Biomaterials 53 (2015) 211–220

Contents lists available at ScienceDirect

Biomaterials

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

Enhanced antibacterial properties, biocompatibility, and corrosion resistance of degradable Mg-Nd-Zn-Zr alloy

Hui Qin [a, 1], Yaochao Zhao [a, 1], Zhiquan An [a, 1], Mengqi Cheng [a], Qi Wang [a], Tao Cheng [a], Qiaojie Wang [a], Jiaxing Wang [a], Yao Jiang [a, **], Xianlong Zhang [a, *], Guangyin Yuan [b, c, ***]

[a] Department of Orthopedics, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China
[b] National Engineering Research Center of Light Alloys Net Forming, Shanghai Jiao Tong University, Shanghai 200240, China
[c] State Key Laboratory of Metal Matrix Composite, Shanghai Jiao Tong University, Shanghai 200240, China

ARTICLE INFO

Article history:
Received 28 November 2014
Received in revised form
15 February 2015
Accepted 21 February 2015
Available online 13 March 2015

Keywords:
Magnesium alloy
Antibacterial properties
Biocompatibility
Corrosion
Biodegradable

ABSTRACT

Magnesium (Mg), a potential biodegradable material, has recently received increasing attention due to its unique antibacterial property. However, rapid corrosion in the physiological environment and potential toxicity limit clinical applications. In order to improve the corrosion resistance meanwhile not compromise the antibacterial activity, a novel Mg alloy, Mg–Nd–Zn–Zr (Hereafter, denoted as JDBM), is fabricated by alloying with neodymium (Nd), zinc (Zn), zirconium (Zr). pH value, Mg ion concentration, corrosion rate and electrochemical test show that the corrosion resistance of JDBM is enhanced. A systematic investigation of the *in vitro* and *in vivo* antibacterial capability of JDBM is performed. The results of microbiological counting, CLSM, SEM *in vitro*, and microbiological cultures, histopathology *in vivo* consistently show JDBM enhanced the antibacterial activity. In addition, the significantly improved cytocompatibility is observed from JDBM. The results suggest that JDBM effectively enhances the corrosion resistance, biocompatibility and antimicrobial properties of Mg by alloying with the proper amount of Zn, Zr and Nd.

1. Introduction

Nowadays, the most extensively used artificial implant materials in orthopedic fields are stainless steels, titanium and titanium alloys [1]. However, implant failures often occur due to the implant-associated bacterial infection as the implant surfaces are in favor of bacterial adhesion, colonization, and biofilm formation [2,3]. Their high elastic modulus leading to stress shielding is another factor [4,5]. In addition, on account of their non-degradability, a second surgical operation for implant removal is often required, which increases costs to the health care system and further morbidity to the patients [6,7]. Therefore, implant materials that possess antibacterial ability, low elastic modulus which is well matched with that of human bone tissue and biodegradability are thus urgently needed.

Among the various biodegradable materials, magnesium (Mg) metal has an elastic modulus similar to that of nature bone [5,8,9] and unique antibacterial properties [10–12]. However, the rapid degradation in the physiological environment hampers its clinical use. To reduce the degradation rate of Mg to match the bone union, alloying and surface coating have been used [13]. But, high corrosion resistance of Mg alloys results in a low alkaline pH, which will compromise the antibacterial activity because the antibacterial property of Mg is attributed to and proportional to the alkaline pH [10], which increases with Mg corrosion [1,5,9]. Thus, to compensate or even improve the antimicrobial activity of the Mg alloy, the elements having antimicrobial property should be chosen as alloy or coating compositions to enhance the corrosion resistance of Mg alloy.

Recently, a novel biodegradable Mg alloy, Mg–Nd–Zn–Zr (Hereafter, denoted as JDBM), has been developed in our laboratory by adding neodymium (Nd), zinc (Zn), zirconium (Zr) element in Mg, which exhibits proper mechanical properties and high corrosion resistance [14–17]. Zn, one of the essential elements in the human body [18,19], has been proved to have antibacterial effects

* Corresponding author. Tel.: +86 21 6436 9183; fax: +86 21 6470 1363.
** Corresponding author. Tel.: +86 21 6436 9184; fax: +86 21 6470 1363.
*** Corresponding author. National Engineering Research Center of Light Alloys Net Forming, Shanghai Jiao Tong University, Shanghai 200240, China. Tel.: +86 21 34203051; fax: +86 21 3420 2794.
E-mail addresses: jiangyao197826@163.com (Y. Jiang), zhangxianl197826@163.com (X. Zhang), gyyuan@sjtu.edu.cn (G. Yuan).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.biomaterials.2015.02.096
0142-9612/

[3,20]. Zr has also been reported to have antibacterial properties [1]. Therefore, in theory, as JDBM degradation in the solution, in addition to the increasing pH value, Zn and Zr on its surface and the release of them will produce antibacterial effect, which needs further explorations.

Accordingly, in this study, three different bacteria: *Escherichia coli* (*E. coli*) (Gram-negative) and *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*) (Gram-positive) were chosen to evaluated the antibacterial effect of JDBM *in vitro*. And *S. aureus* was chose to evaluate it *in vivo*. Moreover, their corrosion resistance and cytocompatibility are also determined systematically.

2. Materials and methods

2.1. Sample preparation and characterization

JDBM alloy was prepared by semi-continuous casting with high purity Mg (≥99.99%), Zn (≥99.995%), Mg−25%Nd and Mg−30%Zr, which was detailed in our previous report [17,21]. For *in vitro* experiments, JDBM, purity Mg and pure titanium (Ti) were fabricated into 10 mm square plates with a thickness of 1 mm. For *in vivo* experiments, the above materials were formed into 150 mm long, 1.2 mm in diameter short Kirschner wires. For later experiments, the samples were polished up to 2000 grid, then ultrasonically cleaned in acetone and alcohol for 10 min, respectively.

The surface morphology and elemental composition of the samples were examined by scanning electron microscopy (SEM; Philips XL30 FEG SEM) coupled with energy-dispersive X-ray spectroscopy (EDS; EDAX Si/Li detector).

2.2. JDBM and Mg degradation in medium

3 ml sterile Trypticase Soy Broth (TSB; BD Biosciences, Franklin Lakes, NJ) was added to each well in 12-well plate contained Mg or JDBM, and incubated at 37 °C for 1 and 3 days. At each time point, the pH of medium was measured using a precalibrated pH meter (Mettler Toledo). The Mg, Nd, Zn and Zr ion concentration in the solutions were determined using inductively coupled plasma-atomic emission spectroscopy (ICP-AES; Perkin Elmer Optima 2000 DV).

After removing from the medium, the JDBM and Mg samples were dried overnight at room temperature and weighted after removal of corrosion products using 200 g/l chromic acid. The loss of weight before and after immersion in TSB determines the sample degradation rate. The corrosion rate was calculated by the following equation: $C = (M_0 - M_x)/\rho At$ (M_0 is the initial mass and M_x is the mass at the respective time points, ρ is the density of materials, A is the initial sample surface area and t is the immersion time).

2.3. Electrochemical test

The electrochemical tests were carried out at room temperature in TSB on a CHI760c electrochemical workstation (CHI Instruments, Inc. Shanghai). The measurements were carried out on a three electrode electrochemical cell with a saturated calomel electrode (SCE) as the reference electrode, a graphite rod as the counter electrode, and the samples (Mg and JDBM) as the working electrode. The tests were performed at a scanning rate of 10 mV/s.

2.4. In vitro cytotoxicity evaluation

The human bone mesenchymal stem cells (hBMSCs) (Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China) were employed to assess the cytotoxicity. The cells were seeded at a density of 1×10^4/well and cultured with 3 ml Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Hyclone) for 24 h in 12-well plates containing samples, then stained with 500 μl of combination dye (Live/Dead cell viability assay, Invitrogen) for 10 min, and examined by confocal laser scanning microscopy (LSM 510 meta; Zeiss, Germany). The viable cells combined with calcein-AM were stained green whereas dead cells combined with ethidium homodimer-1 were stained red.

2.5. Bacteria preparation and characterization

E. coli (ATCC 25922), *S. aureus* (ATCC 43300) and *S. epidermidis* (ATCC 35984) was obtained in a freeze-dried form from the American Type Culture Collection (Rockefeller, MD). The strains were cultured for 24 h at 37 °C on sheep blood agar (SBA) plates. A single colony of each of the cultures was collected and incubated in 6 ml of sterile TSB at 37 °C with agitation at 220 rpm for 12 h. For *in vitro*, the inocula of the strains were prepared to 1×10^6 colony forming units (CFUs)/ml in TSB. For *in vivo*, *S. aureus* (ATCC 43300) was prepared at a concentration of 1×10^5 CFUs/ml.

2.6. Antibacterial effect determination in vitro

3 ml prepared bacteria suspension (1×10^6 CFUs/ml) was added to each well in 12-well plate contained samples, and incubated at 37 °C for 1 and 3 days. At the time points, the planktonic bacteria in the culture medium were analyzed by the spread plate method, the adherent bacteria on the samples' surface were determined by the spread plate method, confocal CLSM and SEM.

2.6.1. Antibacterial activity assay by the spread plate method

At the each time point, the culture medium was collected to determine the viable counts of planktonic bacteria. The samples were gently washed with PBS three times to remove loosely adherent bacteria from the samples surface, the adhered bacteria on each specimen were detached into 3 ml of PBS by ultrasonic vibration (150 W) for 5 min at 50 Hz, then the resulting bacterial suspension was used to count the viable bacteria adhered on the specimens. The above solutions were serially diluted 10-fold, plated in triplicate onto SBA and incubated at 37 °C for 24 h, and then the number of CFUs on the SBA was counted. The antibacterial rates for planktonic bacteria and adhered bacteria were calculated based on the formulas: (1) Antibacterial rate for planktonic bacteria (Rp) (%)=(B−A)/Bx100% and (2) Antibacterial rate for adherent bacteria (Ra) (%)=(D−C)/D × 100%. Here, A indicates the average number of viable bacteria in the culture medium inoculated with the specimen, B is the average number of viable bacteria in the culture medium inoculated without specimen (blank control), C is the average number of viable bacteria on the JDBM or Mg specimens, and D is the average number of viable bacteria on the Ti.

2.6.2. Antibacterial activity assay by CLSM

Following washed with PBS three times, the samples were transferred into a new 12-well plate and stained with 500 μl combination dye (LIVE/DEAD BacLight bacteria viability kits, Invitrogen) for 15 min, and observed with CLSM. The viable bacteria with intact cell membrane stain fluorescent green, whereas nonviable bacteria with damaged membranes stain fluorescent red.

2.6.3. Antibacterial activity assay by SEM

Following rinsed with PBS three times, the samples were fixed with 2.5% glutaraldehyde solution for 4 h, then dehydrated the graded ethanol series (30, 50, 70, 80, 90, and 95 v/v%) for 10 min each sequentially, with the final dehydration conducted in absolute ethanol (twice), freeze dried, coated with gold, and observed using SEM.

2.7. Antibacterial effect determination in vivo

2.7.1. Implant-related femur osteomyelitis model in rats

The *in vivo* experimental protocol was approved by the Animal Care and Experiment Committee of Sixth Peoples Hospital affiliated to Shanghai Jiao Tong University, School of Medicine. Thirty-two male Sprague Dawley rats 3 months in age and weighing an average of 215 g (140–228 g) were used. *S. aureus* (ATCC 43300) was chose to create osteomyelitis. After intraperitoneal injection of 4% chloral hydrate by weight-adopted (0.9 ml/100 g body wt) and sterilization with povidone iodine, the left knee was opened via a medial parapatellar incision, and the patellar was dislocated, then the femoral medullary cavity was opened at the middle of femoral trochlear and widened gradually with Kirschner wires up to a diameter of 1.5 mm. Subsequently, 50 μl of either PBS or PBS containing ATCC 43300 in a concentration of 1×10^5 CFUs/ml was injected into the medullary cavity, and a prepared JDBM or Mg or Ti Kirschner wire (diameter: 1.2 mm) was inserted (Table 1). After the opening sealed with bone wax, the surgical site was irrigated with sterile saline and the soft tissues and skin were closed. After surgery, rats were housed in ventilated rooms and allowed to eat and drink adlibitum. No antibiotic was administered.

2.7.2. Radiographic and micro-computed tomography scanning

At 3, 14, 28 days post-surgery, lateral radiographs of femur were obtained. The animals were euthanized at 28 days post-implantation. Operated femurs were aseptically harvested and scanned using high-resolution micro-computed tomography (microCT; Skyscan 1172, Skyscan, Belgium) at an image resolution of 18 mm (55 kVp and 181 m A radiation source with 0.5 mm aluminum filter). 2D, 3D high-resolution reconstruction images, the cortical bone mineral density and cortical porosity of femur of rats were acquired using the software provided by the manufacture.

2.7.3. Microbiological evaluation

After microCT examination, the Kirschner wires were aseptically explanted and rolled over SBA for semi-quantifying bacteria adhesion on the Kirschner wires. Then,

Table 1
Details of animal experiments.

Group	Number (n)	Implant	Inoculation
I	8	Ti wire	*S. aureus* 10^3 CFU/50 μl
II	8	Mg wire	*S. aureus* 10^3 CFU/50 μl
III	8	JDBM wire	*S. aureus* 10^3 CFU/50 μl
IV	8	Ti wire	PBS/50 μl