Colloids and Surfaces B: Biointerfaces 116 (2014) 303–308

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Effects of material and surface functional group on collagen self-assembly and subsequent cell adhesion behaviors

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ARTICLE INFO

Article history:
Received 4 September 2013
Received in revised form 7 January 2014
Accepted 8 January 2014
Available online 19 January 2014

Keywords:
Hydroxyl group
Collagen self-assembly
Cell adhesion
Collagen fibrous network
Cytoskeleton

ABSTRACT

Collagen fibrous network not only provides structural support for cells but also serves as critical environment modulating various cell functions. Various factors would influence the collagen self-assembly but the effect of substrate surface on such process has been rarely studied. Here we examined the effects of materials (Ti and hydroxyapatite) and their surface characteristics (with and without the enrichment of hydroxyl group) on collagen self-reconstitution and fibrous network formation, and on subsequent cell adhesion and cytoskeleton organization of mesenchymal stem cells (MSCs). For both Ti and hydroxyapatite (HA) substrates, the enrichment of hydroxyl group (—OH) on substrate surfaces promoted the collagen self-reconstitution and facilitated the formation of the fibrous network after 4 h immersion in phosphate buffer solution (PBS), while all samples showed clear fibrous network formation after 2 day soaking in PBS. Compared with the Ti surfaces, the HA surfaces facilitated the self-reconstitution of collagen, leading to a more mature fibrous network with a twisted structure and enhanced lateral aggregation of fibrils. The fibrous network difference resulted in different behaviors of the subsequent MSC adhesion and spreading. The MSCs had the best adhesion and cytoskeleton organization on the —OH enriched HA surface with collagen modification. Our results suggested that both the material selection and the hydroxyl group significantly influenced the collagen self-assembly and fibrous network formation and, as a result, the subsequent cell adhesion behaviors.

1. Introduction

The extracellular matrix (ECM) is a complex organization composed of a wide variety of structural proteins and proteoglycans secreted by the cells [1,2]. The ECM proteins are able to bind growth factors and cytokines and can interact with bone cells via integrins or other specific cell surface receptors, thus controlling cell shape, motility, growth, survival and differentiation [3–6].

Type I collagen, the primary structural element in ECM and the major structural protein of bone, forms a fibrous network that not only provides a structural support for cells but also acts as an important regulator of cell behavior [7,8]. The fibrous architecture can provide a favorable microenvironment for cell attachment, binding of growth factors, and orchestrating signal and cellular events [9,10]. Recent studies have further suggested that the collagen fibers play a critical role for stem cells to sense the mechanical feedback and may ultimately affect the cell-fate decisions [11]. A proper mechanical feedback would be critical for MSC migration [12] and other MSC functions.

It has been well known that solubilized collagen can self-assemble into fibrous structures in vitro with the characteristic axial periodic structure. Various factors would influence the kinetics of the self-reconstitution process, such as collagen concentration, pH value, temperature and ionic strength [9,13–15]. Collagen has often been used with other inorganic materials to form composites for bone regeneration applications [16]. The intrinsic property and surface characteristic of the inorganic material would likely exert a great influence on collagen fibril formation, thus affecting subsequent cellular response to the materials. However, the effects of material and its surface characteristic on the self-assembly of collagen molecules into the fibrils and fibrous network have been rarely studied.

The most commonly used inorganic materials in orthopedic applications are Ti and hydroxyapatite (HA) [17,18]. Widely used in joint prosthesis and dentistry applications, the titanium is bio-inert despite of its excellent mechanical property. Alkali-heat treatment has been commonly used to make titanium surfaces bioactive by forming a bioactive sodium titanate layer on the titanium surface, in association with the increase of —OH groups on the titanium surface [19,20]. HA is the major inorganic component of the natural bone and has been used as bioactive coating on Ti implants in artificial joints. HA coatings with different surface function groups could be synthesized using two different plasma spraying processes: the

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http://dx.doi.org/10.1016/j.colsurfb.2014.01.009

conventional air plasma spray (APS) process and the more recently developed liquid precursor plasma spraying (LPPS) process. During the APS process which uses powder as the feedstock material, serious dehydroxylation would occur due to the high process temperature, leading to the large loss of the —OH group in the APS HA coatings. In the LPPS process which uses liquid precursor as the feedstock, the dehydroxylation effect is suppressed and as a result, the —OH group is largely retained in the resultant LPPS HA coatings [18].

Therefore, Ti and HA substrates were prepared with diminished and enriched —OH surface group respectively, as a result of different treatments applied to the substrates. The aim of this study was to examine the effects of material selection (Ti, HA) and the surface functional group on collagen self-assembly and the formation of the fibrous network, as well as its implication on subsequent cell adhesion behaviors.

2. Materials and methods

2.1. Materials preparation

2.1.1. Preparation of Ti with and without the alkali-heat treatment

The titanium disks (Φ14 mm × 2 mm) with and without alkali-heat treatment were used as the Ti substrates. For the alkali-heat treatment, the titanium disks were first cleaned by 10 min ultrasound treatment in pure acetone, ethanol and distilled water, respectively. The alkali-heat treatment was preformed according to the protocol described by Kim [20], using 10 M NaOH aqueous solution for 24 h at 60 °C. After the alkali treatment, the titanium disks were rinsed with distilled water, dried in an oven at 60 °C, and heat treated at 600 °C for 1 h in air with a heating rate of 5 °C min^{-1}. The functional groups of the pure and alkali treated Ti (Ti and Ti-AH, respectively) substrates were analyzed by X-ray photoelectron spectrometer (XPS, XSAM800, UK).

2.1.2. Preparation of HA coatings through the APS and LPPS processes

HA coatings, with thickness around 100 μm, have been deposited on the Ti–6Al–4V (Φ14 mm × 2 mm) alloy substrate, using the APS and LPPS processes respectively. The liquid precursor was selected as the feedstock during the LPPS process, which was atomized into mists and injected into the plasma jet, instead of the powder feedstock used in the APS process. More detailed information of the APS and LPPS processes can be found elsewhere [18,21]. The functional groups of the APS and LPPS coatings were analyzed by Fourier Transform Infrared Spectroscopy (FTIR, Nicolet, 170SX, Wisconsin, USA), by scratching the powders from the HA coating samples.

2.1.3. Preparation of type I collagen solution and surface modification with collagen

Type I collagen derived from bovine skin (provided by National Engineering Research Centre for Biomaterial, Sichuan University) was dissolved in acetic acid (pH 4.0) at 4 °C with a concentration of 7.0 mg/ml.

The surfaces of the four kinds of samples (referred as Ti, Ti-AH, HA-APS, HA-LPPS hereafter) were modified with the above collagen solution. Onto each sample we added 400 μl collagen solution. First, half of the solution (200 μl) was dropped on each sample, followed by air drying in a laminar flow cabinet at room temperature. Afterwards, the same process was repeated and the remaining half of the collagen solution was applied to the substrate.

2.2. Fibril network formation

The four groups of samples were immersed into Dulbecco's phosphate buffer solution (PBS, pH 7.4, Sigma, USA) to initiate the self-assembly and fibril network formation at 37 °C. After being cultured for 4 h and 2 days respectively, the samples were fixed with 2.5% glutaraldehyde overnight, dehydrated in graded ethanol and isoamyl acetate, and subjected to critical point drying. After gold coating, the morphologies of the collagen fibrils were observed using the scanning electron microscope (SEM, S4800, Tecnai F20, Tokyo, Japan).

2.3. Cell culture

Mesenchymal stem cells (MSCs) were isolated from rabbit bone marrow (1-week-old, New Zealand rabbits), as previously described [22]. MSCs were expanded in 20 ml of a-MEM containing 20% fetal bovine serum (FBS) and 1% antibiotics and cells from passage 3 were used for all the experiments. MSCs were seeded onto the surfaces of the samples that were placed in 24-well plates, with an initial density of 2.0×10^4 cells/well. Each well consisted of 1 ml of PBS supplemented with 20% FBS.

2.4. Cell morphology

2.4.1. Scanning electron microscopy

After the MSCs were cultured for 1 day, the samples were washed twice with PBS. The MSCs were fixed with 2.5% glutaraldehyde buffer, dehydrated by a graded series of ethanol, subjected to critical point drying and gold coating. The cell morphologies were observed using the SEM (S4800, Tecnai F20, Tokyo, Japan).

2.4.2. Confocal microscopy

Following one-day incubation, the MSCs were visualized using a confocal microscope (Leica SP5, Germany). Prior to observation, the cells were fixed in 1 μl/ml fluorescein diacetate (FDA)/PBS (FDA, PBS, Sigma, USA) solution for live cells.

To visualize the actin cytoskeleton, MSCs were fixed in 4% paraformaldehyde solution, treated with 0.1% Triton X-100 and stained with phalloidin Alexa 594 (Sigma) and DAPI (Sigma).

3. Results and discussions

3.1. Material characterizations

3.1.1. XPS analysis for Ti group samples

The XPS spectra of the Ti and Ti-AH surfaces are shown in Fig. 1. Deconvolution of the O 1s peak revealed that the oxygen existed as TiO_2 form in both Ti and Ti-AH surfaces. The oxygen peak (532.6 eV) of chemically adsorbed basic hydroxyl was clearly visible in the Ti-AH spectrum but was barely detectable in the spectrum of the untreated Ti surface, suggesting enrichment of hydroxyl group at Ti-AH surface.

3.1.2. FTIR analysis for HA group samples

Fig. 2 shows the FTIR spectra of the as-deposited HA-APS and HA-LPPS coatings. Strong adsorption bands of H_2O (3434 and 1634 cm^{-1}) and PO_4^{3-} groups (1091, 1044, 960, 601, and 569 cm^{-1}) were observed in both coatings. However, the HA-LPPS coatings exhibited quite sharp and strong adsorption bands of OH^- at 3570 and 630 cm^{-1}, compared with the APS sample. The enrichment of the hydroxyl group is typical to the HA-LPPS coating where dehydroxylation is significantly suppressed likely due to the presence of the water vapor during the plasma spraying process [18].