Osteoblastic cell response on magnesium-incorporated apatite coatings

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\textbf{1. Introduction}

Hydroxyapatite (HA) is the major inorganic component of natural bone and has been used as orthopedic and dental material [1]. As a material for osseous implants, HA shows excellent properties because of its biocompatibility and ability to regenerate calcified tissues [2]. When used to coat an orthopedic or dental implant, synthetic HA provides a surface for the anchorage-dependent osteoblasts to deposit calcium-containing mineral. This promotes osseointegration and stabilization of an implant and prevents motion-induced damage [3].

Despite these bio-advantages, HA is limited in use due to high in vivo solubility and poor mechanical properties. Synthesizing materials that mimic natural bone thus becomes objective of many technological researches [3,4]. Mg is one of the main substitutes for calcium in biological apatite. Enamel, dentin and bone contain 0.44, 1.23 and 0.72 wt.% of Mg, respectively [5–7]. Magnesium indirectly influences mineral metabolism and directly influences or even controls the crystallization processes of mineral substance as well as the pattern of mineral formation [8]. It is reported that magnesium inhibited the crystallization of hydroxyapatite but increased the thermal conversion into β-TCP (Mg-containing β-TCP) [9]. Apatite doped with carbonate and/or Mg ions can improve the behaviors of MSC and MG-63 cells in term of adhesion, proliferation and metabolic activation compared to stoichiometric HA [4]. HA Doped with 2 mol% of Mg\textsuperscript{2+} significantly enhances osteoblast adhesion as compared to pure HA [10]. The adverse effects of magnesium are also reported in the literature. Serre et al. [8] studied the influence of magnesium substitution on a collagen-apatite biomaterial and demonstrated that the apatite containing 4.5 mol\% magnesium adversely affected the osteoinductive properties since too much magnesium intoxicated the apatite thus inhibited osteoblastic phenotype.

Magnesium-containing apatite coatings on metal substrate are expected for the future medical applications. However, the bioreponse of these coatings are not reported in literature. The present study employs the sol–gel dip-coating method to fabricate magnesium-containing apatite coatings to study the dissolution and biocompatibility behaviors of the coatings in physiological saline solution and the response of human osteosarcoma MG63 cells.
2. Experimental

2.1. Deposition of the magnesium-doped apatite coatings

The processing of the dipping sols and deposition of the magnesium apatite coatings are detailed in our previous publications [11,12]. In short, Titanium alloy (Ti-6Al-4V) plates were used as substrates. Calcium nitrate tetrahydrate (Ca(NO₃)₂·4H₂O, Sigma–Aldrich, AR), phosphorus pentoxide (P₂O₅, Merck, GR) and magnesium nitrate hexahydrate (Mg(NO₃)₂·6H₂O, Sigma–Aldrich, AR) were selected to prepare Ca-precursor, P-precursor and Mg-precursor, respectively. The degree of substitution of Ca²⁺ by Mg²⁺ in the mixture was indicated by the $x$ value in the general formula of (Ca₁₀₋ₓMgₓ)₂(PO₄)₆(OH)₂, where $x = 0, 1/2, 2/2, 3/2, 4/2$. The subsequent coatings were labeled as HA, MA1, MA2, MA3, MA4, respectively. The phase characterization of the coatings was conducted by X-ray diffraction (XRD, PW1830). The magnesium concentration in the coating was determined by X-ray photoelectron spectroscopy (XPS, Kratos-Axis Ultra System) using monochromatic Al Kα X-ray source (1486.7 eV).

2.2. Dissolution test

The dissolution behavior of the coatings was investigated by soaking in a Tris-buffered physiological saline solution (0.9% NaCl, pH 7.4) at a constant temperature of 37 °C for fixed periods of time.

At the end of each period, the samples were taken out and the concentration of Ca²⁺ and mg²⁺ in the solution were analyzed with Atomic Absorption spectrometer (AAnalyst 100, PerkinElmer). An average of five measurements was taken for each sample.

2.3. Cell culture

Human osteosarcoma MG63 cells (ATCC, Rockville, MD) were used to assay the osteoblastic cell response on the coating surface.

In the standard incubation condition (5%CO₂, 37 °C), the cells were incubated in a standard culture medium containing Eagle’s Minimum Essential Medium (EMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, ATCC) and 1% penicillin/streptomycin (ATCC). Cells were seeded on the coating surface at a density of 4.4 × 10⁴ cells/cm². In characterization of cell attachment, the cultured cells were detached from the coatings with trypsin/EDTA solution, and then the cell numbers were counted by hemocytometer (Becton Dickinson, Germany). Statistical analysis was carried out on cellular tests using one-way analysis of variance (ANOVA) at an average of 5 replicates. Differences were considered statistically significant at $p < 0.05$. For cell morphology observation, the osteoblast-like cells attached on the coatings were fixed with 2.5% glutaraldehyde for 1 h at room temperature followed by dehydration with a series of graded ethanol/water solutions (50~100%). Then 0.5 ml hexamethyldisilazane was added to each well to preserve the original morphology of the cells. The samples were coated with gold (for conduction) before observation under a Scanning Electron Microscope (SEM, Leica S360).

3. Results and discussion

3.1. Magnesium Incorporation

The surface chemical compositions of the coatings are analyzed with XPS. Fig. 1 presents the XPS survey scan spectra showing that the coating surfaces comprise Ca, P, O, C and Mg. The Mg 2p and Mg 2s peaks become prominent when $x > 1.00$, and the intensity increases with increasing Mg. The Auger peaks of Mg KLL (at about 301 eV [13]) show clearly the increase of magnesium in the coating. The more magnesium in the sols, the stronger the intensities of Mg KLL peaks are observed. The Mg²⁺ concentrations indicated by the $x$ value in the general formula of (Ca₁₀₋ₓMgₓ)(PO₄)₆(OH)₂ in the coatings are shown in Fig. 2. The dash line indicates the ideal situation where all Mg²⁺ in the coatings are completely incorporated in the apatite structure to replace Ca²⁺. The result shows, however, this happens only when $x < 1.00$ (2.4 wt.%), the Mg concentrations in the coatings almost match that designed in the sol. As $x > 1.00$, the difference between that measured in the coating and that designed in the sol aggravates: Mg in the coating becomes more than that designed in the sols. The higher Mg concentrations detected on the coating surfaces when $x > 1.00$ shows that the apatite crystal structure can host Mg only up to $x = 1.00$ (2.4 wt.%).

The differences of magnesium in the designed sols and the measured coatings are from the limitation of the magnesium coating. The more magnesium in the sols, the stronger the intensities of Mg KLL peaks are observed. The Mg²⁺ concentrations indicated by the $x$ value in the general formula of (Ca₁₀₋ₓMgₓ)(PO₄)₆(OH)₂ in the coatings are shown in Fig. 2. The dash line indicates the ideal situation where all Mg²⁺ in the coatings are completely incorporated in the apatite structure to replace Ca²⁺. The result shows, however, this happens only when $x < 1.00$ (2.4 wt.%), the Mg concentrations in the coatings almost match that designed in the sol. As $x > 1.00$, the difference between that measured in the coating and that designed in the sol aggravates: Mg in the coating becomes more than that designed in the sols. The higher Mg concentrations detected on the coating surfaces when $x > 1.00$ shows that the apatite crystal structure can host Mg only up to $x = 1.00$ (2.4 wt.%).

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incorporation in apatite structure. Note that the designed in the sol is the amount of in the crystalline structure. In small amount, magnesium is likely to substitute into apatite. As the amount increases, the apatite crystals rapidly lost their crystallinity and became amorphous [14]. Fig. 3 shows the XRD patterns of MA coatings. Three phases observed: the HA phase (JCPDS file card #9-432, solid circle), the whitelockite (β-TCMP JCPDS file card #9-169, solid star) and the substrate (Titanium alloy, open circle). After incorporation of Mg, the β-TCMP phase formed in the coating and even dominated the coatings as \( x > 1.00 \) (MA2). These results are consistent with that Mg usually stabilizes β-TCP and helps to form Mg-containing β-TCP (β-TCMP) at high Mg/Ca ratios [5,7].

The amount of magnesium that can be hosted in the apatite structure is a subject of heated controversy. Bigi et al. [9] synthesized Mg-containing apatite powders and the results showed that the HA lattice cannot host Mg higher than 1.7 wt.\% of Mg. Mayer et al. [7] reported that Mg was readily incorporated into the apatite structure at levels up to 1.5 wt.\% with or without carbonate added. Suchanek et al. [5], however, claimed that in Mg-containing HA powders, Mg substitution can be as high as 28.4 wt.\%. Our results indicated that the apatite crystal structure can host Mg up to \( x = 1.00 \) (~2.4 wt.\%) beyond which Mg aggregates on the coating surface instead of replacing Ca in the crystal structure.

### 3.2. Dissolution behavior

The dissolution behaviors of magnesium apatite coatings in Tris-buffered physiological saline solution are shown in Fig. 4. All the coatings exhibited dissolutions as confirmed by the presence of Ca\(^{2+}\) and Mg\(^{2+}\) ions in the soaked solutions. The dissolution of Ca\(^{2+}\) and Mg\(^{2+}\) in the solution showed similar behaviors with the soaking time. At the beginning of immersion, dissolution of the Ca\(^{2+}\) and Mg\(^{2+}\) increased rapidly, while after 6 days the dissolution rates decreased gradually. The insets of Fig. 4(a) and (b) show the Ca\(^{2+}\) and Mg\(^{2+}\) concentrations in the solution after soaking for 24 days. In the inset of Fig. 4(b), the Mg\(^{2+}\) concentrations in the solutions increased almost linearly as Mg increases in the coatings. However, the concentrations of Ca in the solution showed different characteristics. As shown in the inset of Fig. 4(a), for \( x < 1 \), Ca concentration followed the order: HA > MA1 > MA2, meaning dissolution of Ca is the fastest in HA, slower in MA1 and the slowest is in MA2. When more Mg is made in the sol, for \( x > 1 \), the order became MA2 < MA3 < MA4, or the dissolution is still the lowest in MA2, higher in MA3 and even higher in MA4. Compare with pure HA, any amount of Mg incorporation brings down the Ca dissolution.

### 3.3. Cell culture results

Fig. 5 shows typical SEM micrographs of MG63 cells after 1 day of culture on the surface of the magnesium apatite coatings. No significant difference is observed in cell morphology on different coatings. All the cells attached to the coating surface and spread well. Typically, the cells were flattened and their pseudopods clearly seen, indicating good livelihood. Cell proliferation was directly monitored by counting cell numbers at culture periods, and the results are plotted in Fig. 6. The same initial cell density of \( 4.4 \times 10^{4} \) cell/cm\(^2\) was implanted on the coatings. After culturing for 1 day, the cell densities on all samples reached \( \sim 5 \times 10^{4} \) cell/cm\(^2\) indicating good attachment of the cells to all samples. After 5 days in the medium, the attached cell concentration on pure HA coating reached \( \sim 1.27 \times 10^{5} \) cell/cm\(^2\). On magnesium-containing coatings, the cell concentrations of MA1, MA2, MA3 and MA4 were \( \sim 1.35 \times 10^{5}, \sim 1.49 \times 10^{5}, \sim 1.39 \times 10^{5} \) and \( \sim 1.27 \times 10^{5} \) cell/cm\(^2\), respectively.

The cell culture results show that all the coatings have good biocompatibility. At least, no adverse effect is observed as Mg is incorporated. The proliferation of cells requires the presence of growth factors in the medium, provided either by serum or purified proteins. Mg\(^{2+}\) plays a key factor in coordinate control of...
metabolism and growth in animal cells [15–17]. It is proven that the reducing of Mg\(^{2+}\) in the medium usually has bad effect for decreasing the number of cells [15]. In present study, the coatings incorporated with magnesium show good bioresponse with MG63 cells just like the HA coating.

4. Conclusion

Magnesium-containing apatite coatings are synthesized on Ti6Al4V substrate by sol–gel dip-coating method. The study shows that the apatite crystal structure can host Mg up to \(x = 1.00\) (\(\sim 2.4\) wt.\%) beyond which Mg aggregates on the coating surface instead of incorporating in the HA crystal structure. Incorporation of Mg decreases the dissolution rate of Ca\(^{2+}\) when the coating is soaked in simulated body fluid, and at the same time provides Mg\(^{2+}\) to the solution. The dissolution of Ca is the lowest at \(x = 1\) (i.e., MA2) or \((\text{Ca}_9\text{Mg})(\text{PO}_4)_6(\text{OH})_2\). Any other Mg amount contributes to higher Ca dissolution. Dissolution of Mg is almost linear with increasing Mg in the coating after long time soaking in the simulated body fluid. The incorporation of magnesium up to \(x = 2\) does not show adverse effect on MG63 cell proliferation.

Acknowledgements

This work is supported by the Agency for Science Technology and Research, Singapore (A*Star) through project 032101 0005 and SIMTech-NTU collaboration project U03-S-389B.

References


Fig. 5. Typical morphology of cells attached on the apatite coatings: (a) HA and (b) MA2.

Fig. 6. Statistics of cell attachment on coatings with different Mg incorporation.

![Fig. 5](image1.png)

![Fig. 6](image2.png)