Materials Chemistry and Physics 221 (2019) 89–98

Contents lists available at ScienceDirect

Materials Chemistry and Physics

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

Bone marrow derived mesenchymal stem cell response to the RF magnetron sputter deposited hydroxyapatite coating on AZ91 magnesium alloy

Maria A. Surmeneva[a], Anna A. Ivanova[a], Qiaomu Tian[b], Rebekah Pittman[b], Wensen Jiang[c], Jiajia Lin[c], Huinan H. Liu[b,c,**], Roman A. Surmenev[a,*]

[a] Physical Materials Science and Composite Materials Centre, National Research Tomsk Polytechnic University, 634050, Lenin Avenue 43, Tomsk, Russia
[b] Department of Bioengineering, University of California, Riverside, 900 University Avenue, MSE 227, Riverside, CA, 92521, USA
[c] Materials Science and Engineering, University of California, Riverside, 900 University Avenue, MSE 313, Riverside, CA, 92521, USA

HIGHLIGHTS

- Nanostructured hydroxyapatite (HA) coating was deposited on AZ91 alloy by RF-magnetron sputtering.
- Multiple grain-boundaries observed at the bias of −25 V and no grain boundaries at the bias of −100 V.
- The release of Mg^{2+} ions was reduced for HA coated samples compared with bare AZ91 alloy.
- HA coating supports adhesion, proliferation of BMSCs compared with uncoated AZ91 alloy.

ARTICLE INFO

Keywords:
Magnesium alloy
RF magnetron sputtering
Hydroxyapatite
Mesenchymal stem cells

ABSTRACT

The aim of this study was to investigate adhesion of bone marrow stromal cells (BMSCs) and *in vitro* degradation of hydroxyapatite (HA) coatings deposited on the surface of magnesium alloy (AZ91). The uniform and pore-free HA coating was successfully obtained via RF magnetron sputter deposition at the substrate bias of −25 and substrate bias of −100 V followed by post-deposition annealing. The structural features of the HA coating have been characterized and biological performance of the HA coating has been examined via *in vitro* test. The X-ray diffraction and Energy dispersive X-ray analysis data revealed that the phase and elemental composition of the deposited HA coatings were similar, but the surface roughness and morphology were influenced by the substrate bias voltage. The coating deposited at −25 V consists of multiple grain-boundaries. In contrast, no grain boundary but rather flat surface was observed for the sample deposited at −100 V. Atomic force microscopy (AFM) was used to obtain topographic images of the obtained coatings and quantitative assessment of the surface roughness. The roughness parameters of the deposited coatings were found to display nanometer scale surface roughness (*Sa* and *Sq*) from 92 to 130 nm over areas of 5 × 5 μm^2. The *in vitro* direct culture experiment showed that the HA coating deposited at the substrate bias of −100 V significantly reduced the release of Mg ions in post-culture media and revealed higher BMSCs adhesion density than that on the HA coating deposited at the bias of −25 V and the uncoated AZ91 alloy. Thus, this study demonstrated that the negative pulse substrate bias applied for HA coating deposition allows to improve the surface features of the AZ91 magnesium alloy and enhance BMSCs adhesion *in vitro*.

1. Introduction

There has been recently a great interest in magnesium (Mg) and its alloys as biodegradable materials for orthopaedic applications, since the elastic modulus and compressive yield strength of Mg alloys are similar to those of bone tissue [1–7]. Moreover, Mg is an essential element for different human metabolic processes, which makes Mg desirable for different orthopaedic applications. The successful application of biodegradable Mg alloys is dependent on the control of degradation rate, which is varied for different material compositions [2,8,9]. The most important disadvantage is the rapid degradation rate of Mg alloys in the physiological environment, which frequently results

* Corresponding author.
** Corresponding author. Materials Science and Engineering, University of California, Riverside, 900 University Avenue, MSE 313, Riverside, CA, 92521, USA.
E-mail addresses: Huinan.liu@ucr.edu (H.H. Liu), rsurmenev@gmail.com (R.A. Surmenev).

https://doi.org/10.1016/j.matchemphys.2018.09.030
Received 26 October 2017; Received in revised form 31 July 2018; Accepted 8 September 2018
Available online 10 September 2018
0254-0584/

in the loss of mechanical integrity of an Mg implant before the complete healing of tissue. In an effort to improve the corrosion resistance of Mg alloys protective coatings can be deposited. The HA [$Ca_{10}(PO_4)_6(OH)_2$] coating allows to control the biodegradation rate of Mg alloys, and also improve surface biocompatibility, because HA is a major inorganic component of bone [10].

RF magnetron sputtering is a promising technique to obtain a highly adhesive, uniform and dense HA coating [10]. It is well known that biological performance of the HA coating is affected by its structure and Ca/P ratio [10,11], which can be adjusted by different RF magnetron sputter deposition parameters, such as discharge power, pressure, gas atmosphere, substrate temperature and bias [10,12,13]. Control over ion bombardment intensity of the substrate via RF magnetron sputtering may lead to the ability to tailor the properties of the HA coating and its behaviour *in vitro*. Based on our previous studies [12,14,15], it is known that a negative substrate bias during RF magnetron sputter deposition of HA coating allowed to vary ion flux density from plasma towards the substrate and, thus, HA coating properties. The effect of pulse negative substrate bias on the cytocompatibility of the HA coated AZ91 prepared by RF magnetron sputtering has never been studied. Therefore, the objective of this paper was to study the performance of the HA coating prepared on the surface of AZ91 alloy by RF magnetron sputtering at different applied substrate biases, in particular, degradation behaviour and cytocompatibility *in vitro* of the prepared biocomposites.

2. Materials and methods

2.1. Samples and coating preparation

The AZ91 Mg alloy plates with the size of 10×10×1 mm^3 (width × length × thickness) were used as substrates. The alloy samples were first polished with 4000 grit SiC emery paper and then with diamond paste with particle size of 3 μm followed by ultrasonic cleaning in acetone and ethanol. The target of a pure HA was prepared according to procedures described elsewhere [14]. A commercially available magnetron sputtering set up with an RF magnetron source (13.56 MHz, COMDEL) was used. The coatings were deposited at an RF-power level of 400 W in Ar atmosphere at 0.4 Pa for 600 min either with substrate pulse biasing at −25 V and −100 V with a duty cycle of 10%. Before coating deposition plasma pre-treatment of the substrate was done (40 W RF-power, −500 V pulsed substrate bias, 0.4 Pa, Ar for 1 h, T = 300 °C). The post-deposition annealing at 400 °C for 2 h at a heating rate of 1 °C/min was used to improve the coatings crystallinity.

2.2. Morphology, structure, and chemical composition of the HA coatings

The surface morphology and chemical composition of the HA coating were investigated using a scanning electron microscope (SEM) FEI Quanta 200 F and ESEM Quanta 400 FEG. To reduce the surface charging effect, a thin Au film was sputter deposited before the analysis by low-vacuum sputtering equipment with a current of 10 mA for 15 s. The structure of the HA coatings was investigated using an X-ray diffractometer (XRD) (D8 Advance Bruker AXS). The measurements were carried out in the Bragg-Brentano configuration with CuKα radiation source (a wavelength of 1.5406 Å) operated at 30 mA and 40 kV. A 1D detector (LynxEye Silicon Strip) with an angular step size resolution and without a monochromator was used. The X-ray diffraction pattern of HA (#09–432) from the ICDD database was analyzed as a reference. The surface roughness parameters were examined using atomic force microscopy (AFM) Certus Standard V operated in tapping mode using a N15/AlBS tip, which allowed to define such surface roughness parameters as S_a and S_q calculated for the surface area of 5×5 μm^2 using Gwyddion software. The surface roughness of each samples was run duplicate at two different spots. Optical ellipsometry (Ellipse 1891-S AG, Institute of Semiconductor Physics, RAS, Siberian Branch) was used to determine the thickness of the deposited coatings. The coating thickness was derived from the changes in ellipsometric parameters between the bare and the coated substrates using a three-phase model (substrate-layer-air).

2.3. The response of BMSCs to in vitro degradation of HA coated AZ91 alloys

2.3.1. Preparation of BMSCs culture

Following a protocol approved by the University of California at Riverside (UCR) Institutional Animal Care and Use Committee (IACUC), BMSCs were harvested from the marrow cavity of the femur and tibia of 1-day-old female Sprague-Dawley rat weanlings that had been euthanized by CO_2 asphyxiation. Specific details regarding BMSCs extraction, isolation and culture have been described elsewhere [16].

2.3.2. Direct culture of BMSCs

The BMSCs direct culture methods were the same as reported elsewhere [46]. In order to prevent degradation of the uncoated sides of AZ91 samples during the BMSC culture, all the non-HA coated sides of AZ91 samples were covered by epoxy resin layers (Pace technologies, USA). Prior to cell culture experiments, the samples were individually weighed (M_0), and sterilized under ultraviolet (UV) radiation for 4 h on each side. Non-culture-treated glass slides (Cat. No. 12-544-1, Fisher Scientific, Hampton, NH, USA) with 1 mm thickness were cut into 10 mm × 10 mm squares, ultrasonically cleaned in acetone and ethanol, sterilized under UV radiation, and used as a reference. All the samples were placed in standard 12-well cell culture treated plates and rinsed with 2 mL of DMEM to calibrate the osmotic pressure. Subsequently, BMSCs (P2) were seeded directly onto the surfaces of the samples at a density of 4×10^4 cells cm^{-2} and incubated in 3 mL of DMEM under standard cell culture conditions for 24 and 72 h. BMSCs only, i.e., BMSCs cultured only with DMEM in the wells without any samples, were also included as a control, designated as the “cells” group. DMEM alone was also used as a blank reference and designated as the “DMEM” group. In order to more closely mimic *in vivo* conditions cell culture media was collected for analysis and replenished with 3 mL of fresh media (pH 7.4) at every 24 h interval.

2.3.3. Quantification of BMSCs adhesion and aspect ratio under direct vs. indirect contact conditions

BMSCs adhesion and morphology on the surface of the samples (direct contact) and on culture plate surrounding the respective samples (indirect contact) was evaluated at 24 h and 72 h of incubation through fluorescence microscopy. The samples in direct contact with BMSCs were removed from the wells and dip-rinsed in PBS to remove non-adherent cells. The corresponding wells were washed separately with PBS to remove non-adherent cells. Adherent cells, both on the sample surface and on the plates, were separately fixed with 4% formaldehyde (10% neutral buffered formalin; VWR, Radnor, USA) and stained with 4,6-diamidino-2-phenylindole dilactate (DAPI; Invitrogen) nucleic acid stain and Alexa Fluor 488 F-actin stain for fluorescence imaging. Adherent cells on the sample surface and on the culture plate surrounding the sample were visualized using a fluorescence microscope (Eclipse Ti and NIS software, Nikon, Melville, USA) with a 10× objective lens at the same exposure condition and analyzed using ImageJ (NIH, Bethesda, USA). Cell adhesion was quantified by counting the DAPI-stained cell nuclei per surface area at each prescribed incubation interval. Cell adhesion density was calculated as the number of adherent cells per unit area. At each time interval, culture media was collected, measured and replaced with fresh media. All the experiments were done in triplicate on five random locations on each sample surface (n = 15), averaged and expressed as the mean ± standard deviation (SD). The aspect ratio of cells under direct and indirect contact were analyzed manually using image J, which denotes the ratio between the longest axis to the shortest axis of each cell.