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In vitro Degradation and Biocompatibility of Ca-P Coated Magnesium Alloy

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Abstract Calcium-phosphate compounds(Ca-P) coating was prepared on an Mg-Al alloy(AZ60). Biodegradation of Ca-P coated magnesium alloy was evaluated in simulated body fluid(SBF) by examining the changes in magnesium ion concentration and pH value, which indicated that the Ca-P coating on magnesium alloy strongly affected the corrosion of magnesium alloy. Osteoblast MC3T3-E1 cells were utilized to investigate the cellular cytocompatibility. The cytocompatibility was measured by carrying out a series of tests, such as cholecystokinin-octapeptide(CCK-8) test, alkaline phosphatase activity(ALP) test, cellular morphology of hematoxylin-eosin(HE) staining and the induction of apoptosis. It was found that the cell function showed better in the Ca-P coated Mg-alloy extract than in the uncoated magnesium alloy extract. In summary, the results indicate that the Ca-P coating can improve the corrosion resistance of magnesium alloy and elevate cellular proliferation and differentiation of osteoblast MC3T3-E1 cells.

Keywords Ca-P coating; Magnesium alloy; *In vitro*

1 Introduction

More and more attentions have been paid to degradable magnesium-based orthopaedic and dental implants in recent years[1—3]. But a significant problem of magnesium materials is the rapid degradation[4]. Surface modification of magnesium alloys has been reported as a valid approach to reduce the degradation[5]. It is also well known that calcium-phosphate compounds(Ca-P), such as the hydroxyapatite(HA), are the major component in human bone and have drawn a great interest in the repair of bone defects. For example, it has been reported that the Ca-P coating effectively improves the corrosion resistance of magnesium alloy and has a good biocompatibility[6—8]. However, in previous investigations, most works on cytotoxicity simply focused the efforts on cell number or cell density instead of nucleus change. The cytotoxicity can be better understood through nucleus change such as nucleus condensation or swelling. Nucleus morphology changes and cell apoptosis caused by nucleus degeneration were observed in this work, and these results provide a way to reflect cytotoxicity much better. In the present study, we prepared a Ca-P coated Mg alloy by two-step chemical processes without pre-treatment. The biodegradation and cytocompatibility on surface modified Mg alloy were investigated in terms of cell morphology, proliferation, differentiation and the induction of apoptosis.

2 Materials and Methods

2.1 Sample Preparation

The die-cast magnesium alloy, which was supplied from Research Institute of Composite Materials of Tianjin University (China), was used as the substrate material. The elements composing the magnesium alloy were Mg(91.2%—94.1%, mass fraction), Al(5.8%—7.2%), Zn(<1.0%), Mn(0.15%—0.5%), Si(≤0.1%), Cu(≤0.05%), Ni(≤0.005%), Fe(≤0.005%). The sample size was 10 mm×10 mm×5 mm and the surface was polished with up to 2000 grit SiC paper followed by sonication cleanout in acetone. The cleaned samples were immersed into a phosphate solution at (37±2) °C for 30 min and the pH was adjusted by adding NaOH to 2.6—2.8, then dried in an attemperator at 60 °C[9]. All the samples were firstly cleaned by ultrasonication in ethanol for 20 min, then sterilized with 29 kGy of gamma radiation prior to experiments[10,11].

2.2 Degradation Test

Ca-P coated samples and uncoated samples were immersed into the simulated body fluid(SBF)[12] according to ASTM-G31-72. The pH value was adjusted to 7.4 and the temperature was kept at 37 °C. The ratio of surface area to solution volume was 1 cm^2/20 mL. The samples were removed from SBF after 1, 3, 7, 14 and 21 d of immersion. The pH value and the magnesium ions concentration of the SBF were monitored by ion detection instrument(Thermo, USA).

2.3 Cytotoxicity Assay

Osteoblast MC3T3-E1 cells were cultured in Dulbecco modified Eagle's medium(DMEM, Gibco USA), containing penicillin/streptomycin(100 U/100 U), with 10% fetal bovine serum(FBS) at 37 °C, 5% CO_2 and 95% humidity. DMEM with

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10% FBS was prepared as the extract medium. Ca-P coated and uncoated Mg alloys were incubated in the extract medium for (24±2) h at (37±1) °C. The ratio of sample surface area to extract medium was 1 cm^2/mL.

Osteoblast MC3T3-E1 cells were incubated in 96-well plates(Corning, NY, USA) at a cell density of 2500 cells/well and allowed to settle for 24 h. Two different extract mediums were added to the cells by replacement of the initial cell culture medium. The group using DMEM medium was negative control. After incubation in a humidified atmosphere for 1, 3, 5 and 7 d, the viability and metabolic activity of the cells were checked by CCK-8 according to the manufacturer's instructions. The orange formazan was quantified by an enzyme linked immunosorbent assay(ELISA) plate reader at a wavelength of 450 nm. Osteoblast MC3T3-E1 cells were seeded in 24-well plates at 1×10^4 cells/well, the wells of which were respectively co- vered with a cover glass. The extract medium was added to the cells by replacement of the initial cell culture medium after seeding for 24 h. The group using DMEM medium was negative control. After the incubation periods of 1, 3, 5 and 7 d, the cover glasses were removed, cold ethanol fixed, hematoxylin-eosin(HE) stained, and observed with an inverted microscope(Olympus, Japan). The alkaline phosphatase(ALP) activity was evaluated on the transformation of *p*-nitrophenyl phosphate(pNPP, Sigma US)[13]. Osteoblast MC3T3-E1 cells were seeded in 24-well plates at 5×10^4 cells/well. The medium was replaced with extract media after culture for 24 h. The group using DMEM medium was negative control. After seeding for 5 d, the differentiation behavior of cells was measured by monitoring ALP activity. The ALP tests were carried out at 410 nm and the optical density(OD) values were compared with the value of a standard series.

Apoptosis tests were carried out in which the two samples were put directly onto the cells. Osteoblast MC3T3-E1 cells were seeded in 6-well plates at a cell density of 1×10^5 cells/well. The group without the samples served as negative control. After seeding for 5 d, samples removed and cells were stained with the apoptosis detection kit according to the manufacturer's instructions(Jiancheng Co., Nanjing, China) before analysis by flow cytometry. Then the samples were analyzed by an FACSCalibur(BD, Franklin Lakes, NJ) flow cytometry with at least 1.0×10^5 events recorded for each condition.

2.4 Statistical Analysis

All the results were expressed as mean values±standard deviation for each group of samples. After the assessment of significant differences by one-way variance analysis(ANOVA), differences among groups were established with *t*-test analysis. Statistical significance was defined as $P<0.05$.

3 Results

3.1 Microstructure and Phase Composition

The surface morphology and energy dispersive spectrometer(EDS) result of the coating on magnesium alloy are shown in Fig.1. A flake-like microstructure with flakes of 10—15 μm in length appears on the surface of the coating. The layer with an average thickness of about 6 μm is mainly composed of dicalcium phosphate dihydrate($CaHPO_4\cdot2H_2O$, DCPD) and a small quantity of magnesium phosphate $Mg_3(PO_4)_2$.

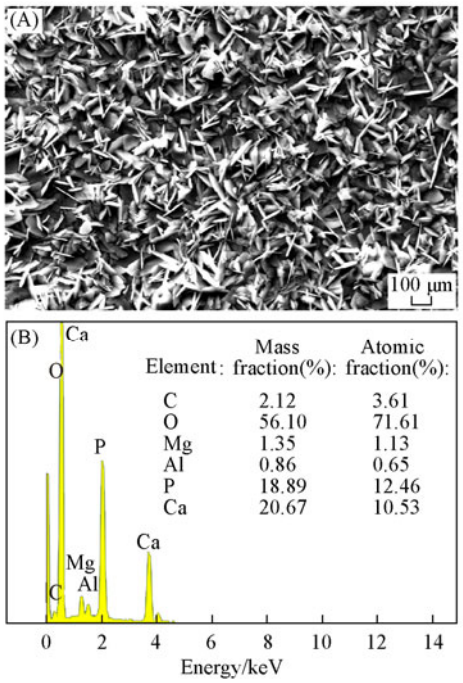

Fig.1 Surface morphology(A) and EDS result(B) of phosphate coating

3.2 Corrosion Behavior

The corrosion degree of the coated and uncoated samples was evaluated based on the changes in magnesium ion concentration and pH value in SBF. Fig.2(A) shows the change in magnesium ion concentration. Uncoated magnesium alloy in

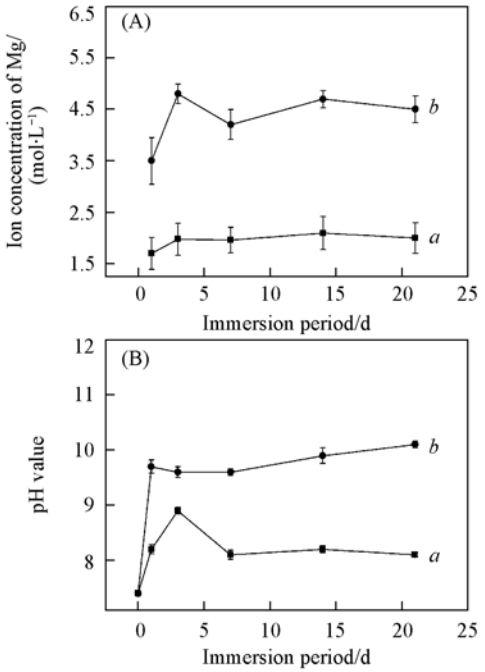

Fig.2 Changes of magnesium ion concentration(A) and the variation of pH value(B) in SBF for different immersion periods

a. Coated Mg alloy; *b*. uncoated Mg alloy.

SBF degraded faster than coated magnesium, and the magnesium ion concentration in SBF increased more quickly than that of coated magnesium alloy in SBF(P<0.05). Fig.2(B) shows the variation of pH value in SBF after different immersion periods. In the uncoated magnesium group, the pH value increased greatly and reached 9.7 after one day's immersion. Subsequently, the pH value kept on increasing slowly. While in the coated magnesium alloy group, the pH value reached a peak of about 8.7 after 3 d and then descended.

3.3 Cell Proliferation Assay

Cell proliferation is analyzed by viability observation of osteoblast MC3T3-E1 cells *via* CCK-8 assay for different culture periods. As shown in Fig.3, the cell viability was no significant difference between the coated magnesium alloy group and the negative control group. But the cell viability of uncoated magnesium alloy group was lower compared with that of negative control group(P<0.05). The difference in cell proliferation in different extract media is shown in Fig.4. The cell morphology in uncoated magnesium alloy group is obvious different from those in the negative control group and coated magnesium alloy group, which showed significant reduction in both the number and the density of the cells and abnormal shape and nucleus of the cells(arrows in Fig.4).

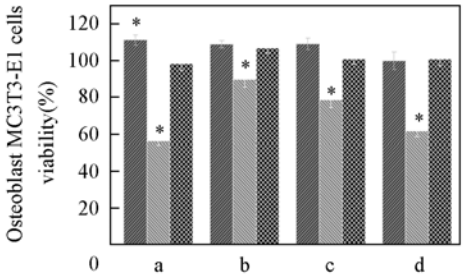

Fig.3 Osteoblast MC3T3-E1 cells viability in different culture periods

Colture period/d: a. 1; b. 3; c. 5; d. 7. Coated Mg alloy; uncoated Mg alloy; negative control. * P<0.05.

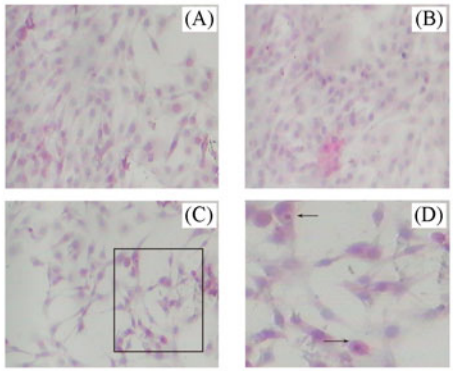

Fig.4 Morphologies of osteoblast MC3T3-E1 cells cultured in different sample extracts after 7 d

(A) Ca-P coated Mg alloy group(×16); (B) negative control group(×16); (C) uncoated Mg alloy group(×40); (D) abnormal cellular nucleus in uncoated Mg alloy group(×40). (D) the enlarged picture of partly (C). Arrows in (D): the abnormal shape and nucleus of cells.

3.4 ALP Tests

The ALP activities of osteoblast MC3T3-E1 cells in extract medium were measured *via* aBCA Protein Assay Kit (Sigma, USA). As exhibited in Fig.5, compared with that in the negative control group, the ALP activity of the cells in uncoated magnesium alloy group was decreased much evidently (P<0.05), while the ALP activity of the cells in coated magnesium alloy group was the same as that in the control group.

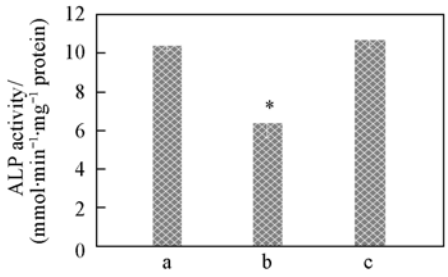

Fig.5 ALP activity of osteoblast MC3T3-E1 cells in cultured extract after 5 d immersion

* P<0.05. a. Coated Mg alloy; b. uncoated Mg alloy; c. negative control.

3.5 Apoptosis Tests

The apoptosis and death of osteoblast MC3T3-E1 cells were detected *via* flow cytometry with Annexin V-FITC/ propidium iodide double staining of cells. Typical figures for the flow cytometric analysis are shown in Fig.6. The cell apoptosis rate is shown in Fig.7. After culturing directly with the two

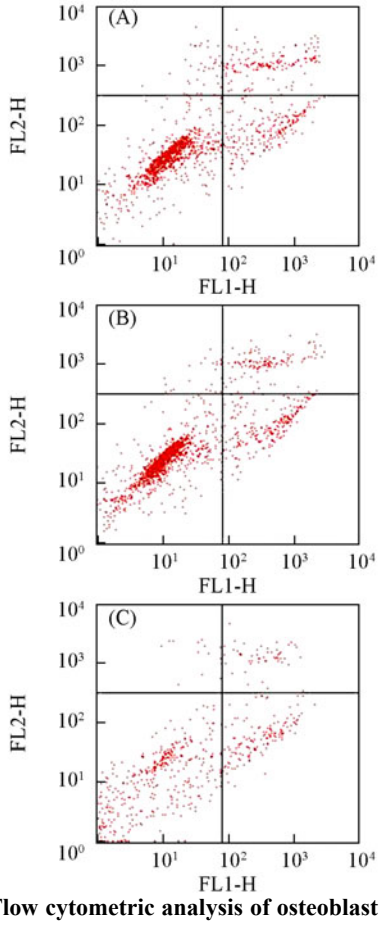

Fig.6 Flow cytometric analysis of osteoblast MC3T3-E1 cells apoptosis after 5 d culturation with the two samples and DMEM medium

(A) Ca-P coated Mg alloy group; (B) negative control group; (C) uncoated Mg alloy group.

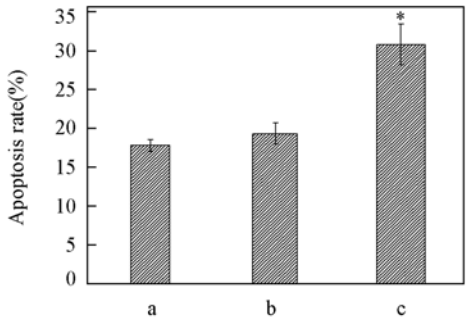

Fig.7 Cells apoptosis rate cultured directly with the two samples and DMEM medium for 5 d

a. Coated Mg alloy; b. negative control; c. uncoated Mg alloy. * P<0.05.

samples and DMEM medium for 5 d, the number of cells in uncoated magnesium alloy group reduced rapidly and the apoptosis rate reached to 30.8%. While in coated magnesium alloy group, the cells apoptosis rate was about 17.82%, which was lower than 19.33% in the negative control.

4 Discussion

To overcome the low corrosion resistance of magnesium alloy, several strategies have been reported in the literature[14,15] to regulate the biocorrosion rate of magnesium alloy. For the addition of other elements, Mn, Zn and Al were generally selected as the alloying elements to develop different alloys in past decades. In recent years, Ca and rare earth elements have been chosen to produce binary magnesium alloys, which show good corrosion resistance but limit the improvement of bone response[16]. Surface modification is another effective method to reduce the corrosion of magnesium alloys[5]. Ca-P coating is recognized as one of the most biocompatible materials for bone replacement and regeneration, and has been widely used as a bioactive coating in clinical orthopedic implants such as titanium alloys[17].

Ca-P coating has been used to protect magnesium alloys from fast corrosion, and have made good results in some *in vitro* experiments[18].

The degradation of magnesium alloys in the SBF is actually caused as following reactions[19,20].

$$Mg+2H_2O=Mg(OH)_2+H_2 \quad (1)$$

$$Mg(OH)_2+2Cl^-=MgCl_2+2OH^- \quad (2)$$

The transformation from $Mg(OH)_2$ into soluble $MgCl_2$ results to excess OH^- ions in the solution so as to eventually elevate Mg^{2+} concentration and pH value in the solution. Therefore, monitoring the changes in Mg^{2+} concentration and pH value within the solution may reveal the corrosion of magnesium alloys.

In our present study, uncoated magnesium alloy reacted with SBF severely and the Mg^{2+} ions concentration and pH value in the solution increased highly after emersion in SBF for 1 d. On the contrary, the Ca-P coated magnesium alloy showed a good corrosion resistance. It is known that Ca-P coating on the magnesium alloy is a porous film, which is permeable to solution ingress and egress. In the SBF, the surrounding solution can continuously penetrate through this kind of porous surface film and react with the inner magnesium alloy to form $Mg(OH)_2$ at the outer surface[21]. The ions in the SBF such as PO_4^{3-}, Ca^{2+} and so on are likely to accelerate the hydroxyapatite nucleation with increasing pH value[22]. Consequently, lots of HA nuclei are formed on the surface film and tightly attached to the matrix so as to increase the corrosion resistance. The tolerable pH value range for most human cells is between 6.0 and 9.0, a significant fluctuation in pH value can hence cause fatal effect on the viability of cell[23]. Furthermore, since the investigated magnesium alloys are usually composed of two or more alloying elements, the influence of other alloying elements should be taken consideration as the effect of corrosion products in SBF is investigated. And examination of other element ion concentration, such as Ca and P, may be helpful to reflect the material corrosion better.

In order to appreciate the biocompatibility of magnesium alloy and preselect appropriate implant candidates, the cytotoxicity tests *in vitro* is needed. Tetrazolium salt based assays are widely used to measure cytotoxicity or cell proliferation[24]. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide(MTT) test is a frequently used assay to quantify cellular growth and cytotoxicity[25,26]. CCK-8 assay resembles MTT assay in appreciating cytotoxicity of cells and is even more sensitive and convenient[27]. The results of the CCK-8 assay show that Ca-P coated magnesium alloy could promoted osteoblast MC3T3-E1 cells viability and had no cytotoxicity, while the uncoated magnesium alloy revealed a significant difference to the negative control(P<0.05) and had obvious cytotoxicity. The severe change in pH value of the extract medium in uncoated magnesium alloy group, which was indicated in the immersion tests, may be a probable reason. In Fig.4, nucleus condensation was observed clearly, and cell apoptosis or death occurred due to nuclear chromatin condensation.

ALP, as we known, is synthesized and secreted by osteoblasts, and also commonly used to investigate the differentiation of osteoblasts on the effects of biomaterials. The other osteogenic markers such as type I collagen, osteonectin, osteocalcin, osteopontin, and bone sialoprotein have been proven to be also useful[28,29]. Maxian *et al*.[30] reported that hydroxyapatite with low crystallinity enhanced ALP activity. Matsuoka *et al*.[31] reported that increased calcium concentration in the culture medium elevated ALP activity of osteoblast cells. From the point of view of ALP expression in our experiments, the Ca-P coating was not harmful to osteoblast MC3T3-E1 cells.

Apoptosis is also known as programmed cell death, and can be triggered by a series of apoptosis signals, such as heat, radiation, nutrient deprivation, viral infection, hypoxia, and increased intracellular calcium concentration[32]. Apoptosis has been reported as an appreciation of cell proliferation induced by biomaterials[33,34]. According to our flow cytometric results, the cell apoptosis rate in Ca-P coating group was lower than that in the control group(P<0.05), while the apoptosis rate in uncoating group was higher than that in the control group(P<0.05).

Some reported studies mentioned that Ca^{2+} and Mg^{2+} ions promoted cell differentiation and dietary Mg deprivation was a risk factor for osteoporosis[35,36]. The ions released during dissolution and degradation of the coated substrate correlated to dynamically influencing cell activity. More accurate test such

as reverse transcription-polymerase chain reaction(RT-PCR) quantitative analysis should be taken to evaluate those expressions.

5 Conclusions

By means of immersion test and osteoblast MC3T3-E1 cells cytotoxicity, Ca-P coated magnesium alloy presented more stimulating effects on corrosion resistance, cell proliferation as well as differentiation than the uncoated one. It is manifested that the Ca-P coating on Mg alloy by two-step chemical processes without pre-treatment is favorable for nucleus stabilization and can down-regulate cellular apoptosis rate. Of course, to deeply understand the effect of the coating on magnesium alloy, more *in vivo* biocorrosion and biocompatibility tests on Ca-P coating should be carried out for a longer time.

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