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Graphene modified titanium alloy promote the adhesion, proliferation and osteogenic differentiation of bone marrow stromal cells

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ABSTRACT

We studied the effects of graphene coating on improving the biological activity of a titanium alloy (Ti_6Al_4V) widely used in hip and knee joint replacements. The experiments included immunofluorescence staining for observing cellular adhesion, Cell Counting Kit-8 (CCK-8) for evaluating cellular proliferation and reverse transcription-polymerase chain reaction (RT-PCR) for detecting the differentiation of bone marrow stromal cells on different scaffolds. The results showed that G-Ti_6Al_4V exhibited a higher mean integrated optical density (IOD) for vinculin and resulted in a higher cell proliferation rate and higher osteoblast-specific gene transcription levels. In summary, graphene could be used as a new nanocoating material for Ti_6Al_4V scaffolds to enhance their surface bioactivity.

1. Introduction

Titanium alloys are often used as metallic biomaterials due to their high mechanical strength, corrosion resistance and excellent biocompatibility [1,2]. Specifically, these alloys are widely used in artificial bones and joints, plates, screws and as substitute materials for other hard tissues [3]. Among these alloys, the most widely used material is Ti_6Al_4V, which accounts for approximately 80% of all titanium alloy products. Titanium alloys are very readily oxidized; in fact, their surface forms a layer of dense and strong TiO_2 film, thereby providing strong corrosion resistance to the underlying substrate. However, the oxidized layer is a biologically inert film and hinders the direct combination of the implant with bone tissues [4], resulting in decreased bioactivity of Ti-based implant outcomes. To address this issue, the coating of implant surfaces and the loading of implants with bioactive substances have been shown to effectively improve the bioactivity of the implants.

Graphene is a two-dimensional atomic crystal comprising a single atomic layer formed by sp2 hybridization. This material, which has unique optical, mechanical, chemical and electrical properties [5], and its applications span from the fields of electronics and chemistry to the biomedical field [6]. Graphene has shown good performance in medical applications, such as for cancer treatments [7], as a drug carrier [8] and a biosensor [9], and in biological imaging [10]. In this study, we prepared graphene coatings on the surface of Ti_6Al_4V and performed *in vitro* experiments. The results suggest that graphene might be a novel type of nanocoating materials that can improve cell adhesion, proliferation and osteogenic differentiation.

2. Materials and methods

2.1. Preparation of graphene coatings

The specifications of the titanium alloy Ti_6Al_4V (Shenyang, China) used in this study are as follows: Ti_6Al_4V sheets ($\Phi = 10$ mm, $\delta = 5$ mm). Graphene films on copper substrates with 100% coverage were prepared using chemical vapor deposition (CVD; Changzhou, China). Briefly, 7% polymethyl methacrylate (PMMA) was evenly coated on the surface of a graphene-coated copper substrate (G-Cu). PMMA-graphene films were then obtained by etching Cu with a $FeCl_3$ solution and transferred to the Ti_6Al_4V surfaces through physical adsorption. In addition, the PMMA was then completely dissolved with acetone to obtain the graphene-coated Ti_6Al_4V alloy (G-Ti_6Al_4V).

2.2. Raman spectroscopy detection

The fine structures of graphene on the G-Cu, G-Ti_6Al_4V and

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Ti_6Al_4V (n = 5) were characterized via Raman spectroscopy (HORIBA JOBIN YVON HR800, Japan) using a laser at a wavelength of 630 nm.

2.3. Hydrophilicity

Contact angle was performed using an optical contact angle measuring instrument (KRUSS, Germany). Three different regions on each sample were selected to determine the contact angle. The results were analyzed, and the hydrophilicity of the samples was evaluated (n = 6).

2.4. Surface roughness

An NT1100 Optical Profilometer (Veeco, USA) was used to determine the surface roughness of three different regions on each sample (n = 6), and the changes in the surface morphology of the scaffolds were compared.

2.5. Culture and seeding of bone marrow stromal cells (BMSCs) of sprague-dawley rats

Animal studies were conducted in strict accordance with the guidelines for the Animal Management Committee of the Fourth Military Medical University and Chinese Animal Research and Management Committee.

BMSCs were extracted from 3-w-old SD rats (98 ± 0.9 g) and were isolated and cultured using a whole bone marrow adherent culture method. Complete Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM-F12; HyClone, USA) was prepared with 10% fetal bovine serum (Gibco, Grand Island, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, USA). The cultures were incubated in an incubator at 37 °C under 5% CO_2 and 95% humidity (Thermo, USA).

The G-Ti_6Al_4V was the experimental group, and the Ti_6Al_4V as the control group. All samples were sterilized by Co^{60} radiation (irradiation dose: 20 kGy). Cells from the P3 passage of the BMSC cultures were seeded on the different scaffolds at a density of 1×10^4/well using a super-clean bench (Wuxi, China).

2.6. Cell adhesion

The P3 passages of BMSCs were seeded at a density of 1×10^4/well on the scaffolds (n = 3) in a 24-well plate. After 1, 3, and 5 d of culture, samples were collected and immunostained. The culture medium was removed, and the cells were washed 3 times with PBS. Then, 4% paraformaldehyde (HEART, China) was added, and the cells were fixed for 15 min. Subsequently, the fixing liquid was removed, and the samples were washed 3 times with PBS. A 0.1% v/v solution of Triton X-100 (Sigma, USA) was added to the culture, and the plates were maintained at room temperature for 3 min to permeabilize the cell membranes. The samples were then washed 3 times with PBS, and 1% w/v bovine serum albumin (BSA, Sigma, USA) was added to the cells. After incubation at room temperature for 3 min to block non-specific binding. Then anti-vinculin antibodies (Abcam, USA; diluted at a 1:100 ratio in PBS) were added to the scaffolds, and the samples were then maintained at 4 °C for 12 h and washed 3 times with PBS. A FITC-labeled goat anti rabbit IgG antibody (Abcam, USA) was diluted 1:50 with PBS and added to the scaffolds, and the samples were allowed to react for 4 h under absolute dark conditions and washed 3 time with PBS. TRITC-labeled phalloidin (Cytoskeleton, USA) was diluted 1:80 in PBS and added to the scaffolds. After 30 min of staining, the samples were washed 3 times with PBS, was dyed with 10 μg/ml 4′6-diamidino-2-phenylindole (DAPI, Beyotime, China) for 5 min and washed with PBS. The samples were then placed on glass slides and observed by laser scanning confocal microscopy (TCS SP5, Leica, Solms, Germany). Statistical analyses were performed using Image-Pro plus (IPP) 6.0 software (Edia Cybernetics Corp., USA) to determine the mean integrated optical densities (IODs).

2.7. Cell proliferation

Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was used to evaluate the proliferation of the cell on the different scaffolds (n = 6). After 1, 3, 5 and 7 d of culture, the culture media were removed from the 24-well plate, and 1000 μl of serum-free medium was added to each well. The cultures were then treated with 100 μl of the CCK-8 reagent for 2 h. The reaction caused the liquid color to change from red to yellow. Following this color change, 100 μl of liquid was sampled and loaded onto a 96-well plate. After a 2-min oscillation, optical density (OD) values were evaluated at 450 nm using a microplate reader (Bio-Tek, USA).

2.8. Live/dead cell test

After 1, 3, 5 and 7 d of culture, a live/dead cell kit (Fisher Thermo, USA) was used for live/dead cells staining and quantitative analysis. Briefly, 1 μl of solution A in the kit (calcein-AM) was added to 1 ml of solution B (ethidium homodimer-1), and 60 μl of the mixture was dropped onto the sample surface. The sample was then maintained in the dark for 20 min and observed under a laser-scanning confocal microscope. IPP 6.0 software was used to analyze the numbers of living cells (n = 3).

2.9. Histochemical and quantitative analysis of the formation of mineralized bone nodules

After osteogenesis induction of the BMSCs on the different scaffolds for 21 d, the samples were fixed with 4% paraformaldehyde for 30 min and washed 3 times with PBS. After the addition of 0.5% alizarin red S (pH 4.2, Sigma, USA), the samples were maintained at room temperature for 1 h. The supernatant was removed, and the samples were washed 3 times with PBS. Then, 10% cetylpyridinium chloride (Sigma, USA) was added to the samples, and the samples were incubated at room temperature for 1 h. The supernatants were aspirated, and the absorbance values of the samples were detected using a microplate reader at 570 nm. Optical microscopy (Nikon, Japan) was performed to observe the alizarin red S-stained bone nodules (n = 6).

2.10. ALP activity analysis

Alkaline phosphatase (ALP) activity was measured using an ALP activity assay kit (JianCheng, China). After 7 and 14 d of the osteogenesis induction of the BMSCs on the different scaffolds, 0.25% trypsin (Corning, USA) was added to digest the cells, and the digested cells were collected by aspirating the supernatant after centrifugation at 1000 rpm for 5 min. After the addition of 200 μl of 0.1% Triton X-100, the cells were resuspended, and the cell masses were subsequently sonicated on ice and placed in centrifuge (Eppendorf, Germany) at 4 °C and 12,000 rpm for 10 min. The supernatant was then collected and saved, and the absorbance of the collected supernatant at 405 nm was measured using a microplate reader. The total protein content of the cell was determined using a bicinchoninic acid (BCA) assay kit for protein concentration quantification (Wanleibio, China), and the absorbance at 570 nm was measured using a microplate reader. ALP activity was determined by calculating the total activity and protein concentration of ALP (n = 6). An ALP active unit was set to 1 U (U/g protein), i.e., 1 mg of

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