**In vitro** and **in vivo** evaluation of the surface bioactivity of a calcium phosphate coated magnesium alloy

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**A B S T R A C T**

Magnesium has shown potential application as a bio-absorbable biomaterial, such as for bone screws and plates. In order to improve the surface bioactivity, a calcium phosphate was coated on a magnesium alloy by a phosphating process (Ca–P coating). The surface characterization showed that a porous and netlike CaHPO\(_4\) \(2\)H\(_2\)O layer with small amounts of Mg\(^{2+}\) and Zn\(^{2+}\) was formed on the surface of the Mg alloy. Cells L929 showed significantly good adherence and significantly high growth rate and proliferation characteristics on the Ca–P coated magnesium alloy \((p < 0.05)\) in in-vitro cell experiments, demonstrating that the surface cytocompatibility of magnesium was significantly improved by the Ca–P coating. **In vivo** implantations of the Ca–P coated and the naked alloy rods were carried out to investigate the bone response at the early stage. Both routine pathological examination and immunohistochemical analysis demonstrated that the Ca–P coating provided magnesium with a significantly good surface bioactivity \((p < 0.05)\) and promoted early bone growth at the implant/bone interface. It was suggested that the Ca–P coating might be an effective method to improve the surface bioactivity of magnesium alloy.

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1. **Introduction**

Recently, magnesium alloys have attracted much attention as potential biodegradable bone implant materials due to their biodegradability in the bioenvironment \([1–3]\) and their excellent mechanical properties such as high strength and possessing an elastic modulus close to that of bone \([4]\). However, the rapid corrosion of magnesium alloys in chloride containing solutions including human body fluid or blood plasma has limited their clinical applications \([4,5]\). Therefore, it is very important to improve the corrosion resistance of magnesium alloys in order for them to be applied clinically.

Element alloying has been studied for developing biodegradable magnesium alloys with good mechanical and corrosion properties. Mn and Zn were selected as the alloying elements to develop Mg–Mn–Zn alloys due to the good biocompatibility of Mn and Zn \([6,7]\). The addition of Mn and Zn improves both the mechanical properties and the corrosion resistance of magnesium alloys. It has been shown that the corrosion resistance of an Mg–1.0 Mn–1.0 Zn alloy in simulated body fluid (SBF) is slightly better than that of the WE43 alloy (containing 3.78 wt.% Y, 2.13 wt.% Nd and 0.46 wt.% Zr), which is one of the best commercially available magnesium alloys \([8]\). **In vivo** experiments indicated that new bone tissue formed around the alloy implant after 6 weeks postimplantation, and about 10–17% of the implant degraded after 9 weeks implantation, and more than 55% degraded after 26 weeks implantation \([3,9]\). No disorders of liver, kidneys and blood composition were caused by the degradation of the Mg–Mn–Zn alloy after 6 weeks, 15 weeks, 26 weeks and 52 weeks postimplantation, respectively \([9]\). Ca was also chosen to produce a binary Mg–Ca alloy \([10–12]\) and Ca–containing AZ91 magnesium alloy (AZ91Ca) \([13]\) for the bone implant due to the beneficial effect of Ca ion on bone growth. It was shown that the addition of Ca increased the corrosion resistance of AZ91 alloy \([13]\). Results on binary Mg–Ca alloys indicated that a Mg–Ca binary alloy with 0.6–1.0 wt.% Ca provides good overall mechanical properties and corrosion resistance \([10,11]\). Further increase in the Ca content would lead to a deterioration in the mechanical properties and corrosion resistance \([14]\).

**In vivo** studies have shown good bone attachment to magnesium implants after 9 and 18 weeks postimplantation and good biocompatibility of magnesium in long-term service \([1,3,14]\). **In vivo**
research on AZ31 [15] reported that osteotylus surrounds the magnesium alloy implant after 2 weeks postimplantation, which was turned into ripe bone tissue after 8 weeks. In contrast, our research on the early bone response to magnesium bone implants found that only 50% of magnesium implants were fixed after 5 weeks postimplantation [16]. Therefore, it is necessary to speed up the early bone response to magnesium. Although the element alloying can increase the bio-corrosion resistance of magnesium alloys to some extent, it is obvious that the alloying cannot significantly improve the bone response to magnesium alloys, especially the bone response at the early stage. Surface modification such as Al coating [17,18], Ti coating [19] and heat treatment [20] have been applied to magnesium alloys to improve the corrosion resistance. However, surface biocompatibility was not enhanced. Bioactive coatings such as various calcium–phosphate compounds (Ca–P) are of importance for modifying the surface of implanted devices, and have been successfully applied to the surface modification of Ti and its alloys in order to promote direct attachment of the surrounding hard tissue and to suppress the release of corrosion products into the human body [21]. Lately, calcium phosphate coatings have been used to protect magnesium alloys from fast corrosion. For example, Cui reported that a Ca–P coating, including hydroxyapatite, octacalcium phosphate and dicalcium phosphate dihydrate protected AZ31Mg alloy from rapid degradation in 3.0% NaCl solution [22]. A previous study has reported the successful preparation of a Ca–P layer on a Mg–Mn–Zn alloy, which effectively improved the corrosion resistance [23]. It was also reported that an electrodeposited HA coating on AZ91D magnesium alloy can obviously slow down the biodegradation rate of AZ91D magnesium alloy in simulated body fluid [24]. However, no bioactivity of the Ca–P coating has been reported so far.

In this paper, a calcium phosphate was coated on an Mg–Mn–Zn alloy by a phosphating treatment in order to improve the surface bioactivity of the magnesium alloy. Cell experiments were used to evaluate the cytocompatibility. Routine pathological examination analysis and growth factor expressions at the interface between magnesium alloy implants and bone were utilized to assess in vivo the surface bioactivity during the early 1–4 weeks postoperation.

2. Experimental

2.1. Sample preparation

Extruded Mg–Mn–Zn (Mg–1.2 Mn–1.0 Zn, in wt.%) bars were prepared in our laboratory [7]. For the calcium phosphating treatment, plate samples with a dimension of 10 mm in diameter and 2 mm in thickness were cut from the extruded bar and moulded into epoxy resin with only one side exposed. Plate samples for the in vitro cell experiments with a dimension of 10 mm in diameter and 2 mm in thickness, and rod samples for in vivo test with 2.8 mm in diameter and 10 mm in length were cut from the extruded bar, respectively. All samples were ground with SiC emery papers of up to 1000 grits, and then ultrasonically cleaned in alcohol for 5 min and dried in warm air. In the cell experiment and the in vivo test, pure titanium samples of the same dimension were also used for comparison after the same surface treatment.

2.2. Calcium phosphate (Ca–P) coating treatment

The samples were first immersed in an alkaline solution at 63 °C for 15 min for degreasing and subsequently immersed in a mixed acid solution (2%) of H3PO4 and H2SO4 at room temperature for 5–10 s for surface activation. Then, the samples were treated in a phosphating bath for 6 min for calcium phosphate (Ca–P) treatment in order to obtain a Ca–P coating on the surface. Table 1 listed the chemical composition of the phosphating bath.

<table>
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<tr>
<th>Table 1</th>
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<tr>
<td>Chemical composition of the phosphating bath.</td>
</tr>
<tr>
<td>Composition</td>
</tr>
<tr>
<td>Concentration</td>
</tr>
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</table>

2.3. In vitro cell experiment

2.3.1. Cell culture

Cell lines LS92 were cultured in a PRMI1640 medium containing 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin sulphate and 10% (vol/vol) FBS. Then, the cells were maintained at 37 °C with 5% CO2 in air in a humidified incubator. Cells LS92 were seeded onto the naked Mg–Mn–Zn samples (without a Ca–P coating) and the Ca–P coated Mg–Mn–Zn samples as well as the pure titanium samples (control sample) at a cell density of 1 × 105 cells/mL. Cultures were incubated for 1 day, 3 days and 5 days at 37 °C with 5% CO2 in air in a humidified incubator in 48 well plates (Corning, NY, USA), respectively. Three parallel samples were used for each experimental condition. After different incubation periods, the samples were washed three times with PBS to remove non-adherent cells.

2.3.2. Cell count

For the cell count, the samples were fixed in 4.0% formaldehyde solution for 24 h, and then used for immunofluorescence staining. Cell counts were conducted at five different locations (four peripherals and one central) of the surface of each sample under a TCS-SP2 laser scanning confocal microscope (LSCM). Each area was about 187.5 μm × 187.5 μm. A mean and a standard deviation were obtained from the five measurements.

![Fig. 1. Implantation of magnesium rod samples with one naked sample and one Ca–P coated sample in one femora of a rabbit.](image)

![Fig. 2. XRD diffraction patterns on the surface of the Ca–P coated Mg–Mn–Zn alloy. The insert figure shows the diffraction peaks of CaHPO4·2H2O in a high magnification.](image)
2.3.3. Morphology

To determine the cellular morphology, the samples were processed for scanning electron microscopy (SEM) by a 24 h fixation in 2.5% (w/v) glutaraldehyde, gradual dehydration in 50–100 vol.% alcohol and coated with Au.

2.4. In vivo study

2.4.1. Surgical procedure

All animal experiments were conducted according to the ISO 10993-2:1992 animal welfare requirements. 18 healthy Japanese big-ear rabbits were used. All

Fig. 3. XPS spectra of the Ca–P coated Mg alloy (a) P2p, (b) Ca2p, (c) O1s, (d) Zn2p and (e) Mg2p: 1: top surface; 2: after ion etching for 300 s; 3: after ion etching for 600 s and 4: after etching for 900 s.
rabbits were anesthetized with 0.5% pentobarbital sodium solution for surgery. One naked Mg–Mn–Zn and one Ca–P coated Mg–Mn–Zn rod sample were implanted into the left femoral shaft of a rabbit after predrilling with a 2.8 mm hand-operated drill, as shown in Fig. 1. After the operation, all rabbits received a subcutaneous injection of gentamycin as an antibiotic prophylaxis. Intravital staining was performed weekly using subcutaneous injections of 1% water solution of calcein (0.3 mg/kg) to observe the newly formed bone. Postoperatively, the rabbits were allowed to move freely in their cages without external support. Three rabbits were sacrificed at 1, 2 and 3 weeks after surgery, respectively. Six rabbits (three for fluorescent observation, another three for routine pathological examination and immunohistochemistry) were sacrificed at 4 weeks after surgery.

2.4.2. Fluorescent observation

For microstructure analysis, the bone samples with magnesium implants were fixed in 2.5% glutaraldehyde solution and then embedded in epoxy resin. Then, the samples were ground by emery paper to a get a ground Mg implant cross-section with bone. The cross-section was observed on an Olympus BX51 microscope.

2.4.3. Routine pathological examination

The bone specimens including implants were taken out to observe osteointegration and evaluate the surface bioactivity of the Ca–P coated samples. Following fixation in 4.0% formaldehyde solution, the specimens were decalcified in 15% EDTA solution for 3 weeks and embedded in paraffin. During this process, the residual magnesium alloy implant was corroded by the solution completely, leaving a hole in the implantation site of the bone sample. The paraffin-embedded bone specimens were used for routine pathological examination and immunohistochemistry. Each bone specimen was consecutively cut into 5-μm-thick slices. Deparaffinized slices were stained with hematoxylin and eosin (HE) stains, and microscopically examined.

2.4.4. Immunohistochemistry

Immunohistochemistry staining was performed using antibodies for BMP-2, TGF-β1 and PDGF respectively. This was developed using a Strept Activdin-Biotin Complex (SABC) method. Deparaffinized sections were pretreated with 0.3% H2O2 for 30 min at room temperature to block endogenous peroxidase activity. After washing three times with distilled water, the sections were blocked with a 10% normal goat serum solution for 40 min at room temperature, and then the redundant solution was discarded. The primary antibodies (Boster, China) diluted in 0.1 M PBS (1:200) were applied onto the sections, and the sections were maintained at 4 °C for 48 h in a humidified chamber. After washing three times with 0.1 M PBS for a total of 6 min, the sections were reacted with the secondary antibody (biotin-goat anti-rabbit IgG, Boster, China) diluted in 0.1 M PBS (1:200) for 20 min at 37 °C. After rinsing three times with PBS for a total 6 min, a strept avidin-biotin complex (Boster, China) was applied onto the sections, and the sections were maintained 30 min at 37 °C. After rinsing the sections four times with PBS for a total 20 min, visualization of the antibody was accomplished by incubating sections in 0.03% 3’-diaminobenzidine (DAR, Boster, China) for 5 min, and the sections were counterstained in hematoxylin. Primary antibody replacement with PBS from the same animal samples was used as the controls. Positive signals were stained brown. The routine pathological examination and immunohistochemistry analysis were all expressed as the mean ± SD. Differences between two groups were analyzed by the paired-samples t-test. Differences of more than two groups were analyzed by an analysis of variance (ANOVA). Statistical significance was defined as p < 0.05.

3. Results

3.1. Microstructure of Ca–P coating

Fig. 2 illustrates the XRD pattern on the Ca–P coated Mg–Mn–Zn alloy. Besides the diffraction peaks from the magnesium matrix, the diffraction peaks of CaHPO4·2H2O were also detected by XRD, indicating that CaHPO4·2H2O was produced as the main new product on the surface of the Mg–Mn–Zn alloy after the calcium phosphate treatment.

Fig. 3 shows P2p, Ca3p, O1s, Zn2p, and Mg2p XPS spectra for the Ca–P coated Mg alloy. It can be seen that the P2p1/2 spectrum was detected as a single peak and the Ca3p spectra were detected as doublet peaks. From the binding energies of P2p1/2 and Ca3p, it can be concluded that the element P exists in the layer in a form of a phosphate. O1s was detected as a single peak. Zn2p spectra were detected as doublet peaks. Small amounts of Mg were also detected by XPS. According to the binding energies, it is deduced that the elements Zn and/or Mg are in the form of Zn2+ and Mg2+. After 300 s, 600 s and 900 s ion etching, respectively, no difference was observed in the P2p, Ca3p, O1s, Zn2p and Mg2p spectra. Combined with the XRD result shown in Fig. 2, it can be concluded that the surface layer is mainly composed of CaHPO4·2H2O with a small amount of Zn and/or Mg containing phosphate compounds precipitated from the phosphating solution. However, the amount of the Zn and/or Mg containing compounds is too small to be detected and identified by XRD.

Fig. 4 shows the surface microstructure and the EDS result of the Ca–P coated Mg–Mn–Zn alloy. A porous and netlike surface...
Fig. 5. Cell morphology at low magnification after the incubation of 1, 3, and 5 days on different samples.

Fig. 6. Cell morphology at a high magnification after 1, 3, and 5 days incubation on different samples.
structure was observed clearly on the surface of the phosphated magnesium alloy, as shown in Fig. 4(a). Small cracks were also found, which were due to dehydration during the SEM sample preparation. EDS analysis on a small square area of the phosphated magnesium, as shown in Fig. 4(b), indicates that the layer was mainly composed of O, P, Ca, Mg and a small amount of Zn, supporting the XPS results in Fig. 3.

3.2. In vitro cell experiments

3.2.1. Cell morphology

Fig. 5 presents the morphology of the cells cultured for 1 day, 3 days and 5 days on the surfaces of the naked Mg alloys, the Ca–P coated Mg alloys and the pure Ti. Differences in the response of the cells to the different surfaces were obvious. For the naked Mg alloy, only a few cells were observed on the surface after a 1-day culture. After 3 days and 5 days, no obvious change in the cell morphology and the cell number was observed in the cultures. For the Ca–P coated Mg alloy, many cells were observed and the cells seemed to be connected with each other after only a 1-day culture. After a 3-day culture, more cells were found on the surface. The cells have spread and connected together and covered most of the sample. After a 5-day incubation, a nearly dense and continuous cell layer covered the whole surface. For the pure Ti, some cells were observed on the surface after a 1-day culture and more cells were seen after a 3-day incubation. After a 5-day culture, a cell layer covered the surface completely. By comparison, more cells were observed on the surface of the Ca–P coated Mg alloy than on the surface of the naked Mg alloy during the whole incubation period, indicating a better cell response to the Ca–P coated Mg alloy. From the point of view of cell morphology, the Ca–P coated Mg alloy shows better cell response than the pure Ti in the first 1 day culture due to the fact that the cells on the surface of the Ca–P coated Mg alloy have been spreading and connecting with each other. However, in the further incubation periods, no obvious difference in the cell morphology was observed at a low magnification compared to the Ca–P coated Mg sample and the pure Ti sample.

In the higher magnification morphology, the differences in the cells response to different surfaces were distinct, as shown in Fig. 6. The cells on the surface of the naked Mg alloys maintained a round or spindle-like morphology during the whole incubation period. For the Ca–P coated Mg alloy, the cells were sail-like, elongated and thicker in the central area of the nucleus and nucleolus and flattened in the peripheral regions after a 1-day incubation. Some cells spread across the surface and contacted with each other. After a 3-day culture, the cells and the excreted matrixes were connected together and it is hard to distinguish between the cells and the matrix. After a 5-day culture, no difference in the morphology of the cells was found in comparison with that incubated for 3 days. For the pure Ti, the cells were spindle-like, and displayed a strong adhesion to the substrate after a 1-day culture. After a 3-day culture, the cells displayed a flattened morphology with numerous filamentous extensions. Further culture did not bring change to the cells morphology. From the morphology of the cell, both the Ca–P coated Mg sample and the pure Ti samples show significantly better cell response than the naked Mg sample during the whole incubation period. In addition, the cells on the surface of the Ca–P coated Mg alloy excreted more matrix than the cells on the surface of the pure Ti did.

3.2.2. Cell proliferation

Fig. 7 illustrates the cell proliferation on the different samples. The cell activity represented by the cell number was found to increase with culture time, indicating that a cell could attach and proliferate on the surface of the samples. For the naked Mg alloys, there is no evident increase in the cell number at all time.
Fig. 9. Pathological photographs of the implant/bone interfaces after 1, 2, 3 and 4 weeks postimplantation (HE stained). (a)-(d) the naked alloy, (e)-(h) the Ca–P coated alloy. I: implant; F: fibroblast band; N: newly formed osteoid tissue; C: connective tissue.
intervals \((p > 0.05)\), indicating that the naked Mg alloy does not promote cell growth and proliferation. For the Ca–P coated Mg alloy and the pure Ti, there is a significant increase \((p < 0.05)\) in the cell number between day 1 and day 3, but no significant difference \((p > 0.05)\) between day 3 and day 5. In comparison with the naked Mg alloy, the cell number on the surfaces of the Ca–P coated Mg alloy and the pure Ti showed a statistically significant increase at all time intervals, indicating that both the Ca–P coated Mg alloy and the pure Ti had a significantly better surface bioactivity than the naked Mg alloy. However, no statistically significant difference in the cell number between the Ca–P coated Mg alloys and the pure Ti was noted at all time intervals \((p > 0.05)\).

### 3.3. In vivo study

#### 3.3.1. Fluorescent observation

The fluorescent images of the cross-section of bone and magnesium implants after 4 weeks implantation are shown in Fig. 8. It can be seen that newly formed osteoid tissue was observed around both the naked Mg alloy implant and the Ca-P coated Mg alloy implant. Compared with the naked Mg alloy implant, the newly formed osteoid tissue around the Ca–P coated Mg alloy implant was compact and uniform. In addition, the outline shape of the magnesium implants was slightly changed, indicating that the implants were corroded by the body fluid, or the implant degraded in the body. However, it is hard to distinguish the difference in the degradation between the naked Mg alloy implant and the Ca–P coated Mg alloy implant after 4 weeks implantation because the duration is not long enough to evaluate the in vivo degradation.

#### 3.3.2. Routine pathological examination

Fig. 9 shows the optical microstructure of the interfaces between the magnesium implants and new bone stained by HE after 1–4 weeks postimplantation. For the naked Mg alloy, lymphocytic infiltration at the interface was observed after 1 week postimplantation, as indicated by “L” in Fig. 9(a). After 2 weeks implantation, there was a continuous fibroblast band between the implant and the bone, as indicated by “F” in Fig. 9(b). However, lymphocytic infiltration and plasmablastic infiltration were not noted. Three weeks later, fibroblast band became thinner, as shown by “F” in Fig. 9(c), and small amount of newly formed osteoid tissue was found, as indicated by “N” in Fig. 9(c). At week 4, the surface of the implant was taken up by newborn bone and bone trabecular, as indicated by “N” in Fig. 9(d). Crowded osteoblasts and bone matrix were observed. For the Ca–P coated Mg alloy, connective tissue was seen at the interface after 1 week postimplantation, as indicated by “C” in Fig. 9(e), but lymphocytic infiltration and plasmablastic infiltration was not noted. At week 2, the connective tissue became thinner, and osteoblasts and bone matrix were noted. Newly formed osteoid tissue with embedded osteocyte formed in some

![Image]

Table 2

Summary of the routine pathological examination of the implant/bone interface.

<table>
<thead>
<tr>
<th>Periods (weeks)</th>
<th>Naked Mg alloy</th>
<th>Ca–P coated Mg alloy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lymphocytic infiltration</td>
<td>Connective tissue</td>
</tr>
<tr>
<td></td>
<td>No lymphocytic infiltration</td>
<td>No lymphocytic infiltration</td>
</tr>
<tr>
<td>2</td>
<td>Continuous fibroblast band</td>
<td>Thinner connective tissue</td>
</tr>
<tr>
<td></td>
<td>No lymphocytic infiltration</td>
<td>Osteoblasts</td>
</tr>
<tr>
<td></td>
<td>No plasmablastic infiltration</td>
<td>Newly formed bone matrix</td>
</tr>
<tr>
<td></td>
<td>Small amount of newly formed osteoid</td>
<td>Newly formed osteoid with osteocyte</td>
</tr>
<tr>
<td>3</td>
<td>Thinner fibroblast band</td>
<td>No connective tissue</td>
</tr>
<tr>
<td></td>
<td>No lymphocytic infiltration</td>
<td>Osteoblasts</td>
</tr>
<tr>
<td></td>
<td>No plasmablastic infiltration</td>
<td>Bone matrix</td>
</tr>
<tr>
<td></td>
<td>Newly formed osteoid</td>
<td>Connecting trabecular</td>
</tr>
<tr>
<td>4</td>
<td>Newbone</td>
<td>More new bones</td>
</tr>
<tr>
<td></td>
<td>Trabecular</td>
<td>New bone trabecular aligned compactly and regularly</td>
</tr>
</tbody>
</table>

Fig. 10. Photomicrographs of BMP-2 expression at the interfaces between the implants and the bone tissue after 1, 2, 3 and 4 weeks postimplantation.

Fig. 11. MOD values of BMP-2 expression at the interface between the implant and the bone tissue after different periods of implantation. *\(p < 0.05\).*

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as indicated by "N" in Fig. 9(f). Three weeks later, the connective tissue was replaced by osteoblasts and bone matrix, as indicated by "N" in Fig. 9(g). Bone trabeculae connected together, but the alignment of the connected bone trabeculae was disorganized. Four weeks later, more newborn bone was observed, as indicated by "N" in Fig. 9(h). Osteoid tissue and newborn bone trabeculae almost covered the implant surface completely. Osteoid tissues connected together, and bone trabeculae aligned compactly and regularly, in which mature osteocyte was embedded. Ossification was manifest. Table 2 summaries the routine pathological examination results.

3.3.3. Immunohistochemistry

3.3.3.1. BMP-2 expression at the implant/bone interface. Fig. 10 shows BMP-2 staining photomicrographs at the interfaces between the implants and bones after different periods of implantation. Fig. 11 shows the MOD values in the BMP-2 expression at the implant/bone interface after different periods of implantation. At week 1, strong positive activities were observed at the interfaces for both the naked alloy implant group and the Ca–P coated alloy implant group. At week 2, positive activities increased and peaked. At weeks 3 and 4, osteoid tissues were clearly observed at the interface, and the BMP-2 expressions gradually reduced. At all time intervals, the Ca–P coated implant group shows a higher MOD value in the BMP-2 expression than the naked implant. A significant positive activity was found at the first 3 weeks as found for the Ca–P coated implant ($p < 0.05$).

3.3.3.2. TGF-β1 expression at the implant/bone interface. Fig. 12 shows TGF-β1 staining photomicrographs at the interfaces between the implants and bones after different periods of implantation. Fig. 13 shows the MOD values from the TGF-β1 expression at the implant/bone interface after different periods of implantation. The TGF-β1 expression has a tendency to give a similar result as that from the BMP-2 expression with the implantation duration. The expression peaked at week 2, and then decreased markedly. At week 4, the TGF-β1 expression became weaker and few positive areas were detectable. The Ca–P coated implant exhibited a higher MOD value in the TGF-β1 expression than the naked implant at all durations and a significant difference in the TGF-β1 positive expression was observed between the naked implant and the coated implant group ($p < 0.05$) at weeks 2, 3 and 4, respectively.

3.3.3.3. PDGF expression at the implant/bone interface. Fig. 14 shows PDGF staining photomicrographs at the interfaces between the implants and bones after different periods of implantation. Fig. 15 shows the MOD values of the PDGF expression at the implant/bone interface after different periods of implantation. A similar tendency of PDGF expression to the BMP-2 and TGF-β1 expressions was observed with implantation duration. Strong positive expressions were observed at week 1 for both the naked and the coated implant. The expressions peaked at 2 weeks, and then the positive reaction diminished at week 3 and became weaker at week 4. The coated implant shows a higher MOD value than the naked implant at nearly all durations, and a significantly high MOD value at weeks 1, 2 and 4, respectively ($p < 0.05$).

4. Discussion

Recently, research on magnesium alloys as biodegradable materials has been conducted extensively. However, the hydrogen release and the alkalization caused by the in vivo corrosion of magnesium alloys are the most critical obstacles to using magnesium alloys as biodegradable implant materials. One of the effective measurements to reduce the corrosion of magnesium alloys is surface modification. For biomaterials, a surface coating is also an effective way to improve the surface bioactivity. Therefore, it is possible to reduce the corrosion of magnesium alloy and improve the surface bioactivity by selecting a proper surface treatment.
The microstructure of the Ca–P coated Mg alloy shows that the coating layer was porous with a netlike surface structure, as shown in Fig. 4(a). EDS and SAXS results indicate that the layer was mainly composed of O, P, Ca, Zn and a little Mg, and the main phase constituent was CaHPO$_4$·2H$_2$O. Besides Ca$^{2+}$ and PO$_4^{3-}$, Mg$^{2+}$ and Zn$^{2+}$ were also detected by XPS on the surface. However, the Mg$^{2+}$ and/or Zn$^{2+}$ containing compounds were not detected by XRD due to their small amounts. Magnesium alloy is a very active alloy. When a Mg alloy is immersed in a phosphating bath, magnesium dissolves and turns into Mg$^{2+}$ and releases H$_2$. The NO$_3^-$ and NO$_2^-$ in the phosphating bath can react with H$^+$ and consume it quickly, so the local pH at the metal-solution interface increases and facilitates the precipitation of insoluble phosphate [25]. At the same time, Ca(H$_2$PO$_4$)$_2$ has the potential to hydrolyse and the hydrolysis product brushite (CaHPO$_4$·2H$_2$O) will precipitate on the surface of the magnesium alloy. During this process, Zn$^{2+}$ in the phosphating bath and Mg$^{2+}$ released from the magnesium alloy could react with any negative ions in the phosphating bath, such as PO$_4^{3-}$ to form small amounts of Zn and/or Mg containing phosphate compounds.

Lots of adhered, sail-like and conjoint cells after a 1-day culture, the connected cells and excreted matrix layer after 3 days and 5 days cultures and the significant increase ($p < 0.05$) in the cell number during the whole incubation on the surface of the Ca–P coated Mg alloy in comparison with the naked Mg alloy indicate the Ca–P coating improves the surface bioactivity of magnesium alloy significantly as proposed.

According to the fluorescent observation, more new osteoid tissues, which are compact and uniform, were observed around the Ca–P coated Mg alloy implant than around the naked Mg alloy implant after 4 weeks implantation, indicating that the Ca–P coated Mg alloy implant is more compatible for bone growth at the early healing process.

Compared with the naked Mg alloy, routine pathological examination analysis results such as no lymphocytic infiltration (inflammation) at week 1, thinner connective tissue and the formation of bone matrix at week 2, more bone matrix and interconnected bone trabecular at week 3, and more newborn bones at week 4, as shown in Fig. 9, reveal clearly that the Ca–P coated Mg alloy exhibits better surface biocompatibility than the naked alloy at the first 4 weeks postoperation.

Numerous growth factors have been implicated in the repair of fracture healing, so expressions of bone growth factors can be used to evaluate osteogenesis at the interface between implant and bone. Among those growth factors, the BMP plays crucial roles in normal skeletal development as well as bone healing, and is able to activate transcription of genes involved in cellular migration, proliferation and differentiation [26]. It was reported that endogenous BMP-2 is an indispensable osteogenic stimulus for initiation of fracture healing in mice [27]. In addition, it is believed that TGF-$\beta$ stimulates osteogenesis, angiogenesis, fibroblast migration, and deposition of matrix [28] and has osteoinductive properties [29–32]. PDGF stimulates osteoblasts proliferation, collagen synthesis, and may play a regulatory role in fracture repair [28,33]. Immunohistochemical analysis of our experiment results demonstrated that the Ca–P coated implant provided a high BMP-2 expression during the first 4 weeks postimplantation, especially statistically significant differences in BMP-2 expression between the Ca–P coated Mg alloy and the naked Mg alloy after 1, 2 and 3 weeks postoperation. The Ca–P coated implant also exhibited a high TGF-$\beta 1$ expression during the first 4 weeks postimplantation, and statistically significant differences in the TGF-$\beta 1$ expression between the Ca–P coated Mg alloy and the naked Mg alloy after 1, 2 and 4 weeks postoperation. Similarly, a statistically significant difference was also observed in PDGF expression between the Ca–P coated Mg alloy and the naked Mg alloy after 2, 3 and 4 weeks postoperation. All the above in vivo results demonstrated significantly good osteoconductivity of the Ca–P coated Mg alloy at the early osseous integration stage.

For biomaterials application, the surface bioactivity is mainly controlled by the physical properties and the chemical properties of the surface. A porous surface at the microscale or nanoscale level
would contribute greatly to the faster adhesion and growth of cells, and a porous coating helps in bone cells growth and proliferation on the surface of the implant, resulting in a significantly stronger bond to the parent tissue [34]. In the surface biomodification of pure titanium and titanium alloys, alkaline treatment has been used to produce a porous surface structure at the micro- or nanoscale level to accelerate the deposition of hydroxyapatite [35,36]. Moreover, the porous structure increases the particle boundaries, creates surface roughness, and may help toward numerous protein interactions and thereby aids cell adhesion, alignment and finally tissue integration [37–42]. Further, these microscale features also mimic the extra cellular matrix (ECM) present in the body to support cells [40,42]. In the present experiments, a porous surface structure was successfully prepared on a magnesium sample by the phosphating treatment, as shown in Fig. 4, which would definitely contribute to the good surface bioactivity.

On the other hand, the surface chemical properties of the biomaterials also play a very important role in good surface bioactivity. Ti and Ti alloys are inert materials to cells. In order to improve the surface biocompatibility, various calcium phosphate coatings including brushite, octacalcium phosphate and hydroxyapatite (HA) have been successfully applied to titanium-based and other alloys [43–45]. In recent studies, it has been described that the same chemical composition or structure as the mineral composition of apatite (HA) have been successfully applied to titanium-based and other alloys [43–45]. In the present experiments, a porous surface structure was successfully prepared on a magnesium sample by the phosphating treatment, as shown in Fig. 4, which would definitely contribute to the good surface bioactivity.

5. Conclusion

A porous and netlike Ca–P coating was successfully prepared on magnesium alloy by a phosphating treatment to improve the surface bioactivity of the magnesium substrate. Microstructure characterization showed the Ca–P coating was mainly composed of CaHPO4·2H2O with small amounts of Zn and Mg ion. In vitro cell tests demonstrated that the Ca–P coating provided the magnesium alloy with a significantly better surface cytocompatibility and in vivo results also confirmed that the Ca–P coating exhibited significantly improved osteoconductivity and osteogenesis in the early first 4 weeks postoperation period.

Acknowledgements

One of the authors (Erlin Zhang) would like to acknowledge the financial support from the Institute of Metal Research (IMR), Chinese Academy of Sciences (CAS), Shenyang Science and Technology Institute (Program No. 1062109–1–00), and Heilongjiang Provincial Natural Funding (No. E2007–18).

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