Research Article

In vitro and in vivo biodegradation and biocompatibility of an MMT/BSA composite coating upon magnesium alloy AZ31

Jian Wang a, Lanyue Cui b, Yande Ren c, Yuhong Zou a,*, Jinlong Ma c, Chengjian Wang c, Zhongyin Zheng d, Xiaobo Chen d, Rongchang Zeng b, e, **, Yufeng Zheng f

a Department of Bioengineering, College of Chemical and Biological Engineering, Shandong University of Science and Technology, Qingdao, 266590, China
b Corrosion Laboratory for Light Metals, College of Materials Science and Engineering, Shandong University of Science and Technology, Qingdao, 266590, China
c Affiliated Hospital of Medical College Qingdao University, Qingdao, 266555, China
d School of Engineering, RMIT University, Carlton, 3053, Victoria, Australia
e School of Materials Science and Engineering, Zhengzhou University, Zhengzhou, 450002, China
f State Key Laboratory for Turbulence and Complex Systems and Department of Materials Science and Engineering, College of Engineering, Peking University, Beijing, 100871, China

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The performance of biodegradable magnesium alloy requires special attention to rapid degradation and poor biocompatibility, which can cause the implant to fail. Here, a sodium montmorillonite (MMT)/bovine serum albumin (BSA) composite coating was prepared upon magnesium alloy AZ31 via hydrothermal synthesis, followed by dip coating. We evaluated the surface characterization and corrosion behavior in vitro, and the biocompatibility in vitro and in vivo. Biodegradation progress of the MMT-BSA coated Mg pieces was examined through hydrogen evolution, immersion tests, and electrochemical measurements in Hank’s solution. In vitro biocompatibility studies were evaluated via hemolysis tests, dynamic crur time tests, platelet adhesion, MTT testing and live-dead stain of osteoblast cells (MC3T3-E1). It was found that the MMT-BSA coating had good corrosion resistance and a marked improvement in biocompatibility in comparison to bare Mg alloy AZ31. In vivo studies were carried out in rat model and the degradation was characterized by computed tomography scans. Results revealed that the MMT-BSA coated Mg alloy AZ31 implants maintained their structural integrity and slight degradation after 120 d of post-implantation. A 100 % survival rate for the rats was observed with no obvious toxic damages on the organs and tissues. Additionally, we proposed a sound coating formation mechanism. Considering the good corrosion protection and biocompatibility, the MMT-BSA coated Mg alloy AZ31 is a promising candidate material for biomedical implants.

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1. Introduction

Because of their advantages of high strength and fatigue resistance, medical metal materials are widely used in orthopedics [1,2]. Commercial medical metallic materials include stainless steels, pure titanium and titanium alloys, and cobalt-based alloys. However, some problems emerge during their clinical services, such as the stress shielding effect stemmed from mismatch in mechanical properties between metal implants and bone, and inflammation caused by the release of toxic ions from bulk implants [3–6]. Meanwhile, most metals are not biodegradable and require secondary surgery for removal, causing additional pain for the patient.

As a new type of medical grade metal, magnesium (Mg) and some of its alloys hold great potential for use as biomaterials owing to their biodegradable features and good biocompatibility [7–10]. Numerous in vivo studies have demonstrated the excellent effects of promoting bone healing of Mg alloys when implanted within or surrounding animal bones [11–14]. However, the corrosion of Mg alloys in human physiological environments do not meet the lifespan requirements for implant materials [15]. In recent years, control of degradation rate of Mg alloys has become a topic of sig-

* Corresponding author.
** Corresponding author at: Corrosion Laboratory for Light Metals, College of Materials Science and Engineering, Shandong University of Science and Technology, Qingdao, 266590, China.
E-mail addresses: zouyh69@126.com (Y. Zou), rczeng@gmail.com (R. Zeng).

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significant interest. Surface modification of Mg alloys may be a good choice to control their degradation rate [16, 17]. Li et al. [18] found that alkali and heat-treated Mg had relatively high corrosion resistance, and after immersion in simulated body fluid (SBF), calcium phosphate was formed on its surface. Cui et al. [19] constructed a SnO2-doped calcium phosphate (Ca-P-Sn) coating on Mg-1Li-1Ca alloy by a hydrothermal process, which exhibited improved corrosion resistance and antibacterial activity. Song et al. [20] prepared a HA coating on the surface of Mg alloy AZ91D via electrochemical deposition, which clearly improved its corrosion resistance in SBF. Liu et al. [21] made a comparison with and without DNA addition on the corrosion resistance and adhesion strength of Ca-P coatings fabricated on Mg alloy AZ21 via hydrothermal deposition. Results showed that the presence of DNA led to the formation of Ca-P coating with improved corrosion resistance and adhesion strength. Wagener et al. [22] investigated a coating consisting of albumin via three different linker molecules (aminopropyl-triethoxysilane ascorbic acid, carbamylidimazole and steearic acid) to improve its corrosion resistance of underlying Mg parts. Turhan et al. [23] demonstrated the corrosion protection ability of polypropylene (PPy) thin films on Mg alloy AZ91D in SBF and found that the sodium salicylate-doped PPy layer afforded significant corrosion protection. It was also demonstrated that the PPy layers could be further functionalized by adsorbed albumin to achieve better corrosion performance.

However, the biological compatibility of Mg alloys is affected by surface modification. Many studies attempted to improve the corrosion resistance and biocompatibility of Mg alloys by preparing composite coatings [19, 24]. Li et al. [25] prepared glucose-induced composite coatings containing crystalline calcium phosphate and Mg(OH)2 interlayer on pure Mg substrate through hydrothermal deposition from alkaline solution. Corrosion resistance of pure Mg specimens was improved by the formation of such a calcium phosphate coating. Zhao et al. [26] constructed the gentamicin-loaded multilayers on Mg alloys through spin-assisted layer-by-layer assembly. Heat treatment was applied for improving the corrosion resistance and prolonging the drug release profile. Chen et al. [27] prepared a polymerized 2-methacryloyloxyethyl phospho-rzycholine coating via surface thiol-ene photopolymerization onto an Mg alloy treated by cathodic plasma electrolytic deposition to improve the corrosion resistance and hemocompatibility. Pan et al. [28] modified Mg alloy AZ31B surface by alkali heat treatment followed by self-assembly of 3-aminopropyltrimethoxysilane to enhance corrosion resistance; then, poly (ethylene glycol) and fibronectin or a fibronectin/heparin complex were sequentially immobilized on the modified surface to improve the hydrophilicity of the substrates. Tian et al. [29] prepared a polycaprolactone layer to seal the plasma electrolytic oxidation coating on Mg alloy AZ31, followed by surface functionalization with polydopamine. The final composite coating presented good corrosion resistance, bioactivity, and cytocompatibility. In conclusion, satisfactory corrosion resistance remains a challenge in terms of development of Mg alloy based implant materials.

Montmorillonite (MMT) is a typical layered phyllosilicate rutilate and its basic unit structure was formed by a sandwich of one aluminum-octahedron between two siloxane tetrahedrons [30]. MMT has excellent intrinsic properties and can be used as an adsorbent, dispersant, suspension, coagulant, and filler [31–33]. Hydration through interlayer inorganic cations, MMT swells and is stripped in an aqueous medium to form MMT suspension [34]. Alibabadi et al. [35] synthesized a chitosan-derivative-modified montmorillonite (HTCC-modified montmorillonite) with excellent biocompatibility, cell growth, and antibacterial efficiency for tissue engineering applications. Lal et al. [36] designed and developed a naturally occurring montmorillonite-based poly lactic-co-glycolic acid nanocomposite as a biocompatible delivery vehicle with the double functionality of low pH stability and mucoadhesivity for oral administration of insulin. Additionally, montmorillonite is currently under investigation for the uses of catalyst [37], drug carrier system [38], additives [39], and implant materials [40–45]. Our previous work indicated that MMT has good biocompatibility with bone cells [46]. A zinc-loaded montmorillonite (Zn-MMT) coating was hydrothermally prepared using Zn2+ ion intercalated sodium montmorillonite (Na-MMT) upon Mg alloy AZ31 as bone repairing materials. Results revealed that both Na-MMT and Zn-MMT coatings exhibited better corrosion resistance than neat Mg AZ31 alloy counterparts. in vitro MTT tests and live-dead stain of osteoblast cells (MC3T3-E1) indicated a significant improvement in cytocompatibility of both Na-MMT and Zn-MMT coatings. Antibacterial properties of two representative bacterial strains associated with device-related infection, i.e. Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus), were employed to explore the antibacterial behavior of the coatings. However, how to improve the biocompatibility of Mg alloy AZ31 with MMT coating and its in vivo performance still needs to be studied.

Sticky and glial albumin is one of the major proteins in human plasma. It can maintain a constant plasma colloid osmotic pressure. When encounters heavy metal ions in human body, albumin will combine with the toxic heavy metal ions for detoxification [47]. When albumin covers the surface of a material, it forms a thin layer that prevents reactions between the blood components and the material, thus significantly reducing the amount of clotting on the surface such as platelets, and increasing the anticoagulant properties of the material. This method is termed “albumin passivation” [48–51] that can be used to modify the surface anticoagulant properties of blood contact materials to improve the biocompatibility of materials. Fibrinogen is a plasma protein closely related to blood coagulation. Adsorption of fibrinogen on the surface of a material not only starts the human coagulation pathway, but also converts into insoluble fibrinogen, which is one of the main components of thrombus. However, on the surface of medical metal materials, the competitive adsorption rate of albumin was significantly lower than that of fibrinogen and other coagulation factors [52]. Therefore, in this work, the corrosion resistance of Mg alloy AZ31 could be improved by pre-treating a feasible MMT coating. To improve the biocompatibility of the coating, the MMT-BSA coating was prepared by the dip coating method. This method can not only increase the albumin concentration on the surface of the material, but also maintain the spatial conformation of albumin. And then we assessed the corrosion resistance and biocompatibility of the MMT-BSA coating. To the best of our knowledge, there have been no studies on improving the corrosion resistance and biocompatibility of Mg alloys through adsorption of albumin on their surface or coatings. This work provides an experimental basis for the formation of the MMT-BSA coating from a fundamental study to clinical applications.

2. Experimental

2.1. Materials and chemicals

Commercially acquired extruded Mg alloy AZ31 was used in this work; its theoretical composition (mass fraction) was 3% Al, 1% Zn, 0.2% Mn, and the balance was Mg. Samples with dimensions of 20 mm × 20 mm × 4 mm were polished with sandpaper up to 1200 grit. The samples were rinsed with acetone and ethanol to remove surface contamination and then dried with hot air [53]. Albumin Bovine V (purity ≥ 96 %) was purchased from Beijing Solarbio Science & Technology Co., Ltd. The chemical composition of Hank’s solution is listed in Table 1.
2.2. Preparation of MMT and MMT-BSA composite coatings

The MMT coating was prepared on the surface of Mg alloy AZ31 via hydrothermal synthesis [54]. A 2% montmorillonite suspension was placed, the pH of the suspension was adjusted to 11 with NaOH solution. The mixture was heated and stirred at 80 °C for 5 h; in the meantime, the pH was adjusted twice to maintain it stability. The treated Mg alloy was placed in a hydrothermal kettle, poured into the suspension, and treated at 130 °C for 36 h. 1 mg mL⁻¹ solution of bovine serum albumin was prepared with a buffered pH of 4.6 through addition of Na₂HCO₃/citric acid buffer solution. Then, the prepared MMT coating was immersed in the prepared solution for adsorption of albumin at 25 °C over 2 h. Afterwards, samples were thoroughly rinsed with deionized water and dried with hot air flow. A schematic illustration of preparation of the MMT-BSA composite coating upon Mg alloy AZ31 is shown in Fig. 1.

2.3. Corrosion behavior

2.3.1. Hydrogen evolution tests

Hydrogen was collected by an inverted funnel connected to a graduated burette [55] and the water level was measured intermittently for 5 d. The apparatus was maintained at a constant temperature water bath of 36.5 °C. The ratio of the exposed sample area to the medium-volume remained constant at 1:40 cm² mL⁻¹. The liquid was renewed every 24 h. Samples were measured in triplicate to ensure reproducibility.

2.3.2. Immersion tests

pH value and the release rates of Mg²⁺ were measured by immersion tests. The samples were vertically immersed in Hank's solution at 36.5 °C. After different immersion periods (1, 5, and 7 d), the samples were used for surface characterization. At the start of the initial 24 h immersion, the pH values were measured by a pH meter once an hour. The test was conducted three times and the average value was calculated. The release of Mg²⁺ ions was quantified via flame atomic absorption spectrometry (FAAS, TAS-986, Beijing Purkinje General Instrument Co., Ltd.) analysis. Each measurement was replicated three times for reproducibility and average values were plotted as cumulative release curves of Mg²⁺ ions.

2.3.3. Electrochemical tests

Potentiodynamic polarization (PDP) curves and electrochemical impedance spectroscopy (EIS) measurements were recorded in Hank's solution at 37 °C using a potentiostat (PARSTAT 2273) connected to a three-electrode cell containing a saturated calomel electrode (SCE) as reference electrode, platinum as counter electrode, and one piece of samples as working electrode. An area of 1.0 cm² was exposed to the solution. The frequency of the EIS measurements ranged between 100 kHz – 10 MHz with an amplitude of 10 mV in relation to the open circuit potential (OCP) [53,55–57].

2.4. Surface characterization

The micro-morphologies of the coatings before and after corrosion were observed with scanning electron microscope (SEM) (Nova Nano SEM 450, USA). The above samples were subjected to spot scanning, line scanning, and surface scanning by energy dispersive spectroscopy (EDS) attached to the SEM to analyze the composition and content changes of each sample. X-ray diffraction (XRD, Rigaku D/Max2500PC, Japan) was used to determine the phase composition of the coatings before and after corrosion withCu-Kα radiation between 2θ values of 2°–80° with a scanning rate of 8° min⁻¹; the X-ray generator was set at 40 kV and 40 mA. The functional groups of the samples before and after corrosion were determined via Fourier transform infrared spectroscopy (FTIR, Nicolet 380, Thermo Electron Corporation, USA) in the wavenumber range of 4000–400 cm⁻¹, each spectrum was recorded with a resolution of 4 cm⁻¹ at room temperature. X-ray photoelectron spectroscopy (XPS) was performed using a Kratos DLD Ulta Spectrometer with Al-Kα irradiation at a pass energy of 160 eV and the data was handled with XPSPEAK4.0.

2.5. In vitro blood compatibility

2.5.1. Hemolysis test

For the hemolysis tests, 4 mL of fresh human blood (voluntary donation by the laboratory, there were no medical ethics issues)
were used and centrifuged at 1500 rpm for 10 min. The supernatant was sucked out, and the erythrocytes deposited at the bottom were washed three times with physiological saline (0.9 wt.%). The obtained erythrocytes were mixed with physiological saline to a 2% suspension. Samples were immersed in physiological saline (sample surface area/extraction medium was 3 cm² mL⁻¹) for 90 min in a constant temperature incubator of 36.5 °C. Subsequently, 1 mL of diluted blood and 7 mL of leach solution were mixed and incubated at 36.5 °C for 60 min. The mixed solutions were centrifuged at 1000 rpm for 10 min and the absorbance of the supernatant at 545 nm was observed using a microplate reader (UNIC-7200, China). The hemolysis ratio (HR) was calculated using the following equation [58]:

\[
HR(%) = \frac{(O_D\text{test} - O_D\text{negative})}{(O_D\text{positive} - O_D\text{negative})} \times 100\%
\]

where \(O_D\text{test}\) was the test sample, \(O_D\text{positive}\) was the positive control (7 mL distilled water with 1 mL diluted blood), and \(O_D\text{negative}\) was the negative control (7 mL physiological saline with 1 mL diluted blood).

2.5.2. Dynamic clouR time test

The dynamic clotting time in vitro could be used to evaluate the blood compatibility of the samples. First, the samples were placed in an incubator at 36.5 °C for 5 min and then 30 μL of anticoagulant blood was dropped onto the surface. After 5, 20, 35, 50, 90, and 130 min, each sample was immediately placed in a beaker containing 15 mL of deionized water for 10 min to allow the uncongealed erythrocyte to fully dissolve in water. Finally, the photometric values of erythrocyte were measured using a spectrophotometer at 545 nm and the dynamic coagulation curve was drawn.

2.5.3. Platelet adhesion test

Platelet adhesion experiments in vitro were used to evaluate the adhesion behavior and the interaction between the platelets and the materials. Platelet-rich plasma (PRP) was obtained by centrifuging fresh anticoagulant blood (donated by laboratory staff) at 1000 rpm for 10 min. Then, 20 μL of PRP was overlaid atop the samples and incubated at 37 °C for 30 min. Platelets that were only weakly adhered were rinsed with PBS (pH 7.4) until clean. Afterward, the samples fixed with 2.5% glutaraldehyde and dehydrated by a series of ethanol/water mixtures (50, 60, 70, 80, 90, and 100 vol.% ethanol). Finally, the samples were dried, sputter-coated with gold, and imaged by SEM.

2.6. Cytotoxicity evaluation

2.6.1. Cell culture

MC3T3-E1 cells were employed to investigate the cell toxicity of the specimens. After sterilization using ultraviolet irradiation for 30 min, the samples were clipped to a 6-well culture plated. The MC3T3-E1 cells in the logarithmic phase were collected and the cell suspension was adjusted. The cells were seeded on the plated
with a density of $1 \times 10^4$ cell per well. The MC3T3-E1 cells were seeded on culture plates and cultured at 37 °C under 5% CO₂.

### 2.6.2. MTT assay

After culturing for 24 h and then washing with PBS twice, 4 mL of the medium containing 0.5 mg mL⁻¹ MTT was added and cultured for 4 h. The culture was stopped and the culture medium was carefully removed. Next, 1.5 mL dimethyl-sulfoxide was added into each hole, and the crystal was fully dissolved by low-speed oscillation for 10 min in a shaker. The absorbance values of each hole were measured at 570 nm using a universal microplate spectrophotometer. A sample of the Mg alloy AZ31 was used as the material control, the cell culture medium as the negative control and the culture medium with 10 % dimethylsulfoxide (DMSO) were added as the positive control.

### 2.6.3. Live/dead cell staining

The MC3T3-E1 cells were cultured on the specimens at a density of $4 \times 10^4$ cells per well. After 24 h of material treatment, the mixture of the material and the culture medium was sucked out and
washed with PBS. Calcein, AM was added first and incubated at 4 °C for 15 min. Then, the cells were washed with PBS and PI was added for staining for 5 min. Fluorescence microscopy was used to take images of the samples. Under fluorescence microscopy, the living cells were yellowish green while the dead cells were red.

### 2.7. In vivo studies

The animal model of paravertebral implantation was utilized to evaluate the corrosion behavior of the Mg alloy AZ31, the Na-MMT coating, and the Na-MMT-BSA coating in vivo. All animal experiments were conducted according to the ISO 10993-2:1992 animal welfare requirements. The protocol for their care and the use of laboratory animals was approved by the Animal Ethical Committee of the Affiliated Hospital of Qingdao University. The SD rats were randomly divided into three groups (n = 5, each group), one for each implant material.

All the SD rats were weighed and anesthetized with pentobarbital sodium (40 mg kg⁻¹) by intraperitoneal injection. The Mg alloy AZ31, MMT coating, and MMT-BSA coating (4 mm × 4 mm × 1.5 mm) were implanted in the paravertebral regions of the rats (Fig. 2). A computed tomography scan (spiral cone beam, General Electric) of the spine was then performed and evaluated for the implant position, degradation, and gas pockets around the implant. The animals were sacrificed 120 d after implantation. The muscle tissues containing the samples were resected, stripped from the samples, and fixed in 10% neutral formaldehyde solution. The samples were cleaned by chromic acid solution (200 g CrO₃, and 10 g AgNO₃ per liter of water). The changes of surface topography of the samples were characterized by SEM. Myocardium, liver, lung, renal cortex, medulla, and local muscle SD rats were collected for slicing and stored in 10% formaldehyde to make paraffin sections. A histological evaluation was performed on the hematoxylin and eosin (HE) stained sections.

### 2.8. Statistical analysis

All the data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by Tukey’s test. When p ≤ 0.05 or p ≤ 0.01, it is defined as a statistically significant difference. Statistical analysis was conducted with SPSS 19.0.

### 3. Results

#### 3.1. Surface analysis

Surface characteristics of the MMT and MMT-BSA coatings are provided in Fig. 3. It shows that the surface of Mg alloy AZ31 is completely covered by a dense and flatten MMT coating layer with small cracks (Fig. 3(a)). Fig. 3(b) shows that O, Na, Mg, Al, and Si are the constitutional elements of MMT coating as determined via EDS. In contrast, MMT-BSA coating is denser and smoother with fewer cracks than MMT coating (Fig. 3(c)). Fig. 3(d) shows that a new element of N, main component of BSA, is evident in the MMT-BSA coating, indicating the incorporation of BSA upon the surface of the MMT coating. FTIR spectra of MMT coating, MMT powder, MMT-BSA coating, and MMT-BSA powder were depicted in Fig. 3. As shown in Fig. 3(e), the MMT coating and the MMT-powder had similar absorption peaks. The absorption peak at 3620 cm⁻¹ could be attributed to the stretching vibration of the hydroxyl bond Al-O-H and the one at 1038 cm⁻¹ was the stretching vibration of Si-O. In addition, the weak absorption bands at 918 and 694 cm⁻¹ were the bending vibrations of Al–OH and Si-O-Mg, respectively. These indicate that the MMT coating had been successfully prepared on the Mg alloy AZ31. BSA had an Amide I band absorption peak at 1652 cm⁻¹ and an Amide II absorption peak at 1545 cm⁻¹. Because of the interferences of water molecules at 1656 cm⁻¹, the peak for Amide II band demonstrated the adsorption of albumin. In addition to the characteristic MMT peaks, the MMT-BSA coating had BSA absorption band, which confirms that albumin was adsorbed on the MMT-BSA coating and the original spatial structure of albumin retains. XRD patterns of the samples are plotted in Fig. 3(f). The peaks at 20° and 22° corresponding to MMT confirm that the MMT coating had been prepared on the surface of Mg AZ31 alloy surface, which is consistent with the MMT powder. Additionally, the characteristic peaks of MMT also appeared on the MMT-BSA coating. However, given the organic nature of albumin molecules, no characteristic peaks are present in the XRD pattern. The peaks at 32°, 34°, 37°, and 71° were characteristic peaks of metallic Mg. Moreover, the characteristic peaks of Mg (OH)₂ were found to indicate the corrosion products of Mg alloys during hydrothermal processes.

The surface composition of the MMT and MMT-BSA coatings were studied by XPS (Fig. 4). The results clearly show the presences of Na 1s, Mg 1s, Al 2p, Si 2p, and O 1s in the MMT coating and Na 1s, Mg 1s, Al 2p, Si 2p, O 1s, and N 1s in the MMT-BSA coating, which was in excellent agreement with the results of the chemical analysis of the coatings. Since N is one of the basic elements of BSA and montmorillonite does not contain N, the surface composition of MMT-BSA coating can be analyzed by XPS to provide direct evidence for the presence of BSA on the MMT coating. The peak at a binding energy of 398.90 eV in the N 1s energy spectrum were generated by the nitrogen of the peptide bond (–CONH–) in BSA, which fully demonstrated that BSA successfully adsorbed onto the MMT coating surface.
3.2. Corrosion behavior

Fig. 5 shows the hydrogen evolution curves, the pH change, and the accumulated Mg²⁺ ions release of the Mg alloy AZ31 and the coated samples in Hank’s solution. It can be seen from Fig. 5(a) that the hydrogen evolution amount (HEA) of Mg alloy AZ31 was significantly higher than the coated samples and was up to 3.1 mL after 5 d of immersion. The HEAs of MMT and MMT-BSA coatings were 1.24 mL and 1.04 mL, respectively. Initially, within 6 h of immersion, the HEA of the Mg alloy AZ31 increased rapidly, which could be ascribed more rapid corrosion by aggressive ions, such as Cl⁻. Then, the markedly reduced corrosion rate can be attributed to the formation of the corrosion products. In general, a lower HEA indicates that the MMT-BSA coating, especially during initial immersion stage, has an obvious protective effect on the substrate. The pH changes of Mg alloy AZ31, MMT, and the MMT-BSA coatings in Hank’s solution are shown in Fig. 5(b). The pH value of Mg alloy AZ31 in Hank’s solution increased rapidly to 8.32 and then remained stable. The values for the MMT and MMT-BSA coating increased slowly to 7.92 and 7.84, respectively. The accumulated Mg²⁺ ion release of the samples (Fig. 5(c)) shows a linear relationship with time up to 5 h after immersion. After that, the release rate of the Mg²⁺ ions gradually reduced and the cumulative released quantity tended to be stable. In addition, both the MMT and MMT-BSA coatings were lower than that of the Mg alloy AZ31. The variation of the Mg²⁺ ions corresponded with that of the pH value. The MMT-BSA coating had a certain protective effect, which effectively slowed the corrosion rates of Mg alloy AZ31.

The FT-IR results of samples immersed in Hank’s solution for 5 days are shown in Fig. 6(a). The absorption peak at 3620 cm⁻¹ can be attributed to the stretching vibration of the hydroxyl bond Al-O–H and the 1038 cm⁻¹ peak is the stretching vibration of Si–O. In addition, the weak absorption bands at 918 and 694 cm⁻¹ were the bending vibration of Al–OH and Si–O–Mg, respectively. The bending vibration of water molecules H–O–H was at 1656 cm⁻¹. The MMT-BSA coating showed an absorption peak at 1545 cm⁻¹, which can be ascribed to Amidell, illustrating that the MMT and BSA coatings were present after immersion. Fig. 6(b) shows the XRD patterns of the samples after immersion for 5 d. The peaks corresponding to Mg(OH)₂ were stronger on the surface of the Mg alloy AZ31 after immersion, which indicates that the surface of the Mg alloy AZ31 corroded and a large number of corrosion products were produced. The characteristic MMT peak was still present, demonstrating that the coating had a good protective effect on the Mg alloy matrix, slowing down the corrosion of the Mg alloy AZ31, which corresponds to the HEA results (Fig. 5(a)).

The SEM morphologies and EDS spectra of the samples immersed in Hank’s solution for 1, 3, and 5 d are shown in Fig. 7. Many corrosion pits and shallow cracks could be observed as a function of the immersion time and corrosion products were layered on the surface of the Mg alloy AZ31. However, the MMT coating had no obvious changes except that its structure was no longer as dense as before. The MMT-BSA coating was much smoother and uniform, with smaller cracks than the MMT coating. The EDS results show the presence of Na, Mg, Si, O, Al, and N on the surface of the coatings, demonstrating that the MMT and MMT-BSA coatings were still present. Furthermore, the Ca and P on each sample increased with immersion time because of the accumulation of phosphate products on the surface. However, the absence of N element can be ascribed to the phosphate products covering the albumin coat.
Fig. 7. SEM morphologies and the corresponding EDS spectra of Mg alloy AZ31 (a-f), MMT (g-l) and MMT-BSA (m-r) coatings immersed in Hank's for 1, 3, and 5 d.
The coating coated the ing. These results reveal that the coatings protected the substrates well.

Fig. 8 shows the potentiodynamic polarization (PDP) curves of the Mg alloy AZ31, MMT, and MMT-BSA coatings immersed in Hank’s solution, and the corresponding parameters were calculated by extrapolation and are summarized in Table 2. The Mg alloy AZ31 had the highest level of $i_{corr}$ ($6.27 \times 10^{-6}$) during the polarization in Hank’s solution. The $i_{corr}$ (2.09 $\times 10^{-6}$) of MMT coating was significantly less than that of the substrate. The MMT-BSA coating exhibited the lowest $i_{corr}$ (7.65 $\times 10^{-7}$), which implied that the coated samples had better corrosion resistance. Moreover, the $E_{corr}$ of the MMT-BSA coating slightly shifted to a more positive potential (10 mV) compared with the Mg alloy AZ31. The $R_p$ of the MMT-BSA coating was greater than the Mg alloy AZ31, which demonstrated that the MMT-BSA coating was able to offer higher corrosion protection.

EIS was carried out to further evaluate the corrosion resistance of the coatings and the data were fitted by ZSimp Win 3.4 software. The higher $Z$ modulus at the lower frequency and the larger radius of curvature indicated better corrosion resistance on the substrates [59]. The Nyquist plot in Fig. 9(a) shows that the Mg alloy AZ31 was characterized by a capacitive loop in the high and medium frequency ranges and an inductive loop in the low frequency range. The capacitive loop was related to the charge transfer process, while the inductive loop was caused by the dissolution and pitting corrosion of Mg [60]. For the MMT coating, a similar evolution of the process for the Mg alloy AZ31 was observed and the corrosion resistance was clearly enhanced because of the larger dimensions of the capacitive loop. For the MMT-BSA coating, the diameters of the capacitive loops were the largest, indicating that the coating possessed the best corrosion resistance.

The corrosion protection performance improves in tandem with an increasing low-frequency impedance modulus [$Z$] [60,61]. Fig. 9(b) shows that the $|Z|$ value of the Mg alloy AZ31 was $1.25 \times 10^3$ $\Omega \ cm^2$, which was lower than that of $8.60 \times 10^3$ $\Omega \ cm^2$ of the MMT coating and was far lower than that of MMT-BSA coating ($1.51 \times 10^4$ $\Omega \ cm^2$). The results reveal that the composite coating enhanced the corrosion protection of the Mg alloy AZ31. In Fig. 9(c), MMT-BSA coating possess the maximum phase angles at an intermediate frequency, which indicates the better corrosion resistance than MMT coating and Mg alloy AZ31. The ECs of the Mg alloy AZ31, MMT, and MMT-BSA coatings are shown in Fig. 9 (d–e) and the fitting data are listed in Table 3. In the ECs, the solution resistance and the charge transfer resistance are expressed by $R_s$ and $R_{ct}$, respectively. The pitting corrosion with inductance is represented by $R_l$ and the resistance of the coating is given by $L$. $R_s$ and the constant phase elements (CPEs) by CPE1 and CPE2. For the Mg alloy AZ31 (Fig. 9(d)), the high-frequency capacitance loop was described by CPE1 and $R_{ct}$, which characterized the loose corrosion product layer. The low-frequency inductive loop represented the onset of pitting corrosion, which was characterized by $R_l$ and $L$. Different from the Mg alloy AZ31, after modified with BSA, MMT coating and MMT-BSA coating showed similar Nyquist plots, which can be fitted with one EC (Fig. 9(e)). The invisible inductance properties indicated that the composite coating had good corrosion resistance. As shown in Table 3, the $R_{ct}$ increased from $2.82 \times 10^3$ $\Omega \ cm^2$ to $8.22 \times 10^3$ $\Omega \ cm^2$ and $1.57 \times 10^4$ $\Omega \ cm^2$, which confirmed that the MMT and MMT-BSA coating effectively protected the Mg alloy AZ31 from corrosion; and the MMT-BSA coating exhibited better corrosion resistance than the MMT coating.

### 3.3. Biocompatibility of Mg alloy AZ31 with MMT-BSA coatings

Fig. 10(a) shows the hemoglobin absorption values of the samples. It was found that the values of the two coatings were significantly lower than that of Mg alloy AZ31. Fig. 10(b) shows that Mg alloy AZ31 has the highest hemolysis ratio (25.6 %), far higher than the recommended value of 5% by the ISO 10993-4 standard [62]. In contrast, the coated samples had acceptable hemolysis ratios of 4.7 % and 3.3 % for the MMT and MMT-BSA coatings, respectively. Compared with the Mg alloy AZ31 and the MMT coating, the MMT-BSA coating had excellent hemocompatibility. This result can easily be understood because the hemolysis of the Mg alloy AZ31 was strongly dependent on the corrosion resistance and the related ion release and pH changes. For the MMT and MMT-BSA coating, the results show that they had good corrosion resistance and slight Mg$^{2+}$ ion release.

The dynamic clotting time in in vitro experiments are commonly used to detect the degree of endogenous coagulation factor activation to observe the effect of the material on the blood clotting time [63]. Here, a longer clotting time indicates better anticoagulant effects, i.e., the material has better blood compatibility. To compare the anticoagulant ability of different materials, the time at which the absorbance value was 0.100 artificially was defined as the clotting time of the material. Fig. 10(c) shows a comparison of the curves of dynamic clotting time among AZ31, MMT, and MMT-BSA coatings. The absorbance of the material gradually decreased as a function of contact time and eventually leveled off. The clotting time of the MMT-BSA coating was significantly improved compared with the MMT coating and Mg alloy AZ31, indicating that the hemocompatibility of the MMT-BSA coating was superior to that of the MMT coating and the Mg alloy AZ31.

The platelet adhesion property is a crucial reaction that corresponds to the formation of thrombus and arterial occlusion [64,65]. The platelets adhered on the Mg alloy AZ31 and coating samples

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**Table 2**

Electrochemical parameters of the polarization curves.

<table>
<thead>
<tr>
<th>Samples</th>
<th>$\beta_s$ (mV dec$^{-1}$)</th>
<th>$-\beta_t$ (mV dec$^{-1}$)</th>
<th>$E_{corr}$ (V SCE)</th>
<th>$t_{corr}$ (A cm$^{-2}$)</th>
<th>$R_p$ (\Omega cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ31</td>
<td>104.98</td>
<td>130.73</td>
<td>−1.41</td>
<td>6.27 $\times 10^{-6}$</td>
<td>4.03 $\times 10^6$</td>
</tr>
<tr>
<td>MMT coating</td>
<td>51.50</td>
<td>168.28</td>
<td>−1.42</td>
<td>2.09 $\times 10^{-6}$</td>
<td>8.20 $\times 10^6$</td>
</tr>
<tr>
<td>MMT-BSA coating</td>
<td>54.42</td>
<td>128.75</td>
<td>−1.40</td>
<td>7.65 $\times 10^{-7}$</td>
<td>2.17 $\times 10^7$</td>
</tr>
</tbody>
</table>

---

Fig. 8. Potentiodynamic polarization (PDP) curves of Mg alloy AZ31, MMT, and MMT-BSA coatings immersed in Hank’s solution.
are shown in Fig. 11. Platelet aggregation begins after activation, which occurs in 3 stages, defined as early dendritic, early spread, and spread accompanied by morphological change. A large number of platelets were found on the Mg alloy AZ31, but most of the platelets remained separated and round (Fig. 11(a)). Compared with the Mg alloy AZ31, the number of platelets on the MMT coating significantly decreased (Fig. 11(c)). After modification with albumin (Fig. 11(e)), the number of platelets decreased further and the platelets had a round morphology. The corresponding EDS results (Fig. 11(b, d and f)) show the presence of S, C, and O and confirm the adhesion of the platelets. The present results suggest that the MMT-BSA coating had good hemocompatibility compared with the MMT coating and Mg alloy AZ31.

The MTT assay was a measure of mitochondrial function, which detects the level of succinate dehydrogenase within the cell. The viability of the MC3T3-E1 cells are shown in Fig. 12(a). The cell viability of the Mg alloy AZ31 (79 %), MMT (85 %) and MMT-BSA (91 %) coatings decreased to a certain degree in 24 h, however, the MMT (87 %) and MMT-BSA (95 %) coatings had a certain rise in 72 h. This indicated that both the MMT and MMT-BSA coatings had a significantly better surface bioactivity than the Mg alloy AZ31. The viability of MC3T3-E1 cells in the Mg alloy AZ31 (75 %) decreased to some extent after 72 h of incubation, which was caused by less alkalization of the cell culture fluid. The results reveal that the coated samples improved the biocompatibility of the Mg alloy AZ31. The toxicity of the Mg alloy AZ31 was low and within an acceptable range (Grade 1 toxicity).

After 24 h of incubation, the main difference between the experimental groups and the negative control group was the number of cells (Fig. 12(b)). A limited number of dead cells (red color) could be randomly observed in the Mg alloy AZ31 (Fig. 12(c)). The number of dead cells on the MMT coating (Fig. 11(d)) further decreased compared with the Mg alloy AZ31. Furthermore, after treatment with albumin (Fig. 11(e)), the amount of the dead cells was minimal. Thus, it could be concluded that the MMT and MMT-BSA display acceptable cytocompatibility to MC3T3-E1 cells.
Fig. 11. Typical SEM images of platelet adhesion and corresponding EDS spectra of different samples: (a, b) Mg alloy AZ31; (c, d) MMT; (e, f) MMT-BSA coatings.

Fig. 12. Viability of MC3T3-E1 cells incubated for 24 h and 72 h (a) and CLSM images of live/dead staining incubated for 24 h with leach liquor of negative control and samples (b, c, d, e).
Fig. 13(a1-c1) and (a2-c2) display the spiral CT scan of the rat paravertebral implantation. The images show that the Mg alloy AZ31, the MTT coating, and the MTT-BSA coating implants were all located at the surgery sites without dislocation. A gas shadow was observed around all the implants; however, the least amount of gas was produced in the MTT-BSA coating implantation area. There...
Fig. 14. H&E staining of myocardium, liver, lung, renal cortex, medulla and local muscle after 120 d of implantation of Mg alloy AZ31, MMT coating and MMT-BSA coating.
was no obvious inflammatory reactions or adverse effects around the implants.

The animals were sacrificed 120 days after implantation; then, the implanted samples were removed from their body. The SEM images and EDS results of the samples are shown in Fig. 13(a3–c3, a4–c4, a5–c5). For the Mg alloy AZ31, a dry riverbed surface morphology was observed (Fig. 13 (a3 and a4)). Interestingly, there were small cracks on the surface of the MMT coating and the MMT-BSA coating. Additionally, some loose materials appeared (Fig. 13(b3)) on the surface of the MMT coating. Fig. 13(b4) shows images at higher magnifications, demonstrating that the loose materials had a platelet-like microstructure. After 120 days of implantation, there were no corrosion products on the surface of MMT-BSA coating (Fig. 13(c3)). The MMT-BSA coating still covered the Mg alloy AZ31 surface, and showed the typical MMT morphology (Fig. 13(c4)). The EDS spectra of the implanted surface for all three specimen groups indicated the presence of C, O, Mg, Al, S, and P, confirming the presence of possible corrosion products, such as Mg(OH)2. Nevertheless, Si was also observed on the surface of the MMT and MMT-BSA coatings (Fig. 13(a5–c5)). The high Si, Al, Mg, O, and C content indicated that the MMT and MMT-BSA coatings were maintained on the Mg alloy AZ31 surface. The in vivo degradation behavior of the MMT-BSA coating could basically fulfill the requirements for implants.

Histology analysis was further conducted to assess systemic toxicity of the samples. The rats implanted with the Mg alloy AZ31, MMT coating, and the MMT-BSA coating were accordingly sacrificed at 120 d following surgery. Representative histological results of the myocardium, liver, lung, renal cortex, medulla, and local muscle are shown in Fig. 14. The H & E stains show that there was no apparent pathological alteration in the livers and kidneys obtained from the experimental animals. All organs show no obvious abnormalities through injection leaking solution of the coatings and the Mg alloy AZ31. The liver was ruddy, shiny, and elastic under the optical microscope, the liver cells are rich in cytoplasm, the nucleus was large and round, the nucleolus was clear, the central vein and the portal area were normal, and the cells were neatly arranged. The structure of the hepatic lobule was clear, the liver cells in the lobule were basically normal, the structure of the portal area was clear, and there was no small bile duct and fibrous tissue hyperplasia around the portal area. Renal tissue HE stains of the Mg alloy AZ31 (Fig. 14(a4, a5)), the MMT coating (Fig. 14(b4, b5)), and the MMT-BSA coating (Fig. 14(c4, c5)) showed that the structure of the renal glomeruli, capsules, and tubules were normal, while no inflammatory lesion was found. Neither the presence of inflammatory cells such as macrophages and neutrophils nor tissue necrosis was observed in pathological sections of local muscle (Fig. 14(a6–c6)). The results indicate that MMT-BSA coating had good biocompatibility with animal organs; hence, it can be inferred that the alloy implants in the animals had good prospects.

4. Discussion

In the present study, the MMT coating was prepared on the surface of Mg alloy via hydrothermal synthesis as a means of promoting its corrosion resistance. Albumin was chosen as a composite coating to enhance the biocompatibility of the samples because it could easily bind covalently with polypeptides and then promote the adsorption and growth of cells on the surface of the implanted material. The corrosion resistance and biocompatibility of the matrix were investigated after preparation of the composite coating.

4.1. Preparation mechanism of the coating

The MMT was prepared on the surface of the Mg alloy AZ31 by removing one molecule of H2O to form the Si-O-Si bond via hydrothermal synthesis [54]. MMT is a kind of layered silicate mineral and its unit cell is composed of two silicon oxide tetrahedral plates and one layer of aluminum oxide octahedral plates. For the crystal structure, the Si4+ in tetrahedral sheets are easily replaced by isomorphisms such as Al3+ and Fe3+. The octahedral head is composed of six oxygen atoms or hydroxide, and the central ions are Al3+ with a hexagonal position, which can also be replaced by lower ions such as Mg2+. When the high cations in the tetrahedral and octahedral bodies are replaced by low cations, an equivalent negative charge is added to the original crystal structure, which makes the MMT crystal layer naturally electronegative and it can absorb the cations in the surrounding environment to balance the increased negative charge.

On the surface of medical metal materials, the competitive adsorption rate of albumin was significantly lower than fibrinogen and other coagulation factors [52]. Moreover, the spatial conformation of the protein’s changes to different degrees after adsorption on the surface of biological materials. Therefore, we fixed albumin on the surface of the MMT via electrostatic adsorption. This could not only increase the concentration of albumin on the surface of the material but also maintain the spatial conformation of albumin to a large extent. According to Bronsted-Lowry’s theory of acid–base protons, dipolar ions of amino acids in water can be viewed as both acids and bases, which are ampholytes, so albumin can be attached to the surface of other substances via electrostatic interactions. The isoelectric point of albumin is 4.8, when the pH of the solution is less than isoelectric point, base ionization occurs. The amino acid cation can be adsorbed on the surface of montmorillonite via electrostatic action.

4.2. Corrosion resistance

Our previous work designated the corrosion resistance of Zn-MMT coating on Mg alloy AZ31 [46]. In this work, the electrochemical behavior of MMT-BSA coating in Hank’s solution was evaluated. The PDP curves of the Zn-MMT coating was shown in Fig. 15. The Ecorr of the Zn-MMT coating was -1.38 V SCE−1 and the icorr was 2.86 × 10−7 A cm−2. Approximately, the Ecorr of the MMT-BSA coating was -1.40 V SCE−1; and the icorr was 9.65 × 10−7 A cm−2. The results indicated that the corrosion resistance of two coatings were much better than that of Mg alloy AZ31. The data and conclusions from other previous investigations are summarized in Table 4. The results of this work indicate that the MMT-BSA composite coating had better corrosion resistance than the other biological coatings. The corrosion resistance of magnesium alloy

Table 4

<table>
<thead>
<tr>
<th>Materials</th>
<th>Coating</th>
<th>Solution</th>
<th>Ecorr (V)</th>
<th>icorr (A cm−2)</th>
<th>Refs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ31</td>
<td>Zn-MMT</td>
<td>DMEM</td>
<td>-1.38</td>
<td>2.86 × 10−7</td>
<td>[46]</td>
</tr>
<tr>
<td>AZ31</td>
<td>(PVP/DNA)−</td>
<td>SBF</td>
<td>-1.53</td>
<td>4.71 × 10−6</td>
<td>[66]</td>
</tr>
<tr>
<td>AZ31</td>
<td>PDA-HA-BMP-2</td>
<td>PBS</td>
<td>-1.53</td>
<td>5.20 × 10−3</td>
<td>[67]</td>
</tr>
<tr>
<td>Mg:4Li:1Ca</td>
<td>MAO/CS-3</td>
<td>Hank’s</td>
<td>-1.93</td>
<td>6.71 × 10−6</td>
<td>[68]</td>
</tr>
<tr>
<td>AZ31</td>
<td>PEO/Mg-Zn-Al LDH</td>
<td>PBS</td>
<td>-0.68</td>
<td>8.59 × 10−6</td>
<td>[69]</td>
</tr>
<tr>
<td>AZ31</td>
<td>MMT-BSA</td>
<td>Hank’s</td>
<td>-1.40</td>
<td>9.65 × 10−7</td>
<td>present work</td>
</tr>
</tbody>
</table>
4.3. Biocompatibility mechanism

The damage of medical materials in erythrocytes include mechanical damage of the surface of materials and chemical effects of material’s soluble ions. The latter and the associated chemistry could change the osmotic pressure and pH of the solution. Sudden hypertonic or hypotonic solution or sudden changes in pH may lead to abnormal protein and lipid content or quality of the erythrocytes, resulting in a destruction of the erythrocytes or increased dissolution brittleness, and thus the occurrence of hemolysis. Therefore, the change of the Mg$^{2+}$ ion concentration in solution during immersion was investigated (Fig. 5(c)). When the erythrocytes were in contact with the Mg alloy AZ31, the combination of a higher Mg$^{2+}$ ion concentration with the higher pH values (Fig. 5(b)) led to different osmotic pressures on the inside and outside of the erythrocytes, resulting in hemolysis. Because of the protective effect of the coating on the matrix, the Mg$^{2+}$ ions concentration and the pH values of the MMT and MMT-BSA coatings displayed little change, which resulted in a lower hemolysis rate.

Based on the biocompatibility experiments, it can be concluded that the MMT-BSA coating shows better biocompatibility, which can be ascribed to three factors:

(a) The hydrophilic enhancement of the surface. The albumin molecules have a large number of hydrophilic groups, so the hydrophilicity of the material could be improved. A surface that has a good hydrophilicity is favorable for cell adhesion and growth.

(b) The changes to the surface microstructure. It can be seen from the SEM images that the MMT coating had a rough surface with obvious cracks. After albumin treatment, the coating became denser and smoother, which was beneficial for the adhesion and growth of the cells.

(c) The corrosion rate of magnesium alloy decreased and the local alkalization weakens. The presence of a coating slows down the formation of Mg(OH)$_2$, which slows down the alkalization of the solution and provides a suitable environment for the cells.

5. Conclusions

The MMT-BSA composite coatings were successfully prepared via hydrothermal synthesis and the immersion method to improve corrosion resistance and biocompatibility. The conclusions are as follows:

1. It was confirmed by characterization that the MMT-BSA coating was successfully prepared on the surface of magnesium alloy and the coating had a smooth surface with no obvious cracks.

2. The coatings had a markedly reduced $i_{corr}$ value ($2.09 \times 10^{-6}$ A cm$^{-2}$ for MMT coating and $7.65 \times 10^{-7}$ A cm$^{-2}$ for MMT-BSA coating), which was one and two orders of magnitude lower than that ($6.27 \times 10^{-6}$ A cm$^{-2}$) of the Mg alloy AZ31. The results indicate that the coatings could enhance the corrosion resistance and slow down the degradation of the Mg alloy AZ31 in Hank’s solution.

3. The MMT-BSA coating had improved hemocompatibility and cytocompatibility compared with the MMT coating and the Mg alloy AZ31. The hemolysis ratio of the coating was 3.3 %, which was lower than that of MMT coating (4.7 %) and Mg alloy AZ31 (25.6 %). The cell viability of the MMT-BSA coating (91 % for 24 h and 95 % for 72 h) was significantly higher than that of the MMT coating (87 % for 24 h and 95 % for 72 h) and the Mg alloy AZ31 (79 % for 24 h and 75 % for 72 h).

4. In vivo studies showed good biocompatibility and controlled degradation of the MMT-BSA coating after 120 d of implantation. The pathological section results showed that the samples had no toxic damages on tissues and organs. Hence, the MMT-BSA coating on the Mg alloy AZ31 is a promising candidate for biodegradable bone implant applications.

Acknowledgements

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