin (Fn) and collagen type I (Col I), modifying their adsorption and competitive behaviors [12,14,17]. Fn and Col I possess RGD (arginine–glycine–asparagine) sequences known to induce cell attachment [18,19]. Integrins bind to the RGD sequence, actin cytoskeletons are assembled within the cell cytoplasm and the associated proteins form focal contacts at the interface of the cell with the material surface [12,14].

The aim of the current investigation was to attest the ability of poly(NaSS) to influence cell spreading and morphology depending on the protein interface. MC3T3-E1 osteoblast-like cells were seeded on Ti6Al4V surfaces ungrafted (control) and grafted with poly(NaSS) and cultured in the presence of different protein regimes including fetal bovine serum (FBS), albumin (BSA), purified Fn and Col I.

2. Materials and methods

2.1. Ti6Al4V surfaces modification and characterization

Ti6Al4V disks of 13 mm diameter and ≈2 mm thickness purchased from CERAVER (France) were used as substrates. The surfaces were initially polished with a series of SiC papers up to 1200 grit, and then cleaned in Kroll's reagent (2% HF, Sigma, and 10% HNO_3, Acros, in 88% H_2O) for 30 s, followed by several washes with distilled water (dH_2O).

Sodium styrene sulfonate (NaSS monomer, Sigma) was purified by recrystallization in a mixture of water/ethanol (10/90 v/v), dried at 60 °C under vacuum, and then stored at 4 °C. Poly(NaSS) chemical grafting was performed in an inert atmosphere (≈99% argon) as previously described [13–15]. The presence of poly(NaSS) on grafted surfaces was determined using the toluidine blue (TB) colorimetric method, X-ray photoelectron spectroscopy (XPS) and Fourier-transformed infrared (FTIR).

Ti6Al4V disks were individually immersed in a TB (Acros) aqueous solution (5×10^{-4} M) at 30 °C for 6 h, allowing TB complexation with the SO_3^- groups from the poly(NaSS). Surfaces were rinsed with 5×10^{-3} M sodium hydroxide in dH_2O to remove the non-complexed dye. Disks were then immersed in a mixture of acetic acid/dH_2O (50/50 v/v, Sigma) for 24 h, inducing TB decomplexation. The concentration of the decomplexed TB was measured by visible spectroscopy at 633 nm using a Perkin-Elmer spectrometer lambda 25.

FTIR spectra, recorded in an attenuated total reflection (ATR), were obtained using a Nicolet Avatar 370 Spectrometer. Spectra were acquired with a 4 cm^{-1} resolution using a 45° Ge crystal (4000 cm^{-1}–600 cm^{-1}). Disks were pressed uniformly against the crystal using a smart Omni sampler.

The surfaces chemical composition was assessed with XPS (K-Alpha XPS Instrument, Thermo Scientific). 50 keV X-ray were used to identify individual elements. Their concentration was determined by detailed scans of each of the elements. The X-ray spot size used was 400 μm. High resolution spectra were profile fitted (Thermo Avantage 4.51 software), and the resulting peaks areas were used to calculate the elemental composition.

Prior to cell culture, all substrates (ungrafted and grafted) were washed with 1.5 M sodium chloride (NaCl, Fisher), 0.15 M NaCl, pure water and phosphate buffered saline solution (PBS, Gibco). This was repeated three times. The surfaces were finally sterilized under ultraviolet light (UV, 30 W) for 15 min.

2.2. Protein adsorption: BSA, Fn and Col I

BSA, Fn and Col I (all from Sigma), were used at different concentrations, mimicking their proportion (Fn/BSA) in human plasma. BSA was used at 4000 ng/mL in PBS, Fn at 20 μg/mL in PBS and Col I at 10 μg/mL in acetate buffer (0.1 M, pH 5.6).

Prior to protein adsorption, surfaces were conditioned in serum free medium (Dulbecco's modified eagle medium, DMEM, with 1% penicillin-streptomycin, 1% fungizone and