Colloids and Surfaces B: Biointerfaces 134 (2015) 370–376

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Bacteria and osteoblast adhesion to chitosan immobilized titanium surface: A race for the surface

Berit L. Foss[a], Niranjan Ghimire[a], Ruogu Tang[b], Yuyu Sun[b,**], Ying Deng[a,*]

[a] Department of Biomedical Engineering, University of South Dakota, 4800 North Career Avenue, Sioux Falls, South Dakota 57107, USA
[b] Department of Chemistry, The University of Massachusetts, One University Avenue, Lowell, MA 01854, USA

ARTICLE INFO

Article history:
Received 6 March 2015
Received in revised form 11 June 2015
Accepted 7 July 2015
Available online 17 July 2015

Keywords:
Anti-infective
Co-culture
Osteoblast
Implant
Chitosan immobilized titanium

ABSTRACT

In order to evaluate the anti-infective efficacy of the titanium implant materials, two co-culture systems, a low-bacteria/osteoblast (L-B) and a high-bacteria/osteoblast system (H-B), were established. Untreated (UN-Ti), sulfuric acid-treated (SA-Ti), and chitosan immobilized titanium (SA-CS-Ti) materials were developed and evaluated. Bacteria and osteoblast behaviors, including initial attachment (evaluated at 30 mins), adhesion (evaluated at 4 h), and osteoblast spreading on each material surface were evaluated using quantification assays, scanning electron microscopy (SEM), and confocal microscopy. Quantification analysis at 30 mins showed significantly higher number of osteoblast present on SA-CS-Ti in both L-B ($10{,}083 \pm 2626$) and H-B ($23{,}592 \pm 2233$) than those on the UN-Ti ($p < 0.05$). SEM observation and confocal microscopy results showed more surface area was occupied by adhered osteoblasts on SA-CS-Ti than UN-Ti and SA-Ti in both co-culture systems at 30 mins. At all time points, SA-CS-Ti had the lowest level of bacterial adhesion compared to UN-Ti and SA-Ti in both co-culture systems. A significantly ($p < 0.05$) lower number of bacteria were recovered from SA-CS-Ti (2233 ± 681) in the H-B system compared to UN-Ti (5367 ± 1662) and SA-Ti (4533 ± 680) at 4 h. Quantitative and qualitative co-culture results show the great potential of chitosan immobilization onto implant materials to prevent implant-associated infections.

1. Introduction

The design of orthopedic implants has focused on the biocompatibility, structural functions, and mechanical properties of the implant. Titanium (Ti) has been a major break through for use in such implants due to its good mechanical strength and osseointegration properties [1–3]. Recently, numerous surface modification techniques, such as biological coatings and acid and alkali etching, have been widely researched for Ti-based orthopedic implants to further encourage bioactivity and osseointegration [4–8]. These modifications modulate the surrounding biological environment of osteoblasts in order to increase protein and cellular adhesion with a goal to facilitate osseointegration. Current *in vitro* and *in vivo* research has shown that an increase in Ti surface roughness increases osteoblast adhesion which is key for osseointegration [1,7,9,10]. However, surface modifications to mitigate possible adverse tissue responses, especially infection, have not been well established.

Patients undergoing implantation surgery are treated with systemic antibiotics, although their efficacy is limited when a biofilm is established on the implant surface [11]. As the World Health Organization [12] has reported in 2014, high rates of antibiotic resistance have been observed in all WHO regions in common bacteria, such as *Escherichia coli* and *Staphylococcus aureus,* causing common nosocomial and community-acquired infections [12]. Because initial adhesion of bacteria to a biomaterial surface is believed to be an important event in the pathogenesis of biofilm-associated infection, various approaches have been tried to modify titanium surfaces to minimize bacterial adhesion [13] and interfere with the microbial colonization process [14,15].

Roughened titanium implant surfaces have been shown to encourage osseointegration properties at the cellular level, such as osteoblast attachment, adhesion, spreading, and proliferation [16]. A consequence of increased titanium surface roughness, shown in the literature [10,14,17], as well as in our study, is that the bacteria attachment rate also increases—leading to a higher risk for infection. In order to prepare for this "post-antibiotic era–in which common infections and minor injuries can kill," [12] we

* Corresponding author. Fax: +1 605 367 7836.
** Corresponding author. Fax: +1 978 934 3013.
E-mail addresses: yuyu_sun@uml.edu (Y. Sun), Ying.Deng@usd.edu (Y. Deng).

http://dx.doi.org/10.1016/j.colsurfb.2015.07.014
0927-7765/

immobilized chitosan onto rough Ti material to prevent infections related to orthopedic implants. The chitosan modification has shown to increase osseointegration of titanium implant material and minimize bacterial attachment in conventional in vitro evaluation systems (signal bacteria or osteoblast culture) [18].

The fate of a biomaterial implant has been hypothesized as a "race for the surface" between bacterial and osteoblast adhesion [19]. This hypothesis aids researchers in the exploration for further surface modification as a means to improve osseointegration and the clinical outcome of orthopedic surgeries [2,14]. Our previous research has combined two surface modifying techniques, sulfuric acid treatment and chitosan immobilization, to address poor osseointegration and implant-related infection, simultaneously [18]. We hypothesize that the increased surface roughness caused by the sulfuric acid treatment will increase osteoblast attachment, while the immobilized chitosan will decrease bacteria attachment. To further test the hypothesis, in this study, we developed two osteoblast/bacteria co-culture systems to evaluate the race for the surface behavior of osteoblast and bacteria towards the unmodified and the modified Ti materials. Osteoblasts and *S. aureus,* a bacteria commonly found in implant-related infections [12,15,20,21], were employed to set up the two osteoblast/bacteria co-culture systems to investigate the behavior of osteoblast and bacteria to Ti surfaces simultaneously. Using the co-culture systems we have investigated the interaction between osteoblast and *S. aureus* in a "race for the surface" scenario and demonstrated the chitosan surface modification aids the osteoblasts to win this race.

2. Materials and methods

2.1. Materials and reagents

The materials and reagents used in this study are from Sigma unless otherwise noted: dulbecco's phosphate buffered saline (PBS), fetal bovine serum, trypan blue, fluorescein diacetate (FDA), propidium iodide (PI), acetone, absolute ethanol, 25% glutaraldehyde, titanium foil (purity: 99.7%; 0.25 mm in thickness), dopamine hydrochloride, glutaraldehyde (25%), and chitosan with low molecular weight (75–85% deacetylated; viscosity: 20–300 cP, 1 wt.% in 1% acetic acid at 25 °C; the molecular weight is approximately 50,000–190,000 da based on viscosity) were purchased from Sigma–Aldrich. Osteoblast-like cells, SaOS-2 (ATCC HTB-85) and *S. aureus* (ATCC 6538) were purchased from American type culture collection. Dulbecco's modified eagle medium (DMEM), non-essential amino acids, and antibiotics were purchased from Fisher Scientific.

2.2. Titanium surface modifications

Titanium samples were prepared and characterized as previously described [18]. To prepare the titanium samples, the Ti foil was cut into $1 \times 1\,cm^2$ and ultrasonically cleaned with acetone, ethanol, and lastly, deionized water. The resulting unmodified samples are referred to as UN-Ti. The Ti samples were then treated in 48% H_2SO_4 at 60 °C under continuous stirring for 3 h. These samples were washed with distilled water and dried under vacuum. These samples were designated as SA-Ti, the intermediate product of the chitosan modification. For the preparation of the chitosan immobilized Ti (SA-CS-Ti) in which the chitosan is covalently grafted on to the Ti surface, the SA-Ti samples were treated with 5 mg/mL dopamine hydrochloride in a 10% 0.1 M Tris–HCl aqueous solution at room temperature for 12 h. This was followed by immersion in 3% glutaraldehyde at 4 °C overnight. Afterwards, the samples were immersed in 0.5% chitosan solution (in 1% acetic acid aqueous solution) at room temperature for 18 h, rinsed with distilled water, and dried under a vacuum. Surface modifications were confirmed through X-ray photoelectron spectroscopy (XPS) analysis.

2.3. Osteoblast cell culture

Osteoblasts were thawed and cultured at a density of 5000 cells/cm^2. The osteoblasts were incubated at 37 °C at 5% CO_2 in culture media of DMEM/High Glucose medium containing 1% non-essential amino acids, 1% antibiotics, and 10% fetal bovine serum. The medium was changed the next day and then every three days until confluency reached 90%. Osteoblasts were then collected using 0.05% trypsin with EDTA. Osteoblasts were quantified using trypan blue and a hemocytometer.

2.4. Bacteria culture

S. aureus was cultured in tryptic soy broth (TSB) for 18 h at 37 °C. The bacteria were centrifuged at 5000 rpm for 5 mins. The supernatant was removed and the bacteria pellet was gently washed with PBS (×3). The pellet was resuspended in PBS and serial dilutions were used to obtain specific bacteria concentrations. Bacteria concentration was determined through agar plate spreading.

2.5. Co-culture system establishment

Titanium samples (UN-Ti, SA-Ti, and CS-SA-Ti) were placed into previously labeled wells of 24 well non-tissue culture plates. Suspended osteoblast cells (100,000/sample) and bacteria (L-B: 10,000/sample or H-B: 100,000/sample) were seeded to their respective wells simultaneously and suspended in a modified medium (98% DMEM w/10% FBS and 2% Tryptic Soy Broth) for a total volume of 500 μL. The co-cultures were incubated at 37 °C in 5% CO_2 for time points determined (at 30 mins to study initial attachment and at 4 h to study steady-state adhesion). At each time point, modified medium was removed and samples were collected for the following quantitative and qualitative assays.

2.6. Scanning electron microscopy preparation

UN-Ti, SA-Ti, and SA-CS-Ti samples were prepared in co-culture conditions as described in the co-culture system establishment section. At each time point, samples were collected for SEM observation. The samples were fixed with 2.5% glutaraldehyde in PBS, dehydrated using a series of ascending ethanol concentrations (70%, 80%, 90% and 100% at 5 mins each), air-dried at 4 °C for 24 h, and sputter-coated (Cressington Scientific) with gold at a thickness of 14–15 nm. The samples were then observed and imaged using a scanning electron microscope (Quanta 450 SEM, FEI).

2.7. Confocal microscope preparation and observation

Overnight-grown bacteria were collected, washed, and fluorescent stained with FDA at a concentration of 1 mg FDA per 10^6 bacteria. The FDA-stained bacteria were then used for the co-culture with the osteoblasts as described in the co-culture system establishment section. At each time point (30 mins and 4 h), samples were washed with assay medium (DMEM without antibiotics) and were fixed with 2.5% glutaraldehyde in PBS for 15 mins, followed by a PBS wash. The osteoblasts were then permeabilized with 0.2% Triton-X 100 in PBS for 10 mins. An aliquot of 80 μL of 100 nM rhodamine phalloidin (Cytoskeleton, Inc.) was used for staining the actin filaments of the osteoblasts. The samples were visualized using confocal microscopy (Nikon A1 TIRF). Images from the confocal microscope at 4 h were used to analyze surface area