Colloids and Surfaces B: Biointerfaces 148 (2016) 200–210

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

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

Dual ions implantation of zirconium and nitrogen into magnesium alloys for enhanced corrosion resistance, antimicrobial activity and biocompatibility

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ARTICLE INFO

Article history:
Received 23 April 2016
Received in revised form 10 August 2016
Accepted 30 August 2016
Available online 31 August 2016

Keywords:
Magnesium alloys
Plasma ion implantation
Antibacterial property
Corrosion resistance
Osseointegration

ABSTRACT

Biodegradable magnesium-based alloys have shown great potential for medical applications due to their superior biological performances and mechanical properties. However, on one hand, some side effects including inferior biocompatibility, a local high-alkaline environment and gas cavities caused by a rapid corrosion rate, hinder their clinical application. On the other hand, it is also necessary to endow Mg alloys with antibacterial properties, which are crucial for clinic orthopedic applications. In this study, Zr and N ions are simultaneously implanted into AZ91 Mg alloys by plasma immersion ion implantation (PIII). A modified layer with a thickness of approximately 80 nm is formed on the surface of AZ91 Mg alloys, and the hydrophobicity and roughness of these AZ91 Mg alloys obviously increase after Zr and N implantation. The *in vitro* evaluations including corrosion resistance tests, antimicrobial tests and cytocompatibility and alkaline phosphatase (ALP) activity tests, revealed that the dual ions implantation of Zr and N not only enhanced the corrosion resistance of the AZ91 Mg alloy but also provided better antimicrobial properties *in vitro*. Furthermore, the formation of biocompatible metal nitrides and metal oxides layer in the near surface of the Zr-N-implanted AZ91 Mg alloy provided a favorable implantation surface for cell adhesion and growth, which in return further promoted the bone formation *in vivo*. These promising results suggest that the Zr-N-implanted AZ91 Mg alloy shows potential for future application in the orthopedic field.

1. Introduction

Mg-based alloys, which possess the ability to biodegrade and have comparable mechanical properties to natural bones, have attracted much attention for orthopedic applications [1,2]. Furthermore, a certain amount of magnesium ions has been demonstrated to stimulate adhesion, proliferation and bone-related gene expression of osteoblasts [3,4]. Unfortunately, the major obstacle hampering clinical application of Mg alloys is their fast corrosion rate, which may result in adverse effects on bone formation and subsequently implant failure [5–7]. Various protective coatings have been prepared using different techniques to improve the corrosion resistance of Mg-based alloys [8,9]. However, an ideal protective coating must primarily have high coating adhesive strength and compactness [10]. Based on the above standpoint, plasma immersion ion implantation (PIII) seems to be an appropriate method to synthesize a dense protective layer in the near surface of Mg alloys without creating an abrupt interface.

Apart from the negative issues related to the fast degradation of Mg alloys, it is also necessary to endow Mg alloys with antibacterial properties and favorable biocompatibility. PIII is an especially promising way to achieve the above requirements because it allows the simultaneous implantation of elements with different attributes into the near surface of the materials. Silver [11], zinc [12,13] and copper [14] are inorganic antibacterial agents that have been incorporated into different alloys to inhibit bacterial adhesion and biofilm formation. However, on one hand, an excess amount of these metal ions may cause deleterious effects. On the other hand, the inclusion of these ions into magnesium alloys will induce more rapid degradation because of the generation of galvanic corrosion

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http://dx.doi.org/10.1016/j.colsurfb.2016.08.056
0927-7765/

[15,16]. Zirconium has good cytocompatibility and antibacterial properties, and recently studies have also reported that physically modifying titanium implants by sputter coating or arc ion plating with ZrN may reduce the adherence of *Streptococcus mutans* while enhancing the adhesion and growth of fibroblasts [17–19]. In addition, previous studies revealed significantly enhanced corrosion resistance after either zirconium or nitrogen plasma treatment on Mg alloys [20,21]. However, the effects of dual zirconium and nitrogen plasma treatment on the antibacterial properties and corrosion resistance of AZ91 Mg alloy, as well as the integration between the magnesium implant and bone have not been studied.

In this work, AZ91 Mg alloys were treated using dual zirconium and nitrogen plasma ion implantation and their *in vitro* corrosion resistance, cytocompatibility and antimicrobial characteristics were determined systematically. In addition, an animal model was established to further investigate the effects of dual zirconium and nitrogen plasma ion implantation on the *in vivo* corrosion resistance and bone formation ability of the AZ91 Mg alloy.

2. Materials and methods

2.1. Sample preparation and characterization

An AZ91 Mg alloy ingot (Mg with Al 9 wt.% and Zn 1 wt.%) was cut into disks (10 mm in diameter and 1 mm thick) for *in vitro* study and rods (2 mm in diameter and 6 mm long) for *in vivo* study. All samples were polished with SiC papers to remove surface oxides, and then ultrasonically cleaned with acetone, and ethanol. Zr plasma ion implantation was performed on a multipurpose plasma immersion ion implanter equipped with a zirconium arc source for 2 h at a pulse voltage of 15 kV with a pulsing duration of 500 μs and a pulsing frequency of 5 Hz. At the same time, nitrogen was bled into the vacuum chamber at a flow of 20 sccm.

X-ray photoelectron spectroscopy (XPS, Physical electronics PHI1800) was employed to determine the surface chemical compositions and elemental depth profiles. The surface topography of the prepared specimens was observed by field-emission scanning electron microscopy (FESEM, FEINOVA NanoSEM). Atomic force microscopy (AFM, Nanoscope 3; Bruker, Germany) was used to determine the surface roughness before and after ion implantation, and the contact angle was determined using a video contact-angle measurement instrument.

2.2. Electrochemical tests

The corrosion resistance of the un-implanted and Zr-N-implanted AZ91 Mg alloys was assessed in a 0.9% sodium chloride solution (0.9% NaCl) and DMEM (Gibco), respectively, by potentiodynamic polarization tests on a CHI760C electrochemical analyzer (Shanghai, China). The potential was referenced to a saturated calomel electrode (SCE) and a graphite rod serving as the counter electrode. The samples with a surface area of $1 \times 1\,cm^2$ were exposed to the two solutions at 37 °C, and polarization curves were obtained at a scanning rate of 1 mV/s. The corrosion potential (E_{corr}) and corrosion current density (I_{corr}) were calculated with the CHI760C software according to Tafel extrapolation.

2.3. Immersion tests

The Zr-N-implanted and un-implanted AZ91 Mg alloys were individually immersed in 10 ml Dulbecco's modified Eagle's medium (DMEM, Gibco) at a temperature of 37 °C for 28 days, and the immersion solution was exchanged every day. The concentration of Mg ions was measured at seven different time points of 1, 4, 7, 10, 14, 21 and 28 days by inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Perkin Elmer Optima 2000 DV). The changes in surface morphology and composition after immersion for 28 days were characterized by SEM and EDS. Furthermore, the pH values were monitored and the weight loss of the samples was measured after removing the corrosion products in chromic acid with an immersion time of 1, 2, 3 and 4 weeks. The average corrosion rates (CR) for the two samples were calculated as follows:

$$C = \Delta m/\rho A t,$$

Where C is the corrosion rate in mm $year^{-1}$, Δm is the weight loss, ρ is the density of the material, A is the initial implant surface area and t is the immersion time.

2.4. Antimicrobial tests

S. aureus (ATCC 43300) was used to evaluate the antibacterial activities of the two samples and pure Ti was used as the control. After the *S. aureus* suspension (1×10^6 CFUs/ml) was prepared, 3 ml of the prepared suspension was seeded on each of the samples in 12-well plates and incubated at 37 °C for 12 and 24 h. At each time point, the samples were gently rinsed with PBS for three times to remove the non-adhered bacteria, and then a LIVE/DEAD BacLight Viability Kit (Invitrogen) was used to stain the bacteria.

The antibacterial rates for adhered bacteria were calculated based on our previous reported method [22]. More specifically, after removing the non-adhered bacteria at each time point, the samples containing adhered bacteria were immersed in 3 ml of PBS. With the aid of ultrasonic vibration, the adhered bacteria were separated into the PBS solutions. The above solutions were serially diluted 10-fold, plated in triplicate onto sheep blood agar (SBA) and incubated at 37 °C for 24 h. Then, the number of CFUs on the SBA was counted. The antibacterial rates for adhered bacteria were calculated based on a formula: antibacterial rate for adherent bacteria (Ra) (%) = (B−A)/B × 100%, where A is the average number of viable bacteria on the un-implanted AZ91 Mg alloy or Zr-N-implanted AZ91 Mg alloy, and B is the average number of viable bacteria on pure Ti.

2.5. Direct cytocompatibility tests

Human MG63 osteoblasts were used in the *in vitro* cell culture experiments. MG63 cells were cultured in DMEM (Gibco) containing 10% FBS at 37 °C in a humidified atmosphere with 5% CO_2. The direct cytotoxicity was evaluated by the LIVE/DEAD assay. MG-63 cells (1×10^5 cells/well) were seeded on the two samples. After 1 and 3 days of incubation, the samples were stained with a LIVE/DEAD Kit (Invitrogen) for 30 min and observed under a fluorescence microscope.

The direct cytotoxicity was further evaluated by SEM observation. Briefly, MG-63 cells (1×10^5 cells/well) were carefully seeded on the two samples. After 1 and 3 days of incubation, samples were fixed by a 2.5% glutaraldehyde solution and then subjected to step dehydration with different concentrations of ethanol. Finally, the samples were sputtered coated with gold and observed with a field emission scanning electron microscope (FESEM, FEINOVA NanoSEM).

2.6. Indirect cell viability and differentiation tests

Indirect cell viability and differentiation were evaluated by an extract assay. The samples were immersed in DMEM containing 10% FBS with a surface area to extraction medium ratio of 1 ml cm^{-2} for preparing extracts in a humidified atmosphere with 5% CO_2 at 37 °C for a 72 h incubation. A MTT assay was used to assess cell viability. MG-63 cells (1×10^4 cells/well) were incubated in 96-