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In-vitro evaluation of Mg-4.0Zn-0.2Ca alloy for biomedical application

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Abstract: The in-vitro degradation and in-vitro cytotoxicity of the as-extruded Mg-4.0Zn-0.2Ca alloy were investigated. The in-vitro corrosion tests indicate that Zn and Ca elements can dramatically increase the corrosion potential of the as-extruded Mg-4.0Zn-0.2Ca alloy in the simulated body fluid and reduce the degradation rate. The cytotoxicity of the as-extruded Mg-4.0Zn-0.2Ca alloy examined by MTT tests on L929 cells suggest that the alloy has eligible toxicity for implant applications.
Key words: magnesium alloy; biodegradation; biocompatibility; cytotoxicity

1 Introduction

Nowadays, magnesium alloys have attracted considerable attention in biomaterials community due to their excellent mechanical performance and outstanding biocompatibilities [1−3]. Magnesium alloys, as a kind of low density metals, have density of 1.7−2.0 g/cm^3, which is much less than that of the biomedical Ti alloy (4.4−4.5 g/cm^3) and close to that of natural bones (1.8−2.1 g/cm^3) [4−5]. Meanwhile, the strength of magnesium alloys is much higher than those of biopolymers. Compared with Ti alloys (110−117 GPa), and stainless steel (189−205 GPa), the elastic modulus of magnesium alloys (40−45 GPa) is much closer to that of human bone (10−40 GPa), hence avoiding the stress shielding effect induced by serious mismatch in the elastic moduli between natural bones and implants [6−8]. However, it should be noticed that most of the reported biomedical magnesium alloys contain Al and/or rare earth (RE) elements, whereas Al is harmful to neurons, and also associated with dementia and Alzheimer's disease; severe hepatotoxicity was detected after the administration of RE elements [9−11]. Therefore, numerous attempts have been devoted to explore novel magnesium alloy containing nontoxic or low toxic element for biomedical applications [3, 12−14]. In biomedical application, Zn and Ca elements can be metabolized and thus are considered to be biocompatible [15]. Previous investigations demonstrated that Mg-Zn-Ca alloys exhibit excellent biocompatibility both in vitro and in vivo [16].

In the present work, 4.0% Zn and 0.2% Ca (mass fraction) were chosen as alloying elements, aiming at improving the corrosion resistance and mechanical properties of magnesium alloy. The microstructure, in-vitro corrodible property and cytotoxicity were analyzed to assess the feasibility of as-extruded Mg-4.0Zn-0.2Ca alloy to be used as bone implant materials.

2 Experimental

2.1 Materials preparation

The fabrication processes of as-extruded Mg-4.0Zn-0.2Ca alloy were previously described in detail [17]. The samples for in-vitro degradation tests with a gauge thickness of 4 mm and diameter of 15 mm were cut from the as-extruded rods. The samples for cytotoxicity tests with a gauge thickness of 4 mm and diameter of 5 mm were also cut from as-extruded rods.

2.2 Composition and microstructure characterization

The microstructure of the as-extruded Mg-4.0Zn-0.2Ca alloy was detected by optical microscopy. The samples were prepared by standard metallographic procedures and etched in a solution containing 3.5 g picric acid, 6.5 mL acetic acid, 20 mL water and 100 mL ethanol. Detailed study on the distribution of secondary phase particles in the alloys was carried out using transmission electron microscope (TEM, PHILIPS CM12) operating at 200 kV. TEM samples were obtained by

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twin-jet electro-polishing using a solution of 5.3 g LiCl, 11.16 g $Mg(ClO_4)_2$, 500 mL methanol and 100 mL 2-butoxy-ethanol at −30 °C and 100 V.

2.3 In-vitro degradation tests

The in-vitro degradation of the as-extruded Mg-4.0Zn-0.2Ca alloy was examined in simulated body fluid (SBF) by electro-chemical measurements including potentiodynamic polarization and electrochemical impedance spectroscopy (EIS) techniques. Table 1 lists the chemical composition of SBF. The samples for in-vitro degradation tests with a gauge thickness of 4 mm and diameter of 15 mm were cut from the as-extruded Mg-4.0Zn-0.2Ca alloy rods. A typical three-electrode system consisting of graphite rod as a counter electrode, saturated calomel electrode (SCE) as a reference electrode and specimen (1 cm^2 exposed area) as a working electrode were used. The samples were carefully polished with SiC paper up to 2 000 grid and then washed with distilled water. Potentiodynamic polarization experiments were carried out at a scan rate of 0.2 mV/s, and pure Mg (>99.99%) was also tested for comparison. The EIS experiments were performed at the open circuit potential with AC amplitude of 10 mV over the frequency range of 10 Hz to 100 MHz. Prior to the polarization and EIS tests, the samples were immersed in the SBF solution for 0.2 h to obtain the free corrosion potential. All the electrochemical tests were carried out at 37 °C, equal to the normal temperature of the human body.

Table 1 Chemical composition of SBF

Order	Reagent	Value	Purity/%
1	NaCl	8.035 g	99.5
2	$NaHCO_3$	0.355 g	99.5
3	KCl	0.225 g	99.5
4	$K_2HPO_4·4H_2O$	0.231 g	99.0
5	$MgCl_2·4H_2O$	0.311 g	98.0
6	1.0 mol/L HCl	39 mL	−
7	$CaCl_2$	0.292 g	95.0
8	Na_2SO_4	0.072	99.0
9	Tris	6.118	99.0
10	1.0 mol/L HCl	0−5 mL	−

2.4 Cytotoxicity assessments

L-929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C under relative humidity of 95% and CO_2 of 5%. The cytotoxicity tests were carried out by indirect contact between cells and samples. DMEM medium was used as negative group. L-929 cells were incubated in 96-well plates for 24 h before cell attachment. Each well was filled with 100 μL medium with 5×10^4 cell/mL. Then the medium was replaced by 100 μL extraction medium. After incubation in a humidified atmosphere for 1, 2, 4 and 7 d, the cell morphology was observed by optical microscopy (Nikon ELWD 0.3 inverted microscope). MTT (Sigma) was then dissolved in phosphate-buffered saline (PBS) at a concentration of 5 mg/mL. After then, 10 μL MTT solutions were added to each well and the samples were incubated at 37 °C for 4 h. Subsequently, 100 μL Formanzan solutions (10% SDS in 0.01 mol/L HCl) were added in each well and optical density optical density (d) measurements were conducted at 490 nm using a Wellscan MK3 spectrophotometer (Lab system). The relative growth rate (R) of cell was calculated by the following formula:

$$R=d_{test}/d_{negative}\times100\%$$

where d_{test} and $d_{negative}$ are optical density for sample and negative control, respectively.

2.5 Statistical analysis

A t-test was used to determine whether any significant differences existed between the mean values of the in-vitro cytotoxicity of the experimental groups. The statistical significance was defined as 0.05.

3 Results

3.1 Microstructure and mechanical properties of extruded Mg-4.0Zn-0.2Ca alloys

Figure 1 shows the typical optical microscopy image of the as-extruded Mg-4.0Zn-0.2Ca alloy. Compared with as-cast alloy [18], the microstructure of the extruded alloy was refined significantly and became more homogeneous, indicating that dynamic recry-stallization occurred during hot extrusion. Once the alloy was hot extruded, the mean grain size of the α-Mg phase

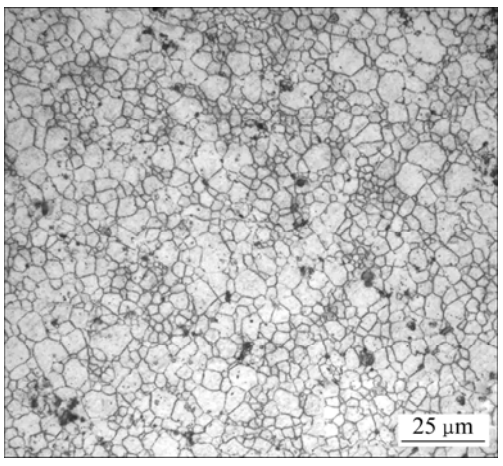

Fig. 1 Microstructure of as-extruded Mg-4.0Zn-0.2Ca alloy

was 6−12 μm and the secondary phase was hardly observed under optical microscopy due to the grain refinement, which contributes to the higher strength and elongation for as-extruded alloy than those for as-cast alloy [17].

In order to further investigate the microstructure, TEM observations were carried out on the as-extruded Mg-4.0Zn-0.2Ca alloy. Figure 2 shows the corresponding TEM bright field image. The bright field image was taken with the incident beam along the zone axis of the Mg matrix. It is evident that the density of dislocations in the as-extruded alloy was kept at a low level. Fine rectangular precipitates could be detected in the matrix, with distribution within the grains of the as-extruded Mg-4.0Zn-0.2Ca alloy, as seen in Fig. 2(a). The selected area electron diffraction (SAED) pattern obtained from the as-extruded Mg-4.0Zn-0.2Ca alloy confirmed that the secondary phases are $Ca_2Mg_6Zn_3$, as seen in Fig. 2(b), and agree well with the previous results [18−19].

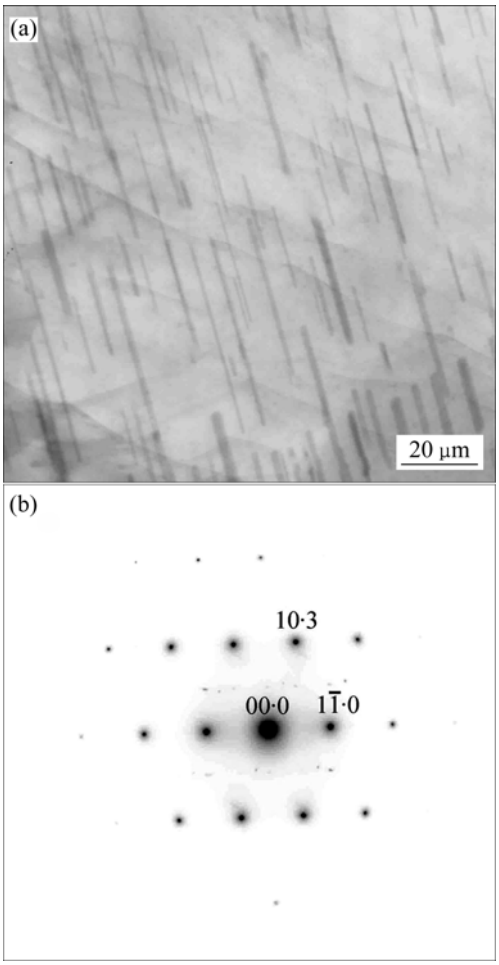

Fig. 2 Bright field TEM image taken along zone axis (a) and corresponding SAED pattern (b) of rectangular precipitates for as-extruded Mg-4.0Zn-0.2Ca alloy

3.2 In-vitro degradation tests in SBF

Figure 3 shows the potentiodynamic polarization of the as-extruded Mg-4.0Zn-0.2Ca alloy. For comparison, the potentiodynamic polarization of pure Mg is also shown in Fig. 3. The pure Mg possesses a corrosion current density of 3.715×10^{-4} A/cm^2 and an associated corrosion potential of −1.906 V. Compared with pure Mg, the corrosion potential (−1.677 V) of the as-extruded Mg-4.0Zn-0.2Ca alloy is much more positive. It is noted that the corrosion current density of the as-extruded Mg-4.0Zn-0.2Ca alloy is much close to that of the pure Mg. Therefore, as-extruded Mg-4.0Zn- 0.2Ca alloy exhibits better corrosion resistance than pure Mg. The enhanced corrosion resistance for as-extruded Mg-4.0Zn-0.2Ca alloy can be attributed to the addition of Zn. Zn is one of the most abundant nutritionally essential elements in the human body. Meanwhile, Zn can accelerate the metabolism of cells. It is reported that Zn can also significantly increase the charge transfer resistance of magnesium and thus dramatically reduce the corrosion rate [20], consistent with the present work.

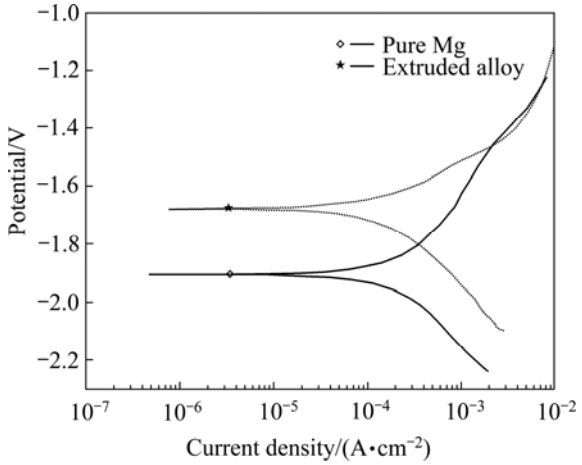

Fig. 3 Potentiodynamic polarization curves of extruded Mg-4.0Zn-0.2Ca alloy and pure Mg in SBF

Figure 4 presents the representative EIS curves of the as-extruded Mg-4.0Zn-0.2Ca alloy. EIS spectrum of the extruded Mg-4.0Zn-0.2Ca alloy can be characterized by three well defined loops: a capacitive loop in the high frequency region, a capacitive loop in the middle frequency region, and an inductive loop in the low frequency region. The capacitive loop in the high frequency region results from both the charge transfer and the film effect. The capacitive loop in the middle frequency is attributed to the mass transportation process in the solid phase and the inductive loop in the low frequency region is probably due to the corrosion process [21]. With the increase of the immersion time, the spectrum obtained from the sample changes. Both capacitive arcs are enlarged and the inductive loop becomes almost invisible, indicating an increasing passivation area. Therefore, the corrosion resistance of

the as-extruded Mg-4.0Zn-0.2Ca alloy can be significantly enhanced with the increase of the immersion times. This will greatly reduce the initial corrosion rate of Mg-4.0Zn-0.2Ca alloy, which is important in maintaining mechanical strength of the implant in the initial bony reunion period and improving the biocompatibility.

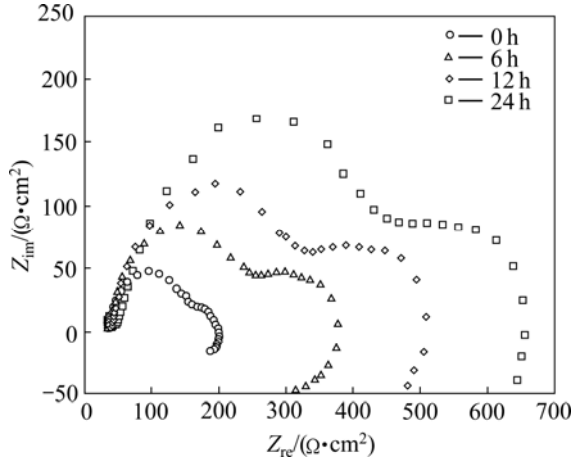

Fig. 4 Representative EIS curves of as-extruded Mg-4.0Zn-0.2Ca alloy

3.3 Cytotoxicity assessments

Figure 5 shows the morphologies of L-929 cells cultured in different extracts after 7 d of incubation. As seen, the morphologies of cells in two different extracts look normal and healthy, similar to those of the negative control. Figure 6 illustrates the RGR values of L-929 cells after 2, 4 and 7 d of incubation. No significant difference can be found between the RGR of cells in the extracts and those in the negative control. The results of the indirect cytotoxicity tests show that the present Mg-4.0Zn-0.2Ca alloy sample exhibits similar cytotoxicity Grade 0−1 (according to ISO 10993—5: 1999 [22]) as the investigated Mg-Ca [23], and Mg-Zn [24] alloys, which proves their biosafety for further biomedical applications.

4 Discussion

4.1 Mechanical properties and microstructure

Compared with degradable polymeric materials, the as-extruded Mg-4.0Zn-0.2Ca alloy had improved mechanical properties [17]. The elastic modulus of the magnesium alloy studied (45 GPa) was also closer to that of human femur bone than those of 316L stainless steel and titanium alloys. The enhanced mechanical properties of the magnesium alloy can be attributed to two factors: alloying and hot working. On one hand, Zn is found to be efficient in strengthening effectiveness as an alloying element in magnesium alloy. Research by YUAN

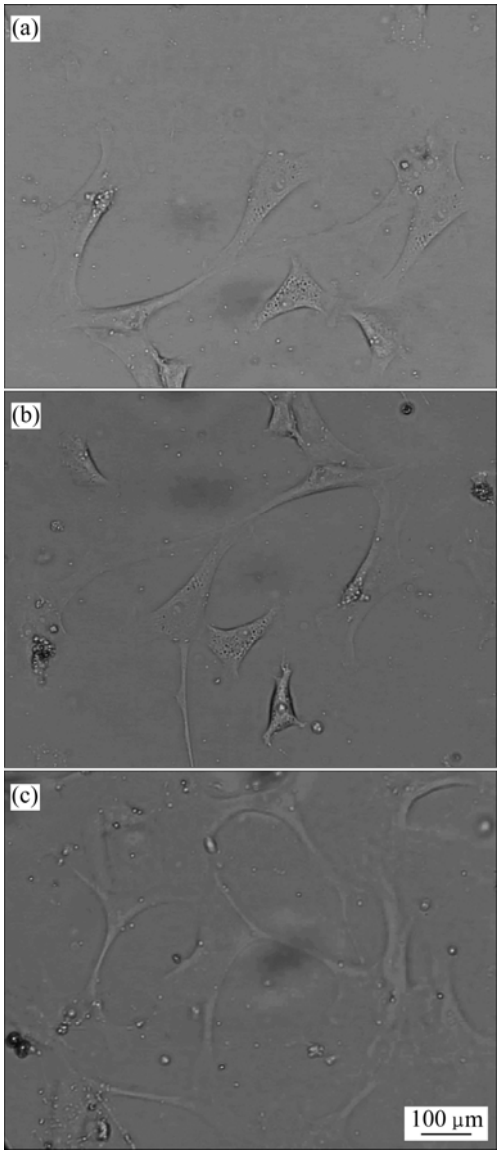

Fig. 5 Morphologies of L-929 cells cultured for 7 d in different extraction media: (a) Negative control; (b) 100% extraction; (c) 50% extraction

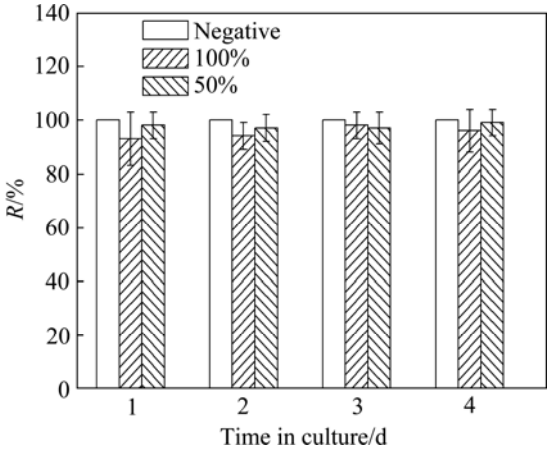

Fig. 6 Cell viability cultured in 100% extraction medium for 1, 2, 4 and 7 d

et al [25] on Mg-Zn-Si alloys revealed that the strength increased with the increase of Zn content. Ca is a healthy element to the human body. Ca was also reported to be an effective grain refiner for magnesium alloy during the solidification and hot working process. At the same time, the proper addition of Ca could lead to the precipitation of desolventizing phase, $Ca_2Mg_6Zn_3$, resulting in the enhanced strength and toughness of the alloy studied. On the other hand, the grain size of as-extruded samples is much finer than that of the as-cast ones, indicating that hot working (hot extrusion in this study) has refined the microstructure. The reduced grain size after extrusion would lead to inhibited crack initiation, inhibited dislocation motion and an increase in the number of barriers to early crack propagation, improving the mechanical properties of the as-extruded alloy sample including yield strength, tensile strength and elongation.

4.2 Degradation process

The as-extruded Mg-4.0Zn-0.2Ca alloy is degraded quickly, accompanied by the rapid formation of an insoluble protective corrosion layer, during the early stage of immersion in SBF. The degradation process of magnesium alloy can be roughly summarized as follows. When the magnesium alloy studied is immersed in SBF or implanted in an animal, it will react with body fluid on the surface and get dissolved in the surrounding body fluid. When the immersion time is prolonged, Mg^{2+}, Zn^{2+} and Ca^{2+} ions can be released from the alloy into the solution. As a result, the local pH of the surrounding fluid near the surface of the Mg alloy can exceed 10 [26]. Consequently, a magnesium containing calcium phosphate would precipitate from the body fluid on the surface of the magnesium alloy implant.

Previous studies showed that this corrosion layer could retard the degradation rate. Therefore, it is proposed that the Mg^{2+}, Zn^{2+} and Ca^{2+} released during degradation should be safe. It can be concluded that degradation of the Mg-4.0Zn-0.2Ca alloy should be harmless and suitable in biocompatibility.

4.3 Cytotoxicity

In order to develop the biomedical magnesium alloy, it is required to assess the cell toxicity of alloying elements. In vitro cell experiment, MTT, as a cheap and convenient measurement, is widely accepted to determine the cytotoxicity. In the present work, the in-vitro cytotoxicity of the as-extruded Mg-4.0Zn-0.2Ca alloy is found to be in Grade 0−1, indicating that the alloy should be biosafe. To further assess the biocompatibility of Mg-4.0Zn-0.2Ca alloy, the in-vivo test is needed.

5 Conclusions

1) The as-extruded Mg-4.0Zn-0.2Ca alloy exhibits a fine microstructure with a mean grain size of 6−12 μm and excellent mechanical performance.

2) Zn and Ca element can effectively increase the corrosion potential of the as-extruded Mg-4.0Zn-0.2Ca alloy. The in-vitro degradation rate of the as-extruded Mg-4.0Zn-0.2Ca alloy in SBF is similar to that of high-purity Mg.

3) In-vitro cytotoxicity experiment indicates that the as-extruded Mg-4.0Zn-0.2Ca alloy has excellent biocompatibility.

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Mg-4.0Zn-0.2Ca 合金生物医用的体外评价

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摘 要：研究了挤压态 Mg-4.0Zn-0.2Ca 合金的体外降解和细胞毒性，通过 MTT 法用 L929 细胞检测挤压态 Mg-4.0Zn-0.2Ca 合金的细胞毒性。研究结果表明：Zn 和 Ca 元素能够显著提高挤压态 Mg-4.0Zn-0.2Ca 合金在模拟体液中的抗腐蚀能力，减慢降解率；挤压态 Mg-4.0Zn-0.2Ca 合金与细胞的相容性良好，可用于骨种植。

关键词：镁合金；生物降解；生物相容性；细胞毒性

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