In vitro and in vivo studies on a Mg–Sr binary alloy system developed as a new kind of biodegradable metal

X.N. Gu,1, X.H. Xie,1, N. Li,1, Y.F. Zheng,*, L. Qin

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ABSTRACT

Magnesium alloys have shown potential as biodegradable metallic materials for orthopedic applications due to their degradability, resemblance to cortical bone and biocompatible degradation/corrosion products. However, the fast corrosion rate and the potential toxicity of their alloying element limit the clinical application of Mg alloys. From the viewpoint of both metallurgy and biocompatibility, strontium (Sr) was selected to prepare hot rolled Mg–Sr binary alloys (with a Sr content ranging from 1 to 4 wt.%) in the present study. The optimal Sr content was screened with respect to the mechanical and corrosion properties of Mg–Sr binary alloys and the feasibility of the use of Mg–Sr alloys as orthopedic biodegradable metals was investigated by in vitro cell experiments and intramedullary implantation tests. The mechanical properties and corrosion rates of Mg–Sr alloys were dose dependent with respect to the added Sr content. The as-rolled Mg–2Sr alloy exhibited the highest strength and slowest corrosion rate, suggesting that the optimal Sr content was 2 wt.%. The as-rolled Mg–2Sr alloy showed Grade 1 cytotoxicity and induced higher alkaline phosphatase activity than the other alloys. During the 4 weeks implantation period we saw gradual degradation of the as-rolled Mg–2Sr alloy within a bone tunnel. Micro-computer tomography and histological analysis showed an enhanced mineral density and thicker cortical bone around the experimental implants. Higher levels of Sr were observed in newly formed peri-implant bone compared with the control. In summary, this study shows that the optimal content of added Sr is 2 wt.% for binary Mg–Sr alloys in the rolled state and that the as-rolled Mg–2Sr alloy in vivo produces an acceptable host response.

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1. Introduction

In recent years magnesium and its alloys have attracted great attention as degradable materials for orthopedic implants due to their degradability, similar mechanical properties to cortical bone and the biocompatibility of magnesium ions [1–5]. A number of Mg alloys had been under investigation for potential clinical applications, such as AZ91 [4,5], WE43 [5,6], Mg–Ca [2], Mg–Zn [7] and Mg–Mn–Zn [6] alloys. Animal tests have proved that these Mg alloys gradually degrade within bone and the corroding products show anabolic effects on peri-implant bone with an appropriate inflammatory response in the short term [2,5,8]. However, the rapid corrosion rate of the above Mg alloys might limit their clinical application [2,5,7].

An approach to this challenge is alloying, which is well known as one of the most effective methods to improve the corrosion resistance as well as mechanical properties of Mg. From the medical point of view, there are only a small number of alloying elements which can be tolerated in the human body and can also retard the corrosion rate of Mg alloys suitable for inclusion in biodegradable Mg alloys, including Ca, Zn, Mn and perhaps a very small amount of low toxicity rare earth elements [2,6,9,10].

Strontium, along with Ca and Mg, belongs to group 2 of the periodic table and shares similar chemical, biological and metallurgical properties. Recently Fan et al. [11] reported that Sr could improve both the mechanical properties and corrosion resistance of alloy AZ91Din NaCl solution. Argo et al. [12] indicated that an increasing amount of Sr addition to Mg–Al alloys could increase the high temperature tensile properties and reduce their corrosion rate.
The good biocompatibility of Sr is a further advantage of adding Sr to Mg for orthopedic applications. Sr is a trace metal in the human body and 99% of the body content of Sr is located in the bones [13]. The body burden of Sr in an adult man is about 140 mg, and the average daily intake is 2 mg [13]. Since Sr is chemically and physically closely related to Ca it is a natural bone seeking element that accumulates in the skeleton, preferably in new trabecular bone [14,15]. Indeed, because of the bone formation stimulation effect of Sr, oral administration of Sr salts is used in the treatment of osteoporotic patients to increase bone mass and reduce the incidence of fractures [15–17]. Additionally, a hydroxyapatite bioactive cement incorporating Sr(Sr-HA) has recently been investigated for bone repair [18–20]. It was shown that the solubility and mineralization ability of Sr-HA exhibited Sr dose-dependent behavior. Both in vitro and in vivo studies indicated that Sr-HA is biocompatible and osteoconductive.

These beneficial effects of Sr prompted us to investigate the feasibility of Sr alloying with Mg and the corresponding effects on the mechanical and corrosion properties and biocompatibility. To date there have been no systematical investigations on Mg–Sr alloy systems for biomedical applications. The purposes of the present study were: (1) to prepare Mg–Sr binary alloys with different Sr contents, ranging from 1 to 4 wt.%, and to study its workability by hot rolling; (2) to seek the optimal amount of added Sr with respect to the microstructure, mechanical properties, corrosion resistance and cytotoxicity; (3) to see what would happen to Mg–Sr binary alloys intramedullary implanted into an animal and the continuing in vivo corrosion process, as well as the resulting biological effects.

2. Materials and methods

2.1. Materials preparation

Mg–Sr alloys with a nominal composition of 1–4 wt.% Sr were prepared from pure Mg (99.98%) and a Mg–10 wt.% Sr master alloy in a high purity graphite crucible under the protection of a mixed gas atmosphere of SF6 (1 vol.%) and CO2 (balance). After being held at 740–760 °C for 30 min the melt was poured into a steel mold preheated to 300 °C. The analyzed chemical compositions of the resulting alloys, measured by inductively coupled plasma atomic emission spectrometry (ICP-AES) (Profile, Leeman Labs, Hudson, NH), are listed in Table 1. The as-cast Mg–Sr alloy ingots with different compositions were further cut into 6 mm thick plates. These plates were pre-heated to 300 °C for 10 min between each pass. The as-rolled pure Mg (99.98%) sheets were removed from Hank’s solution, gently rinsed with distilled water and dried in air. The changes in surface morphology and composition after corrosion were characterized by ESEM, EDS and XRD.

Table 1

<table>
<thead>
<tr>
<th>Alloy</th>
<th>Composition (wt.%)</th>
<th>Sr</th>
<th>Fe</th>
<th>Si</th>
<th>Ni</th>
<th>Cu</th>
<th>Mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg–1Sr</td>
<td>0.98 ± 0.03</td>
<td>&lt;0.005</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>Balance</td>
<td></td>
</tr>
<tr>
<td>Mg–2Sr</td>
<td>1.80 ± 0.05</td>
<td>3.23 ± 0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg–3Sr</td>
<td>3.77 ± 0.12</td>
<td>0.77 ± 0.05</td>
<td></td>
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<tr>
<td>Mg–4Sr</td>
<td>0.77 ± 0.05</td>
<td>0.77 ± 0.05</td>
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</table>

2.2. Microstructure characterization

An X-ray diffractometer (Rigaku DMAX 2400, Japan) using CuKα radiation was employed to identify the crystal structure of the phases. All materials were polished, etched with 3% HNO3 solution and the microstructure then observed by environmental scanning electron microscopy (ESEM) equipped for energy dispersive spectrometry (EDS).

2.3. Mechanical test

The tensile samples were machined according to ASTM-E8-04 [21] from the rolled sheets parallel to the rolling direction. The tensile tests were performed at room temperature in accordance with ASTM-E8-04 [21] using an H25K-S material test machine (Hounsfield Test Equipment Ltd, Redhill, UK). Five parallel specimens were taken for each group.

2.4. Electrochemical test

A three electrode cell, using platinum as the counter-electrode and a saturated calomel electrode (SCE) as the reference electrode, was used for electrochemical measurements. A wire lead was attached to one section of each sample and closely sealed with epoxy resin, leaving one end surface (with a cross-sectional area of 1 cm2) exposed to solution. The electrochemical tests were carried out using an electrochemical workstation (CHI600C, CH Instruments, Shanghai, China) at a temperature of 37 ± 0.5 °C in Hank’s solution (8 gl−1 NaCl, 0.4 gl−1 KCl, 0.14 gl−1 CaCl2, 0.35 gl−1 NaHCO3, 0.2 gl−1 MgSO4·7H2O, 0.12 gl−1 Na2HPO4·12H2O, 0.06 gl−1 KH2PO4, pH 7.4 ± 0.1). The potentiodynamic polarization tests were carried out on a scanning rate of 1 mV s−1 with an initial potential of about 300 mV below the corrosion potential (Ecorr). An average of three measurements were taken for each group.

2.5. Immersion test

Immersion tests were carried out in Hank’s solution according to ASTM-G31-72 [22] at 37 ± 0.5 °C. After 500 h immersion, samples were removed from Hank’s solution, gently rinsed with distilled water and dried in air. The changes in surface morphology and composition after corrosion were characterized by ESEM, EDS and XRD. Both the hydrogen evolution volume and weight loss were measured to calculate the corrosion rate of the samples. The volume of hydrogen released was calibrated following a published method [23]. The weight of the samples after the corrosion test was measured after removal of the corrosion products in chromic acid. An average of five measurements were taken for each group. The in vitro corrosion rate was calculated according to the equation:

\[ C = \frac{\Delta m}{\rho \times t} \]

where C is the corrosion rate in mm year−1, \( \Delta m \) is the reduction in weight calculated from the hydrogen volume (1 ml H2 gas = 1.083 mg of Mg), \( \rho \) is the density of the material, A is the initial implant surface area and t is the implantation time.

2.6. Cytotoxicity and ALP tests

MG63 cells were cultured in minimum essential medium, 10% fetal bovine serum, 100 U ml−1 penicillin and 100 mg ml−1 streptomycin at 37 °C in a humidified atmosphere of 5% CO2. The cytotoxicity and alkaline phosphatase (ALP) tests were carried out by
extract assay. Extracts were prepared with a surface area to extraction medium ratio of 1 ml cm$^{-2}$ in a humidified atmosphere with 5% CO$_2$ at 37 °C for 72 h incubation. The pH and the concentrations of Mg and Sr ions in the extracts were measured by ICP-AES. The osmolality of the extracts was not tested and thus not adapted to the physiological level.

For the cytotoxicity tests cells were incubated in 96-well flat bottomed cell culture plates at 3 x 10^3 cells per 100 μl in each well and incubated for 24 h to allow attachment. The medium was then replaced with 100 μl of extract, 100 μl of a negative control (medium alone) or 100 μl of a positive control (10%DMSO medium). After incubation for 1, 3 or 5 days, 10 μl of MTT was added to each well for 4 h and 100 μl of formazan solubilizing solution (10%SDS in 0.01 M HCl) was added to each well overnight. The spectrophotometric absorbance of each well was measured using a microplate reader (Bio-Rad 680) at 570 nm with a reference wavelength of 630 nm [24].

For the ALP tests, MG63 cells were seeded in 24-well plates at 3 x 10^4 cells ml$^{-1}$ in each well. The medium was replaced with extract after 24 h culture following the above protocol. After 7 days culture with extract, the differentiation behavior of MG63 cells was estimated by measuring ALP activity. The cells were rinsed three times with phosphate buffer solution (pH 7.4) and lysed in 0.1% Triton X-100 by freezing and thawing for two cycles. The ALP activity was measured using p-nitrophenyl phosphate (pNpp) as the substrate (Sigma, St Louis, MO). The reaction lasted for 60 min at 37 °C and was then stopped using 1 M NaOH solution. The quantity of pNpp produced was measured at 410 nm using a microplate reader and the total protein content was measured using a BCA Protein Assay Kit (Sigma).

2.7. In vivo animal implantation study

2.7.1. Surgery

The animal experimental protocol was approved by the Animal Ethics Committee of the Chinese University of Hong Kong (reference no. 10/049/MIS). Eight 3-month-old male C57BL/6 mice were used in this study, randomly assigned to two groups (n = 4). All mice were generally anesthetized with ketamine (75 mg kg$^{-1}$) and xylazine (10 mg kg$^{-1}$) for surgery and the left knee scrubbed with 25 g l$^{-1}$ tincture of iodine and 70% ethanol. For the experimental group the as-rolled Mg–2Sr alloy rods were implanted into predrilled bone tunnels (0.7 mm diameter and 5 mm long) in the femur along the axis of the shaft from the distal femur using established surgical and model protocols [25]. In the control group the predrilled bone tunnel was left empty for comparison. The wounds were then carefully sutured and the mice housed in an environmentally controlled animal care laboratory after surgery. To label the newly formed bone, the fluorescent staining agents calcein green (5 mg kg$^{-1}$) and xylene orange (90 mg kg$^{-1}$) were injected subcutaneously at 14 and 4 days, respectively, before the mice were killed following our published protocol [26]. The change of serum Mg and Sr were measured 2 and 4 weeks post-surgery by ICP-AES.

2.7.2. Radiographic and micro-CT evaluation

Sequential radiographs of distal femora were taken (30 keV, 3 s) for general inspection every week post-surgery. An in vivo micro-computed tomography (micro-CT) set-up (Viva CT40, Scanco Medical AG, Brüttsellen, Switzerland) with a voxel size of 20 μm was also used to monitor the distal femora of mice weekly over the 4 weeks post-surgery. Two-dimensional (2-D) images were acquired directly from the scans and the three-dimensional (3-D) structure was reconstructed using the volume of interest (VOI), with an optimized threshold used to isolate the bone and materials from the background. The density and volume changes of the as-rolled Mg–25Sr alloy implants, as well as the density and the thickness of the peri-implant cortical bone, of the digitally extracted tissue were measured. The in vivo corrosion rate was calculated according to the equation [27]:

$$C = (V_t - V_0)/At$$

where C is the corrosion rate, $V_t$ is the alloy volume measured by micro-CT at different implantation intervals, $V_0$ is the alloy volume measured by micro-CT on week 0, A is the initial implant surface area and t is the implantation time based on our established protocol [25].

2.7.3. Histological evaluations

After the mice were killed 4 weeks post-surgery, the femora were harvested and fixed in 10% buffered formalin. After gradient dehydration, the harvested femora were embedded and polymerized in methylmethacrylate resin. The resin blocks were cut using a Leica SM2500E diamond saw into 120 μm undecalciﬁed sections, perpendicular to the long axis of the femoral shaft. Cross-sections were ground and polished to a thickness of 70 μm, followed by staining with Stevenel’s blue and Van Gieson’s picric fuchsin. The cross-sections were observed by light microscopy and ﬂuorescence microscopy, respectively.

2.7.4. Element distribution analysis

Representative undecalciﬁed sections were ground and polished. Cross-sections were analyzed by element mapping, line scan and EDS point analysis to evaluate the corrosion product layer at the periphery of the implants and release of the alloying element from Mg–Sr alloys compared with the control group.

2.8. Statistical analysis

Statistical analysis was conducted with SPSS 10.0. Differences between groups were analyzed using one-way ANOVA followed by Tukey’s test.

3. Results

3.1. Microstructures and tensile property of Mg–Sr alloys

According to the XRD measurements (Fig. 1), the various as-rolled Mg–Sr alloys (with a Sr content ranging from 1 to 4 wt.%) were composed of α-Mg with a hexagonal close packed crystal structure (a = 0.32 nm, c = 0.52 nm) [28] and Mg$_{17}$Sr$_{2}$ phases with a hexagonal structure (a = b = 10.469 nm, c = 10.3 nm) [28]. The diﬀraction intensities arising from the Mg$_{17}$Sr$_{2}$ phase increased with increasing Sr content. Fig. 2 illustrates the microstructure of the as-rolled Mg–Sr alloys (with a Sr content of 1–4 wt.%), showing the typical metallographic microstructural feature of α-Mg grains with the second phase precipitating along the grain boundaries. The chemical compositions of the α matrix, the second phase at grain boundaries and the white particles (indicated by red arrows in Fig. 2a) were identiﬁed by EDS analysis. The chemical composition of the α matrix was Mg, Sr was predominantly observed in the second phase at the grain boundaries and the white particles. The eutectic phase is indicated by arrows in the microstructure of the as-rolled Mg–45Sr alloy in Fig. 2d. The EDS analysis indicated high levels of Mg (anatom ratio Mg/Sr of approximately 10), which could be due to the existence of α-Mg in the eutectic structure. Moreover, the as-rolled Mg–Sr alloys exhibited ﬁner grain sizes with increasing Sr content. The average grain size was 32.3 ± 6.7 μm for Mg–15Sr, 25.9 ± 8.3 μm for Mg–25Sr, 23.0 ± 8.1 μm for Mg–35Sr and 20.9 ± 8.8 μm for Mg–45Sr alloys.
Fig. 3 presents the mechanical properties obtained by tension testing. The addition of Sr improved both the yield tensile strength (YTS) and the ultimate tensile strength (UTS) of pure Mg. The YTS and UTS values of the as-rolled Mg–Sr alloys increased with increasing amounts of added Sr up to 2 wt.%, whereas YTS and UTS decreased when the amount of Sr was further increased. In contrast, elongation of the as-rolled Mg–Sr alloys decreased with increasing Sr content as the as-rolled Mg–2Sr alloy showed the best combination of strength and ductility, with values of 213.3 ± 17.2 MPa for UTS and 3.2 ± 0.3% for elongation.

3.2. Corrosion behavior

The corrosion rates of the as-rolled Mg–Sr alloys were calculated based on the hydrogen evolution volume, weight loss and potentiodynamic polarization measurements. Fig. 4 shows the potentiodynamic polarization curves and corrosion rates of the as-rolled纯Mg–Sr alloys. The three different methods of measurement indicated similar trends of the corrosion rate for the as-rolled Mg–Sr alloys as a function of Sr content. A reduction in the corrosion rate of pure Mg due to Sr addition was found.
Moreover, the corrosion rates of the as-rolled Mg–Sr alloys decreased with increasing Sr addition up to 2 wt.%, while higher Sr contents (>2 wt.%) led to increased corrosion rates. The polarization curves also suggested that the cathodic branches moved in a positive direction with increasing Sr content from 2 to 4 wt.% (inset in Fig. 4a), demonstrating an accelerated corrosion rate. Both the hydrogen evolution and weight loss measurements revealed almost identical corrosion rates, whereas a significantly higher corrosion rate derived from the polarization curve measurements can be seen in Fig. 4b.

Fig. 5 shows the corroded morphology of the Mg–Sr alloys after 500 h immersion in Hank’s solution, which was in accordance with the corrosion rate results in Fig. 4b. Severe local corrosion with obvious corrosion pits can be seen on the surface of pure Mg, marked by arrows in Fig. 5a. The XRD analysis suggested that the corrosion products were mainly Mg(OH)2 and HA, as shown in Fig. 5f. In contrast, four of the as-rolled Mg–Sr alloys showed a relatively uniform corroded morphology, with only an irregular distribution of precipitates observed on their surface (Fig. 5b–e). The higher magnification of the as-rolled Mg–2Sr alloy showed a compact and smooth surface with a few micro-cracks (inset in Fig. 5c). A representative EDS analysis indicates the presence of the elements C, O, Mg, P, Ca and Sr on the corrosion surface of the as-rolled Mg–Sr alloys (inset in Fig. 5e). The XRD patterns proved the precipitation of Mg(OH)2 on the surface of the as-rolled Mg–Sr alloys, whereas no diffraction peaks arising from calcium phosphate apatite were detected, which might be due to its relatively low content (Fig. 5f).

3.3. Cytotoxicity and ALP activity

The pH values and the ion concentrations of the extracts were measured (Fig. 6a and b). The Mg and Sr concentrations as well as the pH values of the as-rolled Mg–1Sr and Mg–2Sr alloy extracts were significantly lower than those of the other three groups (P < 0.05). Additionally, we observed that the as-rolled Mg–2Sr alloy extract exhibited a similar pH value and Mg concentration to the as-rolled Mg–1Sr alloy extract, although it had a significantly higher Sr concentration. Significant differences were found among the groups in terms of cytotoxicity (Fig. 6c). The values recorded for the as-rolled pure Mg, Mg–1Sr and Mg–2Sr alloy extracts were significantly higher than those for the Mg–3Sr and Mg–4Sr alloys ("P < 0.05,""P < 0.01). The as-rolled Mg–1Sr and Mg–2Sr alloys showed Grade 1 cytotoxicity according to ISO 10993-5 [29], suggesting acceptable biosafety for cellular applications. Compared with as-rolled pure Mg, increasing protein normalized ALP activity was observed for alloys with increasing Sr content up to 2 wt.%. However, further increasing the Sr content to 3–4 wt.% resulted in significantly reduced ALP activity compared with the as-rolled pure Mg control (Fig. 6d). The significant reduction in cell viability and ALP activity for as-rolled Mg–3Sr and Mg–4Sr alloy might be attributed to the high pH as well as the high ion concentration of their extracts [30,31] (see Fig. 6a and b).

3.4. Animal experiment

In the 3-D micro-CT reconstruction of the mouse distal femur with an as-rolled Mg–2Sr alloy implant and 2-D tomographs of cross-sections, we can observe degradation of the as-rolled Mg–2Sr alloy and new bone formation during the 4 week implantation period. Local corrosion of the implants at the rod surface occurred in both the trabecular and cortical bone areas, while the center of the rod maintained its integrity (as shown in Figs. 7 and 8). Corrosion of the Mg–2Sr alloy implants differed in different regions within the femur, exhibiting faster corrosion in the distal metaphyseal region with rich trabecular bone) than proximal femur, i.e., bone marrow cavity of the diaphyseal region (Figs. 7b and 8). Along with degradation of the implant, new bone formation and bone remodeling at the surface of the distal femur was observed from week 0 to week 4. The surface of the distal femur was smooth at week 1, with some new bone formation was seen during the next two weeks. By week 4 we found that the new bone had been integrated and formed a relatively smooth surface (Fig. 7a). During the 4 weeks implantation period, a significant increase in the peri-implant bone mineral density (BMD) of the as-rolled Mg–2Sr alloy group was observed as compared with the non-implanted control group (Fig. 9a). The peri-implant cortical bone thickness of the as-rolled Mg–2Sr alloy implantation group was significantly higher
than that of the non-implanted control since 1 week post-surgery ($P < 0.05$ and $P < 0.01$) and the bone thickness increased with increasing implantation period (Fig. 9b). In addition, gas evolution was detected immediately after surgery in the 2-D micro-CT tomo-graphs (black shadows in the first column in Fig. 8), which could not be observed 1 week post-surgery.

Figs. 10 and 11 show the histology of cross-sections of the distal femora and diaphyseal region of the mice. The implanted Mg–2Sr alloy rod maintained its central integrity, with the debris from the corroded implants dispersed in the trabecular area (indicated by yellow dots in Fig. 10d), some of which was trapped within fibrous tissue (orange triangles in Fig. 10c). More bone trabeculae could be seen in the as-rolled Mg–2Sr alloy group (Fig. 10b) in comparison with the non-implanted control group (Fig. 10a). In the diaphyseal region new bone formation was observed in the fluorescence images of both the as-rolled Mg–2Sr alloy group and the non-implanted control group, especially beneath the periosteum (Fig. 11c and d). Thicker cortical bone occurred in the as-rolled Mg–2Sr alloy group (Fig. 11b) than the non-implanted control (Fig. 11a). Both the experimental and control groups showed periosteal bone formation, whereas only the as-rolled Mg–2Sr alloy group showed endosteal new bone formation (blue

![Graph](image_url)
arrows in Fig. 11b and d and red arrows in Fig. 11e). The newly formed periosteal bone in the control group (Fig. 11a and c) could have been induced in part by mechanical stimulation during the reaming or drilling procedure [32]. The serum Mg and Sr concentrations of mice at 2 ($24.71 \pm 0.133 \, \text{mg} \cdot \text{ml}^{-1}$ for Mg and $0.216 \pm 0.005 \, \text{mg} \cdot \text{ml}^{-1}$ for Sr) and 4 ($23.59 \pm 0.189 \, \text{mg} \cdot \text{ml}^{-1}$ for Mg and $0.221 \pm 0.007 \, \text{mg} \cdot \text{ml}^{-1}$ for Sr) weeks post-surgery showed no significant differences from the values for the control group ($25.16 \pm 0.224 \, \text{mg} \cdot \text{ml}^{-1}$ for Mg and $0.217 \pm 0.007 \, \text{mg} \cdot \text{ml}^{-1}$ for Sr).

Elemental distributions in cross sections of the mouse femoral shaft for the as-rolled Mg–2Sr alloy 4 weeks post-surgery are presented in Fig. 12. The element mapping analysis showed a high concentration of Mg in regions containing residual implant, with the concentration decreasing towards the periphery of the implant.
A homogeneous distribution of Mg was found within the bone tunnel, suggesting that Mg could be readily and rapidly absorbed by the surrounding tissue. A high density of Ca and P was found in the white fraction region within the bone tunnel, indicating the new bone formation, which is in accordance with the histological results in Fig. 11e. A Sr map of this area could not be obtained because of the relatively low Sr concentration in the implant (<2 wt.%) and the resolution limits of the EDS mapping technique.

Higher magnification images of the residual implants and peri-implant region showed that only a small fraction of the as-rolled Mg–2Sr alloy rod was left in the bone tunnel (marked by the red dotted circle in Fig. 13a). A thick corrosion product layer (marked by the yellow dotted circle) at the periphery of the residual implant was clearly visible, which was composed mainly of Mg, C and O, with relatively low amounts of Ca, P and Sr (as shown in Table 2). The EDS results showed that the atom ratio O/Mg in the corrosion

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Fig. 6. (a) Mg and Sr concentrations and (b) pH value of as-rolled pure Mg and the various Mg–Sr alloy extracts (Sr content ranges from 1 to 4 wt.%). (c) MG63 cell viability and (d) ALP activity of as-rolled pure Mg and the Mg–Sr alloy extracts. n = 5, *P < 0.05, **P < 0.01.

Fig. 7. 3-D micro-CT topographies of a representative mouse femur with an intramedullary as-rolled Mg–2Sr alloy implant scanned in vivo at different post-implantation time points. The three lines in the first image are the locations used for 2-D cross-sectional imaging of the mouse femur shown in Fig. 8. (a) Complete outline of the distal femur of a mouse; (b) complete outline of the Mg–2Sr alloy implant. Bar 1.0 mm.
product layer was approximately 2.35, indicating that the main corrosion product is Mg(OH)$_2$. Line scanning determined the distribution of elements from newly formed bone to the corrosion product layer (Fig. 13b and c). The area around the as-rolled Mg–2Sr alloy could be divided into three layers according to the distribution of elements: a corrosion product layer, a transition layer and a new bone layer (as marked in Fig. 13b and c). A significant increase in Mg concentration and decrease in Ca and P was observed in the transition layer compared with newly formed bone. Given the relatively low Sr concentration and the limits of resolution of line scanning we could not clearly distinguish the changes in Sr concentration from Fig. 13b and c. Therefore, an EDS point analysis was carried out in different regions, including the residual sample, corrosion product, transition layer, newly formed bone and the control group, as shown in Table 2. It was shown that both the area in the vicinity of the residual implant and the newly formed periosteal bone exhibited higher amounts of Sr, 0.26–0.93 wt.%, in comparison with the bone of the control group (0.07 wt.%). In addition, a low atom ratio Ca/P was found in the new periosteal bone and new peri-implant bone (Ca/P = 1.39–1.58) as well as the control group (Ca/P = 1.57) than in HA (Ca$_{10}$(PO$_4$_3)(OH)$_2$)(Ca/P = 1.67), which might be due to bone remodeling.

4. Discussion

Sr is a known osteopromotive element which can activate osteoblastic cell replication through the calcium sensing receptor (CaSR) and signal-regulated kinase (ERK) 1/2 phosphorylation, inhibit bone resorption by increasing osteoprotegerin (OPG) and decreasing receptor activator of nuclear factor kappa B ligand (RANKL) expression by osteoblasts [18,20,33]. Hereafter whether biodegradable and osteopromotive binary Mg–Sr alloys meet the requirements of applicable biodegradable orthopedic implants, or not, and what is the optimal amount of Sr added with respect to the mechanical properties and corrosion resistance, as well as the resulting in vitro and in vivo biological responses, will be discussed.

4.1. The optimal amount of Sr in Mg–Sr binary alloys

An increase in strength (YTS and UTS) occurred on increasing the amount of Sr from 0 to 2 wt.%, [28,34,35]. With increasing amounts of Sr, the grain size of as-cast Mg–Sr alloys was significantly reduced, mainly due to the growth restriction factor (GRF) mechanism. Since the solubility of Sr in α-Mg is relatively limited...
Sr will be rapidly enriched in the liquid ahead of the growing interface, thus restricting grain growth during solidification [28,34]. According to the Hall–Petch law the strength of Mg is grain size sensitive, i.e. finer grains will lead to a higher strength. Fan [11] and Zeng [35] also found that an increase in Sr addition resulted in a decrease in grain size and thus increasing strength of the alloys AZ91D and AZ31. However, the increase in strength does not improve the elongation to fracture. The formation of Mg17Sr2 intermetallic phases can be a crack source with a harmful effect on the ductility of Mg alloys. Additionally, when the Sr content increased from 3 to 4 wt.%, the grain size is not obviously refined while the volume fraction of Mg17Sr2 phase increases, which may be the reason for the dramatic decrease in strength and elongation to fracture of the alloys. From the viewpoint of the mechanical properties, the optimal amount of Sr in Mg–Sr alloys is 2 wt.%.

The determination of corrosion rates from hydrogen evolution, weight loss and potentiodynamic polarization measurements

Fig. 10. Representative histology of the cross-sections of the mouse distal femora (metaphysical region) from (a) a negative control without implantation and from (c–d) the as-rolled Mg–2Sr alloy group at week 4 after operation. (b) The outline of a bone tunnel (red dotted circle) could be observed with the Mg–2Sr implant (red arrow). The bone volume around the bone tunnel was more than that in the negative group. (c) A magnified image of (b). The outline of the Mg–2Sr alloy implant (red dotted circle) is clear. The degraded material at the edge of the implant dispersed into the bone tunnel (orange triangle) and new bone was formed adjacent to the implant (blue arrows). (d) A magnified image of the green frame in (c). The degraded material dispersed into the surrounding bone and was distributed directly next to it (yellow dots).

Fig. 11. Representative histology of cross-sections of the mouse femoral shaft from both the control group and the as-rolled Mg–2Sr alloy group (a, b) stained with Stevenel’s blue and Van Gieson’s picro fuchsin staining and (c, d) observed by fluorescence microscopy. (a) Normal cortical bone and bone marrow were observed in the control group, while (b) thicker cortical bone was observed in the as-rolled Mg–2Sr alloy group, with dispersed implant debris at the edge of the tunnel (blue arrow) and some fibrous tissue (red arrow) around the implant. The density and the area of fluorescence (red arrows) at the periosteum in the Mg–2Sr alloy group (d) were higher than those in control group (c) and fluorescence at the endosteum (blue arrow) was also found in the Mg–2Sr alloy group. (e) A magnified image of (c). New bone formation was observed around the implants (red arrows).
show similar trends with respect to the amount of Sr added to binary Mg–Sr alloys. With addition of 0–2 wt.% Sr both the corrosion resistance and uniformity of corrosion were improved (as shown in Figs. 4 and 5), which could be attributed to the reduced micro-shrinkage porosity as well as reduced grain size by Sr addition [37]. Micro-pores in Mg alloys reduce their corrosion resistance in two ways: (1) a high porosity density results in a greater exposed area; (2) micro-pores are easily blocked by the corrosion products, which will form an auto-catalytic corrosion cell inside the pores leading to severe localized corrosion [38]. With Sr addition greater than 3 wt.%, the corrosion rate of the as-rolled Mg–Sr alloys increased due to the increased formation of galvanic couples between the α-Mg and Mg17Sr2 phases. In our study of the Mg–4Sr alloy in the as-cast state we observed α-Mg in the eutectic structure completely dissolved in the Mg17Sr2 phases at the grain boundary on the surface of the sample after 12 h immersion in Hank’s solution (as shown in Supplementary Fig. 1). Although no standard electrode potential of the Mg17Sr2 phase is available, it was reasonable to conclude from the above experimental results that the Mg17Sr2 phase was more stable than α-Mg and should have a higher corrosion potential than α-Mg. Therefore, when Mg–Sr alloys are exposed in solution, the internal second phase Mg17Sr2 acts as the cathode and the anodic dissolution of Mg occurs. Since an increase in Mg17Sr2 would lead to an increase in the area ratio of cathode to anode, this could explain why the corrosion rate of the as-rolled Mg–Sr alloys increased with increasing Sr content (2–4 wt.%) [39]. Brail [40] and Niu [41] also found that the corrosion resistance of Mg–Al-based alloys was sensitive to the amount of Sr addition, indicating that appropriate addition of Sr could improve the corrosion resistance of Mg–Al-based alloys, while excessive Sr addition led to an increase in corrosion rate. From the viewpoint of corrosion resistance, the as-rolled Mg–2Sr alloy exhibited the slowest corrosion rate and thus the optimal amount of Sr of binary Mg–Sr alloys is 2 wt.%.  

4.2. Positive cellular and tissue response to the Mg–Sr alloys

In examining the cellular response to the Mg–Sr alloys, the experimental extracts showed an increase in Sr concentration with increasing amount of Sr added up to 2 wt.%, and MG63 cells exhibited significantly increased ALP activity accordingly ($P < 0.05$). These results are in accordance with those of other studies reporting a stimulatory effect of Sr on osteoblastic cell behavior and a dose-dependent effect on osteoblasts [18,19]. Sr-HA nanocrystals increased the ALP activity of MG63 cells with increasing Sr concentration in the range 3–7 at.% [42]. Qiu et al. [43] reported that the optimal Sr concentration in calcium polyphosphate scaffolds incorporating Sr is 1%. Below 1% Sr incorporation, increasing cell viability and ALP activity of ROS17/2.8 cells was found, while these values decreased when higher concentrations of Sr were incorporated. In the present study the concentrations of Sr in the as-rolled Mg–1Sr and Mg–2Sr alloys (0.19–0.38 μg ml$^{-1}$ from Fig. 6a) was even lower than the lower limit (17.5 μg ml$^{-1}$) strontium ranelate concentration stimulating osteoblastic cell proliferation and differentiation [44,45]. This may be due to the co-release of Mg ion from Mg–Sr alloys, which will act in conjunction with Sr ions, decreasing the limit value. Park et al. [44] also reported that a relatively low concentration of Sr ions released from a Ti64/Sr surface (103–135 ng ml$^{-1}$) enhanced osteoblastic cell differentiation. However, further investigation is needed to explore whether and how the presence of Mg ions act in conjunction with Sr ions. Additionally, the inhibitory effect of Sr on osteoclast proliferation will be helpful in preventing bone resorption [42].

In the in vivo tests we observed a significantly higher cortical bone thickness and bone mineral density, as well as newly formed endosteal bone, around the as-rolled Mg–2Sr alloy compared with the control. First, this might be attributed to the release of Mg ions. The EDS line scan in Fig. 13 demonstrates the release of Mg ions as a gradient distribution between the implant and new bone. Witte et al. [5] indicated that a high Mg concentration could lead to bone resorption. Our previous work [2] found increased activity of osteoblasts and osteocytes around Mg–1Ca pins. Second, it could be assumed that corrosion of the as-rolled Mg–2Sr alloy increased the local pH value around the rod, providing a favorable environment for mineralization [46]. Bartsch [47] demonstrated localized alkalization around a Mg implant within muscle. The simultaneous presence of P and Ca in the corrosion product and transition layer, indicating the beginning of bone formation, could contribute to
this assumption to some extent [48,49]. Third, locally released Sr ions might have a more direct effect on implant osseointegration than systemic administered Sr and avoid potential adverse reactions. The assumption was that Sr, sharing the same physiological pathway as Ca, can be deposited and substitute for Ca in the mineral structure of bone, especially in regions of high metabolic turnover (i.e. newly formed bone) [18,33,50]. In the present study we observed that Sr was present at higher concentrations in newly formed peri-implant and periosteal bone than in the non-implanted control group (Table 2), suggesting that Sr released from the as-rolled Mg–2Sr alloy implant might be deposited in new bone. Moreover, as the locally released Sr doses (approximately 0.016 mM kg\(^{-1}\) Sr would be released after total degradation of the as-rolled Mg–2Sr alloy rod) is well below the oral administration threshold dose for mice (4 mM kg\(^{-1}\) day\(^{-1}\)) and the therapeutic Sr dose (2.4–8.75 mM kg\(^{-1}\) day\(^{-1}\) [15]) for human the safety of Sr should not be a question. The stable Sr serum level during the 4 weeks of implantation and the bone reaction also demonstrate the safety of Sr release from the as-rolled Mg–2Sr alloy implant. However, before Mg–Sr-based alloys can be applied as biomedical
implants, further studies are needed to demonstrate an absence of systemic toxicity (e.g. organ pathology), especially after long-term implantation.

4.3. Comparison with other binary Mg alloy systems

Table 3 presents the mechanical and corrosion properties of the as-rolled Mg–Sr alloy in comparison with previously reported binary Mg alloys for biomedical applications (including Mg–Y [51], Mg–Gd [52], Mg–Ca [2,49] and Mg–Zn [7] alloys). The as-rolled Mg–2Sr alloy exhibits comparable YTS and UTS values to as-extruded Mg–0.8Ca and Mg–6Zn, however, with a low elongation to fracture. This may be attributed to the rolling procedure and the machining direction (parallel to the rolling direction). It is known that a strong texture is readily formed during rolling [53], usually leading to poor ductility of Mg alloys. Our previous work [2] showed that as-rolled Mg–1Ca exhibited an elongation to fracture value one quarter of that of as-extruded Mg–1Ca. On the other hand, rolled sheets normally exhibit strong mechanical anisotropy, for instance AZ31 machined along the transverse direction (TD) exhibited higher strength and almost twice the elongation to fracture than AZ31 machined along the rolling direction (RD) [54]. Kaiser et al. [54] assumed that the RD AZ31 exhibited a higher probability of the activation of slip systems according to the Schmid factor than transverse direction. Therefore, improved strength and elongation to fracture can be expected on changing the machining direction for the as-rolled Mg–2Sr alloy samples.

The 3-D micro-CT reconstruction shows that the as-rolled Mg–2Sr alloy implants exhibited faster corrosion in the distal femur than in the bone marrow cavity of the diaphyseal region, which might be due to the rich blood supply in the trabecular bone region. Erdmann et al. [55] also reported a higher corrosion rate of an Mg–0.8Ca alloy in close contact with blood vessels and body fluid than in cortical bone. Xu et al. [6] reported that Mg–Mn–Zn was degraded more rapidly in the medullary cavity than in cortical bone, due to the large amount of body fluid within bone marrow. The in vivo corrosion rate of the as-rolled Mg–2Sr alloy was calculated based on the volume change measured by micro-CT. From a comparison with other binary Mg alloys (Table 3) it can be seen that only as-extruded Mg–0.8Ca exhibited a slower in vivo corrosion rate than the as-rolled Mg–2Sr alloy, and the as-rolled Mg–2Sr alloy had a comparable or better corrosion resistance than other binary Mg alloy systems. However, the binary Mg–2Sr alloy system may not satisfy the requirements for practical usage and further investigation of strategies to improve the mechanical properties and slow down the corrosion rate are needed, e.g. alloying, heat treatment or coating.

5. Conclusions

This is the first study to develop binary Mg–Sr alloy systems in the as-rolled state as biodegradable materials for skeletal applications. The optimal Sr content was 2 wt.%. Sr concentrations below 2 wt.% improved the strength and corrosion resistance of Mg, whereas excessive Sr addition resulted in poorer mechanical properties as well as increased the corrosion rate of the as-rolled Mg–Sr alloys. The in vitro cell experiment indicated that as-rolled Mg–2Sr alloy exhibited Grade I cytotoxicity and increased ALP activity. The in vivo results showed that the degrading as-rolled Mg–2Sr alloy promoted bone mineralization and peri-implant new bone formation without inducing any significant adverse effects. Therefore, the as-rolled Mg–2Sr alloy is the most promising material considering the preliminary results for different Mg–Sr alloys (with Sr contents ranging from 1 to 4 wt.%). Further research will be needed before Mg–Sr–based alloys can be applied as orthopedic implants, including demonstrating the absence of systemic toxicity (e.g. organ pathology), especially after long-term implantation, and improving the mechanical properties and further reducing the corrosion rate.
Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1–7 and 9–13, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.actbio.2012.02.018.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2012.02.018.

References


