Available online at www.sciencedirect.com
ScienceDirect
Biomolecular Engineering 24 (2007) 549–554
Biomolecular Engineering
www.elsevier.com/locate/geneanabioeng

Ti-Cp functionalization by deposition of organic/inorganic silica nanoparticles

Clément Roux [a,b], Feng Chai [b], Nathalie Ollivier [a], Anne Ochsenbein [c,d], Stefan Winter [c], Oleg Melnyk [a], Hartmut F. Hildebrand [b,*]

[a] CNRS UMR 8161, 1 rue du professeur Calmette, F-59021 Lille Cedex, France
[b] Faculté de Médecine (GRB), 1 place de Verdun, F-59045 Lille Cedex, France
[c] Lehrstuhl Materialwissensch. Metalle, Universität des Saarlandes, D-66041 Saarbrücken, Germany
[d] CNRS UMR 8008, PERF-ENSCL, BP 108, 59652 Villeneuve d'Ascq, France

Abstract

In orthopaedics and cardiovascular surgery, titanium has become the metal of choice, due to its excellent mechanical properties and biocompatibility. In many surgical operations, chemicals and/or biomolecules (such as antibiotics or growth factors) are used in conjunction with prostheses, so as to avoid or stimulate targeted biological events. Often, immobilization instead of release of such molecules is preferred to optimize their effects, thus avoiding ectopic transformations. A versatile method for the functionalization of pure Ti is shown here, which allows the covalent immobilization of polypeptides.

In order to avoid the hydrolysable Ti–O–Si bond found in directly silanized Ti, we use organic/inorganic silica colloids, derived from commercially available 25 nm Ludox® silica nanoparticles. Prior to deposition onto Ti-Cp, the silica nanoparticles are functionalized by a propylsemicarbazide moiety by silanization. After spin-coating onto the Ti substrates, the colloids were shown by SEM to form a uniform layer, and to be very strongly adsorbed; the reactivity of the supported semicarbazide (Sc) functionalities being maintained. Chemoselective reaction of semicarbazide groups on the surface with aldehyde moieties present on the polypeptide of interest was chosen in this work due to its efficiency, to its compatibility with the proteinogenic amino acids and in particular cystein and to the use of mild experimental conditions. Aldehyde groups are also easily introduced onto polypeptides by synthesis, oxidation of N-terminal Ser residue or polysaccharide moieties of glycoproteins.

Biological assays with MC3T3-E1 osteoblasts revealed an excellent cytocompatibility as shown by the assessment of cell viability, vitality and morphology.

Keywords: Titanium; Silica nanoparticles; Functionalization; Osteoblasts; Biomolecules

1. Introduction

Titanium is nowadays the metal of choice for biomedical applications. Surgeons use Ti in a whole variety of applications, including hard tissue implants, osteosynthesis and cardiovascular applications (stents, occlusion coils), bone plates and screws, etc. Although it combines good properties both in terms of mechanics and biocompatibility, bare Ti lacks bioactivity. Modification of titanium surface is a way to favor or inhibit its interactions with surrounding tissues, accelerate its integration or disfavor the development of local infections, whilst retaining its advantageous strength and resistance properties. Various methods are now available for titanium and titanium alloys surface modification. The field has been reviewed recently (Liu et al., 2004). On the other hand, titanium surface functionalization is a way to bring more chemical or biochemical diversity onto the surface. Covalent attachment of organosilanes was often used to enhance surface properties, such as wettability, adhesiveness, and surface activity (Porte-Durrieu et al., 2004). Recently, self-assembled monolayers of phosphonic acid have been used to functionalize titanium surface (Viornery et al., 2002). Self-assembled monolayers can then be utilized to graft polypeptides onto the titanium surface in order to improve biocompatibility, but usually the grafting method utilizes functional groups already present in polypeptides such as thiols

* Corresponding author. Tel.: +33 320 62 69 75; fax: +33 320 62 68 54.
E-mail addresses: oleg.melnyk@ibl.fr (O. Melnyk), fhildebrand@univ-lille2.fr (H.F. Hildebrand).

1389-0344/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bioeng.2007.08.005

(Xiao et al., 1997), amino- or carboxylate moieties, that may potentially result in the formation of several bonds between the polypeptide and the surface and thus impair its accessibility. We have examined an alternative method for the functionalization of titanium surface by polypeptides, based on the use of functional organic/inorganic silica colloids derived from commercially available 25 nm Ludox® silica colloids. The nanoparticles are adsorbed on the titanium surface and allow the site-specific immobilization of polypeptides through the formation of a semicarbazone bond. This chemistry has been successfully used for the attachment of peptides or DNA molecules to silica surfaces (Coffinier et al., 2005; Melnyk et al., 2002; Blanchet et al., 2003; Olivier et al., 2003; Duburcq et al., 2004). The method uses mild experimental conditions, is independent of the polypeptide sequence and is compatible with the presence of thiols either as an additive in the buffer or as part of Cys residues.

In this context, we describe the preparation of a new type of modified titanium surfaces, where hybrid organic/inorganic silica nanoparticles are used to functionalize commercially pure titanium (Ti-Cp). The nanoparticles bear a nucleophilic semicarbazide group, brought about by silanization of commercially available silica colloids with a functional silane. The colloids are then deposited by spin-coating onto mirror-polished Ti-Cp surfaces, thus forming a uniform layer of nanoparticles. Surfaces were analyzed by means of electron microscopy and reflection infrared spectroscopy. The reactivity of the semicarbazide functionality towards aldehyde-containing polypeptides was evaluated using model fluorescent peptides. The biocompatibility of our surfaces was assessed on MC3T3-E1 osteoblast-like cells. Cell proliferation, differentiation, and morphology were studied on a 6-day trial.

2. Materials and methods

2.1. Particle synthesis

A detailed account of the synthesis of Sc silica nanoparticles will be published elsewhere. Briefly, Ludox silica colloids (25 nm in diameter) were silanized with Fmoc-protected silane **1** in *N*,*N*-dimethylformamide (DMF). The deprotection was achieved *in situ* with piperidine which also catalyzed the formation of siloxane bonds on the silica nanoparticles. The colloids were then rinsed thoroughly by centrifugation/redispersion cycles to remove the excess of reagents, and stored at 4 °C as a 1% (w/v) suspension in DMF (stable for months). Concentration was determined by evaporating DMF and weighting the resulting residue. Nanoparticles were characterized by transmission electron microscopy, ^{29}Si and ^{13}C CP MAS NMR, FT-IR and elemental analysis.

2.2. Titanium discs

Commercially pure titanium (Ti-Cp) was cut into discs 1–2 mm thick and 15 mm in diameter. They were then automatically mirror-polished with 0.25 μm diamond paste. The discs were cleaned with 30% HNO_3 (30 min), deionized water (5× 5 min) and dried in an oven at 50 °C for 1 h.

2.3. Particles spin coating

Each disc was spin-coated with 75 μL of a 1% (w/v) nanoparticles suspension in DMF (acceleration: 3500 rpm^2; speed: 3500 rpm; 45 s).

2.4. Characterization of the surfaces

Coated titanium discs were observed with a Zeiss 820 scanning electron microscope at an accelerating voltage of 10 kV.

Reflection IR spectra were recorded on a Perkin-Elmer infrared spectrometer.

2.5. Reaction with peptides

The preparation of peptides **2** and **3** labelled with 5(6)-carboxytetramethylrhodamine is described in detail elsewhere (Olivier et al., 2003). The reactivity of Sc nanoparticles immobilized on titanium was evaluated using fluorescence.

Titanium discs were cleaned as described above. On each disc was printed an array of nanoparticles spots (three drops per spot, 1 nL overall, concentrations ranging from 0.1 to 1% (w/v) in DMF/water: 1/1 (v/v)) using a non-contact piezoelectric printer (BCA1 arrayer, Perkin-Elmer, four replicates per concentration). Both peptides were dissolved at a concentration of 10^{-4} M in pH 5.5 0.1 M sodium acetate buffer. The discs were incubated under cover-glass for 1 h at 37 °C with 20 μL of peptide **2** or **3** solution. After thoroughly rinsing the discs with PBS, the substrates were rinsed in deionized water and absolute ethanol, and dried in air. The discs were then visualized with an optical microscope equipped with a filter for 5(6)-carboxytetramethylrhodamine and photographs were taken with a Coolsnap digital camera (Photometrics). The fluorescence intensity of each spot was quantified and the mean background was subtracted from the mean spot signal.

2.6. Biological evaluation

2.6.1. Cell culture

The MC3T3-E1 cells, an immortalized cell-line that derived from mouse calvarium tissue, are cultured in T75 plastic flasks (Nunc) in alpha-MEM medium with glutamax (Gibco BRL) supplemented with 10% foetal calf serum (FCS, Eurobio) (Chai et al., 2006). The medium also contained penicillin (100 IU mL^{-1}), streptomycin (0.1 g L^{-1}) and mycostatin (100 IU mL^{-1}). All *in vitro* cell incubations were performed in a CO_2 incubator (Binder, CB 150/APT-line) at 37 °C with 5% CO_2 atmosphere and 100% relative humidity.

2.6.2. Proliferation test

The growth period for cell proliferation tests was 3 and 6 days without renewal of the medium following the ISO 10993-5 standard (Hornez et al., 2002). Ti-Cp or coated titanium (Ti/Sc) disc-samples were placed in the bottom of 24-well plates (Nunc) after UV sterilization. Then 10^4 logarithm-growing cells were gently seeded in each well. Wells which were filled only with cell suspension, served as negative control (tissue culture polystyrene, TCPS). Three and 6 days after the cell seeding, cells were detached by a trypsine-EDTA solution; the cell quantity was determined by using a cell counter Z1 (Coulter Electronics). Relative proliferation rates were calculated as the quantity of living cells on samples divided by that on the negative control. Test samples for each group were triplicated. Final results were rated as the mean percentage of four of such separate triplicate assays. One-way ANOVA was applied to determine the statistical significance of the differences observed between groups: $P < 0.05$ were considered as significant.

2.6.3. Vitality test

Cell vitality test was assessed with the non-toxic Alamar Blue dye simultaneously conducted with the above proliferation test (Hamid et al., 2004). This vitality test gives a precise indication on the cell function due to the reduction of Alamar blue by intracellular enzyme activity (O'Brien et al., 2000). Three and 6 days after the cell seeding, and just before cell counting, the culture medium was removed from each well. Five hundred microliters of medium containing 10% fluorescent dye (Interchim Ref. UP669413) were deposited in each well. After 3-h incubation, the solutions were transferred into 96-well titer-plates (Nunc), and the fluorescence intensity was measured by a fluorometer (Twinkle™ LB 970, Berthold Technologies) at 530 nm and 590 nm for excitation and emission, respectively. Cell vitality rate was calculated as the fluorescence of living cells on samples divided by that of negative control. Data were expressed as the mean percentage ± S.D. with respect to the control culture (100%).