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In vitro metal ion release and biocompatibility of amorphous $\text{Mg}_{67}\text{Zn}_{28}\text{Ca}_5$ alloy with/without gelatin coating

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ABSTRACT

Amorphous zinc-rich Mg-Zn-Ca alloys have exhibited good tissue compatibility and low hydrogen evolution *in vivo*. However, suboptimal cell-surface interaction on magnesium alloy surface observed *in vitro* could lead to reduced integration with host tissue for regenerative purpose. This study aims to improve cell-surface interaction of amorphous Mg₆₇Zn₂₈Ca₅ alloy by coating a gelatin layer by electrospinning. Coated/uncoated alloys were immersed and extracted for 3 days under different CO₂. The immersion results showed that pH and metal ion release in the alloy extracts were affected by gelatin coating and CO₂, suggesting their roles in alloy biocorrosion and a mechanism has been proposed for the alloy-CO₂ system with/without coating. Cytotoxicity results are evident that gelatin-coated alloy with 2-day crosslinking not only exhibited no indirect cytotoxicity, but also supported attachment of L929 and MG63 cell lines around/on the alloy with high viability. Therefore, amorphous Mg₆₇Zn₂₈Ca₅ alloy coated with gelatin by electrospinning technique provides a useful method to improve alloy biocompatibility.

KEYWORDS: Magnesium alloys; gelatin; amorphous; biocompatibility; electrospinning

INTRODUCTION

Mg and Mg alloy has been a promising candidate for orthopaedic and cardiovascular prosthetic materials due to its strength and relatively good biocompatibility, but high corrosion (e.g. high hydrogen evolution and local pH alkalization) is the drawback for its use [1-7]. Recently, zinc-rich amorphous Mg-Zn-Ca alloys, which contains more than 28 at. % Zn, have shown to form passivating layer in simulated body fluid (SBF) and result in low hydrogen evolution and good tissue compatibility *in vivo* [6]. However, further *in vitro* studies found that Zn-rich Mg-Zn-Ca alloys resulted in reduced cell viability and suboptimal cell attachment [2]. Coating of biocompatible synthetic polymers such as polycaprolactone (PCL) and poly(lactide-co-glycolide (PLGA) has been shown to greatly improve biocompatibility of Mg alloys [3, 5]. However, a lack of biological signals could be a drawback of using synthetic polymers as coating. Therefore, naturally derived proteins such as gelatin could be beneficial to improve alloy biocompatibility in terms of cell-surface interaction [8, 9]. Gelatin is a hydrolyzed collagen derived from animal

skin, tendon and bone which has been widely used in biomedical applications such as wound dressing (e.g. Gelfoam®, Spongostan® and Surgifoam®)[10, 11].

Dip coating [3] and pressurized spraying [5] have been employed in alloy coating. These methods might not be applicable to gelatin since exposure of aqueous gelatin solution could induce corrosion. Alternatively, electrospinning could be a potential coating method. It is a technique that has been used to create tissue engineering scaffolds with a porous nanofibrous structure that mimics native tissue [12-14]. When used in coating, it could further increase the biocompatibility of the alloy. Furthermore, the process provides sufficient time for solvent drying before fibre lands on the sample, thereby minimizing undesired alloy corrosion.

In this study, zinc-rich amorphous $Mg_{67}Zn_{28}Ca_5$ alloy was synthesized and gelatin-coated by electrospinning. The gelatin layer on Mg alloy was then dehydrothermally crosslinked for 2 or 5 days. The alloys, both coated and uncoated, were subjected to 3-day immersion in the culture medium at various CO_2 . The various CO_2 conditions (0.03, 5 and 10%) were tested due to its substantial presence in the body fluid [15] and its observed effect on biocorrosion of Mg and Mg alloys at 5% CO_2 [16, 17]. pH and metal ion release were measured at the end of the test and the extracts resulted from the immersion test was exposure to two cell lines to evaluate indirect cytotoxicity. To complete this study, direct cytotoxicity test was carried out by seeding cell lines on the alloys to evaluate cell-material interaction.

METHODS

1. Preparation of magnesium alloys

Amorphous $Mg_{67}Zn_{28}Ca_5$ alloy (abbreviated as A) was synthesized by disintegrated melt deposition (DMD) technique as described by Fu *et al.*[1]. Briefly, pure Mg tubes are melted and superheated with Zn pellets and Ca shots between 750 and 800°C under argon. The molten was then mixed homogeneously followed by a proprietary melt-spinning process (Magnequench, Singapore) to produce alloy ribbons. Amorphous nature was confirmed by X-ray diffraction [1]. To coat amorphous alloy, 10% (w/w) gelatin (Sigma) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Merck)[9] was electrospun at voltage of 25kV for 1 hour onto the Mg ribbons. The coated alloys were then freeze-dried overnight followed by dehydrothermally (or DHT) crosslinked at 140°C

under vacuum pressure of 10^{-3} mmHg[18] for duration of (i) 2 days (abbreviated as G2) or (ii) 5 days (abbreviated as G5). All alloys were cleaned in ultrasonic bath with 100% ethanol for 10 minutes followed by rinses with sterile deionised water thrice before testing in the biosafety cabinet.

2. Immersion test at various gaseous CO₂ (%)

Alloy strips ($10 \times 2 \times 0.1$ mm³) from each group (A, G2 and G5; n=3) were immersed in 2.5 ml culture medium for 3 days in 37°C humidified incubators (SANYO MCO-17AIC) adjusted at 0.03, 5 and 10% CO₂, respectively. Culture medium used is Dulbecco's Modified Eagle Medium (DMEM, HyClone) containing pH indicator, phenol red, supplemented with 10% fetal bovine serum (FBS, HyClone) and 1% antibiotics-antimycotic solution (Sigma) and the ion concentration of DMEM is shown in Table 1. Control media (media without alloys) were also incubated similarly. On each day, 400 µl of alloy extract/blank was withdrawn from each sample and replenished by fresh medium to maintain the volume. Measurements of pH and metal ion concentrations were carried out at the end of the test. pH measurement was adapted from the spectrophotometric method for determining pH in the culture medium containing phenol red [19]. Briefly, 2×200 µl of withdrawn extracts were quickly transferred into a 96-well microplate using a multichannel pipettor. The absorbance of the medium was measured at a wavelength of 560nm by a microplate reader (Tecan Evolution) and correlated with a standard curve prepared by a series of pH standards (from pH 7 to 10) containing phenol red. Metal ion concentrations were quantified by Quantichrom assay kits (Hayward, CA) according to the company instructions.

3. Indirect cytotoxicity

Human MG63 osteosarcoma and mouse L929 fibrosarcoma cell lines were purchased from ATCC and routinely cultured in complete medium. 5000 cells/ 0.2 ml culture medium were seeded into a 96-well microplate and incubated overnight. On the following day, medium was removed from each well and replaced by 0.2 ml alloy extracts/control media (n=3) collected from the immersion test. Fresh medium (n=3, negative control) and medium containing 0.01%

Triton X-100 (n=3, positive control), were also added to cells. On Day 3, cell viabilities were measured by MTS assay according to company instructions (Promega) and calculated relative to the negative control (expressed as mean $\pm$ SD, in %).

4. Direct cytotoxicity

Alloy strips ($10 \times 2 \times 0.1 \text{ mm}^3$) from each group (n=4) were secured on a 12-well plate and 12×10^4 cells/ 2.5ml medium (both L929 and MG63 cells) were added into each well with alloy and cultured for 3 days. On each day, cells around the alloy were observed by inverted microscope (Olympus LX40) and 400 μl of medium was withdrawn from each sample and replenished by fresh medium to maintain the volume. Measurements of pH and metal ion concentrations were carried out at the end of the test (Day 3), as described in the immersion test. After the final medium withdrawal on Day 3, cells (n=4 for each group) were stained with 2 μM calcein AM and 4 μM ethidium homodimer-1 (Invitrogen) for 15 minutes. Stained cells around the alloys and on top of the alloys were observed by inverted fluorescence microscope at 4 $\times$ magnification (Olympus LX40) and fluorescence microscope at 5 $\times$ magnification (Carl Zeiss Axioskop 2 Mot Plus), respectively.

5. Statistical analysis

Student t-test with $p = 0.05$ was carried out for comparison of the quantitative data.

RESULTS

1. Surface morphology of uncoated and coated Mg alloy

Figure 1 shows the morphologies of amorphous $\text{Mg}_{67}\text{Zn}_{28}\text{Ca}_5$ alloy before (Figure 1a) and after coating with gelatin by electrospinning (Figure 1b). On the coated alloy, a porous/fibrous structure was laid on the alloy surface dramatically reducing alloy areas exposed to the external environment. The coating has the porous nanofibrous nature similar to the conventional electrospun structure. Furthermore, the porous nature of the gelatin coating was not altered after DHT crosslinking.

2. pH and metal ion release in culture medium after 3-day immersion

Culture medium contains sodium bicarbonate that works with gaseous CO_2 (usually 5% to 10%) to buffer the culture environment within physiological pH. When CO_2 was reduced to 0.03%, pH of control medium was shifted beyond 9, as shown in Figure 2a. When comparing between control medium and alloy extracts at fixed CO_2 , pH of all alloy extracts, except for coated alloys at 0.03% CO_2 , were statistically similar to the control. For the exception cases, it is interesting to observe that coated alloys resulted in a small but significant pH lowering at low CO_2 ($p < 0.05$). Provided with the current data, pH of alloy extract was mainly governed by $\text{CO}_2/\text{HCO}_3^-$ equilibrium in the culture medium.

Although alloy biocorrosion did not significantly increase extract pH, significant Mg^{2+} release was observed in all cases, when compared with control medium ($p < 0.05$) at fixed CO_2 , as shown in Figure 2b. Mg^{2+} release appears to be proportional to CO_2 level for both coated and uncoated alloys. It was also noticed that coated alloys at 10% CO_2 resulted in significantly lower Mg^{2+} release than uncoated counterparts ($p < 0.05$), suggesting the role of gelatin in reducing Mg dissolution at high CO_2 . When comparing between coated alloys, G5 alloy which was crosslinked to a higher degree, led to a significantly higher Mg^{2+} release at all levels of CO_2 ($p < 0.05$). On the other hand, $[\text{Ca}^{2+}]$ in all alloy extracts was statistically similar to control medium ($p > 0.05$) at various CO_2 , as shown in Figure 2c, implying no noticeable release of Ca^{2+} from the alloys. Furthermore, it is apparent that $[\text{Ca}^{2+}]$ in medium is pH-dependent. As CO_2 was lowered from 10% to 0.03%, $[\text{Ca}^{2+}]$ dropped significantly below 1mM, suggesting calcium precipitation occurred in culture medium at alkaline condition with the available anions. The enrichment of Zn in the alloy composition was found to form a protective $\text{ZnO}/\text{Zn}(\text{OH})_2$ on the Mg alloy[6]. From Figure 2d, CO_2 did not show to contribute to significant Zn^{2+} release to the culture medium ($p > 0.05$) except for the coated alloys immersed at 10% CO_2 ($p < 0.05$), implying coating effect on accelerated Zn dissolution at high CO_2 .

3. Indirect cytotoxicity

Test samples including alloy extracts and control media collected from the immersion test were exposed to L929 and MG63 cells to examine indirect cytotoxicity. Fresh culture medium without

incubation was served as negative control. Figure 3a and 3b are the cell viability after 3-day exposure to the test samples and negative control. It was found that the cellular sensitivity of the alloy extracts is cell type-dependent: on one hand, MG63 cell viability was unaffected by all test samples when compared to negative control (all $p > 0.05$); on the other, although L929 cell viability was $>90\%$ in all test samples, 5 out of 9 alloy groups showed significantly lower cell viability when compared to the negative control ($p < 0.05$).

Lower L929 viability compared to negative control could be interpreted as toxicity/growth inhibiting effect of these alloy extracts on L929 cells. However, when compared with control medium in the absence of alloy, all alloy extracts resulted in either similar ($p > 0.05$) or even higher cell viability ($p < 0.05$). This suggested that all alloy extracts actually did not exhibit cytotoxicity to L929 cells and the incubation process itself imposed a negative impact on the culture medium that reduced L929 viability. Furthermore, 5 out of 9 alloy groups at higher CO_2 showed significantly higher cell viability than the control medium implying that the increased metal ion concentrations in these alloy extracts could promote cell proliferation. In this case, initial extract pH does not predict cell viability as alkaline samples derived from $0.03\% \text{ CO}_2$ did not cause substantial cell death. Furthermore, even when pH was buffered at similar pH in all alloy extracts and control medium during immersion, difference in cell viability was still observable. When cell viability of L929 cells was plotted against metal ion concentrations of corresponding alloy extracts, R^2 values were 0.512 against $[\text{Mg}^{2+}]$, 0.092 against $[\text{Ca}^{2+}]$ and 0.0014 against $[\text{Zn}^{2+}]$, as shown in Figure 3c, 3d and 3e. A higher R^2 against $[\text{Mg}^{2+}]$ suggested that increased $[\text{Mg}^{2+}]$ due to alloy corrosion could be a possible factor for higher L929 proliferation. This could also explain why coated alloys, which released lower Mg^{2+} at high CO_2 (Figure 2b), resulted in significantly lower cell viability ($p < 0.05$) than its uncoated counterpart.

4. Direct cytotoxicity

L929 and MG63 cells were seeded on the culture well containing alloy samples and cultured for 3 days at $5\% \text{ CO}_2$ and 37°C . When compared to the immersion results at $5\% \text{ CO}_2$ without cells (Figure 2), alloys seeded with cells led to a lower mean pH and generally higher Mg^{2+} and Zn^{2+} release. However, Ca^{2+} release was not noticeably different. In the presence of cells, it was found

that most of the coated alloy groups (3 out of 4) showed similar pH and $[Ca^{2+}]$ ($p>0.05$) but significantly lower $[Mg^{2+}]$ compared with their uncoated counterparts ($p<0.05$). For $[Zn^{2+}]$, the trend was cell-type dependent: when seeded with L929 cells, all coated alloys showed higher Zn^{2+} release than uncoated alloy ($p<0.05$) whereas when with MG63 cells, coated samples either released similar (G2, $p>0.05$) or lower Zn^{2+} (G5, $p<0.05$) compared with uncoated sample. Furthermore, only $[Zn^{2+}]$ was different between G2 and G5 alloys that G5 alloys consistently released more Zn^{2+} into the medium than its less crosslinked G2 counterpart ($p<0.05$). Figure 5 and 6 are the live/dead cell staining of L929 and MG63 cells either around (a, c & e) or on the alloy (b, d & f), respectively. Both cell types were able to proliferate and maintain high viability around all alloys. However, both cell lines showed poor attachment and survival on the uncoated alloys: only one in four uncoated samples were observed with a few L929 cell patches on the alloys (Figure 5b) or with only a small number of MG63 cells scattered on the alloy (Figure 6b). In comparison, gelatin-coated alloys, especially G2 alloy, dramatically improved cell attachment on the alloys. A highly viable cell population of both cell lines was found over the G2 alloys (Figure 5g and 6g) while G5 only showed improvement in overall attachment and distribution of L929 cells but not MG63 cells (Figure 5h and 6h).

DISCUSSION

Direct cell interaction with alloy surface has been an issue for zinc-rich Mg-Zn-Ca alloy although it has shown to be tissue-compatible *in vivo* [6]. In this study, naturally derived gelatin was proposed as coating material for its bioactivity. Gelatin is a hydrolyzed collagen, which is more stable and economical in terms of process and preservation compared to collagen but still retains good bioactivity to promote attachment of many cell types. However, since gelatin is water-soluble but insoluble in most of the common organic solvents (e.g. acetone), coating with aqueous gelatin solution without rapid drying could initiate unwanted corrosion. Therefore, HFIP was chosen as solvent since it dissolves gelatin but evaporates faster than water (159 mmHg vs. 23.8 mmHg in vapor pressure at 25°C). Electrospinning was selected for coating since the process renders sufficient time for the solvent to evaporate before gelatin fibre lands on the alloy. Coating thickness is also controllable via electrospinning duration. More importantly,

electrospinning creates a nanofibrous structure that resembles tissue architecture which could further improve biocompatibility [12, 14]. However, water-soluble gelatin coating is susceptible to hydrolysis and therefore was subjected to crosslinking by dehydrothermal method (DHT) [18] before use. DHT is advantageous since it is relatively simple and does not involve reaction in aqueous media.

In this study, medium pH and metal ion release were focused since these parameters not only provide a measure for biocorrosion but also could allow correlation with cell cytotoxicity.

Duration of 3 days was chosen for immersion test because it represents the onset period of alloy biocorrosion which caused a rapid change of these measured parameters in the aqueous environment. Although the test setup was not under continuous perfusion, a partial medium removal/replenishment was carried out daily to introduce an interstitial fluid exchange. Previous alloy studies consistently observed a pH rise when pure Mg and Mg alloys were immersed in test medium [2, 17]. At Mg alloy surface, Mg reacts with water to form hydrogen gas (H₂) together with insoluble Mg(OH)₂, which becomes more dissociative in the presence of aggressive Cl⁻:

Mg dissolution results in rapid pH rise near the alloy surface, which also causes formation of insoluble hydroxides [2, 17] and phosphate and carbonate salts [6, 16, 17]. Immersion results suggested that pH and metal ion release to culture medium were dependent of CO₂ and coating. For pH, since pHs of most of the alloy extracts were found to be similar to those in the control media, CO₂/HCO₃⁻ equilibrium [15] in the bulk medium could be the pH-governing step:

At 5-10% CO₂, alloy extracts were buffered near the physiological level with HCO₃⁻ in the medium. As CO₂ was reduced to 0.03%, CO₂/HCO₃⁻ equilibrium is driven backward consuming free HCO₃⁻ (and also H⁺) in the culture medium to give off CO₂. H⁺ removal action causes an overall rise in extract pH. A proposed biocorrosion mechanism of coated and uncoated Mg₆₇Zn₂₈Ca₅ alloys in relation to pH, metal ion release and precipitation under different CO₂ condition is illustrated in Figure 8. Although corrosion products were not studied in this study,

previous studies discovered that formation of corrosion products are linked to the available anions and cations in the medium [20]. In poor CO₂ condition, H₂PO₄²⁻ and HCO₃⁻ formulated in the culture medium together with increasing OH⁻ are the main anions involved in the formation of insoluble phosphate and carbonate salts and hydroxides with free metal ions (Figure 8a and 8c). A great amount of free Ca²⁺ in the bulk medium also takes part in the precipitation process leading to lower [Ca²⁺], as implied by the immersion results. Due to alkaline pH and smaller pH gradient between alloy surface and the bulk medium at low CO₂, all the precipitated salts are less prone to dissolution and therefore, lower Mg²⁺ release was observed for both uncoated and coated alloys. Furthermore, solubility products (k_{sp}) of carbonates/phosphates of Ca and Zn are at least three orders of magnitude lower than that of Mg, so Ca/Zn precipitates are believed to be far more insoluble than Mg salts and Ca²⁺/Zn²⁺ release should be less detectable, as supported by the ion concentration data. This result also showed that gelatin coating could lower extract pH in poor CO₂ condition, probably due to the amphoteric nature of its chemical structure [21]. Figure 8c illustrates that as pH is increased, free OH⁻ could bind to positively charged sites (-NH₃⁺) of gelatin that led to a slight pH lowering effect.

When supplemented with 5-10% CO₂, not only phosphate but also more carbonate salts are expected to form due to increased carbonate species [16, 17] (Figure 8b and 8d). However, rich CO₂ environment buffered near neutral pH caused calcium precipitation to a less degree, as interpreted from a higher [Ca²⁺] in the alloy extracts and also control medium. It is not surprised with the case for control medium since k_{sp} for calcium phosphate is as low as 2×10^{-33} , Ca²⁺ in the medium slowly precipitates into calcium phosphate with PO₄³⁻ when exposed to air in day-to-day practice that could cause increased conversion of phosphate from dihydrogen phosphate.

Although previous studies showed an increase of CaP precipitate with increased HCO₃⁻, it could be due to synergistic action together with alkaline pH [16, 20]. Furthermore, increasing pH gradients facilitates dissolution of more soluble magnesium salts and its hydroxide (with higher k_{sp}) to the medium [22] (Figure 8b). Wong *et al.*[5] demonstrated AZ91 Mg alloy coated with porous PCL released lower Mg²⁺ than uncoated alloy by > 3 folds over 60 days. Although it is still unclear, alloy surface that is covered by coating material can be isolated from the aqueous environment such that biocorrosion could be slowed down by limiting ion exchange and

precipitation at the exposed sites, as proposed in Figure 8c and 8d. In this study, protection effect of gelatin may be less prominent at low CO₂ since higher pH could sufficiently stabilize the insoluble precipitates formed on the alloy surface, which slows down biocorrosion similar to uncoated alloy. As CO₂ was increased, an increase of [Mg²⁺] was observed from gelatin-coated alloys, a trend that is similar to uncoated alloys; but at 10% CO₂, gelatin-coated alloys exhibited a significant increase of [Zn²⁺], which was not found in the uncoated counterparts. It is probably due to local acidification effect by reaction of amino group (-NH₂) on protein with increased CO₂: [15]

The release of H⁺ from gelatin near the alloy surface could lower local pH to the extent that not only dissolves Mg precipitates but also less soluble Zn precipitates resulting in elevation of [Zn²⁺] for gelatin-coated alloys at high CO₂, as shown in Figure 8d. However, due to coating-limited corrosion sites that could reduce formation of Mg precipitate for dissolution (illustrated in Figure 8c and 8d), Mg²⁺ release from coated alloys was significantly lower than its uncoated counterpart.

Indirect cytotoxicity study revealed that both uncoated/coated Mg₆₇Zn₂₈Ca₅ alloys exhibited no cytotoxicity to MG63 cells when compared to both control mediums and negative control (fresh medium without incubation). However, although alloy extracts resulted in lower cell viability in L929 cells than negative control, they all either exhibited similar or even higher viability when compared to control medium. These findings can be interpreted as follows: (1) cytotoxicity of alloy extract is cell-type dependent; (2) initial pH of medium extract might not be a determining factor for cell viability; (3) incubation itself could degrade growth-related factors in the medium that reduce cell proliferation and (4) metal ion stimulate cell proliferation.

Nutrient requirements and resistance to environment change are known to be cell-type dependent and this result showed MG63 cells is less sensitive to the medium variation due to alloy biocorrosion than L929 cells. It would be important to test biocompatibility with the cell types that are relevant to the biomedical applications in the future test. It was once thought that alloy extracts with pH > 9 could cause significant growth aberration and cell death [23, 24] but this result did not show such dramatic reduction in cell viability. This could be due to the test volume

required for cytotoxicity. A 96-well cytotoxicity test requires about 200µl sample for each test in 96-well format. Even though the initial pH is high for the extracts/controls derived from 0.03% CO₂, 5% CO₂ supply in the incubator could allow rapid buffering in small samples during the test. On the other hand, even with buffering during immersion test, significant difference in cell viability of L929 cells was still observed when exposed to alloy extracts with similar initial pH at physiological level. Therefore, initial extract pH cannot predict cytotoxicity. When viability of L929 cells was plotted against metal ion concentration, it revealed that the increased level of [Mg²⁺] in the medium could contribute to L929 cell proliferation. It is not surprised since many studies showed that extracellular Mg²⁺ is essential for cell proliferation of certain cell types such as L929 cells [7] and vascular endothelial cells [4, 25]. However, it should be noticed that increasing [Mg²⁺] beyond physiological level in the culture environment could induce cell death [26, 27]. With regards to the medium used in the indirect cytotoxicity test, a blank test medium incubated under the same condition as for the alloy extraction is always appropriate for comparison purpose. This study demonstrated a CO₂-dependent biocorrosion process by varying CO₂ at three different levels. Therefore, CO₂, which usually contains > 5-6% (40-45mmHg) in the blood [15], should be included in the *in vitro* immersion test to create a more *in vivo*-like immersion environment, rather than using artificial buffers [16, 17].

Previous SEM results showed increased cell attachment of L929 and MG63 cells on the Mg₆₆Zn₃₀Ca₄ alloy compared to Mg₇₀Zn₂₅Ca₅ and pure Mg after a 5-day culture [2]. However, pH measurement in this study showed that cells were exposed in an alkaline environment (pH >8) over 5 days. Previous mechanism study demonstrated that alkaline pH at 8.3 has shown to cause cell death after 24 hours by increasing oxidative stress through alteration of mitochondria potential [24]. Therefore, whether the cells attached on the alloys are viable or healthy requires further investigation. In this study, pH and metal ion release were monitored during the culture. Live/dead cell staining was carried out to identify viable/dead cells attached around or on the alloy since attached cells may not be all viable and observation of cells around the alloy also facilitates a more complete understanding on the cell-material interaction.

With cells attached around and on the alloy, pH was reduced and in addition, Mg²⁺ and Zn²⁺ release were increased when compared to the alloy immersion without cells at similar CO₂

condition (i.e. 5%). Interestingly, Zn^{2+} release on the gelatin-coated alloy was cell-type dependent, suggesting cell-mediated action in Zn^{2+} release specific to cell types. Although these events are not fully understood, it appears that the microenvironment is actively altered by interaction between alloy biocorrosion of Mg alloy and surrounding cells. Around all the alloys tested, both cell types were highly viable and proliferated to confluency but only G2 alloys promoted attachment of both cell types in high viability. Therefore, gelatin coating with 2 days of DHT crosslinking is beneficial in attachment of viable cells. Although with similar gelatin coating, G5 alloy with higher crosslinking apparently reduced bioactivity and cytocompatibility. It could be due to the modification of the gelatin structure by DHT process which involves removal of bound water and condensation reaction between carboxyl ($\text{C}=\text{O}$) and amino groups ($-\text{NH}_2$) [18]. Cell attachment on natural biopolymers is based on peptide sequence such as RGD [28]. If the structure is overly modified by crosslinking, the peptide sequence and conformation could be altered to the extent that bioactivity is reduced or lost. This study demonstrates gelatin coating plays a differential role in alloy biocorrosion at various CO_2 and gelatin coating with 2-day crosslinking improves biocompatibility of the Mg alloy. It will be of interest to investigate the formation of corrosion products on the coated alloys in terms of quantity, composition and location. Process of gelatin coating has to be optimized in terms of coating thickness and degree of crosslinking for different biomedical applications. As coating detachment is another concern [3], a long-term study will be required to verify the stability gelatin coating over time.

CONCLUSION

Metal ion release and biocompatibility of amorphous $\text{Mg}_{67}\text{Zn}_{28}\text{Ca}_5$ alloy with or without gelatin coating was evaluated in this study. By varying CO_2 in the alloy immersion, pH, $[\text{Mg}^{2+}]$ and $[\text{Ca}^{2+}]$ were significantly affected in different mechanism. In addition, alloy coated by electrospinning of gelatin exhibited differential effect on pH, Mg^{2+} and Zn^{2+} release at different CO_2 level. All alloy extracts derived from the immersion test were nontoxic to two cell types tested but only gelatin-coated alloys with 2-day crosslinking promoted attachment of both cell types. In conclusion, Gelatin coating by electrospinning provides a useful technique that improves biocompatibility of amorphous $\text{Mg}_{67}\text{Zn}_{28}\text{Ca}_5$ alloy.

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Figure 1. SEM micrographs (3000 $\times$) of amorphous $\text{Mg}_{67}\text{Zn}_{28}\text{Ca}_5$ without coating (a) and with coating of gelatin (b). Coating was carried out by electrospinning of 10% gelatin solution in HFIP at 30kV with a tip-to-alloy distance of 12 cm, followed by crosslinking. The electrospun coating is a porous nanofibrous structure similar to the conventional electrospun structure.

Figure 2. Alloy extract parameters (pH and metal ion concentrations) after three-day immersion of alloys in culture medium under 0.03% (dots), 5% (diagonal lines) and 10% (vertical lines) CO₂ at 37°C: (a) pH, (b) [Mg²⁺], (c) [Ca²⁺] and (d) [Zn²⁺]. Three samples were tested: Amorphous Mg₆₇Zn₂₈Ca₅ alloy without coating (A), with gelatin coating/2-day crosslinking (G2) and with gelatin coating/5-day crosslinking (G5). Control medium (Ctrl) is culture medium incubated without alloy under designated CO₂ condition. *, % and ^ indicate $p < 0.05$ for alloy group vs. control medium, for coated vs. uncoated alloy group and for G2 vs. G5 group at fixed CO₂, respectively. For CO₂, 0.03% vs. 5% and 5% vs. 10% were found to be $p < 0.05$ for pH,

[Ca²⁺] and [Mg²⁺] in all alloy groups and control medium; 5% vs. 10% was found to be $p < 0.05$ for [Zn²⁺] in coated alloys only.

Figure(s)

Figure 3. Cell viability of (a) L929 cells and (b) MG63 cells three days after incubation in the alloys extracts derived from the three-day immersion test under 0.03% (dots), 5% (diagonal lines) and 10% (vertical lines) CO₂ at 37°C. A, G2 and G5 represent amorphous Mg₆₇Zn₂₈Ca₅ alloys without coating, with gelatin coating/2-day crosslinking and with gelatin coating/5-day crosslinking. Control medium (Ctrl) = culture medium incubated without alloy under designated CO₂ condition. Negative control (Neg) = fresh medium without incubation. Plots of cell viability of L929 cells against metal ion concentrations in the corresponding alloy extracts: (c) against [Mg²⁺]; (d) against [Ca²⁺] and (e) against [Zn²⁺]. *, %, ^ and # indicate $p < 0.05$ for alloy group vs. control medium, for coated vs. uncoated alloy group, for G2 vs. G5 group, all at fixed CO₂, and also for each sample (alloy or control medium) vs. negative control, respectively.

Figure 4. Medium parameters (pH and metal ion concentrations) three days after incubation with the alloys with L929 cells (dots) and MG63 cells (diagonal lines) at 5% CO₂ and 37°C: (a) pH, (b) [Mg²⁺], (c) [Ca²⁺] and (d) [Zn²⁺]. Three samples were tested: Amorphous Mg₆₇Zn₂₈Ca₅ alloy was tested without coating (A), with gelatin coating and 2-day crosslinking (G2) and with gelatin coating and 5-day crosslinking (G5). A control medium (Ctrl, grids) is culture medium incubated without alloy under designated CO₂ condition. *, % and ^ indicate p < 0.05 for alloy group vs. control medium, for coated vs. uncoated alloy group and for G2 vs. G5 group, respectively.

Figure 5. Live (green)/dead (red) cell staining of attached L929 cells around magnesium alloys at 40 $\times$ magnification (a, c & e) and on their surfaces at 50 $\times$ magnification (b, d & f). Three samples were tested: Amorphous $\text{Mg}_{67}\text{Zn}_{28}\text{Ca}_5$ alloy without coating (a & b), with gelatin coating/2-day crosslinking (G2, c & d) and with gelatin coating/5-day crosslinking (G5, e & f). White bar = 100 μm .

Figure 6. Live (green)/dead (red) cell staining of attached MG63 cells around magnesium alloys at 40 $\times$ magnification (a, c & e) and on their surfaces at 50 $\times$ magnification (b, d & f). Three samples were tested: Amorphous $\text{Mg}_{67}\text{Zn}_{28}\text{Ca}_5$ alloy without coating (a & b), with gelatin coating/2-day crosslinking (G2, c & d) and with gelatin coating/5-day crosslinking (G5, e & f). White bar = 100 μm

Figure(s)

Low CO₂

a

High CO_2

b

Low CO_2

c

High CO_2

d

Figure 7. Proposed biocorrosion and ion release mechanism of amorphous $\text{Mg}_{67}\text{Zn}_{28}\text{Ca}_5$ alloy without coating (a & b) and with gelatin coating (c & d) at low CO_2 (a & c) and high CO_2 (b & d) conditions. (----) bulk medium interface; ($\blacktriangle$) phosphate precipitates and ($\triangle$) carbonate precipitates. (a) At low CO_2 ($= 0.03\%$), pH of bulk medium reaches as high as 9.3 due to gas-off of HCO_3^- . Increase in pH is conducive to CaP precipitation by tapping into free Ca^{2+} and PO_4^{3-} in the medium, thereby reducing $[\text{Ca}^{2+}]$. Increase of pH also promotes other precipitate formation (e.g. hydroxides and phosphates) and slows down dissolution of precipitates. (b) At high CO_2 ($= 10\%$), pH of bulk medium is buffered near 7.4. Reduction in pH tends to accelerate dissolution of Mg precipitates compared to Ca/Zn precipitates due to lower k_{sp} values. Lower degree of CaP precipitation occurs at neutral pH, resulting in higher free $[\text{Ca}^{2+}]$ in the bulk medium. (c & d) In the presence of gelatin coating, biocorrosion sites for ion exchange and precipitate formation mainly are limited at the exposed areas. At low CO_2 in (c), positively charged amino groups in the gelatin molecule could bind with OH^- significantly reducing pH in the bulk medium. At high CO_2 in (d), amino groups could react with CO_2 to release H^+ that reduces local pH near the alloy surface. Local pH could be sufficiently low to promote dissolution of Zn precipitate, resulting in increased Zn^{2+} release to the medium. Although local pH reduction should also increase release of Mg^{2+} , lower Mg precipitate formation on the gelatin-coated alloy could reduce Mg^{2+} release compared to uncoated alloy at high CO_2 .

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Table 1: Ion concentration in Dulbecco's Modified Eagle Medium (DMEM)

	Cations				Anions			
Ions*	[Mg ²⁺]	[Ca ²⁺]	[K ⁺]	[Na ⁺]	[Cl ⁻]	[HCO ₃ ⁻]	[SO ₄ ²⁻]	[H ₂ PO ₄ ⁻]
(mM)	0.813	1.80	5.33	155	118	44.0	0.813	0.916

*DMEM also contains 0.248μM Fe(NO₃)₃