Acta Biomaterialia xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Contribution of fibronectin and vitronectin to the adhesion and morphology of MC3T3-E1 osteoblastic cells to poly(NaSS) grafted Ti6Al4V

Helena P. Felgueiras [a], Margaret D.M. Evans [b], Véronique Migonney [a,*]

[a] Laboratory of Biomaterials and Specialty Polymers, LBPS-CSPBAT CNRS UMR 7244, Institut Galilée, Université Paris 13, Sorbonne Paris Cité, 93430 Villetaneuse, France
[b] CSIRO Biomedical Materials Manufacturing Program, 11 Julius Avenue, North Ryde, Sydney, NSW 2113, Australia

ARTICLE INFO

Article history:
Received 1 June 2015
Received in revised form 27 August 2015
Accepted 25 September 2015
Available online xxxx

Keywords:
Ti6Al4V
Poly(NaSS)
Fibronectin
Vitronectin
Depleted serum
Osteoblast
Cell adhesion

ABSTRACT

This study is focused on understanding the underlying mechanisms involved in the improved *in vitro* and *in vivo* responses of osteoblasts on poly(sodium styrene sulfonate) (poly(NaSS)) functionalized Ti6Al4V surfaces. We probed the contribution of cell-adhesive glycoproteins fibronectin (Fn) and vitronectin (Vn) in the initial adhesion of MC3T3-E1 osteoblastic cells to poly(NaSS) functionalized and control Ti6Al4V surfaces. Firstly, culture media containing serum depleted of Fn and Vn (DD) were used to establish the contribution of Fn and Vn in the adhesion and spreading of cells on poly(NaSS) grafted and control surfaces. Compared to ungrafted surfaces, poly(NaSS) grafted surfaces enhanced the levels of cell adhesion, cell spreading and the formation of intracellular actin cytoskeleton and focal contacts in serum treatments where Fn or Vn were present (FBS, DD + Fn, DD + Vn). Cell responses to Fn were more significant than to Vn. Secondly, blocking Fn and Vn integrin receptors using antibodies to $\alpha_5\beta_1$ (Fn) and $\alpha_v\beta_1$ (Vn) showed that adhesion of cells to poly(NaSS) grafted surfaces principally involved the Fn integrin receptor $\alpha_5\beta_1$. Thirdly, blocking of the heparin and cell-binding regions of Fn molecule (RGD, C-HB, N-HB) showed that grafting with poly(NaSS) altered the conformation of Fn. Together these outcomes explained why the presence of sulfonate (SO_3^-) groups grafted on the Ti6Al4V surface enhanced the early cell adhesion and spreading processes which determine clinical success for applications that require osseointegration.

Statement of Significance

This study is devoted to the basic analysis of the mechanism at the origin of the improved *in vitro* and *in vivo* osteoblast cell responses exhibited by poly(sodium styrene sulfonate) (poly(NaSS)) functionalized Ti6Al4V surfaces. The aim was to probe the contribution of cell adhesive glycoproteins fibronectin and vitronectin in the initial adhesion of MC3T3-E1 osteoblastic cells to poly(NaSS) functionalized Ti6Al4V surfaces. The outcomes of this research explained why the presence of SO_3^- (sulfonate) groups grafted on the Ti6Al4V surface enhanced the early cell adhesion and spreading processes which determine clinical success for applications that require osseointegration. This work is a step further in the research of poly(NaSS), a very promising bioactive polymer with potential to the orthopedic and dental fields.

1. Introduction

Ti6Al4V, the most common titanium (Ti) alloy, is well established as a primary metallic material for orthopedic implants. It possesses excellent corrosion resistance, low toxicity, acceptable compatibility with the living tissue and good mechanical properties namely high tensile strength, high ductility, and low density [1,2]. Despite its use for permanent implants, there is often insufficient integration with surrounding bone. A fibrous layer may form at the material–bone interface preventing the formation of a stable chemical bond between the implant and the host tissue, which may lead to implant failure [3]. By altering the surface chemistry of Ti, it is possible to improve the short and, most importantly, the long-term performance of Ti-based implants.

* Corresponding author.
E-mail address: veronique.migonney@univ-paris13.fr (V. Migonney).

http://dx.doi.org/10.1016/j.actbio.2015.09.030
1742-7061/

Chemical and physical methods are commonly used to modify the surface properties of implanted materials in an attempt to promote bone growth and improve osseointegration. Grafting of bioactive polymers bearing anionic functions is an efficient approach to improve clinical outcomes. Many polymers with carboxylate (COO^-) or sulfonate (SO_3^-) chemical groups have been used to modulate the biological response [4]. In our laboratory, we have successfully grafted poly(sodium styrene sulfonate) (poly(NaSS)) onto polymeric (such as poly(ethylene terephthalate)) and model metal surfaces (such as Ti), with promising *in vitro* and *in vivo* results [5–8]. We have shown that poly(NaSS) is stable in physiological environments, prevents bacterial adhesion, and improves bone anchorage through enhanced osseointegration [9–11]. More recently, it was shown that poly(NaSS) alters the conformation of important proteins from the extracellular matrix (ECM), such as fibronectin (Fn) and collagen type I, modifying their adsorption and competitive behaviors with beneficial outcomes to cellular activities [12].

The response of osteoblasts to biomaterial surfaces is mediated by the adsorption of proteins from the local environment to the surface of the implanted material [13]. Ligands in the ECM molecules interact with cell membrane receptors on the surface of the bone cells via integrins that influence cell adhesion, growth and differentiation [14–16]. Fn and vitronectin (Vn) are glycoproteins found in both serum and the ECM that are known to play a significant role in these cellular activities, particularly in initial cell attachment. These two proteins can induce the reorganization of the actin microfilaments and the creation of new focal contacts, which together promote cell adhesion and spreading at early stages of the cell–material interaction. It has been shown that enhanced adsorption of Fn and Vn on nanoporous TiO_2 stimulates the attachment of human fetal osteoblasts by increasing the cells access to binding motifs within these proteins [17]. Moreover, the early binding of osteoblasts to implant surfaces is strongly associated with the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ which primarily recognize binding sites on the Fn and Vn molecules, respectively [18,19]. Functionalization of surfaces to promote the adsorption of these important proteins with cell binding regions exposed or peptides that mimic the native ECM such as the RGD sequence (Arg-Gly-Asp) can improve cell adhesion [20]. RGD is the principal ligand for the integrin family of adhesion receptors on Fn and Vn that include the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ [18,19]. Integrin receptors also recognize other protein domains, such as the heparin-binding (HB) sites, that contribute to the cell adhesion and the formation of the cytoskeleton [21].

The adsorption of Fn and Vn to a material surface is a very complex process when cells are cultured in media supplemented with serum. Protein adsorption is a dynamic and competitive process involving other proteins (including non adhesive ones) that compete for the active/available sites at the surface [22]. The contribution of the serum Fn and Vn to the attachment and spreading of cells varies from one study to another. In early work, serum Fn was found responsible for most of cell adhesion and spreading that occurred in poly(NaSS) grafted polymethylmethacrylate (PMMA) surfaces by enhancing the exposure of the cell binding sites on these proteins [12,23]. More recently, it was reported that serum Vn can be significantly more effective for initial cell attachment than serum Fn on the same substrates, since Vn promotes normal cell spreading, even on bare PMMA, which was found to be independent of Fn [24].

It is widely accepted that the initial interactions between osteoblasts and Ti6Al4V surfaces are crucial for successful clinical outcomes in orthopedics. Previous work has shown that levels of cell adhesion can be increased by chemically grafting poly(NaSS) to the material surface, which may lead to increased rates of bone formation [5–8], yet there is still much to understand about the underlying biological mechanisms taking place at the cell–material interface.

The current investigation pursued this issue and has examined the role of Fn and Vn in the initial attachment and spreading of MC3T3-E1 osteoblastic cells to poly(NaSS) grafted Ti6Al4V surfaces. Firstly, the contribution of Fn and Vn in early cell adhesion and spreading process was explored by examining cell adhesion to grafted and ungrafted surfaces in serum treatments where Vn and Fn had been intentionally removed and then added back individually. Secondly, Fn and Vn integrin receptors were blocked using antibodies to $\alpha_5\beta_1$ (Fn) and $\alpha_v\beta_1$ (Vn) to explore the role of these integrin receptors in the adhesion of cells to grafted surfaces. To allow direct correlation with previous work (current and soon to be published), the current study focuses on the β_1 integrin subunit that is constant in both Fn and Vn proteins. On that basis, $\alpha_v\beta_3$, the integrin receptor recognized by Vn is not the subject of investigation in the current study. Thirdly, the heparin and cell-binding regions (RGD, C-HB, N-HB) of the Fn molecule were blocked to see if the conformation of the Fn molecule adsorbed to the grafted surfaces was altered by the presence of the grafted sulfonate groups.

2. Materials and methods

2.1. Substrate preparation

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 from Sigma, and 10% HNO_3 from Acros, in 88% H_2O) for 30 s, followed by several washes in 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 a inert atmosphere (≈99% argon) as previously described [5,25]. Briefly, Ti6Al4V substrates were chemically oxidized by immersion in sulfuric acid/dH_2O (50/50 v/v, Sigma) for 1 min and then in hydrogen peroxide (Sigma), for 3 min. The oxidized substrates were placed in a 0.7 mol/L NaSS solution in dH_2O and left for 15 h at 70 °C.

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 (4000–600 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.

Please cite this article in press as: H.P. Felgueiras et al., Contribution of fibronectin and vitronectin to the adhesion and morphology of MC3T3-E1 osteoblastic cells to poly(NaSS) grafted Ti6Al4V, Acta Biomater. (2015), http://dx.doi.org/10.1016/j.actbio.2015.09.030