Biomaterials 35 (2014) 4441–4453

Contents lists available at ScienceDirect

Biomaterials

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

The effect of cerium valence states at cerium oxide nanoparticle surfaces on cell proliferation

Tamaki Naganuma [a,*], Enrico Traversa [b]

[a] Smart Biomaterials Group, Nano-Life Field, Biomaterials Unit, International Center for Materials Nanoarchitectonics (MANA), National Institute for Materials Science (NIMS), 1-1 Namiki, Tsukuba, Ibaraki 305-0044, Japan
[b] Physical Science and Engineering Division, King Abdullah University of Science and Technology (KAUST), Thuwal 23955-6900, Saudi Arabia

ARTICLE INFO

Article history:
Received 22 November 2013
Accepted 30 January 2014
Available online 5 March 2014

Keywords:
Cell proliferation
Adhesion
Scaffold
Surface modification
Nanoparticles

ABSTRACT

Understanding and controlling cell proliferation on biomaterial surfaces is critical for scaffold/artificial-niche design in tissue engineering. The mechanism by which underlying integrin ligates with functionalized biomaterials to induce cell proliferation is still not completely understood. In this study, poly-L-lactide (PL) scaffold surfaces were functionalized using layers of cerium oxide nanoparticles (CNPs), which have recently attracted attention for use in therapeutic application due to their catalytic ability of Ce^{4+} and Ce^{3+} sites. To isolate the influence of Ce valance states of CNPs on cell proliferation, human mesenchymal stem cells (hMSCs) and osteoblast-like cells (MG63) were cultured on the PL/CNP surfaces with dominant Ce^{4+} and Ce^{3+} regions. Despite cell type (hMSCs and MG63 cells), different surface features of Ce^{4+} and Ce^{3+} regions clearly promoted and inhibited cell spreading, migration and adhesion behavior, resulting in rapid and slow cell proliferation, respectively. Cell proliferation results of various modified CNPs with different surface charge and hydrophobicity/hydrophilicity, indicate that Ce valence states closely correlated with the specific cell morphologies and cell–material interactions that trigger cell proliferation. This finding suggests that the cell–material interactions, which influence cell proliferation, may be controlled by introduction of metal elements with different valence states onto the biomaterial surface.

1. Introduction

Understanding interaction of cells with biomaterial surfaces is essential to the design of scaffolds/artificial-niches that can control cell adhesion, proliferation and differentiation in tissue engineering. Research exploring facets of biomaterial surface functionalization suggest that adequate hydrophilicity, positive charge and given surface functional groups contribute to cell attachment and differentiation [1–4]. However, the mechanism by which underlying integrin bonds with surface molecules adhering on the functionalized biomaterial surfaces is still not well understood. Likewise, the complexity of substrate features makes it difficult to identify the effect of specific biomaterial surface features on cell functions. For these reasons, it is challenging to develop insight regarding functionalization of biomaterial surfaces to induce cell function.

New insight has emerged regarding the ability of polymer composite scaffolds mixed with cerium oxide nanoparticles (CNPs) to promote cell proliferation [5]. Surface features of CNPs on the composite scaffolds may trigger integrin-mediated signal transduction, which induces accumulation of specific intracellular proteins, resulting in rapid cell proliferation. However, the interaction mechanism between CNPs and cell proliferation is still unclear. Different Ce valence states of extracellular CNPs may contribute to control of cell functions through cell-material interactions. Assessing the influence of Ce valence state on cell adhesion is, therefore, paramount for understanding the mechanisms of interaction between cells and extracellular CNPs.

Altering the valence states of metal ions *in vivo* acts as a catalytic agent, such as in enzymes. Since valence states of metal oxide nanoparticles have similar catalytic ability, cerium oxide nanoparticles (CNPs) have recently attracted attention for biomedical therapy. Mixed valence state CNPs facilitate the expression of redox cycling in pharmacological applications [6,7] and therapeutic potential by scavenging noxious reactive oxygen species (ROS) in cells [8–12]. CNPs uptaken in cells (intracellular CNPs) act as a catalytic

* Corresponding author. Tel.: +81 298 60 4800; fax: +81 298 60 4799.
E-mail address: NAGANUMA.Tamaki@nims.go.jp (T. Naganuma).

http://dx.doi.org/10.1016/j.biomaterials.2014.01.074
0142-9612/

antioxidant to reduce oxidative stress [13]. With regard to CNPs in vitro experiments, the reversible redox cycle allows CNPs to react catalytically with superoxide and hydrogen peroxide, mimicking the behavior of superoxide dismutase (SOD) [10,11] and catalase enzymes [12]. In particular, the Ce^{3+} and Ce^{4+} sites associated with CNP surfaces exhibit SOD [11] and catalase [12] mimetic activity, respectively. The Ce^{3+} sites in CNPs have shown the ability to inhibit redox-dependent apoptosis [9]. Another report suggests that the Ce^{3+}/Ce^{4+} ratio and particle size of the CNPs trigger and induce pro-angiogenesis, and that this activity is critically dependent upon the surface valence state of CNPs [13]. Based on this evidence, different Ce valence states have the potential to induce physiological functions. Stimulation of extracellular CNPs also affects cell functions, as described above regarding the composite scaffolds mixed with CNPs. For this reason, CNPs-functionalized biomaterial surfaces with different Ce valence states may influence cell functions such as cell motility, attachment and proliferation.

Tetravalent Ce is typically the stable Ce valence state in cerium oxide, due to its electronic configuration with the loss of a single electron of 4*f* orbital in trivalent Ce. By contrast, trivalent Ce ions in cerium oxide facilitate oxidation in ambient air. We established, however, that stable trivalent-Ce can be created in CNP layers by Ar^{+} irradiation [14]. Using this method with a mask, CNP layers (CLs) with dominant regions of Ce^{4+} or Ce^{3+} (IV or III) can be formed on biodegradable polymer surfaces, and subsequently may be used to examine the effect of Ce valence states on cell function. Since the surface treatment with Ar ions on CLs retains similar surface stiffness, roughness and topology of CLs, the influence of these physical surface characteristics on cell behavior can be disregarded. However, isolating the effect of Ce valence states in CNPs on cell functions requires clear demonstration of the relationship between cell function and surface features of CLs with dominant Ce^{4+} and Ce^{3+} regions. To clearly isolate the effect of Ce valence states in CLs on cell proliferation, we used additionally modified CLs with different surface charges and hydrophobicity/hydrophilicity. In the present work, we investigated the morphology and proliferation of human mesenchymal stem cells and osteoblast-like cells by examining cell adhesion behavior on CLs with different Ce valence states. We perform this research to gain new insights useful to the design of biomaterial surfaces that can control cell proliferation.

2. Materials and method

2.1. Fabrication of CLs with dominant Ce^{4+} and Ce^{3+} regions (A-IV and B-III)

A poly L-lactide acid (PLLA, Sigma–Aldrich Co., USA) dissolved in dehydrated chloroform (Wako Pure Chemical Industries, Ltd., Japan) was spin-coated on a glass substrate treated by silane coupling agent (1% 3-Methacryloxypropyltrimethoxysilane, KBM-503, Shin-Etsu Chemical Co. Ltd., Japan) in acetic acid solution (pH 4.2) after SPM (Sulfuric acid/Peroxide Mix, H_2O_2:H_2SO_4 = 1:1) treatment. Non-porous PLLA layers (PL samples) were obtained by a spin coating process using the following parameters: <3000 rpm at < 25 °C with <60% RH. Cerium oxide nanoparticles with dominant Ce^{4+} sites (CNPs-1, Nanotek, C. I. Kasei Co. LTD.) were suspended in milli-Q water (18.2 MΩ·cm). After surface modification of PL surface by oxygen plasma etcher at 300 W for 5 min, CNP layers (CLs) with Ce^{4+} sites were formed on the PL surface by dip-coating with aqueous colloidal suspensions of CNPs-1 (PL/CNPs-1 with dominant Ce^{4+}, named as A-IV). The A-IV samples were rinsed in milli-Q water to eliminate the non-adherent CNPs. The A-IV surface was irradiated by Ar ions at RF bias power at 200 W for 1 min, resulting in the formation of PL/CNPs-1 with high concentration of Ce^{3+} sites (named as B-III). The detailed fabrication conditions are described in an earlier work [14]. Since the backside of glass substrate should be covered for protection of adherent cells, all samples were fixed on culture plates with a PDMS thin sheet synthesized by silicone elastomer base with curing agent (SYLGARD® 184, Dow Corning Co., USA).

2.2. Additionally modified surface features of CLs (C-IV and D-IV)

To compare the influence of surface features of CL samples on cell adhesion and proliferation, additional CLs with different wettability and surface charge were prepared by water exposure treatment and precipitation synthesis of CNPs. Hydrophilic CLs with a majority of Ce^{4+} sites (C-IV) were created via the re-oxidation of B-III samples, which were immersed in water for 1 day. Cerium oxide nanoparticles with isoelectric points differing from CNPs-1 were synthesized from Ce $(NO_3)_3 \cdot 6H_2O$ (99.99%, Aldrich) at pH = 9.0 using precipitation method at room temperature (named as CNPs-2). 1.0 M NH_4OH was poured into 0.1 M Ce $(NO_3)_3 \cdot 6H_2O$ solution until pH = 9.0. The CNP-2 solution was stirred for 4 h and settled overnight. The CNP-2 particles were collected after centrifugation, washed in milli-Q water several times to remove any dissolved ions, and then formed on the surfaces of PL samples (PL/CNPs-2, named as D-IV) in a similar manner for the CLs of CNP-1, as described above.

2.3. Characterization of CLs

Static water contact angle and surface charge of PL and CL samples were measured by contact angle analysis (VCA Optima-XE, AST PRODUCTS, INC., USA) and zeta potential measurements (Delsa™ Nano C, Particle analyzer, Beckman Coulter, Inc. USA. and ELSZ-1000, Photal, Otsuka electronics Co., Ltd., Japan, with 10 mM NaCl in milli-Q water). X-ray photoelectron spectroscopy (XPS, Theta Probe, Thermo Fisher Scientific K.K., Japan) was used to identify the Ce valence state on CLs. To determine the specific elements absorbed on CLs with dominant Ce^{4+} and Ce^{3+} sites (A-IV and B-III) during cell culture experiments, both A-IV and B-III samples were incubated in the basal medium for hMSC (MSCGM BulletKit, B3001, Lonza) at 37 °C for 30 min. After rinsing twice with milli-Q water, zeta potential and XPS analysis were carried out on both A-IV and B-III surfaces. A specimen of CLs was milled by a focused ion beam (FIB-2000, Hitachi High Technologies Co., Japan) to obtain a slice sample for transmission electron microscopy (TEM) observation. Cross-section and diffraction pattern of CLs were observed by TEM (JEM-2100F, JEOL, Japan). Surface roughness of CLs was measured by atomic force microscope (Nanowizard® II AFM, JPK Instruments AG) with a super-sharp tip cantilever (Tip radius: 2–3 nm; SI-DF20S; Seiko Instruments Inc.).

2.4. Cell culture

All samples were preliminarily sterilized by UV irradiation for at least 15 min. In brief, osteoblast-like cells (MG-63) were seeded onto sample substrates in the same dishes at 5×10^3 cells/cm^2 and cultured in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and 1% penicillin. As provided in culture protocols for human mesenchymal stem cells (hMSCs), hMSCs were seeded at 5×10^3 cells/cm^2, and subsequently cultured in a recommended culture medium (MSCGM BulletKit, B3001, Lonza) with L-glutamine and mesenchymal cell growth supplement (MCGS). Cell cultures were maintained in an incubator equilibrated with 5% CO_2 at 37 °C. After 24 h, the culture medium was replaced with fresh culture medium and incubated. All cell studies were performed using cell passages below 20 for MG63 and 5 for hMSCs. The cells were incubated with Calcein-AM and propidium iodide (PI) in PBS at 37 °C, and then observed by florescence microscope with filters at λ = 490 and 545 nm. Cell adhesion and migration behavior on sample substrates was observed by phase difference microscope with a temperature/humidity/CO_2-gas control system and a time-lapse CCD camera.

2.5. Cell proliferation assay

Cells were seeded on PL and CL sample substrates (10 × 10 mm) in one dish and at the same cell density, as described above. After incubation for 1, 3 and 7 days (hMSCs) and for 1, 3, and 5 days (MG63), a cell counting kit-8 (CCK-8) assay was used to determine cell viability in cell proliferation. Absorbance of the supernatant of each sample in 24 well plates was measured by microplate reader at λ = 450 nm. Control for background absorbance was the unseeded medium. Repetition of proliferation assay was done at least 2 times.

2.6. Immunofluorescence staining for confocal microscope observation

In brief, cells cultured on the CL samples were fixed with a 3.7% formaldehyde solution in 20 mM HEPES buffer. After soaking in PBS containing 0.2% Triton X-100, the cells were blocked with 3% albumin from bovine serum (BSA) in PBS and subsequently incubated with 1/50 hVIN-1 (ab11194, abcam). The cells were stained with a fluorescence-conjugated goat anti-mouse second antibody (Cy3-labeled IgG, Alexa Fluor® 488, A11001, Invitrogen), 1/40 Alexa Fluor® 555 Phalloidin and 0.1% DAPI. The immunostaining cells were observed with a confocal microscope (TCS SP5, Leica Microsystems, Japan).

2.7. Total amount of proteins adsorbed on CLs and electrophoresis

In brief, PL and CL samples were immersed in cell culture medium (MSCGM for hMSCs), and incubated at 37 °C for 30 min. After rinsing 5 times with 20 mM HEPES, the dried samples were immersed in 1 wt% SDS solution, and then ultra-sonicated for 5 min. Total amounts of proteins in this SDS solution were assayed by Nano-Orange® Protein Quantitation Kit (invitrogen). All data was normalized by the value of SDS solution immersed with each sample substrate without medium-incubation. For electrophoresis assay, fixed amounts of the SDS solution containing proteins were freeze-dried, and re-dissolved with milli-Q water. The solution samples were heated at 70 °C for 10 min before sodium dodecyle surface-polyacrylamide gel