Microstructure, Mechanical Properties, In Vitro Degradation and Cytotoxicity of Mg-4Zn-3HA Alloy for Biodegradable Implant Materials

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Abstract: In this study, a kind of Mg-4Zn-3HA alloy was investigated as a biodegradable material. Magnesium alloys can be totally degraded in the body, and their corrosion products are not deleterious to the surrounding tissues. We conducted a comprehensive investigation of the microstructure, mechanical properties, in vitro degradation assessments, and in vitro cytotoxicity evaluations of Mg-4Zn-3HA alloy. Our observations of the microstructure show that the Mg-4Zn-3HA alloy is porous, and its structural characteristic resembling nature bone make this alloy suitable for use in implants. The results of in vitro degradation indicate that the corrosion resistance is improved with the extension of immersion time, and XRD and EDS analysis proves that the corrosion products on the surface of Mg-4Zn-3HA alloy contain hydroxyapatite (HA), Mg (OH)2, and other magnesium-substituted calcium phosphate, which could reduce the degradation rate. The degradation process of magnesium alloy and the formation of corrosion layer are also discussed in this work. The corrosion products contain HA and Mg (OH)2, which can promote good biocompatibility. No significant cytotoxicity to MC3T3-E1 cells is detected in the extraction medium, and MC3T3-E1 cells are able to adhere and spread on the corrosion layer of the Mg-4Zn-3HA alloy. Mg-4Zn-3HA alloys have the potential to be used for biomedical applications.

Keywords: Biocompatibility, Biodegradable, Biomaterial, Magnesium alloy

Original

Introduction

As a biodegradable implant material, magnesium and its alloy has attracted much people’s attention. In clinical application, the characteristics of the biodegradable can be from secondary surgery in patients with pain. Magnesium is one of the essential elements in the human body and involved in many energy metabolic reactions, nucleic acid and protein synthesis and enzyme activity. Approximately half of the total physiological magnesium stores in bone tissue, in addition magnesium has stimulatory effects on the growth of new bone tissue. The density (1.74-2 g/cm³), Elastic modulus (41-45 GPa), Compressive yield strength (65-100 MPa) of magnesium are closer to the bone tissue (1.8–2.1 g/cm³, 3–20 GPa, 130-180 MPa) than that of the conventional implant. Ti alloy is 4.4-4.5 g/cm³, 110-117GPa, 758-1117MPa. Co-Cr alloy is 8.3-9.2 g/cm³, 230GPa, 450-1000MPa. Stainless steel is 7.9-8.1 g/cm³, 189-205GPa, 170-310MPa. So magnesium alloys as implant materials can effectively reduce stress shelter effect because of the dissimilarity in elastic modulus between the implant and nature bone. But unfortunately pure magnesium corrodes too fast, especially in a large number of the presence of chloride ions. The degradation of magnesium alloys will always lead to hydrogen evolution and alcalization of solution. Now, kinds of methods, such as alloying, surface treatments and high purity alloys, have been used in order to improve the corrosion resistance of magnesium. Reported that many magnesium alloys contain HA, Zn, Ca, Mn, Al, Re, Y, Li and Zr. It is obvious that Al is harmful to neuron and Re will lead to hepatotoxicity. Zn and Ca are important elements in the body. Ca is also reported to be an effective grain refiner for magnesium alloy. HA has similar chemical and crystallographic structures to bone and Zn participates in the syntheses of enzymes. So they have excellent biocompatibility. During the corrosion process, HA would adsorb Ca²⁺ and PO₄³⁻ ions efficiently and induce the deposition of Ca-P compounds on the surface of composites. In the design of biodegradable materials, elements with potential toxicity should be avoided if possible. Therefore, in this work, Zn and HA were selected as the alloying elements for the biodegradable implant materials. Besides the alloying, hot extrusion was applied to the fabrication of magnesium alloys.
Material preparation

The metal matrix composites were produced by mixing 4 wt % Zn (99.9 % purity), 3 wt % HA and other balance powders are Mg (99.9 % purity). Powder mixtures with designed composition were mixed by planetary ball milling for 8 h, and cooled pressed into cylindrical performs at pressures of 400 MPa. The diameter is 52 mm and holding time is 2 min. Cold isostatic pressing was used to composite at pressures of 400MPa and holding time is 200 sec, which could make the material denser. Then, these composites were placed into a small sealed Al tube, and heated to 250 °C, 300 °C and 350 °C separately for 20 min. Finally, the heat treated alloy sample was hot extruded with an extruded ratio of 16:1.

Disk samples for in vitro degradation and cell biology experiment, having a diameter of 13 mm and a height of 2.0 mm and 1.0 mm, were machined from the extruded composites. All samples were polished with SiC paper 800 to 2000 grit, then, followed by ultrasonic cleaning in acetone, ethanol and distilled water, and then dried in open air. For cell biology experiments, every aspect of the samples was sterilized by ultraviolet radiation for at least 30 min.

Microstructure and mechanical properties

Microstructure was characterized using scanning electron microscopy (SEM). The compression samples had diameter of 10 mm and a height of 15 mm. A crosshead speed of 0.5 mm min⁻¹ was used. Three specimens for each alloy were tested.

Immersion testing

In order to evaluate the in vitro degradation properties, immersion test was performed in simulated body fluid (SBF), containing NaCl 8.035 g/L, NaHCO₃ 0.355 g/L, KCl 0.255 g/L, K₂HPO₄ •3H₂O 0.231 g/L, MgCl₂ •6H₂O 0.311 g/L, 1.0M-HCl 39 ml, CaCl₂ 0.292 g/l, Na₂SO₄ 0.072 g/l, Tris 6.118 g/l, 1.0M-HCl 0-5 ml[16]. The pH of SBF was adjusted with HCl and Tris solution to 7.4 and the temperature was maintained at 37 ±0.5 °C. According to ASTM G31-72[17], the radio of surface area to solution volume is 1 cm²:20 ml. The immersion test lasted for 28d, and the SBF was renewed every 48 h in order to keep a relatively stable pH value.

Samples were removed after 2, 4, 6, 8, 10, 14 and 28 days from the solution, rinsed with distilled water and dried at room temperature. Then the surface morphology after immersion was observed using scanning electron microscopy (SEM). XRD was used to determine the corrosion products. Lastly, the samples were cleaned in 200 g/l chromic acid solution with 10 g/L silver nitrate for 5 min to remove surface degradation products. The corrosion rates were determined by weight loss. The pH value of the solution was also recorded during the immersion test. Three specimens for each alloy were tested. The corrosion rates were calculated by the weight loss according to the following equation[14]: CR= Δ W/ (A×T) where Δ W is the weight loss (g), A is the sample area exposed to solution (cm²), T is the exposed time (d).

Biocompatibility

Cell toxicity test

Cell Counting Kit-8 (CCK-8) test was used to test the cell toxicity and mouse osteoblast-like cells MC3T3-E1 were used. The MC3T3-E1 were cultured in modified Eagle’s medium alpha (α-MEM, Hyclone), supplemented with 10% fetal bovine serum (FBS, Gibco), 100U ml⁻¹ penicillin and 100μg ml⁻¹ streptomycin at 37 °C in a humidified incubator 5% CO₂. According to ISO 10993-5[19], extracts were prepared using α-MEM contained 10% FBS, 100U ml⁻¹ penicillin and 100μg ml⁻¹ streptomycin at 37 °C in a humidified incubator 5% CO₂ for 72h, the specimen with the surface area of extraction medium ratio is 3 ml/cm². The supernatant fluid was withdrawn to prepare the extraction medium, and then stored at 4 °C before the cytotoxicity test. The extracts were sterile filtered through 0.2 μm syringe filters before being added to cells. Firstly, the MC3T3-E1 cells were seeded in 96-well culture plates at 1×10⁴ cells/ml medium in each well and incubated for 24 h to allow cell attachment. The α-MEM medium acted as a negative control while α-MEM medium containing 0.64% phenol as a positive control. After 24 h incubation, medium was replaced with 100 μl of extraction medium incubated for 1, 2 and 3 days. At the end of each incubation time, 10 μl CCK-8 solution was added to each well. The samples were incubated with CCK-8 solution for 2 h at 37°C. The absorbance of the samples was measured by microplate reader at 450 nm with a reference wavelength of 600 nm. The results were directly shown by the optical density (OD). All experiments were carried out in triplicate. The cell viabilities were expressed as “relative growth rate” (RGR) determined by RGR (%) = (OD sample/OD negative control) ×100%.

Cell morphology

The MC3T3-E1 cells were cultured on each specimen for up to 6 h at a seeding density of 1×10⁵ cells/ml. Following incubation, the samples were washed with phosphate buffer solution (PBS), fixed in 2.5% glutaraldehyde for a night at 4 °C, dehydrated with graded ethanol (10, 30, 50, 70, 90 and 100%) every 30 min, dried in open air and gold sputter coated in the end. Then they were examined using SEM.

Statistical analysis

Statistical differences were compared by one-way ANOVA analysis and defined as p < 0.05.

Results

Microstructure and Mechanical properties

The microstructure of the Mg-4Zn-3HA composites at 250 °C, 300 °C and 350 °C are shown in Fig.1. The powder metallurgy method is one of the best methods for magnesium matrix composite preparation, and the HA and Zn particles were successfully retained by the Mg matrix. Fig.1 shows that the density of magnesium alloy by powder metallurgy method is less than that of cast alloys, the magnesium alloy is usually loosened and porous. To contrast, the distribution of HA and Zn particles are uniform and dispersed in
Fig. 1 (b). Generally, many conglomerations particles are undesirable for the alloys because heterogeneous distribution of the reinforcement material leads to spatial heterogeneity of its properties.

The compressive yield strength, elastic modulus and compressive strength of the Mg-4Zn-3HA alloy at different temperature are shown in Table.1. Compared with Ti alloy, three kinds of magnesium alloy in the compressive strength and elastic modulus are very close to natural bone. With the increase of extrusion temperature, the compressive strength increased slightly. But, for the compressive yield strength and elastic modulus, the extruded magnesium alloy at 300 °C has better performance.

Immersion testing

Fig.2 illustrates the pH variation of the immersion tests within 28 days. It can be seen that the pH value rose rapidly during the first 4 days. About at the sixth day, the pH values began to fall, and about at the eighteenth day, the pH values stabilized. At the end of the immersion tests, the pH values reached about 8.3.

The relationship between the corrosion rates and the immersion times is shown in Fig. 3. The curves reveal that the corrosion rates of all specimens decreased with the increasing of immersion time ultimately. The extruded magnesium alloy at 300 °C had the lowest corrosion rate and the extruded magnesium alloy at 250 °C had the highest corrosion rate. The corrosion rate of the extruded magnesium alloy at 350 °C was slightly higher than that of the extruded magnesium alloy at 300 °C. All groups of corrosion rate basically stable in about ten days.

To identify the phase of material degraded from specimens, we chosen the degradation products of the extruded magnesium alloy at 300 °C after 28 days immersion, XRD analysis (Fig. 4) was carried out and the results showed strong Mg(OH)₂ peaks and a number of additional weak peaks, such as HA and Mg-Zn compound.

The surface morphology of three specimens soaked in SBF solution for 28 days were shown in Fig. 5 (a-c) and Fig. 5 (d-f) showed the EDS results for the surface corrosion products on the Mg-4Zn-3HA alloy. A number of cracks and precipitates were observed on the surface of the specimens after 28 days immersion. The EDS results revealed that the surface corrosion products were rich in Mg, Ca, P and O and the degradation specimen surface was rough and there were a lot of pores.

Biocompatibility

Cell toxicity test

To further evaluate the biocompatibility of the Mg-4Zn-3HA alloys, specimens of different complex temperature were selected through examining the cytotoxicity of the different extracts to MC3T3-E1 cells. Fig.6 shows the viability of MC3T3-E1 cells expressed as a percent in the negative control after 1, 2 and 3 days incubation respectively. It is revealed that compared with the negative control and positive control, the different extracts do not show significant cytotoxicity towards the cells. The cells can survive and proliferate in all three of these extracts in a cell culture system. According to the ISO 10993-5, the cytotoxicity of these extracts was grade 0-1. Thus, the three kinds of Mg-4Zn-3HA alloys are innocuous. At three time points, the data all of the magnesium alloys at 300 °C were very significant difference compared with other groups.

Cell morphology

Fig. 7 is SEM image showing the earlier adhesion of the MC3T3-E1 cells on the surface of three kinds of alloys for 6h. Some precipitates and cracked corrosion layer on the alloys can be seen, on which the cells have been spread. There is no obvious cell morphological difference between the three kinds of samples. Furthermore the cells spread out along the surface and the filopodia reaches the surrounding cells.

Discussion

Mechanical properties

From Fig. 1, we can see that the Mg-4Zn-3HA alloy is not tight. It is like nature bone make alloy and more suitable to implant materials. Zn is a common alloying element for magnesium and has a solid solution strengthening effect on magnesium alloys. Hot working (hot extrusion in this paper) can also refine the grain and improve the strength and hardness according to the Hall-Petch relationship. The elastic modulus of the magnesium alloy in this paper is also closer to that of nature bone than that of stainless steel and titanium alloys.

The degradation process

The magnesium alloy in SBF will dissolve according to the following reactions:

Anodic reaction: \[ \text{Mg} \rightarrow \text{Mg}^{2+} + 2e \] (1)

Cathodic reaction: \[ 2\text{H}_2\text{O} + 2e \rightarrow \text{H}_2 + 2\text{OH}^- \] (2)
The existence of chloride ions (Cl\(^-\)) transforms Mg(OH)\(_2\) into soluble MgCl\(_2\), and then MgCl\(_2\) will dissolve to Mg\(^{2+}\) and 2Cl\(^-\), resulting in extra OH\(^-\) in the solution, eventually the pH value of the solution will rise. And it is possible for phosphate ions (such as H\(_2\)PO\(_4\)\(^-\), HPO\(_4\)\(^{2-}\), or PO\(_4\)\(^{3-}\)) and Ca\(^{2+}\) in the SBF to react with OH\(^-\) to form HA (Ca\(_{10}\)(PO\(_4\))\(_6\)(OH)\(_2\)). This phenomenon explains the existence of HA by XRD in this study (Fig. 4). Furthermore, Mg\(^{2+}\) is one of the main ionic substitutions in biological apatites and then the magnesium-substituted calcium phosphate is also possible to precipitate on the surface of alloy\(^{20}\). At last, corrosion products containing HA, Mg(OH)\(_2\) and other magnesium-substituted calcium phosphate and form a layer covering the surface of the alloy that can reduce the corrosion rate. This can explain why the corrosion rate (Fig. 3) and pH value (Fig. 2) back to slow down. Previous research\(^{21}\) has shown that such magnesium–calcium contained phosphates can promote osteoinductivity, osteoconductivity and are beneficial for biocompatibility of magnesium alloys. In addition, HA particles are insulated, and the solution could not be corroded during the immersion, so HA particles could improve the corrosion resistance of magnesium alloy. Meanwhile, HA particles could promote Ca and P ions deposit on the surface of magnesium alloy. This result was shown in Fig. 5 (d-f).

Mg\(^{2+}\) +2OH\(^-\) → Mg(OH)\(_2\) (3)

Figure 5. SEM/EDS of the Mg-4Zn-3HA alloy specimens soaked 28 days in SBF: (a) SEM micrograph of the Mg-4Zn-3HA alloy at 250 °C; (b) SEM micrograph of the Mg-4Zn-3HA alloy at 300 °C; (c) SEM micrograph of the Mg-4Zn-3HA alloy at 350 °C; (d) EDS spectrum of the Mg-4Zn-3HA alloy at 250 °C; (e) EDS spectrum of the Mg-4Zn-3HA alloy at 300 °C; (f) EDS spectrum of the Mg-4Zn-3HA alloy at 350 °C.

Figure 6. RGRs of the MC3T3-E1 cells expressed as a percentage of the cells in the control after 1, 2 and 3 days incubation.

Figure 7. SEM images of Mg-4Zn-3HA alloys cultured with MC3T3-E1 cells for 6h: (a) the Mg-4Zn-3HA alloys at 250 °C; (b) the Mg-4Zn-3HA alloys at 300 °C; (c) the Mg-4Zn-3HA alloys at 350 °C.
Biocompatibility

When magnesium alloy is implanted in vivo or immersed in the simulated body fluid in vitro, magnesium alloy will react with body fluid. As a result, pH value of the surrounding fluid will increase and metal ion will be released from the alloy. Therefore, it is necessary to assess the cell toxicity of alloying elements for development of biodegradable magnesium alloy. In vitro cell experiment, for example CCK-8, a cheap and convenient measurement, is widely accepted to determine the cytotoxicity. The cytotoxicity of Mg-4Zn-3HA was found to be Grade 0–1, indicating that the magnesium alloy is safe as an implantable material. So, even though the Mg-4Zn-3HA alloy degraded rapidly, it also had good compatibility. The better cell proliferation could be attributed to the appropriate magnesium ions released into the culture medium by the degradation of the Mg-4Zn-3HA alloy\(^1\). A protective corrosion layer appeared on the surface is rough and benefits the adhesion of cells, as shown in Fig. 7. Cell morphology and adhesion are very good.

In conclusions, in this paper the Mg-4Zn-3HA alloy was investigated as a biomedical degradable material in vitro immersion test and cell toxicity test. The following conclusions can be drawn.

1. The Mg-4Zn-3HA alloy has similar mechanical properties with nature bone. In the process of degradation, the rough surface porous material is conducive to pre-osteoblastic MC3T3-E1 cell adhesion.

2. With the increasing of immersion time, the pH value rises, ions release, surface is stripped, degradation products deposit, but MC3T3-E1 cell is not affected too much, the proliferation and adhesion ability perform well.

3. The mechanical properties, corrosion resistance and biocompatibility of magnesium alloys extruded at 300°C were superior to that of the magnesium alloys extruded at 250°C and 350°C.

In summary, the Mg-4Zn-3HA alloy has the potential to biodegradable implant materials. However, in order to improve the degradation rate, there’s a lot of work to do.

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