Corrosion resistance and antibacterial activity of zinc-loaded montmorillonite coatings on biodegradable magnesium alloy AZ31

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Abstract

A Zinc-loaded montmorillonite (Zn-MMT) coating was hydrothermally prepared using Zn$^{2+}$ ion intercalated sodium montmorillonite (Na-MMT) upon magnesium (Mg) alloy AZ31 as bone repairing materials. Biodegradation rate of the Mg-based materials was studied via potentiodynamic polarization curves, electrochemical impedance spectroscopy (EIS) and hydrogen evolution tests. Results revealed that both Na-MMT and Zn-MMT coatings exhibited better corrosion resistance in Dulbecco’s modified eagle medium (DMEM) + 10% calf serum (CS) than bare Mg alloy AZ31 counterparts. Hemolysis results demonstrated that hemocompatibility of the Na-MMT and Zn-MMT coatings were 5%, and lower than that of uncoated Mg alloy AZ31 pieces. 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. The measured inhibitory zone and bacterial growth rate confirmed that Zn-MMT coatings exhibited higher suppression toward both E. coli and S. aureus than that of Na-MMT coatings. The investigation on antibacterial mechanism through scanning electron microscopy (SEM) and lactate dehydrogenase (LDH) release assay manifested that Zn-MMT coating led to severe breakage of bacterial membrane of E. coli and S. aureus, which resulted in a release of cytoplasmic materials from the bacterial cells. In addition, the good inhibition of Zn-MMT coatings against E. coli and S. aureus might be attributed to the slow but sustainable release of Zn$^{2+}$ ions (up to 144 h) from the coatings into the culture media. This study provides a novel coating strategy for manufacturing biodegradable Mg alloys with good corrosion resistance, biocompatibility and antibacterial activity for future orthopedic applications.

Statement of Significance

The significance of the current work is to develop a corrosion-resistant and antibacterial Zn-MMT coating on magnesium alloy AZ31 through a hydrothermal method. The Zn-MMT coating on magnesium alloy AZ31 shows better corrosion resistance, biocompatibility and excellent antibacterial ability than magnesium alloy AZ31. This study provides a novel coating on Mg alloys for future orthopedic applications.

1. Introduction

Magnesium (Mg) and its alloys, as promising biodegradable materials, encounter a series of critical challenges, such as rapid degradation [1–3], dramatic local alkalization and vigorous hydrogen gas evolution [4–6], which impede their clinical deployment.
Microbial infection after clinical device-implantation [7] is also an issue. Existing research mainly focused on improving corrosion resistance, biocompatibility and antibacterial activity [8,9]. In general, degradation rate of Mg alloys can be tailored through metal-lurgical alloying [10–13], post-treatment [14,15], surface coatings [16–18] and modification [19–21], and so on. Though addition of appropriate contents of Zn, Ca, Mn, Sr, Li, and rare elements (e.g. Y and Nd) into Mg matrix improves mechanical strength and corrosion resistance [22–26] to a certain degree, localized corrosion and subsequent loss of mechanical integrity in Mg-based implants in physiologic environments can inevitably occur during bone recovery progress. Surface modifications or/and coatings, in contrast, turn out to be simple and efficient options to protect Mg alloys against corrosion and thus satisfy the requirements for a great number of engineering applications, including biomedical devices [27–30].

A large body of reported publications demonstrates that fluoride conversion coating [31], Si-containing coating [32], and polycaprolactone (PCL) coating [33] are feasible to optimize corrosion resistance and biocompatibility of Mg alloys. Our previous work [34] reveals that micro-arc oxidation (MAO)/polymethylmethacrylate yslane (PMMTS) composite coatings offer a superior protective function and a self-healing ability to Mg alloy AZ31 in Hank’s solution, which is attributed to the formation of calcium phosphate precipitates and amorphous silica film. Recently, we developed a saline-TiO2 based hybrid coating through layer-by-layer (LBL) assembly followed by saline treatment [35] to impose tangible protection on Mg-11Li-1Ca alloy. In addition, Ca-doped ZnPO4 chemical conversion coating [36], polysiloxane modified layer-by-layer assembled self-healing coating [37], layer-by-layer assembled DNA coating [38] and spin-assisted layer-by-layer assembled coating [39] have been proposed by our group to optimize corrosion resistance and biocompatibility of Mg alloy AZ31.

In addition, device-related infection may occur after surgical management with implantation in the patient, leading to an earlier failure of implanted devices [40,41]. In recent years, antibacterial and biodegradable Mg alloys have attracted enormous interest from both academia and industry. To date, mainstream studies regarding antimicrobial performance of biomedical implants are employing copper (Cu), silver (Ag) and antibacterial organic compounds as key antibacterial agents in bulk and coatings for metallic implants [42–44]. However, it is not a feasible way to utilize the inherent germ-killing ability of Cu and Ag in bulky Mg parts, which will form micro-galvanic couples and accelerate corrosion kinetics of Mg [45]. As such, it is of great significance to develop coatings upon Mg alloys with both corrosion-protective and antibacterial functions. It was reported that fluorine [46] and nano Ag particles [47] based conversion coatings are promising candidates to kill E. coli on the surface Mg alloy AZ31. However, Cu+, Ag+ and F− ions play a detrimental role in surrounding tissues and cells, which incurs severe safety concerns [48]. It remains a technical challenge to yield a single coating system for Mg alloys that could play a multiple role in mitigating corrosion, inhibiting pathogens and providing sufficient support to new bone-tissue growth [49].

Over the past few years, a number of new strategies were attempted to tackle such issues, including tannic acid (TA) [50], chitosan (CHI) ammonium salt coating [51], plasma electrolytic oxidation coating (PEO)/polycaprolactone (PCL) [52], MAO chitosan [53], and gentamicin sulfate (GS)/poly(sodium 4-styrene sulfo-)nate (PSS) hybrid coating [54]. Zinc ions containing coatings with a comparative antibacterial role but least detrimental to DNA or the immune system were developed as a replacement of toxic Cu2+ and Ag+ counterparts [55,56]. Of the various antibacterial materials, Zn2+ ions exhibit unique biodegradability in the physiological environments, and play diverse biological roles in facilitating DNA synthesis, enzyme activity, and biomineralization.

A number of research groups have been attempting to incorporate Zn2+ ions into phosphate conversion coatings upon Mg alloys to enhance their corrosion resistance [57,58]. However, the antibacterial activity and bone cell compatibility Zn-related coatings are rarely investigated together.

To carry on and release Zn2+ ions in a safe and sustainable manner, montmorillonite (MMT, Na0.7[Al33Mg0.7]Si8O20(OH)4·nH2O) was selected as a carrier [59] owing to its excellent biocompatibility and extensive uses in a broad range of medical and pharmaceutical applications [60–62]. MMT is a classification of natural clay and a safe carrier material with layered silicate structure, featuring with a large surface area, high adsorption capacity and ion exchanging properties [63,64]. In particular, nano-sized MMT has been successfully used as drug carrier [65], catalyst [66], food additives [67], and issue engineering materials [68]. It has also been reported that Na-MMT is non-cytotoxic to a variety of cell lines, such as caco-2, human skin fibroblast (CRL2522), saos-2 and L929 cells [69–72]. In recent years, the immobilization of Ag+, Cu2+ and Zn2+ ions with MMT molecules for potent antimicrobial activity has been widely documented [73]. Therefore, the feasibility of Zn-MMT coatings for excellent antibacterial activity and bone cell compatibility on Mg alloys was explored in this study. Herein, MMT coatings incorporated with Zn2+ ions (Zn-MMT) were prepared through a simple hydrothermal approach. Immersion and electrochemical tests were performed in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% calf serum (CS). This study aims to understand the degradation behavior, antibacterial performance and cytocompatibility of Zn-MMT coating on AZ31 alloy, and to take insights into in vitro degradation and antibacterial mechanisms.

2. Materials and methods

2.1. Materials and chemicals

Extruded AZ31 sheets with a nominal chemical composition (in wt%), Al 2.5–3.0, Zn 0.7–1.3, Mn > 0.2 and balanced Mg, was used as starting material. Na-MMT powder, zinc nitrate (Zn(NO3)2·6H2O, analytical reagent, 99.0%) and all substances for Dulbecco’s modified eagle medium (DMEM, Table 1) were supplied by Qingdao Jingke Chemical Reagent Co. Ltd., China. Metal specimens were machined off the sheets into dimensions of 20 mm × 20 mm × 4 mm for following coating growth and characterization. Mechanical grinding was carried out with SiC papers progressively up to a 1500-grit surface finish, followed by ultrasonic cleaning in ethanol (analytical reagent, 99.7%) and distilled water before drying in a cold air flow and ultraviolet sterilization.

2.2. Preparation of Na-MMT and Zn-MMT coatings

Zn-MMT powder was fabricated by ion exchange between Na-MMT and zinc nitrate in deionized (DI) water. 5.0 g Na-MMT powders were suspended in 95 mL DI water in a 250 mL conical flask. Then, 1.168 g (100% cation exchange capacity (CEC) times of pristine MMT) Zn(NO3)2·6H2O intercalation agent was added and followed by vigorous stirring at room temperature for 5 h. The suspensions were centrifuged at 1500 rpm for 10 min, then washed several times using DI water until Zn2+ ions were not detectable by sodium sulfide (Na2S) [74]. The washed cake was dried at 65 °C for 24 h.

In brief, Zn-MMT powder (2.0 g) was added into 100 mL DI water in an ultrasonic bath for 30 min. The above solutions were maintained at 130 °C for 5 h with magnetic stirring at pH 10.5. The resulting slurry and Mg alloy AZ31 specimen were transferred to a Teflon-lined autoclave and heated in an oven at 130 °C for 36 h


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to yield Zn-MMT coating upon the AZ31 pieces. Na-MMT coatings were hydrothermally prepared on Mg alloy AZ31 substrate at pH 10.5, 130 °C for 5 h and used as control group. The procedures of preparation of Zn-MMT and Na-MMT coatings are schematically illustrated in Fig. 1 [75].

2.3. Surface characterization

Surface and cross-sectional morphologies of Zn-MMT and Na-MMT coatings were observed by means of scanning electron microscopy (SEM, Nova NanoSem 450) equipped with energy-dispersive X-ray spectroscopy (EDS). Chemical bonding states of the coatings were identified by X-ray photoelectron spectroscopy (XPS, ESCALAB250, USA) and Fourier transform infrared spectroscopy (FT-IR, Nicolet 380, Thermoelectron, USA). Crystallographic structure was examined by X-ray diffraction (XRD, Rigaku D/MAX2500PC, Japan) with a Cu Kα (λ = 0.15406 nm) source operated at 40 kV and 40 mA.

2.4. Electrochemical tests

Potentiodynamic polarization curves and electrochemical impedance spectroscopy (EIS) were recorded on a classical three-electrode cell at room temperature. The cell contains 80 mL DMEM + 10% CS solution with a sample piece as working electrode and a platinum plate as counter electrode and a saturated calomel electrode (SCE) as reference electrode cell at room temperature. The cell contains 80 mL DMEM + 10% CS solution with a sample piece as working electrode.

<table>
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Table 1

Chemical compositions of DMEM solution (mg L⁻¹).

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Fig. 1. Schematic illustration of preparation of Zn-MMT and Na-MMT coated Mg alloy AZ31.
diameter of 25 μm. The scratch tests were performed at a scan rate of 5 μm s\(^{-1}\). And the load was linearly increased up to 1000 mN, with an increased speed of 10 mN s\(^{-1}\) applied after 50 μm displacement, until the total scratch reached a length of 200 μm.

2.7. In vitro biocompatibility evaluation

2.7.1. Hemolysis tests

Preparation of normal saline extracts: samples (with dimensions of 20 mm × 20 mm × 4 mm) were placed into 25 mL beakers according to the ISO 10993-1 standards: Biological evaluation of medical devices Part 1: Evaluation and testing within a risk management process (ISO 10993-1: 2009). The sample surface area/extraction medium volume equals 3:1 cm\(^2\) mL\(^{-1}\). 10 mL saline was added into the beakers, and then maintained in a shaking bath at 36.5 °C for 30 min, followed by centrifuging at a rate of 1500 rpm for 10 min to yield supernatants.

Hemolysis tests were conducted as described previously [79]. Anticoagulant citrate dextrose (ACD) human blood was obtained from a healthy volunteer and with an addition of 8% sodium citrate in proportions. Then, 0.2 mL diluted ACD whole blood (8 mL ACD whole blood was diluted by 10 mL normal saline) was drop wisely added into the material extracting solution. Similarly, normal saline solution was used as a negative control and DI water as a positive control. Absorbency of the solutions was measured with an ultraviolet spectrophotometer (UNIC-7200, China) at 545 nm. The hemolysis ratio (HR) can be derived from Eq. (3) [26]:

\[
HR = \frac{A_S - A_N}{A_P - A_N} \times 100\%
\] (3)

where, \(A_S\) is the absorbency of the samples; \(A_P\) and \(A_N\) denote the absorbency of the positive control and the negative control, respectively.

2.7.2. In vitro cytotoxicity evaluation

Cytotoxicity of the samples towards mouse osteoblasts cells (MC3T3-E1) (Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China) was quantitatively determined for 24 h and 72 h by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay. Forty wells with cell suspension were divided into four groups (n = 5): bare AZ31 piece, Na-MMT coating, Zn-MMT coating and control group (culture medium containing cell suspension). The MC3T3-E1 cell suspension was adjusted to a cell density of 1 × 10\(^4\) mL\(^{-1}\), and then 200 μL was added into each well of 96-well plates, and 20 μL MTT solution was injected on each of 24 h and 72 h. The 96-well plates were placed in an incubator for 4 h. After incubation, the medium was removed, and formazan crystals were dissolved in DMSO. Absorbance (A) was measured by an automatic microplate reader (SPECTRA, MAX M5, USA) at a wavelength of 570 nm. Cell viability rate was calculated as follows:

\[
\text{Cell viability} = \frac{A_{t}}{A_{c}} \times 100\%
\] (4)

where, \(A_{t}\) is the absorbance of testing specimens and \(A_{c}\) is the absorbance of control groups. Results were expressed as mean ± standard deviation (n = 5).

MC3T3-E1 cells were seeded at a density of 4 × 10\(^4\) per well, cultured in 3 mL DMEM, supplemented with 10% fetal bovine serum (FBS) for 24 h and 72 h in 6-well plates containing samples, stained with 200 μL of combination dye (Calcein-AM/PI-DNA) for 10 min, and examined by confocal laser scanning microscopy (Olympus IX83; USA). Viable cells combined with calcein-AM were stained green whilst dead cells combined with ethidium homodimer-1 were stained red.

2.8. Antibacterial assays

Antibacterial properties of bare, Na-MMT and Zn-MMT coated Mg alloy AZ31 specimens were evaluated through using E. coli (ATCC 25922) and S. aureus (ATCC 25923) as model Gram (-) and Gram (+) bacteria. E. coli was cultured in Luria-Bertani (LB) broth; and S. aureus was cultured in tryptic soy broth.

2.8.1. Inhibition zone

Antibacterial activities of bare, Na-MMT and Zn-MMT coated AZ31 specimens against E. coli and S. aureus were evaluated by inhibition zone tests. S. aureus and E. coli bacteria strains were cultured in agar plates at 37 °C overnight. The optical density of the tested organism was adjusted to 0.699 at 650 nm (S. aureus) and 0.428 at 410 nm (E. coli), which indicates the content of bacteria reached approximately 10\(^8\) colony-forming units (CFU) mL\(^{-1}\). Freshly grown bacteria were diluted by phosphate buffered saline (PBS) to an approximate concentration of 5 × 10\(^7\) CFU mL\(^{-1}\) of S. aureus or E. coli. 10 mL of the stock solution was spread onto the surface of an agar plate. After sterilization, bare, Na-MMT and Zn-MMT coated Mg alloy AZ31 specimens were directly used as antibacterial pieces. The plates were incubated at 37 °C for 24 h prior to observation of inhibition zones.

2.8.2. Bacterial growth curves

First of all, E. coli and S. aureus were cultured to logarithmic phase and vaccinated to broth liquid medium according to 1% of the quantity (10\(^8\) bacteria per mL). The samples (i.e. bare AZ31, Na-MMT coating, and Zn-MMT coating) were placed into the culture medium and incubated in a conical flask in a rocking bed at 37 °C with a shaking speed of 150 rpm. For controls, E. coli and S. aureus were cultured in the same conditions. The pH value and absorbency of the culture medium with spectrophotometer (UNIC-7200, China) at 410 nm for E. coli and 650 nm for S. aureus were measured every 2 h. And then, pH variation and bacterial growth curves were plotted accordingly. All experiments were performed in triplicates and average values were reported.

2.8.3. Bacterial morphology

SEM was performed to observe morphology and surface structure of the bacterial cells on Mg alloy AZ31 substrate, Na-MMT and Zn-MMT coatings by using Nova Nano SEM 450. SEM micrographs were taken using the following procedures: after culture on AZ31 substrate, Na-MMT and Zn-MMT coatings for 12 h, cells from the samples were fixed with 2.5% glutaraldehyde overnight at 4 °C. And then, washed with 0.1 M PBS (pH 7.4) and dehydrated with a graded ethanol series (50, 60, 70, 80 and 90%). Gold was sputtered on the sample surfaces for SEM observation.

2.8.4. Measurements of releasing Zn\(^{2+}\) and Mg\(^{2+}\) ions

Mg alloy AZ31 substrate, Na-MMT and Zn-MMT coatings were immersed in PBS solution with a pH of 7.4 at 36.5 °C and shaken at 100 rpm throughout the entire process. 1 mL of PBS solution was removed at a regular interval and 1 mL of freshly pre-heated PBS solution was added. Concentration of Zn\(^{2+}\) and Mg\(^{2+}\) ions released from bulk samples in the removed liquid was measured by atomic absorption spectrometry (AAS). Triplicates were performed for each measurement to yield accumulative release curves of Zn\(^{2+}\) and Mg\(^{2+}\) ions.

2.8.5. Membrane integrity

LDH assays (Baomianbo Co., Ltd. Shanghai, China.) were used to evaluate cell membrane integrity. 200 μL sample was added to 1 mL reagent containing Nicotinamide adenine dinucleotide (NADH) and incubated at 25 °C. LDH activity was then determined by measuring the rate of decrease in the NADH concentration which
was monitored by recording the change of absorbance at 334 nm. Three parallel tests were carried out to yield mean values (n = 3).

2.9. Statistical analysis

Statistical analysis was conducted with SPSS 19.0. Difference between groups was analyzed using one-way ANOVA followed by Tukey’s tests. p ≤ 0.05 is defined as a statistically significant difference.

3. Results

3.1. MMT coating characterizations

Surface morphology of the Na-MMT and Zn-MMT coatings are presented in Fig. 2. Na-MMT coating showed uniform porous features with either irregular, curly or pedal edges (inset of Fig. 2a). The overall morphology of Zn-MMT coating was similar to that of Na-MMT; however, smaller pores and a more homogenous layered structure was observed. The incorporation of Zn²⁺ ions in the interlayers of MMT had a slight impact on the layered structure of the Na-MMT coating. Zn-MMT coating was much denser than Na-MMT coating. Cross-sectional images (Fig. 2 c and d) demonstrate that the thickness of Na-MMT and Zn-MMT coatings were 40 and 42 μm, respectively. EDS data in Fig. 2e shows that Mg, Al, O, Si, and Na were the main components of Na-MMT coating; while Zn, Al, O, Si, and Mg were the key components of Zn-MMT coating and the results are in agreement with the EDS analysis. The Al2p peak of the Zn-MMT coating was observed at 75.04 eV and was shifted higher by ~0.54 eV compared to the Na-MMT coating. The binding energies of O1s, Si2p, and Mg1s for the Zn-MMT coating are similar to that of Al2p, which shifted higher by 0.44 eV, 0.24 eV and 0.24 eV, respectively. The peak shifts of Al2p, O1s, Si2p, and Mg1s imply a change in the basal spacing, which is consistent with the XRD patterns (Fig. 3b).

XRD patterns of AZ31 substrate with Na-MMT and Zn-MMT coatings are shown in Fig. 3a. XRD pattern of Na-MMT powder displays a peak at 2θ of 5.98°, which is assigned to d (0 0 1) basal spacing of 1.48 nm. XRD pattern of AZ31 alloy with Na-MMT coating displays same characteristic peaks to those of Na-MMT phases over a 2θ range of 20°–25°, which confirms that Na-MMT coating (Fig. 3a) was deposited on AZ31 substrate using the hydrothermal process. Regarding Zn-MMT powder, characteristic diffraction peaks at lower 2θ of 5.64°, corresponding to the increased d (0 0 1) basal spacing of 1.57 nm. Zn-MMT powder prepared by ion exchange displays a typical layered structural characteristic of Na-MMT powder with obvious peaks, corresponding to the diffraction peaks of (0 2 0) and (0 0 4) planes (Fig. 3b).

XPS analysis was employed to identify the chemical states of the elements in the Na-MMT and Zn-MMT coatings (Fig. 4). From the results we can see that Na, Al, O, Si, and Mg were present in Na-MMT coating; while Zn, Al, O, Si, and Mg were the key components of Zn-MMT coating and the results are in agreement with the EDS analysis. The Al2p peak of the Zn-MMT coating was observed at 75.04 eV and was shifted higher by ~0.54 eV compared to the Na-MMT coating. The binding energies of O1s, Si2p, and Mg1s for the Zn-MMT coating are similar to that of Al2p, which shifted higher by 0.44 eV, 0.24 eV and 0.24 eV, respectively. The peak shifts of Al2p, O1s, Si2p, and Mg1s imply a change in the basal spacing, which is consistent with the XRD patterns (Fig. 3b).

Fig. 4g shows the presence of Na peaks in the Zn-MMT coating, revealing that Na⁺ ions were partially replaced by Zn²⁺ ions during the ion-exchange process. The observed 2θ increase after the Zn²⁺ ion exchange could be derived from the difference in the size of the hydrated forms between Zn²⁺ and Na⁺ ions. Despite the ionic radius of Zn (0.074 nm) being much smaller than that of Na (0.095 nm), the Na⁺ ions in Na-MMT were exchanged by [Zn(H₂O)₆]²⁺ rather than the Zn²⁺ ions [80].

Fig. 2. SEM Surface morphology (a) Na-MMT coating, (b) Zn-MMT coating; and cross-sectional micrographs of (c) Na-MMT coating, (d) Zn-MMT coating; corresponding EDS plots and elemental fraction of (e) Na-MMT and (f) Zn-MMT coatings.
The FT-IR spectra (Fig. 5) of the Na-MMT and Zn-MMT coatings exhibit the characteristic MMT absorption band at 3620 cm⁻¹, which corresponds to the stretching vibration of the -OH group arising from adsorbed water on the surface, interlayer water, and magnesia. The absorption peaks at 3240 cm⁻¹ and 1644 cm⁻¹ are attributed to the stretching and bending vibrations of the interlayer H₂O, respectively. The major peaks at 1038 cm⁻¹ (stretching vibration of Si-O), 915 cm⁻¹ (Al-OH bending vibration), 850 cm⁻¹ (stretching vibration of Mg-OH), 694 cm⁻¹ (Si-O-Mg bending vibration), and 464 cm⁻¹ (Si-O bending vibration) are characteristics of the clay structure. For the modified Zn-MMT, the O-H stretching vibration band increased slightly and this can be attributed to the hydrated Zn²⁺ ions.

3.2. Corrosion performance

3.2.1. Electrochemical behavior

As shown in Fig. 6a, samples were exposed to electrolyte and stabilized at open circuit potential (OCP) for 600 s. OCP curves as a function of the immersion time demonstrate that OCPs of bare Mg alloy AZ31 substrate and coatings can be ranked in a decreasing order as: Na-MMT coating > Zn-MMT coating > AZ31 substrate. Na-MMT coating has the most passive OCP and this scenario indicates that it has the lowest corrosion tendency. Fig. 6b shows the polarization curves for the bare AZ31, the Na-MMT, and the Zn-MMT after immersion in DMEM + 10% CS solution. The corresponding samples parameters are shown in Table 2. The free corrosion potential (Ecorr) of the bare AZ31, Na-MMT, and Zn-MMT are -1.60, -1.49, and -1.38 V vs. SCE, respectively. The corrosion current density (Icorr) of the bare AZ31, Na-MMT, and Zn-MMT are 2.81 × 10⁻⁶, 2.90 × 10⁻⁷, and 2.86 × 10⁻⁷ A cm⁻², respectively. This indicates that the Icorr values for the Zn-MMT and Na-MMT-coated AZ31 samples decreased by about two orders of magnitude compared to the AZ31 substrate alone. The anodic Tafel slope (βa = 238.3 mV decade⁻¹) of the AZ31 substrate decreased to 153.7 and 119.2 mV decade⁻¹ after modification with the Na-MMT and Zn-MMT coatings, respectively. The good corrosion protection of the Na-MMT and Zn-MMT coatings are also confirmed by the increasing Rct from 1.2 × 10⁶ to 7.6 × 10⁶ and 8.4 × 10⁶ Ω cm², respectively. It should be noted that the Ecorr value is in agreement with the Rct for both the Na-MMT and Zn-MMT coatings.

EIS spectra for bare Mg alloy AZ31 substrate and Na-MMT/Zn-MMT coatings in DMEM + 10% CS solution are shown in Fig. 7a–c. The Nyquist plot (Fig. 7a) shows that all the samples are characterized by two capacitive loops in both high and medium frequency ranges and an inductive loop in low frequency range. The capacitive loops can be related to a charge transfer process and formation of a corrosion product layer and/or due to the presence of the coatings: while the inductive loop can be attributed to the dissolution of Mg and peeling off of the coatings. However, corrosion resistance is clearly enhanced due to the larger diameter of the capacitive loop for the coated samples than for the bare AZ31. Moreover, the larger low-frequency impedance modulus, |Z|, indicates a better corrosion protection performance [81,82]. From Fig. 7b, it can be observed that |Z| value of the bare AZ31 is 147.5 Ω cm², which is far lower than that of Na-MMT (2.73 × 10⁴ Ω cm²) and Zn-MMT coatings (3.23 × 10⁴ Ω cm²). The results demonstrate that the MMT coatings significantly enhanced corrosion resistance of Mg alloy AZ31 substrate. Fig. 7c shows bode phase plots for the samples. In general, phase angle of the bare AZ31 substrate is lower. The phase angles were similar for Na-MMT and Zn-MMT coatings and reached approximately 65°. The curves in the middle frequency range are smooth, demonstrating that the surface of the samples was compact and uniform.

ECS for bare AZ31 substrate, Na-MMT, and Zn-MMT coatings are shown in Fig. 7d and e [83] with fitting data in Table 3. In ECs, Rct and Rs are the solution resistance and the charge transfer resistance, respectively. Rct is a resistor at low frequency with an inductance (L). R1 shows the resistance of the coatings. Furthermore, CPE1 and CPE2 represent constant phase elements (CPEs) that are related to the double layer capacitance. For the AZ31 substrate (Fig. 7d), the high-frequency capacitance loop is described by CPE1 and R1, and characterizes the low corrosion product layer, and CPE2 and Rct present the charge transfer process between the substrate and corrosion products layer. The low-frequency inductive loop represents the dissolution of Mg and is characterized by Rct and L. The Na-MMT coating exhibits similar corrosion behavior to bare AZ31, while CPE1 and R1 mean the resistance of Na-MMT coating, and the inductive loop suggests that pitting corrosion occurs during the electrochemical testing due to its porous structure (Fig. 2a). In addition, for Zn-MMT coating (Fig. 7e), the values of Rct and Rs are lower than those for the Na-MMT coating, indicating better corrosion resistance of Na-MMT coating. Rs and L demonstrate the peeling of coating and formation of corrosion products. From the fitting results shown in Table 3, the increase in the Rs from 31.55 Ω cm² to 3.62 × 10⁴ Ω cm² and 2.94 × 10⁴ Ω cm² for Na-MMT and Zn-MMT, respectively, confirms that the both the coatings effectively protect Mg alloy AZ31 from corrosion.

3.2.2. Immersion tests

The SEM images in Fig. 8 illustrate the morphology and the corresponding EDS spectra for the bare AZ31, Na-MMT, and Zn-MMT coatings after immersion in the DMEM + 10% CS solution for 1 and 3 days. For the AZ31 substrate, a dry riverbed surface morphology...
was observed (Fig. 8a and b). Interestingly, there was no visible corrosion on the surface of Na-MMT coating (Fig. 8c and d); while corrosion was comparatively slight for Zn-MMT coating (Fig. 8e and f). EDS spectra of the corroded surface for all three specimen groups indicated the presence of C, O, Mg, Al, N, S, and Cl, confirming the presence of possible corrosion products, such as Mg(OH)$_2$ [84,85]. Nevertheless, Si was also observed on the surface of the Na-MMT and Zn-MMT coatings (Fig. 8d and f); the high Si, Al, Mg, O, and C content indicates that the Na-MMT and Zn-MMT coatings remained on the AZ31 alloy surface. Note that the Al content of the Na-MMT and Zn-MMT coatings retained a higher value than the AZ31 alloy, this is due to the presence of Al$_2$O$_3$ in the coatings and Al$^{3+}$ release from the corroded AZ31 substrate. The Na-MMT and Zn-MMT coatings had a much greater corrosion resistance because a certain coating thickness could block the migration of H$_2$O and chlorides into the Mg substrate and inhibit attack from the solution. The corrosion of the Zn-MMT coating was initiated from the surface to the bulk resulting in the formation of cracks. From the observed cross-sectional morphology (Fig. 8d and f), it can be seen that Na-MMT and Zn-MMT coatings were strongly

Fig. 4. (a) XPS broad survey of Na-MMT coating and high-resolution of (b) Na, (c) Al, (d) O, (e) Si and (f) Mg. (g) XPS broad survey of Zn-MMT coating and high-resolution of (h) Zn, (i) Al, (j) O, (k) Si and (l) Mg.
adhered to the AZ31 substrate. Further localized corrosion or pitting corrosion occurred after immersion. The micro-cracks on the Zn-MMT coating (Fig. 8f) could be attributed to a decrease in the coating density due to ultrasonic processing and ZnO growth during the immersion tests. The results demonstrated that the compact and uniform Na-MMT and Zn-MMT coatings could provide effective protection for Mg alloy AZ31 specimens.

As indicated from the HER curves (Fig. 9a) of the AZ31 substrate, Na-MMT and Zn-MMT coatings in DMEM + 10% CS solution, there are three distinct stages for the HER of the Mg alloy AZ31: at the initial soaking of 10 h, the HER rapidly slowed down to 0.08 mL cm\(^{-2}\) h\(^{-1}\). After an immersion of 11–60 h, the HER slowly reduced, indicating the formation of the Mg(OH)\(_2\) film on the AZ31 substrate. Then, HER remained stable due to the formation and protection of the corrosion products on the AZ31 substrate.

For both Na-MMT and Zn-MMT coatings, the HER curves were very similar. There were two stages: the HERs increased slightly in initial immersion of 10 h, then kept steady (as shown in the inset of Fig. 9a). It was clear that the HER of the Na-MMT and Zn-MMT coatings were significantly lower than that of the AZ31 substrate. The average HER of the Na-MMT and Zn-MMT coatings was approximately 0.010 mL cm\(^{-2}\) h\(^{-1}\) and 0.015 mL cm\(^{-2}\) h\(^{-1}\), respectively. And the HER of the AZ31 substrate was 0.06 mL cm\(^{-2}\) h\(^{-1}\). These results confirmed that the Na-MMT and Zn-MMT coatings could significantly improve the corrosion resistance of AZ31 substrate. It is noteworthy that no significant differences in the average HER were observed for both Na-MMT and Zn-MMT coatings, which might be associated with their similar structures.

It is noted that the HER of AZ31 substrate was obtained in DMEM + 10% CS solutions in the study was higher than that obtained in NaCl or Hank’s solutions. The difference in HER might be ascribed to the different chemical compositions of the solutions and methods. The hydrogen evolution tests were performed by a replacement of the DMEM + 10% CS solutions every 24 h. The amount of Cl\(^-\) in DMEM led to faster corrosion of Mg alloy AZ31.

The pH value (Fig. 9b) of the immersion solution increased rapidly in the first 5 h in DMEM + 10% CS solution for AZ31 alloy; whereas a slow increase in pH followed, and which reached up to approximately 8.9 after an immersion of 24 h. However, the pH value increased slowly for Na-MMT and Zn-MMT coatings, and went up 7.82 and 7.91, respectively, after an immersion of 24 h. Therefore, the corrosion of the AZ31 alloy was alleviated with the protective coating Na-MMT and Zn-MMT. It is noticeable that the pH value for Zn-MMT coating was slightly higher than that for Na-MMT coating. The insignificant increase in pH could provide a suitable microenvironment for cell growth.

XRD patterns of the immersed samples in Fig. 9c show peaks associated with Mg(OH)\(_2\), which indicate corrosion between coatings and AZ31 substrate. It can be seen that the peaks associated with Na-MMT and Zn-MMT coatings on Mg alloy AZ31 were still

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**Table 2**

Electrochemical parameters of the polarization curves in DMEM + 10% CS solution.

<table>
<thead>
<tr>
<th>Samples</th>
<th>(E_{corr}(V_{oc}))</th>
<th>(i_{corr}(\text{A}\ cm^{-2}))</th>
<th>(j_{a} (\text{mV dec}^{-1}))</th>
<th>(-j_{c} (\text{mV dec}^{-1}))</th>
<th>(R_{p} (\Omega \text{ cm}^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare AZ31</td>
<td>-1.60</td>
<td>2.81 \times 10^{-5}</td>
<td>238.3</td>
<td>115.3</td>
<td>1.2 \times 10^{6}</td>
</tr>
<tr>
<td>Na-MMT coating</td>
<td>-1.49</td>
<td>2.90 \times 10^{-7}</td>
<td>153.7</td>
<td>76.8</td>
<td>7.6 \times 10^{7}</td>
</tr>
<tr>
<td>Zn-MMT coating</td>
<td>-1.38</td>
<td>2.86 \times 10^{-7}</td>
<td>119.2</td>
<td>100.9</td>
<td>8.4 \times 10^{7}</td>
</tr>
</tbody>
</table>
present after 5 days of immersion. It indicates that the coatings had good corrosion resistance. Several weak peaks could be observed in the Zn-MMT coating and can be ascribed to the possible formation of a small amount of ZnO.

Fig. 9d shows the FT-IR spectra of the Na-MMT and Zn-MMT coatings immersed in DMEM + 10% CS solution for five days. The characteristic bands assigned to MMT remained after the immersion test, demonstrating that the Na-MMT and Zn-MMT coatings had a very stable structure. Interestingly, the band at 1408 cm$^{-1}$ in the FT-IR spectrum of Zn-MMT coating can be attributed to C=O. This finding designates the presence of CO$_3^{2-}$ ions, indicating the formation of Zn carbonated compounds on the coating surface in DMEM + 10% CS solution for five days.

3.3. Nanoscratch tests

The adhesion strength between the coatings and AZ31 substrate was investigated via the nano-scratch tests, which could be used to evaluate the critical load (L) during the adhesive failure of the coating/substrate system. Fig. 10 showed the relationships among the depth, load and sliding displacement. The critical load of the Zn-MMT coating is 134.93 mN (Fig. 10b), representing the adhesion between the coating and the AZ31 alloy. The adhesion strength of the coating is almost equal to 134.16 mN for the Na-MMT coating because of the same hydrothermal process (Fig. 10a). Therefore, in the process of preparing Zn-MMT coating, ultrasound treatment for 30 min had no evident effect on the adhesion strength between the coating and the substrate.

In addition, in combination with hydrogen evolution, electrochemistry, and the SEM morphologies after immersion, it could be seen that the Zn-MMT coating had almost the same corrosion resistance as Na-MMT coating. It might be the reason for the lamellar structure, which was more or less damaged by ultrasound treatment for 30 min during the preparation of Zn-MMT, and then rearranged during water heat treatment. It is, however, beneficial for sustained release of zinc-ions and strong antibacterial property in this way [79].

3.4. In vitro biocompatibility evaluation

3.4.1. Hemolysis tests

The hemolysis test is regarded as a common screening test, in particular for medical materials that come in to direct contact with blood. A higher degree of hemolysis indicates a higher risk of broken red blood cells (RBC) occurring on the material surface. Fig. 11 shows the absorbency (Fig. 11a) and HR (Fig. 11b) of the AZ31 alloy, and the Na-MMT and Zn-MMT coatings. After being in contact with the erythrocytes for 30 min, the HR of the Mg substrate was 41.62% (Fig. 11b) and the HR for the Na-MMT and Zn-MMT coatings were 4.81% and 3.99%, respectively, which is lower than 5%. The results indicate that the Na-MMT and Zn-MMT coatings had an acceptable HR.

3.4.2. In vitro cytotoxicity evaluation

The cytotoxicity of the Na-MMT and Zn-MMT coatings were determined using MC3T3-E1 cells and an MTT assay (Fig. 12).
The cell viability in the three groups was less than 100% after 24 h and 72 h of culture; however, the Na-MMT and Zn-MMT coatings had values that were significantly higher than those of the AZ31 substrate (p < 0.05). The viability of the cells with the Na-MMT and Zn-MMT coatings decreased to some extent, but remained above 75%, which suggested acceptable biosafety for cellular applications (ISO-10993:5). There were no statistically significant differences in cell proliferation between the Na-MMT and Zn-MMT coatings after culturing for 24 h and 72 h (p > 0.05). Among all the groups, the Zn-MMT coating group displayed the lowest cytotoxicity. In summary, the Zn-MMT coating was able to improve the cytocompatibility of the AZ31 substrate.

Live/dead staining was carried out to further investigate the effects of the sample surface on the viability of the MC3T3-E1 cells. Compared to the AZ31 substrate, the number of dead cells declined, while the number of live cells significantly increased on the Na-MMT and Zn-MMT coatings after 24 h (Fig. 13a-d) and 72 h (Fig. 13e-h) of culture. The results indicate that the Na-MMT and Zn-MMT coatings improved the cytocompatibility compared to the Mg alloy AZ31. The release of Ca²⁺, Mg²⁺, and Sr²⁺ ions from MMT can considerably improve MC3T3-E1 cell growth; allowing cells to be well attached and spread on MMT surface [69,86]. Particularly, the improved cellular compatibility of Zn-MMT coatings might be ascribed to the beneficial influence of Zn²⁺ ions, which promotes the proliferation and activity of bone cells [87-91]. The addition of Zn to a composite coating could be helpful to adhesion and spread of cells [92].

3.5. Antibacterial ability

3.5.1. Inhibition zone

The results indicated that no inhibition zones were produced for the AZ31 substrate and with the Na-MMT coating against E. coli (Fig. 14a and b) and S. aureus (Fig. 14d and e). This implied that the AZ31 substrate and the Na-MMT coating had no antibacterial effects. As expected, obvious inhibition zones (Fig. 14c and f) could be discerned for the Zn-MMT coating for both E. coli and S. aureus, the diameters of the inhibition zone were 22 mm and 32 mm, respectively. Hence, the Zn-MMT coating demonstrated a
Fig. 9. HER, pH, XRD and FTIR for bare, Na-MMT and Zn-MMT coated Mg alloy AZ31 specimens in DMEM + 10% CS solutions.

Fig. 10. Scratch results of (a) Na-MMT and (b) Zn-MMT coated Mg alloy AZ31.

Fig. 11. Absorbency (a) and HR (%) (b) for bare, Na-MMT and Zn-MMT coated Mg alloy AZ31.
superior activity and a higher biocidal effects against *S. aureus* than *E. coli* [93,94].

3.5.2. Inhibited growth of bacteria

The effect of the Zn-MMT coated Mg substrate on the growth curve of *S. aureus* and the pH variation are presented in Fig. 15a. The *S. aureus* growth curves for the control and Na-MMT coating groups display a typical S shape, indicating that the Na-MMT group had no inhibitive effect on *S. aureus*. No obvious changes in the OD value of the bacterial solution could be identified for both the AZ31 substrate and the Zn-MMT coating, implying that *S. aureus* growth was inhibited by the Zn-MMT coating. PH of the culture media with AZ31 substrate increased rapidly during the first 5 h immersion, and reached 8.73 after 24 h. However, pH value increased more slowly for Na-MMT and Zn-MMT coatings, and reached 7.38 and 7.43 after 24 h of immersion, respectively. The lower observed pH in the coated samples was due to the protective effects of the coatings.

The effects of pH variation and the Zn-MMT coating on the growth curve of *E. coli* are shown in Fig. 15b. The observed growth curve of *E. coli* is a typical bacterial growth curve with an S shape. The group with the Na-MMT coating showed a short delay period, quickly followed by a logarithmic growth phase and then finally by a stable phase. The pH value increased slowly for the Na-MMT coating and reached 7.40 after immersion for 24 h. The *E. coli* growth curve for the AZ31 substrate had a delay period of ~10 h, which was followed by a normal growth curve. The pH value for the AZ31 substrate increased rapidly in the first 5 h and reached 8.84 after 24 h. These observations may be due to the alkalization of the culture medium and the formation of Mg(OH)₂ caused by the degradation of the Mg alloy. During the gradual adaptation of *E. coli* to the conditions, the growth curve became logarithmic in nature. The pH value for the Zn-MMT coating rose to 7.42 after 24 h. No changes in the growth curve for the Zn-MMT coating were identified, indicating that *E. coli* growth was obviously inhibited.

3.5.3. Bacterial morphology

SEM was used to investigate the effects of the Na-MMT and Zn-MMT coatings on the bacterial morphology and the membrane integrity. The *E. coli* bacterial cells (Gram-negative) cultured on the control and the Na-MMT coating were mostly rod-shaped (Fig. 16a and c). The *S. aureus* (Gram-positive) cells displayed a smooth and intact surface on the control and the Na-MMT coating (Fig. 16b and d). As shown in the SEM images in Fig. 16b and d, the bacterial cells for both *E. coli* and *S. aureus* that were cultured in the presence of the Na-MMT coating were viable with no observed membrane damage or cell death. While the bacteria on the Zn-MMT coating (Fig. 16e and f) displayed obvious morphological change; their surface was coarse and distorted and the cell membrane was damaged with intracellular material leaking out. Zn²⁺ ions are essential for the metabolism of microorganisms; however, a high concentration could lead to cytotoxicity in prokaryotic cells. A previous study also observed cell debris and bacteria lysis on the Zn-doped coating surface [95].

3.5.4. Accumulated Zn²⁺ and Mg²⁺ release

The accumulative release curve of the Zn-MMT coating in a phosphate buffer is presented in Fig. 17a. The release curve can be divided into two stages: Zn²⁺ ions were released at a constant rate during the early immersion of 96 h (the first stage), followed by a decreased release rate; and the release amounts were significantly reduced (the second stage). The release rate of Zn²⁺ ions from Zn-MMT coating did not display a trend similar to previously conducted research [78] on the MMT-sustained release devices with a fast-stable-slow trend. The difference might be related to the thorough cleaning of Zn²⁺ ions physically-absorbed on the surface of the MMT coating during its preparation. Therefore, no rapid releases occurred during the initial release phase; whereas, only the chemically-absorbed Zn²⁺ ions and Zn²⁺ ions between the layers of MMT were released slowly, lasted for 96 h. Although the release of Zn²⁺ ions was significantly reduced, while a small amount of Zn²⁺ ions was still positioned in the Zn-MMT coating. Thus, Zn²⁺ ions from Zn-MMT coating were released slowly; its antibacterial effect lasted for 144 h. This result designated MMT is a drug sustained-release material carrier and shows strong antimicrobial ability against *Gram-positive S. aureus* [93].

During the immersion, Mg²⁺ ions would be released from the bare AZ31, Na-MMT and Zn-MMT coatings into the phosphate buffered solution. The change in Mg²⁺ ions is showed in Fig.17b. The release curve of Mg²⁺ ions from the AZ31 substrate had a flexion point around 12 h. Mg²⁺ ions as a function of time shows a linear relationship before an immersion of 12 h. After then, the release rate for Mg²⁺ ions gradually reduced. In addition, the release rate of Mg²⁺ ions from both Na-MMT and Zn-MMT coatings were very lower, suggesting that both Na-MMT and Zn-MMT coatings have better corrosion resistance than the substrate. It is noted that the curve of Mg²⁺ as a function of time (Fig. 17b) is similar to that of pH in Fig. 4b.

3.5.5. LDH assay

Membrane integrity of bacteria was assessed with LDH assay (Fig. 18) after incubation on the Na-MMT, Zn-MMT coatings, AZ31 and the control at 37 °C for a culture time of 15 h. The bacterial cells cultured on the AZ31 and Na-MMT coating exhibited minimal LDH activity for both *E. coli* and *S. aureus*. However, LDH activity increased significantly when bacterial cells cultured on the Zn-MMT coating, which showed an activity of 107.4 ± 8.6 U/L and 79.4 ± 3.7 U/L for *E. coli* and *S. aureus*, respectively. In comparison to the control group, LDH activity of Zn-MMT coating was approximately 9 times higher for *E. coli* and increased by approximately 3–4 times for *S. aureus*. Moreover, the LDH activity for Zn-MMT coating was significantly higher than those for Na-MMT coating and Mg alloy AZ31 (P < 0.05). This LDH assay shows that Zn-MMT coating led to the damage of both the walls and the inner contents of the *E. coli* and *S. aureus*. 

![Fig. 12. Cell Viability incubated for 24 h and 72 h with bare, Na-MMT and Zn-MMT coated Mg alloy AZ31.](image-url)
4. Discussion

It is well-known that layered double hydroxides (LDH) \cite{96,97} with the same laminar structure as MMT \cite{98}. A comparison of corrosion resistance of recently reported LDH coatings on Mg alloy in various solutions is summarized in Table 4. The corrosion current density, $i_{corr}$, for the LDH-coated AZ31 \cite{19,99,100}, AZ91D \cite{101} and pure Mg \cite{97} decreased by one or two orders of magnitude compared to their substrates. It is noteworthy that the current density of the MMT-coated (Na-MMT and Zn-MMT) samples in the study decreased by two orders of magnitude compared with that of the AZ31 substrate. The results demonstrate that the corrosion resistance of the MMT coatings far exceeded that of the LDH coatings on Mg alloys. The MMT coatings in this experiment have a thickness (approximately 40–42 \mu m) that is higher than LDH coatings of 2–25 \mu m. The MMT coating have a more compact morphology than the LDH coatings. The difference in corrosion resistance may be attributed to the solutions besides the compactness and thickness of the coatings. In most cases, in vitro corrosion tests have been performed in simulated body fluids (SBF) such as 0.9 wt % NaCl, phosphate buffered solution (PBS) and Hank’s solution, in which only inorganic species (e.g., Cl\textsuperscript{−}, HCO\textsubscript{3}{}, HPO\textsubscript{4}{2−} and H\textsubscript{2}PO\textsubscript{4}{}) exist. In this paper, the experiments were performed in DMEM + 10% CS solution. DMEM contains amino acids, glucose, etc. besides inorganic salt, is more realistic than dilute chloride or Hanks type solutions. In our previous work \cite{78,102}, the effects of glucose...
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MMT has the advantages of a high cation exchange and adsorption capacity [105,106] and is an ideal carrier material suitable for pharmaceuticals [107,108]. In the present study, two of the most important factors for utilizing Zn as an antimicrobial agent in MMT coatings are the ability for long-term sustained action while minimizing the potential cytotoxic effects of the cation. According to inhibition zone tests and bacterial growth, no antibacterial activities were observed for Na-MMT coating. Similar findings have been previously reported [109]. Nevertheless, Zn-MMT coating displayed an obvious antibacterial effect. Overall, the observed inhibition zone (Fig. 14) and the growth curve (Fig. 15) showed that $\text{S. aureus}$ instead of $\text{E. coli}$ was more sensitive to the Zn-MMT coating. By analyzing the aforementioned antibacterial experiments,
Fig. 16. Morphology of bacteria after co-incubation with samples. Scanning electron microscopy images of bacteria in the control (a, b), Na-MMT (c, d), and Zn-MMT coated Mg alloy AZ31 (e, f) after incubation at 37°C for 24 h.

Fig. 17. Accumulated Zn²⁺ and Mg²⁺ ions that were released from the samples.
antibacterial mechanisms of the Zn-MMT coating are proposed as follows: Zn$^{2+}$ ions could exchange with the inter-layer cations of Na-MMT and consequently be adsorbed into the inter-layer space. This results in a valence imbalance in Na-MMT and a positively charged coating. Bacteria with negative charges could be attracted electrostatically to the coating, which resulted in a high number of bacteria being adsorbed to the surface of Zn-MMT coating [110]. Zn$^{2+}$ ions, released on the surface of Zn-MMT coating and slowly released between Zn-MMT layers, could inhibit bacterial proliferation. Similar to our results, previous reports demonstrated that a Cu/Zn-MMT composite had excellent antimicrobial activities against S. aureus and E. coli.

Furthermore, a small amount of ZnO was developed on the coating surface during immersion and also played a synergistic antibacterial role. Although the exact mechanisms for the inhibitory effects of Zn$^{2+}$ on the microorganisms remain unknown, the leakage (Fig. 16) of intracellular constituents might be one of the key factors in cell de-activation. Gram-positive cell walls consist of a much thicker layer of peptidoglycan than Gram-negative cell walls; however, the constituents of Gram-positive cell walls, teichoic acids (TA) and lipoteichoic acids (LTA), are more vulnerable to Zn$^{2+}$ ions [111–113]. Therefore, the enhanced inhibitory activity towards S. aureus can reasonably be attributed to membrane damage caused by Zn$^{2+}$ ions resulting from the Zn-MMT coating. On the other hand, Gram-negative E. coli have an extra (outer) membrane that contains lipopolysaccharide (LPS) molecules which act as a hydrophobic barrier against Zn$^{2+}$ ions. It has been reported that the higher susceptibility of Gram-positive bacteria than Gram-negative bacteria is related to the differences in the cell wall structure, the cell physiology, metabolism or the degree of contact [114,115]. The exact mechanisms that causes the membrane damage are not clear. Furthermore, the most likely antibacterial mechanism is the production of a large amount of bactericidal reactive oxygen species (ROS) [116] (including H$_2$O$_2$, OH$^-$, O$_2$$^-$) through introduction of Zn$^{2+}$ ions into MMT coating.

Colinas et al. [119] reported that the release of XXX likely results in a relatively high concentration of intercellular Zn$^{2+}$. This causes a rapid disruption of the metabolic activity through extracellular cation competition, subsequently causing oxidative stress. The ability of ZnO to inhibit bacterial growth by generating free oxygen radicals has been demonstrated by many reports [91,117,118]. A study also indicated that Zn can bind to bacterial DNA; hence, inhibiting replication or deactivating bacterial protein [119–121]. In this study, it was shown that Zn$^{2+}$ ions caused damage to bacterial cellular membrane, which in turn led to the leakage of intracellular enzymes such as LDH (Fig. 16). In addition, Zn$^{2+}$ ions were slowly released from the MMT laminar structure and resulted in long-lasting antibacterial activity.

5. Conclusions

An anticorrosive, antibacterial activity and sustained-releasing potential Zn-MMT coating was successfully prepared on the surface of Mg alloy AZ31 by the hydrothermal method.

(1) SEM images indicated that the lamellar Na-MMT and Zn-MMT coatings are tightly bound to Mg alloy AZ31 with a thickness of 40 μm and 42 μm, respectively. Na-MMT coating on Mg alloy AZ31 results in a marked decrease in the corrosion current density from 2.8 × 10$^{-5}$ to 2.9 × 10$^{-7}$ A cm$^{-2}$; Zn-MMT coating from 2.8 × 10$^{-5}$ to 2.8 × 10$^{-7}$ A cm$^{-2}$, indicating that the two coatings possess excellent corrosion resistance in DMEM + 10% CS. The results might be ascribed to the thickness of the coatings and organic compositions, including amino acids and glucose.

(2) Na-MMT and Zn-MMT coatings could significantly improve hemocompatibility and cell cyto-compatibility of Mg alloy AZ31. Zn-MMT coating inhibited growth of S. aureus and E. coli significantly, showing significant antibacterial effect. Zn$^{2+}$ ions acted on the cellular membrane of the bacteria, leading to a leakage of intracellular substance and the death of the bacteria. And the release of Zn$^{2+}$ ions from the coating could last for approximately 144 h, exhibiting enough time to combat early infection with medical implants.

(3) This study indicated the potential applications of Zn-MMT coating in reducing corrosion and infections of the implants. It is expected to be an ideal and promising coating for biomedical magnesium alloys.
Declaration of Competing Interest

The authors declare no competing financial interest.

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