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Surface chemical functionalities affect the behavior of human adipose-derived stem cells *in vitro*

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ABSTRACT

This study examines the effect of surface chemical functionalities on the behavior of human adipose-derived stem cells (hASCs) *in vitro*. Plasma polymerized films rich in amine (—NH_2), carboxyl (—COOH) and methyl (—CH_3), were generated on hydroxyapatite (HAp) substrates. The surface chemical functionalities were characterized by X-ray photoelectron spectroscopy (XPS). The ability of different substrates to absorb proteins was evaluated. The results showed that substrates modified with hydrophilic functional group (—COOH and —NH_2) can absorb more proteins than these modified with more hydrophobic functional group (—CH_3). The behavior of human adipose-derived stem cells (hASCs) cultured on different substrates was investigated *in vitro*: cell counting kit-8 (CCK-8) analysis was used to characterize cell proliferation, scanning electronic microscopy (SEM) analysis was used to characterize cell morphology and alkaline phosphatase (ALP) activity analysis was used to account for differentiation. The results of this study demonstrated that the —NH_2 modified surfaces encourage osteogenic differentiation; the —COOH modified surfaces promote cell adhesion and spreading and the —CH_3 modified surfaces have the lowest ability to induce osteogenic differentiation. These findings confirmed that the surface chemical states of biomaterials can affect the behavior of hASCs *in vitro*.

1. Introduction

In tissue engineering and regenerative medicine, as in many other areas, material plays a cornerstone and an important role not only as inert mechanical supports or cell and drug delivery vehicles but also to direct and guide the interaction at the tissue or cell–material interface [1]. Materials can provide cues that control cell adhesion, proliferation, differentiation and migration. These cues include the material physical and chemical properties such as surface topography [2–4], mechanical properties [5,6], surface chemical states [7–9], *etc.* Understanding these interactions is important for the future materials design that will facilitate and promote tissue repair and regeneration [10].

Stem cells based therapies have generated significant interest and are regarded as the future of various medical procedures and treatments. Human adipose-derived stem cell (hASC) is a type of mesenchymal stem cell which has the potential to undergo multilineage differentiation: adipogenesis [11], osteogenesis [12], chondrogenesis [13], neurogenesis [14], angiogenesis [15] and so on. Compared with other sources of mesenchymal stem cells such as bone marrow, the most commonly used in tissue engineering, adipose tissue is available in relatively large quantities using liposuction procedures which the patients are ready to accept [16]. Accordingly, over the last 10 years, an increasing number of researchers have started using hASCs as seeding cells for bone tissue engineering instead of human bone marrow-derived mesenchymal stem cells (hMSCs) [17–19].

In the context of stem cell based thereapies, it is important to understand how material surface properties influence the differentiation of hASC cells. Hydroxyapatite (HAp), which is the inorganic constituent of human bones and widely used in bone tissue engineering because of its good biocompatibility, was selected as the substrates. It is very interesting to know if the surface modification can affect the biocompatibility and osteoinduction of HAp. Plasma polymerization is usually employed as a surface modification method. Compared with other methods, for example, silanization or self-assembling, plasma polymerization can effectively provide a pinhole free film with high retention of functional groups and good surface coverage with relatively low cost [20].

In this study, a range of surfaces of engineered surface functionalities were prepared to explore how the surface chemical states influence hASC morphology, proliferation and differentiation *in vitro*. Amine (—NH_2), carboxyl (—COOH) and methyl (—CH_3) were generated on HAp substrates by plasma polymerization. These

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functional groups were chosen because they can be found naturally within biological systems [21]. It is a good first step to mimic in a simple way the chemical functionalities that comprise the microenvironment of hASCs using these functional groups. Previous studies have demonstrated that the surface functional groups can influence the morphology, adherence, proliferation and differentiation of human mesenchymal stem cells (hMSCs) [22], osteoblasts [23] and endothelial cells [24]. The influence of these functional groups on attachment and morphology of hASCs was also explored before, however how these functionalities affect hASCs differentiation is unknown [25]. Thus, the major objective of this study is to understand the behavior of hASCs on HAp substrates modified with amine, carboxyl and methyl.

2. Material and methods

2.1. Materials and plasma polymerization

The nano hydroxyapatite (HAp) powder (obtained from Institute of Nuclear and New Energy Technology, Tsinghua University) was consolidated into cylindrical substrates (15 mm in diameter and 2.5 mm in height) *via* uniaxial loading and sintered for 1 h at 600 °C and 2 h at 1200 °C.

Plasma polymerization was carried out in a reactor previously described [26,27]. Allylamine, acrylic acid and 1,7-octadiene were used as precursors for plasma polymerization in order to generate thin films rich in amine, carboxyl and methyl chemical groups, respectively. Before deposition, the substrates were cleaned with oxygen plasma using power of 50 W for 2 min. Then, the chamber was evacuated to pressure of 1×10^{-3} mbar. In order to provide optimum quality of plasma polymer films, the following deposition conditions were used: (i) Allylamine was deposited at pressure of 2.1×10^{-1} mbar, using power of 40 W and deposition time of 30 s. The resulting coating had thickness of 24 nm as measured by ellipsometry. (ii) Acrylic acid was deposited at pressure of 4.2×10^{-2} mbar, power of 20 W for 30 s. The coating thickness was 36 nm. (iii) 1,7-octadiene was deposited at pressure 5.2×10^{-2} mbar, power of 20 W for 60 s. The coating thickness was 46 nm.

2.2. HAp substrate surface characterization

The HAp substrate surface chemical states were determined by X-ray photoelectron spectroscopy (XPS, ESCALAB-250Xi) using a monochromatic Al Kα X-ray source. XPS C 1s and N 1s high resolution spectra were recorded using pass energy of 20 eV. The energy resolution was 0.05 eV. Binding energy is calibrated with C 1s = 284.8 eV. The spectra were fitted using XPSPEAK 4.1 software.

2.3. Evaluation of ability to absorb proteins

Before cell culture, the ability to absorb proteins of the HAp substrates modified with different plasma polymerized films was evaluated. Bovine serum albumin (BSA) was selected as a model protein. The substrates were immersed in 200 μg/mL BSA solution (in PBS containing 25 ppm sodium azide) for 7 days. Then the BSA concentration (s, with a unit of μg/mL) of the supernatants was determined with the BCA Assay Kit (Beyotime, China) according to the guideline of the company. The percentage of adsorbed protein (a) was determined by the formula of $a = (200 - s)/200 \times 100\%$.

2.4. Human adipose-derived stem cells (hASCs) culture

Human adipose-derived stem cells (hASCs, obtained from Chinese Academy of Military Medical Sciences) were expended in low glucose Dulbecco's modified Eagle's medium (LG-DMEM, Invitrogen), containing 10% fetal bovine serum (FBS, Invitrogen, USA), 100 μg/mL streptomycin, 100 U/mL penicillin (growth medium, GM) and used at passage 3. The substrates were placed in a 24-well plate (Nunc, Denmark) and exposed to ^{60}Co irradiation before use. hASCs (10^4 cells/well) were seeded on HAp substrates and allowed to adhere under standard culture condition (5% CO_2, 37 °C) for 24 h. Then the supernatant medium was replaced with osteogenic medium (OM, GM containing 10^{-7} M dexamethasone, 10 mM β-glycerophosphate disodium, 50 μg/mL ascorbic acid). The supernatants were replaced with fresh medium every 3 days.

2.5. Evaluation of adipogenesis and osteogenesis

The adipogenesis and osteogenesis of hASCs were evaluated to explore the stemness of hASCs. hASCs were seeded in a 48-well plate (Corning, USA) at a density of 5000 cells/well. After 2 days of culture in GM, described above, the medium was changed into lineage specific media to induce adipogenesis and osteogenesis. The supernatants were replaced with fresh medium every 3 days for another 2 weeks. All cultures were maintained in 37 °C, 5% CO_2 environment.

The adipogenic medium (AM, GM containing 10 μg/mL insulin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine and 200 μM indometacin) was used to induce adipogenesis. After 14 days of induction, Oil Red-O was used to stain the adipocytes. Briefly, the cells were washed with PBS twice, fixed for 10 min with 4 (w/v%) paraformaldehyde in PBS and stained for 30 min with the Oil Red-O staining solution (3 vol. of a 0.5 (w/v%) stock solution of Oil Red-O in isopropanol plus 2 vol. of distilled water). To quantify retention of Oil Red-O, stained cells were extracted with 500 μL of isopropanol for 15 min at room temperature, and the absorbance (OD) was measured by microplate reader at 570 nm.

The osteogenic medium (OM, mentioned above) was used to induce osteogenesis. After 14 days of induction, Alizarin Red was used to stain the calcium nodules. Briefly, the cells were washed with PBS twice, fixed for 10 min with 4 (w/v%) paraformaldehyde in PBS and stained for 30 min with 0.1 (w/v%) Alizarin Red staining solution. To quantify retention of Alizarin Red, stained cells were extracted with 500 μL of 10 (w/v%) cetylpyridinium chloride in trisodium phosphate for 15 min at room temperature, and absorbance (OD) was measured by microplate reader at 570 nm.

2.6. CCK-8 assay for proliferation

A cell count kit-8 (CCK-8, Dojindo, Japan) was used to quantitatively evaluate the cell proliferation. At the days 1 and 7 after seeding, CCK-8 with a 10 vol.% of the medium was added into the wells and incubated for 4 h at 37 °C. CCK-8 was transformed into orange-colored formazan by the activity of dehydrogenases in cells. The amount of formazan is directly proportional to the number of living cells. The absorbance (OD) of the solution was measured by microplate reader at 450 nm.

2.7. Scanning electron microscopy assay for morphology

At the days 1, 7 and 21 after seeding, the cells on the substrates were washed with PBS and fixed with 2.5 (w/v%) glutaraldehyde in PBS overnight at 4 °C. After fixation, they were washed twice with PBS for 10 min each wash. Then the cells were dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95% and 100%) for 20 min each and replaced with graded series of tert-butyl alcohol (25% for once, 50% for once, 75% for once and 100% for twice) for 10 min each. After vacuum freeze drying for an hour, the samples were surface metalized by sputter-coating with gold for scanning