A pH-sensitive self-healing coating for biodegradable magnesium implants

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ABSTRACT

Self-healing coatings have attracted attention on surface modification of magnesium alloys, as it can recover the barrier ability of the coatings from corrosion attack. Nevertheless, previous works on this aspect are not suitable for biomedical magnesium alloys owing to the lack of biocompatibility. In this study, we fabricated a self-healing coating on biomedical Mg-1Ca alloy by compositing silk fibroin and K3PO4. PO43−/Co2+ ions act as corrosion inhibitor, while K+ ions help to regulate the secondary structures of silk fibroin. The scratch test, scanning vibrating electrode technique (SVET), and electrochemical impedance spectroscopy (EIS) provide comprehensive results, confirming the pH-sensitive self-healing capacity of the composite coating. Moreover, cells’ (MC3T3-E1) multiple responses including spreading, adhesion, proliferation, and differentiation illustrate the preferable biocompatibility as well as the osteogenic activity of the coating. These primary findings might open new opportunities in the exploration of self-healing coatings on biomedical magnesium alloys.

Statement of significance

Biomedical magnesium alloys surface modifications have been studied for years, which however the biomedical self-healing coatings were rarely involved. In this work, silk fibroin and phosphate (K3PO4) were composited to fabricate coating on biomedical magnesium alloys. The coating not only owned the self-healing ability with pH sensitivity, but also endowed the substrate preferable corrosion resistance as well as osteogenic activity. This work gives a new insight into surface modification for biomedical Mg alloys.

1. Introduction

Recently, the temporary implant concept of magnesium (Mg) and its alloys has attracted increasing attention on orthopedics of special clinical applications, but the speedy corrosion rate still limits their practical implementation [1,2]. Rapid degradation in physiological ambient can lead to absence of mechanical integrity, local alkalosis, and release of copious hydrogen [3,4]. Surface modification is a well-known technology to efficiently improve the corrosion resistance of Mg alloys [5,6]. Presently, various surface modification methods have been developed, such as phosphate coating [7,8], microarc oxidation (MAO) [9,10], fluoride conversion film [11,12], and polymer coatings [13–15]. These methods mainly improve the corrosion resistance of Mg alloys by playing the role of barriers to corrosion electrolyte.

Self-healing coatings have been studied in the industry in recent years [16]. For these coatings, once the defects appear on the surface and the protective barrier is destroyed, they can recover the protection without external help. The self-healing coatings are constructed by two parts, namely, skeleton coatings derived from polymers such as PSS [17], polyester [18], and PEI
and corrosion inhibitors such as Ce$^{2+}$ ion [20], 8-hydroxyquinoline [21,22], and benzotriazole [23,24]. These methods are efficient for industrial magnesium alloys but not suitable for biomedical materials, as both skeleton coatings and corrosion inhibitors lack biosafety and biocompatibility.

Silk fibroin is a typical natural protein extracted from the silk-worm moth Bombyx mori [25]. The main elements of silk fibroin are repeats of the GAGAGS motif, composed of a heavy chain (325 kDa) and a light chain (25 kDa), which are connected by a disulfide bond and complexed by a small glycoprotein, P25 (30 kDa) [26]. The most significant feature of silk fibroin is the controllable secondary structures. Generally, the secondary structures of silk fibroin contain random coils, $\alpha$-helices, $\beta$-sheets, and $\beta$-turns, which can be adjusted by metal ions [27], solvent [28], post-treatment [29], shearing force [30], etc. The proportion of various secondary structures is related to mechanical properties, stability, wettability, degradability, and biocompatibility, which are key factors for silk fibroin in biomaterial applications [31]. Furthermore, silk fibroin has been investigated for the loading capacity, especially for drugs [32,33], hydroxyapatite [34,35], and BMP-2 [36,37]. Silk fibroin has limited inflammatory responses when compared with other polymers evaluated in biomaterials such as PLA, PGA, and PLGA, which degrade into acid monomers [38].

In the present work, inspired by the self-healing coatings on industrial magnesium alloys, we establish self-healing coating on biomedical Mg-1Ca alloy by compositing silk fibroin and K$_3$PO$_4$. Mg-1Ca alloy is a newly designed promising magnesium alloy for bone implants because of the preferable mechanical properties and release of Mg$^{2+}$ and Ca$^{2+}$ ions during degradation that can promote bone healing [39]. However, the corrosion rate of the Mg-1Ca alloy is too rapid for application in the clinic. Silk fibroin acts as the skeleton coating, while the PO$_4^{3-}$ ions serve as the corrosion inhibitor because of the lower solubility of the formed Mg$_6$(PO$_4$)$_3$. Furthermore, silk fibroin is known as an excellent biomaterial with osteogenic activity, and the PO$_4^{3-}$ ion is the inorganic ingredient of bone. Thus, the composite coating is expected to possess self-healing ability as well as osteogenic activity, which can be a new option for surface modification of magnesium alloys.

2. Materials and methods

2.1. Substrate preparation

Mg-1Ca alloy was extruded according to our previous work [40]. The grain size of the Mg-1Ca alloy was approximately 70.5 $\pm$ 1.5 mm, and its roughness was 51.3 $\pm$ 2.5 mm. It was cut into discs ($\Phi$ = 12 mm, h = 2 mm); ground using 2000-grit SiC papers; then ultrasonically washed in acetone, ethanol, and deionized water, respectively; and dried in cold air sequentially.

2.2. Fluoride pretreatment

The fluoride treatment (step 1 in Fig. 1) was processed by two steps. Briefly, the ground samples were immersed in boiling NaOH (5 M) solution for 3 h and then rinsed with deionized water and immersed in HF (40%) solution for 6 h at 60 °C. The as-prepared samples were rinsed and dried at room temperature.

2.3. Self-healing coating formulation

The silk fibroin solution was prepared following the previous protocol [25]. Cocoons were boiled in 0.02 M Na$_2$CO$_3$ aqueous solution for 30 min and then rinsed with DI water to extract sericin. After drying in room temperature overnight, the degummed fibroin was dissolved in 9.3 M LiBr solution for 4 h at 60 °C. Subsequently, the solution was dialyzed against DI water for 3 days. Finally, silk fibroin with a preliminary concentration of approximately 8% (m/v) was obtained.

The obtained silk fibroin solution was diluted, and the K$_3$PO$_4$ solution was added to the silk fibroin. The final concentration of silk fibroin was 2 wt%, and the concentration of K$_3$PO$_4$ was 0.2 M. A spin-coater (Model KW-4A Spin Coater, Sijyouyen", Beijing, China), with a low rotation speed of 500 rpm for 12 s and a high rotation speed of 4000 rpm for 10 s at room temperature, was employed to fabricate the coating by the spin-coating method. Fifty microliters of the solution was added to the specimen for a cycle, and this was repeated five times for each side of the sample. The target coatings were formed by three silk fibroin and K$_3$PO$_4$ composite coatings and two pure silk fibroin coatings, and the coated samples were denoted as "Silk-KP" (shown in Fig. 1 Steps 2 and 3). The pure silk fibroin coated samples were used as the control and denoted as "Pure Silk" (Fig. 1 Step 2'). The fabrication process is illustrated in Fig. 1.

2.4. Coating characterization

To evaluate the structure of K$_3$PO$_4$-treated silk fibroin, the surface coating obtained in Step 2 (denoted as "Silk-K$_3$PO$_4$ coating") and Step 2' ("Pure Silk" as control) of Fig. 1 was characterized. The chemical composition and states of the designed coatings were determined by X-ray photoelectron spectroscopy (XPS; AXIS Ultra, Kratos) with Al K$_\alpha$ irradiation ($h\nu = 1486.71$ eV). The surface functional groups of the coatings were recognized by Attenuated Total Reflectance-Fourier Transform Infrared analysis (ATR-FTIR, Thermo Fisher Scientific), with spectra recording from 4000 cm$^{-1}$ to 400 cm$^{-1}$. Peak Fit software was used to fit and calculate the secondary structures of silk fibroin.

The Nanoindentor System (Hysitron, USA) was used to measure the adhesion resistance and mechanical properties (hardness, Young’s modulus, and wear resistance). Adhesion resistance was measured by the ramp-load scratch procedure with a diamond cone tip ($r = 1\ \mu$m) under a loading force of 0 $\mu$N to 2000 $\mu$N. Wear resistance was measured by the vertical-load scratch procedure with the diamond cone tip ($r = 1\ \mu$m) under a loading force of 100 $\mu$N. Hardness and Young’s modulus were measured by the vertical-load scratch procedure with a diamond triangular pyramid tip ($r = 50\ \mu$m) poking at a depth of 60 nm. Five duplicates were measured in each test.
2.5. In vitro corrosion evaluation

Hank’s solution (NaCl 8.00 g/L, KCl 0.40 g/L, CaCl₂ 0.14 g/L, NaHCO₃ 0.35 g/L, MgSO₄·7H₂O 0.20 g/L, Na₂HPO₄·12H₂O 0.12 g/L, KH₂PO₄·0.06 g/L, pH = 7.4) was adopted for immersion experiment according to ASTM-G31–72 at 37 °C. The hydrogen evolution volume and pH value of the solution were recorded for 14 days of immersion. After immersion for 7 days, samples in Hank’s solution were taken out, rinsed with deionized water, and air-dried, sequentially. Changes in surface morphologies were assessed by SEM (FE-SEM, S4800, Hitachi), and the main elements of the surface were detected by EDX, XPS, and ATR-FTIR. Three duplicates were taken for each group.

2.6. Self-healing capability

2.6.1. Scratch tests

Artificial scratches were measured using the Nanoindentation System (Hysitron, USA) with a diamond cone tip (radius of 1 μm). To obtain a similar scratch on each sample, the tip was plunged into the surface and loaded with a constant force of 100 μN. The 3-dimensional size of the scratches was recorded and imaged. The samples with the scratches were immersed in Hank’s solution at 37 °C. After immersion for 24 h, the size of the scratches was recorded and imaged again. Furthermore, the elements in scratches after immersion were detected by EDX.

2.6.2. Scanning vibrating electrode technique (SVET) measurements

SVET measurements were made on M370 Scanning Electrochemical Workstation (Ametek, USA). Silk and Silk-KP samples with artificial defects were fixed on an epoxy resin holder and immersed in Hank’s solution. The measurement was controlled using ASET software, and the system recorded the real-time vector current density of the assigned surface. The Pt-blackened electrode tip (15 μm diameter) was situated on the tested surface at a distance of 100 ± 2 μm. The electric current densities of the exposed area (1.5 × 1.5 mm²) were scanned with a step size of 60 μm on a lattice of 25 × 25 points and presented in the form of 3D maps. Scans were performed after 40 min of immersion and automatically collected every 40 min during the experiments. The samples were monitored for 3 h.

2.6.3. Electrochemical impedance spectroscopy (EIS) measurements

The EIS measurement was performed using an electrochemistry workstation (PGSTAT 302N, Metrohm Autolab) connected to a standard three-electrode cell with a saturated calomel electrode (SCE) as the reference electrode and a platinum piece as the counter electrode in Hank’s solution at 37 °C. The samples immersed in Hank’s solution at various time points served as the working electrode, and the area exposed to the electrolyte was 0.20 cm². The study was conducted at OCP in the frequency range of 100 mHz to 100 kHz. The EIS plots that were generated were analyzed using Nova 2.1 software, and they were best-fitted to the appropriate equivalent circuit (EC) models. An average of three measurements was taken for each group.

2.6.4. pH stimuli-responsive ability

To evaluate the effect of the pH value on the designed coating, the Silk-KP was coated onto glasses (the same size as that of the Mg-1Ca alloy sample) by the same method to exclude the impact of the substrate. DI water of pH values 7.4 and 10.0, which were adjusted by NaOH (0.1 mol/L), was employed as the immersion medium to exclude the influence of other substances. Samples were immersed in the as-prepared DI water with a solution volume-to-surface area of the sample ratio of 20 ml/cm². At predetermined time points, the entire volume of the solution was collected and refilled with fresh DI water of pH values 7.4 and 10.0. The concentration of the element P (derived from PO₄³⁻) in the solution was detected by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7700X, USA). An average of at least five parallel samples was taken for each group.

2.7. Cytocompatibility and osteogenic activity

2.7.1. Cell culture and seeding

For in vitro biocompatibility measurements, mouse osteoblast-like cells (MC3T3-E1) were adopted and cultured in α-MEM (minimum essential medium alpha, HyClone, Beijing) containing 10% FBS (fetal bovine serum, HyClone, Beijing) and 1% penicillin and streptomycin (HyClone, Beijing) in a humidified incubator with 5% CO₂ at 37 °C. The contents of α-MEM are listed in Table S1. The extracts of each sample were obtained following the instruction of ISO10993-12. For all cell response experiments, α-MEM alone containing 10% FBS was added per well into TCPS as the control. The culture medium was refreshed every two days during the culture period. Each group had at least five duplicate wells in each evaluation.

2.7.2. Cell spreading morphology

Cells were cultured in extracts or on the samples directly for 6 h and 24 h, respectively. At both these time points, on the one hand, the cells were sequentially rinsed with PBS, fixed using 4% (w/v) paraformaldehyde for 10 min, permeabilized with 0.1% (v/v) Triton X-100 (Sigma) for 10 min, stained with 1.0% (v/v) FITC-phalloidin (Sigma) for 30 min and 1 mg/mL DAPI (Sigma) for 5 min, and imaged using an LSCM (using a laser scanning confocal microscope, Nikon ALR-SI; identical apparatus throughout the experiments); on the other hand, the samples were rinsed with PBS, fixed with 2.5% glutaraldehyde solution for 2 h at room temperature, dehydrated in a gradient ethanol/DI water (ethanol concentrations of 50%, 60%, 70%, 80%, 90%, 95%, and 100%), and observed using an SEM (FE-SEM, S4800, Hitachi).

2.7.3. Cell viability

A mitochondrial activity-based cell counting kit (CCK-8, Dojindo, Japan) was adopted to determine cell viability. Cells were cultured for 1, 3, and 5 days in extracts for measurements, and the α-MEM with 10% FBS was used as the negative control, while α-MEM containing 10% DMSO (dimethyl sulfoxide) was used as the positive control. Cells were cultured in the 96-well plate, and 5 duplicates were taken for each sample. At scheduled time points, 10% CCK-8 solution was added to the medium and incubated for another 2 h to generate formazan. The OD values ([A]) at 450 nm were read on a microplate spectrophotometer (Bio-Rad, USA). Cell viability was calculated using the formula

\[
\%\text{cell viability} = \frac{[A]\text{Sample} - [A]\text{Negative}}{[A]\text{Positive}} \times 100\%
\]

2.7.4. Osteogenic differentiation studies

ALP activity was measured by the colorimetric production of p-nitrophenol (p-NP) through p-nitrophosphine (p-NPP)/endogenous ALP enzymatic reaction (Jiancheng, Nanjing, China). After culturing for 7 days and 14 days, the cells were lysed in 1% Triton X-100 for 1 h. Then, 30 μL of the cell lysates was cultured with carbonate buffer solution (50 μL) and substrate solution (4-aminooantipyrine, 50 μL) for 15 min at 37 °C. Afterwards, a chromogenic agent (potassium ferricyanide, 150 μL) was added into the mixed solution. The absorbance of the mixed solution was read at 520 nm using the microplate spectrophotometer (Bio-Rad, USA). Moreover, the ALP activity was normalized using the Micro BCA
Hank’s solution at various time points served as the working electrode in Hank’s solution at 37 °C (SCE) as the reference electrode and a platinum piece as the counter electrode. The workstation (PGSTAT 302N, Metrohm Autolab) connected to a computer was used to collect data every 40 min during the experiments. The samples were immersed in as-prepared DI water with a solution adjusted by NaOH (0.1 mol/L), which was employed as the immersion medium for 10 days. A Mg-1Ca alloy sample was immersed in the mixed solution. The absorbance of the mixed solution was then measured.

For collagen secretion and ECM mineralization assays, cells that were cultured for 28 days were rinsed with PBS and fixed in 4% PFA for 15 min. For staining, the cells were stained in Sirius Red (SR, 0.1%, Sigma) overnight or Alizarin Red S (ARS, Sigma, 2%, pH = 4.3) for 10 min at 4 °C and washed until no more color appeared. Optical images for each well were acquired using an optical microscope with a camera. Moreover, the stain was eluted in 200 µL of 50% 0.2 M NaOH/methanol solution or 10% cetylpyridinium chloride, and the absorbance of the resulting solution was measured at 570 nm for quantification.

2.8. Statistical analysis

Quantitative data, presented as means ± standard deviations, were obtained for n ≥ 3. Statistical analysis was performed using...
one-way analysis of variance (ANOVA), and the significance was regarded at p value < 0.05.

3. Results

3.1. Chemical composition of the surface coating

The chemical and structural changes after K$_3$PO$_4$ was added to the silk fibroin were determined by XPS and ATR-FTIR. As shown in Fig. 2 (A1-A2), the survey spectrum of XPS results showed that the main elements on the surfaces of both the Silk-K$_3$PO$_4$ coating and the Silk were C, N, and O, which were the main elements of silk fibroin. Additionally, the P element was detected on the Silk-K$_3$PO$_4$ coating, which confirmed the addition of K$_3$PO$_4$. Furthermore, the C1s spectrum for the Silk-K$_3$PO$_4$ coating (Fig. 2 (A3)) showed characteristic peaks at 285 (C–C), 286 (C–H), and 288 (C@O), corresponding to different chemical environments of silk fibroin [41,42].

As shown in Fig. 2(B1), the functional groups of both the Silk-K$_3$PO$_4$ coating and the Silk were detected by FTIR, and two peaks at 1655 cm$^{-1}$ and 1540 cm$^{-1}$ were found for these two samples, respectively. It was attributed to the amide I (N–H deformation) and amide II (C–N stretching) [43], respectively, which were characteristic peaks of silk fibroin. Moreover, the peaks at 1065 cm$^{-1}$, 982 cm$^{-1}$, and 824 cm$^{-1}$ were assigned to $v_3$ vibration peak of PO$_4^{3-}$ [44], which was obtained for the Silk-K$_3$PO$_4$ coating only. To further realize the secondary structural changes of silk fibroin after K$_3$PO$_4$ was added to the silk fibroin, the amide I region (1590–1700 cm$^{-1}$) was further characterized by deconvolution of the ATR-FTIR spectra. The proportion of the main secondary structures was calculated, and the data are given in Table 1. The content of $\beta$-sheets was more than 30% in both the Silk and Silk-K$_3$PO$_4$ coating, suggesting water insolubility [45]. Notably, random coils transformed into silk I ($\beta$-turns) when K$_3$PO$_4$ was added to the silk fibroin.

3.2. Adhesion and mechanical property

Nano-indentation is widely used to measure the surface mechanical properties [46,47]. The results of the adhesion resistance are shown in Fig. 3 (A-C). In the loading procedure, the first plateau of the vertical displacement was at approximately 260 nm, which implied that the tip pierced the Silk-KP coating, according to the thickness of the Silk-KP coating (Fig. S1). At that point, the loading time was 19 s, and its corresponding lateral force was 820 mN. This lateral force was the indicator of adhesion resistance and much higher than that of the other polymer coatings on magnesium alloys [46], inferring preferable adhesion of the Silk-KP coating. The hardness, Young’s modulus, and friction coefficient are

<table>
<thead>
<tr>
<th>Assignments</th>
<th>Silk</th>
<th>Silk-K$_3$PO$_4$ coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Side chains (1590–1605 cm$^{-1}$)</td>
<td>0.6% ± 0.1</td>
<td>1.0% ± 0.1</td>
</tr>
<tr>
<td>Silk II, $\beta$-sheets (1610–1635 cm$^{-1}$)</td>
<td>32.8% ± 0.2</td>
<td>33.9% ± 0.3</td>
</tr>
<tr>
<td>Random coils (1635–1645 cm$^{-1}$)</td>
<td>15.9% ± 0.3</td>
<td>10.1% ± 0.1</td>
</tr>
<tr>
<td>Silk I, type II $\beta$-turns (1647–1654 cm$^{-1}$)</td>
<td>15.8% ± 0.3</td>
<td>22.4% ± 0.2</td>
</tr>
<tr>
<td>$\alpha$-Helices (1658–1664 cm$^{-1}$)</td>
<td>13.9% ± 0.2</td>
<td>10.2% ± 0.4</td>
</tr>
<tr>
<td>Turns and bends (1666–1695 cm$^{-1}$)</td>
<td>21.0% ± 0.3</td>
<td>22.4% ± 0.2</td>
</tr>
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</table>

Fig. 3. (A) Vertical displacement and (B) Lateral force changed with time during the nano-scratch resistance, (C) deformation morphology after nano-scratch, and (D) mechanical property parameters of the Silk-KP (n = 5).
The characteristic peaks at 285 (C–C), 286 (C–H), and 288 (C–S) correspond to different chemical environments of silk fibroin [41,42].

Fig. 3. (A) Vertical displacement and (B) Lateral force changed with time during the nano-scratch resistance, (C) deformation morphology after nano-scratch, and (D) optical images of the tip penetration into the coatings.

EDX results of each group immersion in Hank's solution for 7 days.

<table>
<thead>
<tr>
<th>Atom</th>
<th>Mg-1Ca Alloy</th>
<th>Silk</th>
<th>Silk-KP</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>–</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>O</td>
<td>59</td>
<td>21</td>
<td>30</td>
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<td>Ca</td>
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<tr>
<td>F</td>
<td>–</td>
<td>22</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2

Table 3

<table>
<thead>
<tr>
<th>Atom</th>
<th>Mg-1Ca alloy</th>
<th>Silk</th>
<th>Silk-KP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ length (μm)</td>
<td>+0.15</td>
<td>-0.30</td>
<td>-1.47</td>
</tr>
<tr>
<td>Δ width (μm)</td>
<td>-0.40</td>
<td>+0.07</td>
<td>-0.47</td>
</tr>
<tr>
<td>Δ depth (μm)</td>
<td>+0.27</td>
<td>+0.14</td>
<td>-0.87</td>
</tr>
</tbody>
</table>

Table 3

Changes in the length, width, and depth of the scratches on the surface of the Silk and Silk-KP immersion in Hank’s solution for 24 h; the “+” represents increase, while the “-” represents decrease.

Fig. 4. Variation of (A) hydrogen evolution volume and (B) pH values, (C) surface morphologies of each group immersed in Hank’s solution for 7 days, (D) ATR-FTIR spectra and XPS survey spectra of samples after immersion in Hank’s solution for 7 days, and (E) XPS co-responding core-level spectra for C 1s and P 2p of the Silk-KP after 7 days of immersion in Hank’s solution (n = 3).

Corrosion of magnesium alloys in the physiological environment was accompanied by hydrogen release and pH value increase. The corrosion process occurred as per the following equation:

\[ \text{Mg} + 2\text{H}_2\text{O} \rightarrow \text{Mg}^{2+} + 2\text{OH}^- + \text{H}_2 \uparrow \]

Thus, the hydrogen release volume and pH value changes reflected the corrosion status of each group during 14-day immersion in Hank’s solution. As shown in Fig. 4(A, B), the hydrogen release of the Silk-KP was an average of 1.19 ml/cm², and the pH value was increased to 8.73 at 14 days. However, the bare Mg-1Ca alloy showed a quick and copious accumulation of hydrogen...
evolved with time (5.75 ml/cm² at 14 days), and the pH value was higher than 9 in the first three days and reached to 10.13 at 14 days.

The surface morphologies of experimental samples after 7-day immersion in Hank’s solution were studied by SEM, as shown in Fig. 4(C). The Silk and the Silk-KP groups showed uniform surface morphologies with a few corrosion products on the surface. After removing from Hank’s solution and air-drying, cracks appeared; the cracks on the Silk group were wider than those on the Silk-KP group. On the other hand, the bare Mg-1Ca alloy was covered with thick corrosion products.

The surface elements (orange block region framed in Fig. 4(C)) were detected by EDX, and the results are given in Table 2. The surface of the Silk-KP had more P and C elements but fewer Mg and F elements. Furthermore, the corrosion products were analyzed by ATR-FTIR and XPS. As displayed in Fig. 4(D), the characteristic peaks of CO₃²⁻, PO₄³⁻, and HPO₄²⁻ appeared in the infrared spectra, implying mainly corrosion products of carbonate, phosphate, and hydrophosphate. Notably, the characteristic peaks of amide were detected (marked in red circle) in the Silk-KP group. XPS results revealed that the main elements on the surface were Mg, O, P, C, and N, which only appeared on the surface of the Silk and Silk-KP, thereby confirming the existence of the silk fibroin coating. The C1s spectrum of the Silk-KP showed characteristic peaks of silk fibroin (C–C, C–H, and C=O) and carbonate. The P 2p spectrum was a single peak, implying PO₄³⁻. Overall, Silk-PK
were detected by EDX, and the results are given in Table 2. The corrosion of the bare Mg-1Ca alloy was covered with thick corrosion products. On the other hand, the bare Mg-1Ca alloy was covered with thick corrosion products.

The cracks on the Silk group were wider than those on the Silk-KP. After immersion for 120 min, both the anodic and cathodic current density verged to zero plane (Fig. 6), verifying that the designed self-healing function operated normally. The maps of the Silk-KP group were similar to those given in the previous reports of self-healing coatings on metals [49]. However, during immersion, the Silk group sustained a negative peak evolution, representing significant cathodic current density. It was illustrated that the material chose anodic sacrifice protection to prevent further corrosion [50]. Furthermore, when immersion for 160 min, the Silk demonstrated a deeper cathodic current peak, signifying more serious corrosion on the defect region.

The EIS measurement was a nondestructive and powerful technique for the assessment of corrosion resistance properties. The Nyquist plots and Bode magnitude plots of the Silk and Silk-KP groups in Hank’s solution with different immersion time points are presented in Fig. 7 (A for the Silk group, and B for the Silk-KP group). To further interpret the EIS results, the equivalent electrical circuit (EEC) models (Fig. 7 (A) and (B)) were proposed for fitting the data. Constant phase element (CPE) was used in the fitting procedure, and Rs was the solution resistance. The EEC model for the Silk-KP group included two parameters and expressed as Rs(Qdl(QbRbRct)). The Silk group displayed one more inductive semicircle arc at low frequency, and the EC was denoted as Rs(QdlQbRsRct). Based on the circuits, the double-layer capacitance (Qdl) and Rs can be determined at high frequency. The capacitive (Qb) and resistance (Rs) were indicative of the coatings on the surface of Mg-1Ca alloy. The pseudo-inductive loop at low frequency appearing in the Silk was related to two parameters, Rs and L, which implied that the dissolution and pitting corrosion occurred during immersion [51]. Moreover, polarization resistance (Rp) was defined as the difference between the real impedance of the Nyquist plot. While the frequency was equal to zero with solution resistance (Rs), Rp was proportional to the corrosion resistance [52] and could be

3.4. Self-healing capacity

Artificial scratch was made on the bare Mg-1Ca alloy, the Silk, and the Silk-KP samples using a Nanoindenter System. The depth, width, and length of the resulting scratch were measured and compared with values before and after immersion in Hank’s solution for 24 h. The changes occurring in the 3-dimensions were analyzed using supporting software, which are given in Table 3. As shown in the images (Fig. 5), the scratch on the Silk-KP sample was shorter and narrower. The measurement of 3-dimensional size also confirmed the healing of the crack on the Silk-KP. By EDX analysis at the position of scratches after immersion, it was revealed the scratch region of the Silk-KP was covered with more phosphate than the top portion of the Silk because of the higher P element concentration of the Silk-KP than that of the Silk.

Scanning vibrating electrode technique (SVET), which serves as a nondestructive tool to visualize current densities and accurately express the localized cathodic and anodic activities, is employed to further evaluate the self-healing ability of the experimental samples in the electrolyte solution. The artificial dot defects were approximately 200 ± 5 μm in diameter and depth. While immersion in Hank’s solution, the evolution of ionic flux signals around artificial defects was recorded and transformed to current density data, which are presented in Fig. 6 as 3D maps. For the Silk-KP group, after immersion for 40 min, the anodic current peak appeared, thus implying that the corrosion occurred around the defects. While immersion for 80 min, the anodic current peak increased, illustrating progressive propagation of localized corrosion. After immersion for 120 min, both the anodic and cathodic current density verged to zero plane (Fig. 6), verifying that the designed self-healing function operated normally. The maps of the Silk-KP group were similar to those given in the previous reports of self-healing coatings on metals [49]. However, during immersion, the Silk group sustained a negative peak evolution, representing significant cathodic current density. It was illustrated that the material chose anodic sacrifice protection to prevent further corrosion [50]. Furthermore, when immersion for 160 min, the Silk demonstrated a deeper cathodic current peak, signifying more serious corrosion on the defect region.

The EIS measurement was a nondestructive and powerful technique for the assessment of corrosion resistance properties. The Nyquist plots and Bode magnitude plots of the Silk and Silk-KP groups in Hank’s solution with different immersion time points are presented in Fig. 7 (A for the Silk group, and B for the Silk-KP group). To further interpret the EIS results, the equivalent electrical circuit (EEC) models (Fig. 7 (A) and (B)) were proposed for fitting the data. Constant phase element (CPE) was used in the fitting procedure, and Rs was the solution resistance. The EEC model for the Silk-KP group included two parameters and expressed as Rs(Qdl(QbRbRct)). The Silk group displayed one more inductive semicircle arc at low frequency, and the EC was denoted as Rs(QdlQbRsRct). Based on the circuits, the double-layer capacitance (Qdl) and Rs can be determined at high frequency. The capacitive (Qb) and resistance (Rs) were indicative of the coatings on the surface of Mg-1Ca alloy. The pseudo-inductive loop at low frequency appearing in the Silk was related to two parameters, Rs and L, which implied that the dissolution and pitting corrosion occurred during immersion [51]. Moreover, polarization resistance (Rp) was defined as the difference between the real impedance of the Nyquist plot. While the frequency was equal to zero with solution resistance (Rs), Rp was proportional to the corrosion resistance [52] and could be

![Fig. 6. SVET 3D maps of the electric current density measured above the defected surface of the Silk (A1-A4) and the Silk-KP (B1-B4).](image-url)
Fig. 7. EIS and the fitted results for the Silk (A1-A4) and the Silk-KP (B1-B4) (1–4: Nyquist plots, Bode plots of |Z| vs. frequency, Bode plots of phase angle vs. frequency, respectively, and equivalent circuit model immersion in Hank's solution at various time points.)
calculated with the following equations for the corresponding models of the Silk and Silk-KP groups immersed for various time points:

\[
\frac{1}{R_p} = \frac{1}{R_{ct} + \left( R_{ct}^{-1} + R_b^{-1} \right)^{-1}}
\]

and

\[
\frac{1}{R_p} = \frac{1}{R_{ct} + R_b}
\]

\[\text{Fig. 7 (A4):}\]

\[\text{Fig. 7 (B4):}\]

The fitting results of EIS data for the Silk and the Silk-KP immersion in Hank’s solution at various time points are listed in Table 4. It was found that the \( R_p \) of the Silk group decreased with prolonged immersion time, but the \( R_p \) of the Silk-KP group just decreased within immersion for 40–80 min and increased later.

### 3.5. pH Stimuli-Responsive ability

The release profiles of \( \text{PO}_4^{3-} \) are displayed in Fig. 8. While immersion for only 30 min, the Silk-KP immersed in the DI water with a pH of 10.0 released much more \( \text{PO}_4^{3-} \) (55 ± 2 \( \mu \text{g/cm}^2 \), \( n = 5 \)) than that immersed in the neutral solution (18 ± 2 \( \mu \text{g/cm}^2 \), \( n = 5 \)). During the first 6 h, the cumulative release of \( \text{PO}_4^{3-} \) at pH = 10.0 (260 ± 20 \( \mu \text{g/cm}^2 \), \( n = 5 \)) was more than two times of that at pH = 7.4 (107 ± 12 \( \mu \text{g/cm}^2 \), \( n = 5 \)). It was observed that the Silk-KP more rapidly released the \( \text{PO}_4^{3-} \) in an alkaline environment, which was deemed as pH stimuli-responsiveness.

### Table 4

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time (min)</th>
<th>( R_s (\Omega \text{ cm}^2) )</th>
<th>( R_{ct} (\text{k}\Omega \text{ cm}^2) )</th>
<th>( Q_{dl} (\text{mF sn} \cdot \text{cm}^{-2}) )</th>
<th>( n_1 )</th>
<th>( R_0 (\text{k}\Omega \text{ cm}^2) )</th>
<th>( Q_{b} (\text{mF sn} \cdot \text{cm}^{-2}) )</th>
<th>( n_2 )</th>
<th>( R_L (\text{k}\Omega \text{ cm}^2) )</th>
<th>( L (\text{kH cm}^2) )</th>
<th>( R_p (\text{k}\Omega \text{ cm}^2) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silk</td>
<td>40</td>
<td>65.02</td>
<td>56.70</td>
<td>4.49</td>
<td>0.86</td>
<td>10.82</td>
<td>205.77</td>
<td>0.49</td>
<td>26.69</td>
<td>283.67</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>65.04</td>
<td>45.70</td>
<td>4.57</td>
<td>0.86</td>
<td>9.02</td>
<td>228.66</td>
<td>0.50</td>
<td>28.65</td>
<td>298.96</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>69.76</td>
<td>31.39</td>
<td>5.51</td>
<td>0.91</td>
<td>6.26</td>
<td>16.77</td>
<td>0.73</td>
<td>1091.15</td>
<td>1786.86</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>60.81</td>
<td>19.00</td>
<td>10.34</td>
<td>0.77</td>
<td>4.83</td>
<td>9.38</td>
<td>0.92</td>
<td>3563.90</td>
<td>1748.13</td>
<td>24</td>
</tr>
<tr>
<td>Silk-KP</td>
<td>40</td>
<td>140.09</td>
<td>88.44</td>
<td>5.34</td>
<td>0.82</td>
<td>6.77</td>
<td>5.45</td>
<td>0.72</td>
<td>–</td>
<td>–</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>142.42</td>
<td>65.45</td>
<td>5.14</td>
<td>0.83</td>
<td>5.00</td>
<td>5.55</td>
<td>0.79</td>
<td>–</td>
<td>–</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>134.74</td>
<td>160.99</td>
<td>5.21</td>
<td>0.81</td>
<td>14.96</td>
<td>5.22</td>
<td>0.68</td>
<td>–</td>
<td>–</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td>133.36</td>
<td>170.57</td>
<td>5.28</td>
<td>0.81</td>
<td>14.23</td>
<td>5.64</td>
<td>0.32</td>
<td>–</td>
<td>–</td>
<td>176</td>
</tr>
</tbody>
</table>

**Fig. 8.** The release behavior of \( \text{PO}_4^{3-} \): (A) release amount at different time points, (B) cumulative release amount of the Silk-KP-coated glass immersion in DI water with varying pH values for 6 and 12 h (\( n = 5 \), **p < 0.005, ***p < 0.001**).

### 3.6. In vitro biocompatibility and osteogenic activity

#### 3.6.1. Cell attachment

Cell attachment was investigated through F-actin/FAK staining (indirect contact culture) and SEM observation (direct culture), respectively. As displayed in Fig. 9 (A) and (B), more cells and actin filaments were found in cells cultured in the Silk and Silk-KP extracts. Additionally, cells of the Silk and Silk-KP groups exhibited fusiform-spreading morphologies with elongated filopodia (white arrows in Fig. 9 B), presenting tight adhesion to the substrates. For the bare Mg-1Ca alloy, especially for cells directly cultured on the substrate, a few near-round dead cells and cracks were detected on the surface.

#### 3.6.2. Cell cytotoxicity

The CCK-8 cell viability tests were used to evaluate the cytotoxicity of each group for cells being cultured for 1, 3, and 5 days (Fig. 9 (C)). The results were expressed as average activity with standard deviation. At 1 and 3 days, the cell viability of each group showed no significant difference. At 5 days, the cell viability of the Silk-KP group reached 114% of the negative control, revealing that the Silk-KP group was noncytotoxic, and when prolonging the culture time, it might promote cell proliferation.

#### 3.6.3. Osteo-differentiation

ALP activity, as an early hallmark for osteogenic differentiation potential, was stained with BCIP/NBT, as shown in Fig. 10 (A), and was normalized and calculated, as shown in Fig. 10 (B). The ALP activity for all groups increased from 7 days to 14 days. While cultured for 7 days, the ALP activities of all groups showed no statis-
tical difference. Nevertheless, the Silk-KP group showed a higher ALP activity expression while cultured for 14 days. Additionally, the staining results coincided well with the ALP activity results. The Silk-KP group displayed deeper staining feature. The ECM collagen was secreted at a similar level among all experimental groups. Moreover, as for ARS staining of calcium, the Silk and Silk-KP groups demonstrated more locally better stained spots/nodules and higher absorbance in quantitative analysis. It was revealed that both the Silk and Silk-KP groups could facilitate matrix mineralization.

4. Discussion

Drawing inspiration from the self-healing coatings on magnesium alloys in the industrial field, a biocompatible self-healing coating on the biomedical magnesium alloys was developed in this work. Compositing the silk fibroin and K$_3$PO$_4$ inorganic salt to form the self-healing coating could combine loading capacity, biocompatibility, and pH sensitivity from silk fibroin and the insolubility and osteogenesis from phosphate. Hence, it was promising to serve as a self-healing anticorrosion coating on biodegradable magnesium alloys, especially for use within the bone.

The coating composition characteristics confirmed that the designed coating could be coated on the magnesium alloy substrate successfully. Moreover, the structures of the resulting coatings kept stable for a certain period, which was a crucial property for anticorrosion coating on magnesium alloys. The fabrication method of spin-coating and the addition of K$^+$ ions assisted in adjusting the secondary structures of silk fibroin. Previously, the spin-coating method provided the shear force to regulate the content of $\beta$-sheets higher than 30\% [30]. In other works, a silk fibroin film fabricated by casting was soluble (content of $\beta$-sheets below 25\%) [45]. Notably, random coils transformed into silk I ($\beta$-turns), while K$_3$PO$_4$ was encapsulated in silk fibroin. A previous work showed that the K$^+$ ion regulates the secondary structure of silk fibroin [53]. It was suggested that K$^+$ ions, at a high concentration in the silk fibroin solution (higher than 3.7 mg/g), transform random coils to silk I because of the interaction of the K$^+$ ions with carbonyl oxygen atoms from amino acid palindrome sequences (illustrated in Fig. 2(C2)). Moreover, the comprehensive mechanical performance tests displayed that the Silk-KP was robust and had a preferable adhesion to the substrate. These properties are beneficial for the coatings of bone implants. The higher Young’s modulus and preferable adhesion make it possible to be coated on the surface of a variety of shaped substrates, such as screws, plate,
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**Fig. 10.** Osteogenic activity of MC3T3 cultured in different extracts. (A) Optical imaging of the coloration of ALP activity (deep purple) for 7 and 14 days, collagen (COL) secretion (purplish red) for 28 days, and calcium (CAL) deposition (red or purplish red) for 28 days; (B) the degree of ALP activity for 7 and 14 days; (C) quantification of collagen secretion and calcium deposition for 28 days (n = 5, **p < 0.001). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Fig. 11.** Schematic of the self-healing mechanism upon Silk-KP exposure in the immersion environment.
needles, etc. The hardness and wear resistance can avoid physical damage to the coating during the implantation, to some extent. According to the immersion testing results in vitro, the Silk-KP showed preferable corrosion resistance as compared to the bare Mg-1Ca alloy. Although the cracks appeared on the Silk-KP after immersion for 7 days, which might be due to air-drying before SEM observation, the increase in pH value could be kept at a limited range and low hydrogen release volume. It was observed that the coatings could avoid the foremost issues presenting in biomedical magnesium alloys [54]. Moreover, the cracks appearing on the coatings were common for polymer coatings on the magnesium alloys during immersion [55].

Self-healing capacity was one of the main parameters focused in this work, and the self-healing process could be speculated as illustrated in Fig. 11. Once the magnesium alloy was corroded, defects appeared on the surface with increase in local pH value and release of Mg2+ ions (diagramed in Fig. 11(B)), presenting a decrease in corrosion resistance (expression in the increase of anodic current density in SVET and decrease of Rp in EIS results). As described in section 3.5, the Silk-KP could release the PO4−3 ions rapidly in an alkaline environment. This was attributed to the residual random coils and α-helical structures in the Silk-KP, which endowed the structure reversibility of the Silk-KP. While in a microenvironment with high pH, these structures of the silk fibroin were of negative charge and the interactions were disconnected, resulting in a likely rather elongated molecular conformation [56]. Thus, more PO4−3 ions were released in the local defects (illustrated at the bottom of Fig. 11). The released Mg2+ ions would react with PO4−3 ions to yield the product Mg3(PO4)2, as per the equation:

\[ 3\text{Mg}^{2+} + 2\text{PO}_4^{3-} \rightarrow (\text{Mg}_3\text{PO}_4)_2 \downarrow \]

Generally, PO4−3 ions and the Mg3(PO4)2 salt are stable in an alkaline environment, and the solubility product constant of Mg3(PO4)2 is as low as 1.04 × 10−24. Thus, the Mg3(PO4)2 salt could deposit a passive film on the defects, healing the barrier property and preventing further corrosion (Fig. 11(C)). This was demonstrated by the diminished scratch size on the surface, the current densities verged to zero plane in SVET and increased Rp of the EIS.

The cytocompatibility and osteogenic activities were evaluated by the multiple cell responses in vitro. The causes for the improvement of biocompatibility of the Silk-KP could be attributed to the following factors: (1) the improvement in corrosion resistance. Rapid corrosion is accompanied with locally unduly high pH values and Mg2+ concentration, which could destroy the protein-based transport channels and then disabling active ionic transport [57]. (2) Biocompatibility and osteogenic activity of the silk fibroin coating itself. Previous works confirmed the silk fibroin would enhance the expression of ALP and Runx2 mRNA by significantly downregulating Notch signaling [58]. (3) Addition of phosphate. Phosphate was the inorganic ingredient of natural bone. A previous study indicated good osteoinduction ability of phosphate [59].

5. Conclusions

The biocompatible pH-sensitive self-healing anti-corrosive Silk-KP was fabricated on the Mg-1Ca alloy by a convenient method, and it yielded the following advantages over the bare Mg-1Ca alloy.

(1) Corrosion resistance was enhanced by the self-healing function of Silk-KP, and Silk-KP showed more stable structure by compositing silk fibroin and K+ ions.

(2) PO4−3 ions existed in Silk-KP, serving as the corrosion inhibitor, and the scratch tests, SVET, and EIS were performed to confirm the self-healing ability of the Silk-KP coating on top of the bare Mg-1Ca alloy.

(3) Biocompatibility evaluation in vitro revealed the designed Silk-KP not only was biocompatible but also exhibited osteogenic activity.

This work introduces self-healing coating on biodegradable magnesium alloys and provides a new candidate coating for surface modification of biodegradable metals for potential use within the bone.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.actbio.2019.04.045.

References

by the multiple cell responses in vitro demonstrated by the diminished scratch size on the surface, the current charge and the interactions were disconnected, resulting in a likely yield of Fig. 11. The released Mg$^{2+}$ ions would react with PO$_4^{3-}$ as per the equation: 

$$\text{Mg} + 2\text{PO}_4^{3-} \rightarrow \text{Mg}_3(\text{PO}_4)_2$$

SEM observation, the increase in pH value could be kept at a limit to yield the product Mg$_3$(PO$_4$)$_2$, as per the equation:

$$\text{Mg} + 2\text{PO}_4^{3-} \rightarrow \text{Mg}_3(\text{PO}_4)_2$$

Rapid corrosion is accompanied with locally unduly high pH values shown preferable corrosion resistance as compared to the bare top of the bare Mg-1Ca alloy.

According to the immersion testing results in the study, the corrosion resistance and Coatings Technology 350 (2018) 281–297.

Appendix A. Supplementary data to this article can be found online at

www.sciencedirect.com/science/article/pii/S2352079317300514

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