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UNIVERSITY OF CALIFORNIA
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Controlling the Biodegradation of Magnesium Implants Through Nanostructured
Calcium-Phosphate Coating

A Thesis submitted in partial satisfaction
of the requirements for the degree of

Master of Science

in

Bioengineering

by

Maria Emil Iskandar

June 2012

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The Thesis of Maria Emil Iskandar is approved:

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ABSTRACT OF THE THESIS

Controlling the Biodegradation of Magnesium Implants Through Nanostructured Calcium-Phosphate Coating

by

Maria Emil Iskandar

Master of Science, Graduate Program in Bioengineering
University of California, Riverside, June 2012
Dr. Huinan Liu, Chairperson

Magnesium (Mg) alloys, a novel class of degradable, metallic biomaterials, have attracted growing interest as a promising alternative for medical implant and device applications due to their advantageous mechanical and biological properties. Moreover, Mg is biodegradable in the physiological environments. However, the major obstacle for Mg to be used as medical implants is its rapid degradation in physiological fluids. Therefore, the present key challenge lies in controlling Mg degradation rate in the physiological environment. The objective of this study was to develop a nanostructured-hydroxyapatite (nHA) coating on polished Mg implants to control the degradation and bone tissue integration of the implants.

The nHA coatings were deposited on Mg using the Spire's patented TPA process to moderate the aggressive degradation of Mg and to improve quick osteointegration between Mg and natural bone. Nanostructured-HA coatings mimic the nanostructure and chemistry of natural bone, which will provide a desirable environment for bone tissue regeneration. Surface morphology, element compositions, and crystal structures were

characterized using scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS), and x-ray diffractometry (XRD), respectively. SEM images of the deposited nHA-coating was analyzed using ImageJ's quantitative image analysis tool, to determine the nHA-coating particle size and thickness. The degradation of nHA-coated and non-coated Mg samples was investigated by incubating samples in phosphate buffered saline (PBS) and revised simulated body fluid (r-SBF), under standard cell culture conditions. To mimic the *in vivo* cell response in the physiological environment, rat bone marrow stromal cells (BMSC) were harvested and cultured with nHA-coated and non-coated polished Mg samples to determine cytocompatibility.

The degradation results suggested that the nanocoatings positively mediated Mg degradation. It can therefore be concluded that nHA-coatings show promise for controlling the biodegradation of Mg-based orthopedic implants and devices. Cell studies indicated significantly improved BMSC adhesion on the surfaces of the nHA-coated and non-coated Mg samples, in comparison to the cells surrounding the Mg samples. These results indicated that the nHA-coated and non-coated Mg samples promote cell activity on the surface. However, cell experiments must be repeated on a larger number of samples with extensive short and long term cell studies, to achieve more verifiable results.

Keywords: calcium-phosphate; hydroxyapatite; nanostructure; orthopedic/medical implant; degradation; magnesium; coating; bone marrow; mesenchymal stem cells; hematopoietic stem cell

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1. Introduction

1.1 Current metallic biomaterials for orthopedic applications

Bone fractures can occur when a physical force exerted on the bone is greater than the bone's strength. Upon fracture, a bone can heal, but inadequately if not properly attended. A fracture can heal properly with a surgically inserted supported implant. However, current metallic implanted biomaterials remain permanent in the body and require a second surgical procedure for removal after fulfilling their functions (eg. to secure fractures).¹ This secondary surgical removal results in increased health care costs,² extended hospitalization, and more notably, pain and loss of productivity for patients.¹ Therefore, a desirable implantable biomaterial should have the ability to degrade in the body after the bone tissue has healed.

1.2 Benefits of Mg and its biodegradability

One such biomaterial is magnesium (Mg). Mg alloys are a novel class of biodegradable metals and have attracted growing interest as a promising alternative for medical implant and device applications due to their advantageous mechanical, physiological and degradation properties.^{1,3-21}

In addition to Mg's biodegradability in the physiological environment, Mg possesses many advantages over the currently used metallic implants. It is more favorable due to its light weight, and compressive yield strength and elastic modulus being similar to cortical bone.^{1,2,22} Thus, Mg can decrease the likelihood of stress shielding and the possible loss of bone density, as well as provide optimal initial fixation without breakage during implantation.^{1,22-24} It is also both osteoconductive^{1,25} and can promote new bone

growth,^{15,26} which can enhance osteointegration, a necessity for implant stability.

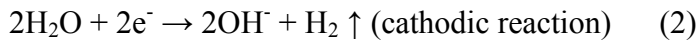
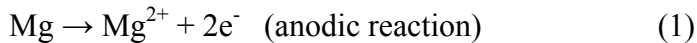
Additionally, it is naturally found in bone tissue, and it is the 4th most abundant cation in the human body, with almost half of the entire physiological Mg stored in bone.^{1,27}

Moreover, the degradation by-products of Mg are non-toxic and are readily excreted and metabolized through the kidney.²²

1.3 Mg's rapid degradation and challenges

Although Mg possesses many desirable properties, its rapid degradation rate is an obstacle that needs to be overcome in order to make it optimal for implant applications. In the atmosphere, Mg forms a porous oxide layer, which provides acceptable protection in a dry environment.²⁸ However, in the body, the oxide layer is permeable to water, allowing for Mg dissolution through the following degradation reaction: $\text{Mg} + 2\text{H}_2\text{O} \rightarrow \text{Mg}(\text{OH})_2 + \text{H}_2$.

The degradation process of Mg is summarized in the following reaction scheme:^{29,30}



The degradation behavior involves micro-galvanic coupling between anodic and cathodic regions.²⁸ The sum of the above partial reactions give the overall reaction of Mg degradation: $\text{Mg} + 2\text{H}_2\text{O} \rightarrow \text{Mg}(\text{OH})_2 + \text{H}_2$.^{28,31} In summary, Mg dissociates by reacting with water to produce a porous, crystalline film of Mg hydroxide ($\text{Mg}(\text{OH})_2$), and hydrogen gas (H_2).¹⁹ This $\text{Mg}(\text{OH})_2$ layer acts as a passivate that slows further degradation to some extent. However, *in vivo*, chloride in the physiological fluid can

cause Mg to degrade at a faster rate by forming highly soluble MgCl_2 ,^{30,32} producing pitting corrosion³³ and therefore, eventual mechanical failure and implant loosening.^{19,28}

Moreover, during degradation of pure Mg, 1 mole of H_2 is produced for every 1 mole of dissolved Mg.³⁰ The H_2 formation during degradation,^{9,16,19,22,27} can cause tissue necrosis, delaying its healing process.²⁷ Additionally, hydroxyl groups (OH^-) formed during degradation increases the pH around the implantation site,^{17,22,27,34} thus adversely affecting cell proliferation and new tissue formation.¹⁹

Therefore, Mg's rapid degradation produces many adverse affects including hydrogen gas cavities, local pH increase, and mechanical failure. In order to be functional, an implant must maintain its mechanical integrity during the period necessary for bone tissue to heal. However, due to Mg's rapid degradation, there is not a sufficient amount of time for bone to heal completely before the implant loses its mechanical integrity.^{10,16,22,35} Therefore, the present key challenge for utilizing Mg alloys for medical applications is in controlling their degradation rate in the physiological environment.

1.4 The need for surface coating

Research has shown that surface coating can delay the onset of degradation.³⁴ In recent work, calcium-phosphate have been considered as possible coating materials for Mg due to its similarity to the chemistry of natural bone.^{1,2,8,36,37} Moreover, calcium-phosphate has not shown toxicity during degradation.³

Calcium-phosphate coatings have shown to slow down the diffusion of water and other ions to the Mg surface, thereby decreasing the degradation rate,^{7,17,34} which, as a result, will improve the pH of the surrounding fluid. Particularly, the phosphate ions have

shown to decrease Mg degradation and delay pitting corrosion due to the precipitation of Mg phosphate.^{7,28,33} Moreover, precipitation of calcium-phosphate coatings have been observed on the surfaces of Mg-based metals immersed in physiological medium and when implanted *in vivo*; these coatings have shown to improve biocompatibility of metallic implants and to enhance osteoblast response, and therefore, promote osteointegration.^{1,2} Moreover, hydroxyapatite (HA, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), one type of calcium-phosphate, is the main inorganic component of bone tissue; therefore it is a natural part of our body.^{38,39}

1.5 Benefits of nanostructured-HA surface coating

The precipitation of HA mineral crystals on organic protein collagen matrix constructs bone connective tissue.³⁹ Moreover, natural bone tissue is a nanostructured composite material, composed of 70% nanostructured inorganic HA crystals of approximately 50 nm in length and 5 nm in diameter, and 22% organic Type I collagen fibers.^{40,41} Thus, bone cells are naturally accustomed to interact with nanostructured materials. Therefore, it stands to reason that mimicking the nanostructure of natural bone will provide a desirable environment for bone tissue regeneration. Surface structure alone has shown to influence cellular adherence, migration, proliferation and differentiation.⁴² Moreover, for orthopedic implant applications, Mg implants become integrated into bone tissue. Therefore, osteointegration is critically needed to stabilize orthopedic implants for clinical success; the success of an implant is dependent on its ability of inducing osteointegration at the implant-bone interface. In addition to Mg's osteoconductivity, nanostructured-HA (nHA) coatings will also induce osteointegration and osteogenesis.

Studies have shown that osteoblast adhesion increased significantly on nHA than on conventional micron-sized HA after 4 hours of culture.⁴³ Similarly, osteoblast proliferation increased significantly on nHA than on conventional HA after 3 and 5 days of culture.⁴⁴ Therefore, nanostructured materials have the potential to enhance bone cell functions.^{40,43,44} As a result, nHA-coatings are not only a promising candidate to delay the biodegradation process, but also in enhancing osteointegration, and in improving overall orthopedic implant efficacy.

1.6 Bone marrow stromal cell's and their involvement with implants

Implants come in direct contact to bone marrow stromal cells (BMSC). BMSCs play a significant role on implant and tissue integration. An implant is successful when it promotes osteointegration. BMSCs primarily contain mesenchymal and hematopoietic stem cells (MSC and HP, respectively). MSC's and HP's are precursor cells that are responsible for osteogenesis and inflammatory response, respectively. MSC's harvested from bone marrow are commonly used in experimental procedures to promote bone regeneration. Yet, other bone marrow cellular constituents in combination with MSC's, play a significant role in osteogenesis.⁴⁵

Orthopedic surgeries are known to cause damage to the surrounding tissue, prompting a wound healing response to activate various inflammatory cells at the implant site.³⁹ HP's, precursors of inflammatory cells, are located near the endosteal surface of trabecular bone, in close proximity to the mesenchymal population.⁴⁵ Therefore, it stands to reason that both cell types play an interactive role in influencing the other's functions.⁴⁵ Compared to cultures with MSC's alone, studies have shown enhanced MSC osteogenic

differentiation (induced by dexamethasone treatment) when co-cultured with HP's, as verified by early alkaline phosphatase synthesis and enhanced calcium deposition (early differentiation markers).⁴⁵ The inclusion of HP's provide an environment for cell-cell interactions and paracrine signaling that enhance differentiation.⁴⁵

Moreover, since bone is composed of HA crystals in the nanostructure scale, studies have also revealed that nano-sized-HA can enhance osteogenic differentiation of bone marrow derived mesenchymal stem cells.⁴⁶ Therefore, implant-bone interface that mimic the interfaces naturally occurring between bone, will enhance bone formation. In this study, bone marrow derived stromal cells containing MSCs and HPs were harvested and cultured on the nHA-coated and non-coated Mg samples, in order to mimic the *in vivo* physiological environment to which implants are exposed. These cells cultured in combination will affect the overall cell response and, therefore, will help provide an understanding of how these different cells types respond collectively in the body.

1.7 Objective of thesis study

It typically takes 4 to 12 weeks for bone tissue to heal, depending on the type and anatomical location of bone. Therefore, it is desirable for Mg to maintain its mechanical properties over a time scale of 12 to 18 weeks, until the bone tissue regenerates.¹ The objective of this study is to develop a nHA-coating on Mg implants to control the degradation rate and improve the tissue integration of implants.

2. Materials and Methods

2.1 Fabrication of nHA-coated Mg samples

Pure (99.9%), polished magnesium (Mg) from Goodfellow Inc. (Oakdale, PA, USA) was used as the substrate. The Mg substrates were 250 μm in thickness and cut into 10 mm x 10 mm squares. Each sample was ultrasonically cleaned with ethanol for 10 minutes before the deposition process. The non-coated Mg substrates were used as the control. The nHA-coatings were deposited using the Spire Biomedical Inc. patented transonic particle acceleration (TPA) deposition process on Mg to moderate the aggressive degradation of Mg and to improve osteointegration between Mg and natural bone. The nHA-coating was applied with a thin coating on both sides.

2.2 Surface Characterization

Morphology, element compositions, and crystal structures of the nHA coated and non-coated Mg samples were characterized using scanning electron microscopy (SEM, Philips XL30), energy dispersive x-ray spectroscopy (EDS), and x-ray diffractometry (XRD, Model D8/Advanced), respectively. An accelerating voltage of 10-15 kV at a spot size of 3, and working distance of 9.5-10.5 mm were used for SEM and EDS analysis. Copper $K\alpha$ radiation produced at 40 kV was used to scan the diffraction angles (2θ) between 10° to 70° (2θ) at a step size of 0.02° for XRD analysis. SEM images of the nHA surface coating and cross section were analyzed using ImageJ's quantitative image analysis tool to determine the HA particle size and thickness, respectively. The frequency of particle size was calculated by dividing the number of particles within each range by

the total number of particles; this value was then multiplied by 100 to obtain a percentage.

2.3 *In vitro* degradation studies

In vitro degradation of nHA-coated and non-coated Mg samples were investigated by immersing the samples in standard corrosion mediums of phosphate buffered solution (PBS) and revised simulated body fluid (r-SBF), for two experiments. The degradation studies in PBS was initially investigated to determine if the nHA-coatings could, in fact, improve degradation. A more in-depth degradation study was performed using the r-SBF solution as the immersion medium.

PBS is a standard buffer solution containing sodium chloride (NaCl), potassium chloride (KCl), sodium hydrogen phosphate (Na_2HPO_4), and potassium hydrogen phosphate (KH_2PO_4) and was buffered to the physiological pH of 7.4. The ion concentrations are given in Table 1. The r-SBF solution was prepared according to the method described in Oyane et al, containing ion concentrations equal to the levels of human blood plasma, to mimic the body's physiological environment.⁴⁷ The ion concentrations are given in Table 2. The pH was adjusted to 7.4, and the temperature was kept at 36.5-37°C in a water bath.

PBS and r-SBF have an ionic strength of 0.174 and 0.149, respectively. Therefore, PBS is a more reactive solution than r-SBF.

Prior to immersion, the non-coated samples were immersed in ethanol for 30 minutes, while the nHA-coated samples were ultrasonically washed with ethanol for 10 minutes. Thereafter, the samples were further sterilized under ultraviolet (UV) light for at least one

hour. Each sample was then individually immersed in a 12 well polystyrene non-tissue cultured plates containing 3 ml of PBS, and 3 ml of r-SBF in a separate 12 well plate, for full submersion, then incubated under a sterile, 37 °C, 95/5% air/CO₂, humidified environment in PBS for a total of 28 days and in r-SBF for 31 days. All experiments were performed in triplicate. The r-SBF solution, without Mg, was used as a control for pH.

The degradation of the nHA-coated and non-coated Mg samples immersed in PBS was monitored after 28 days of incubation. The mass was recorded after 28 days, when the PBS solution dried out. Change in mass (ΔM) was calculated according to the following equation:

$$\Delta M = M_{\text{final}} - M_{\text{initial}}$$

Positive and negative mass represents mass gain and loss, respectively.

Photographs of the degradation appearance, mass, r-SBF pH and release of mg ions from the nHA-coated and non-coated Mg samples immersed in r-SBF, were recorded at different time points (i.e. 1, 2, 4, 8, 16, 24 hours, 2, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, and 31 days). The ΔM with respect to incubation time was calculated.

The data is presented as the mean of three measurements. Contrary to the procedure used on the nHA-coated and non-coated Mg samples immersed in PBS, the r-SBF solution was replaced with fresh solution after every immersion period. Inductively-coupled plasma mass spectrometry (ICP-AES) (Optical Emission Spectrometer) was used to determine the concentration of Mg ions in the r-SBF media that was dissolved from the nHA-coated and non-coated Mg samples after each prescribed immersion period. The concentration of Mg ions contained within the r-SBF media was subtracted from the

concentration of Mg ions dissolved from the nHA-coated and non-coated samples. This was done to monitor the release of Mg ions from the samples only.

2.3.1 Statistical analysis of degradation data

Quantitative data were analyzed using standard two-tailed T-test and an $\alpha = 0.05$ indicated statistical significance.

2.3.2 Surface Characterization after degradation

Morphology and element compositions of the nHA coated and non-coated Mg samples after degradation were characterized using scanning electron microscopy (SEM, Philips XL30) and energy dispersive x-ray spectroscopy (EDS) operated with the same parameters used to characterize the nHA-coated and non-coated samples before degradation.

2.4 *In vitro* cytocompatibility with BMSC culture

2.4.1 Isolation and culture of BMSCs

The BMSCs were harvested from the femur and tibia of one 19 day-old male Sprague Dawley rat weanling, after euthanasia by CO₂, following an approved IACUC protocol. Under standard sterile conditions, the ends of the bones were dissected and the bone marrow was flushed out of the bone cavity using Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin-streptomycin (P/S, Invitrogen), and then collected. The collected cells in DMEM were then filtered through a 70 μ m nylon strainer, to remove any cell aggregates and tissue debris. The filtered cells were cultured in T75 tissue culture treated flasks with

the supplemented DMEM under standard cell culture conditions (37 °C, 95/5% air/CO₂, humidified environment) until the cells reached confluency.

2.4.2 Stromal cell seeding and adhesion on Mg samples

Before seeding, the nHA-coated and non-coated Mg samples were sterilized according to the method used for the degradation studies. The samples were photographed to record surface appearance before cell culture. When the bone marrow cells reached 90% confluency, they were detached using trypsin and seeded onto the the nHA-coated and non-coated Mg samples in a 12 well polystyrene tissue culture plate (PSTC) at a density of 10,000 cells/cm² and incubated in DMEM supplemented with 10% FBS and 1% P/S, under standard cell culture conditions, for 24 hrs. The supplemented DMEM was then collected to monitor change in pH after 24 hr culture with the nHA-coated and non-coated Mg sample. The supplemented DMEM with cells and supplemented DMEM alone (without cells) were used as controls. After that time period, phosphate buffered solution (PBS) was used to remove non-adherent cells. The adherent cells were then fixed with 4% paraformaldehyde and stained with DAPI nucleic acid stain and Alexa Flour® 488 cytoskeleton stain. Thereafter, the adhered cells were imaged and counted using a fluorescence microscope. Due to difficulty in differentiating between the different cell types, the stromal cell nuclei were counted and cell adhesion density (cells per cm²) were averaged based on six or more random fields on each Mg sample and on the PSTC surface surrounding each Mg sample, and on the control. Cell adhesion density was divided by seeding density to obtain the percentage of adhered cells per cm².

The cell culture experiments were conducted on one sample of each nHA-coated and non-coated Mg sample.

2.4.3 Statistical Analysis of BMSC adhesion data

Quantitative data were analyzed using the Dunn test with appropriate pairwise comparisons and a $p < 0.05$ indicated statistical significance.

3. Results and Discussion

3.1 Surface characterization before degradation and cell culture studies

3.1.1 Surface microstructure and composition of nHA-coated and non-coated Mg

The scanning electron micrographs (SEM) showed that the polished Mg surface appeared smooth before coating and EDS analysis confirmed it was pure Mg (Fig. 1A, B). The nHA coating deposited on the polished Mg surface was uniform with a particulate-like microstructure, typical for HA coating (Fig. 2A). HA contains Ca, P, and O, which were all observed in the EDS spectrum (Fig. 2B). A Mg peak was also observed due to the Mg substrate. The EDS analysis showed the coating had a Ca/P ratio of 1.6 (Fig. 2B, Table 3), close to that of natural HA (1.66).³

It was noted that some carbon was detected in the nHA-coated Mg surface layer (Table 3) which may be due to some organic contamination,⁴⁸ or to the carbon tape used during sample mounting.

3.1.2 nHA-coating particle size and thickness

The SEM images of nHA surface coating and cross section were analyzed using ImageJ's quantitative image analysis tool to determine the HA particle size and thickness, respectively. The results showed a 45 μm thick HA coating on both sides of the Mg

samples, with a broad size distribution of particles ranging from 50-1400 nm in long axis, with the most frequent particle size between 300-500 nm (Fig. 3A). The short axis particle size ranged from 35-900 nm with the most frequent short-axis particle size between 200-300 nm (Fig. 3B).

3.1.3 Phase analysis of HA

XRD results indicated the deposited coating consisted of HA, with some peaks of octacalcium phosphate (OCP, $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$), another phase of calcium-phosphate (Fig. 4). Some background noise was observed between 10° to 20° , which may have indicated some amorphous HA.

3.1.4 Discussion of nHA-coating characterization

The calculated Ca/P ratio of the nHA-coating is 1.6 (Fig. 2B) as shown in the results, similar to the natural HA ratio of 1.66^3 . Therefore, Ca and P were deposited in accordance with the appropriate stoichiometry of natural HA of bone during nucleation. XRD also indicated the deposited coating consisted of HA. The OCP peaks observed in the nHA-coated Mg sample (Fig. 4) may be attributed to the coating process or initial HA nanopowder material used for the deposition.

Moreover, concerning the nHA particle size, the HA nanopowder used as the depositing material may not have been initially fine enough. Trabecular bone is composed of HA minerals of 30 to 120 nm in diameter.³⁹ Therefore, more improvements need to be made to develop nHA coating with particle size in the range of natural bone. However, the TPA technology patented by Spire Biomedical Inc. is state of the art and

approved technology for orthopedic applications. The method is a propriety process, therefore no detailed information is provided about the process.

3.2 Degradation results of nHA-coated and non-coated Mg in PBS

3.2.1 Photographs of degradation appearance and ΔM of nHA-coated and non-coated Mg samples after immersion in PBS

After 28 days immersion in PBS, the non-coated Mg sample showed larger area of degradation and more degradation products of white precipitates on the sample (Fig. 5D, D') compared to nHA-coated Mg (Fig. 5C, C'). The nHA-coated sample gained 19 mg of mass, while the non-coated sample gained 15.1 mg. The mass gain was due to the salt precipitation from the PBS solution and accumulating on the sample. However, the non-coated sample showed larger area of degradation with much more severe localized degradation attack manifested as pitting corrosion and more degradation products of white precipitates on the surface (Fig. 5D, D'), compared to the nHA-coated Mg sample (Fig. 5C, C').

3.2.2 Surface characterization after immersion in PBS

After 28 days of immersion in PBS, SEM image of the nHA-coated Mg showed that the surface feature changed to plate-shaped crystals (Fig. 6A). The EDS analysis suggested the surface composed of Mg, O, Na, C, Cl, and a small amount of P (Fig. 6B, Table 3), in comparison to the nHA-coated sample before degradation (Fig. 2B). The O came from the chemical composition of HA and the Mg degradation reaction with water. Na and Cl precipitated from the PBS solution during incubation. No Ca was detected in the degradation layer, indicating dissolution of HA.

After 28 days of immersion in PBS, SEM image of the non-coated Mg showed that the surface morphology changed to a scale-like porous structure (Fig. 7A). EDS analysis suggested the surface composed of Mg, O, Na, C and a significant amount of Cl, in comparison to the nHA-coated sample after 28 days of degradation, indicating aggressive Cl attack (Fig. 7B, Table 3).

It was noted that some carbon was always detected in the Mg surface layer. The C may be due to the carbon dioxide in the incubator, which could have been dissolved in the solution and incorporated into degradation layer during incubation. It could also have come from some organic contamination⁴⁸ or to the carbon tape used during sample mounting.

3.2.3 Discussion of the degradation of nHA-coated and non-coated Mg in PBS

Surface elemental atomic percentage compositions (atomic %) of the nHA-coated and non-coated Mg samples before and after degradation in PBS are summarized in Table 3. It is important to note the abundance of Cl that accumulated on the surface of the non-coated sample after 28 days of degradation in PBS. A much lower Cl content was observed on the nHA-coated Mg sample. Although MgCl_2 is highly soluble, over saturation and prolonged exposure to the PBS media contributed to the accumulation of Cl on the non-coated Mg surface. Cl can penetrate into the porous $\text{Mg}(\text{OH})_2$ degradation layer, attacking the Mg. This explains the obvious pitting corrosion observed on the non-coated sample. This also justifies the non-coated sample's quicker degradation due to more chlorides reacting with Mg on the non-coated Mg sample.

3.3 Degradation results of nHA-coated and non-coated Mg in r-SBF

3.3.1 Photographs of degradation appearance after immersion in r-SBF

The photographs of degradation appearance of the nHA-coated and non-coated Mg samples in r-SBF after each prescribed immersion period, are shown in Fig. 8 and Fig. 9, respectively. Over time, the nHA-coated and non-coated Mg samples showed significant ($\alpha = 0.05$) degradation on the edges of the samples. By 15 days, the non-coated sample showed breakage (Fig. 9). Conversely, the nHA-coated sample remained maintained its structural integrity (Fig. 8). By 17 and 19 days, half of the non-coated sample remained, and by 21 days the non-coated sample completely degraded while the nHA-coated sample maintained its structural integrity.

3.3.2 Sample mass loss after immersion in r-SBF

The mass loss of the nHA-coated and non-coated Mg samples after each prescribed immersion period is shown in Fig. 10. Over time, the non-coated Mg samples showed significantly more mass loss than the nHA-coated Mg samples, indicating a faster degradation. The average mass loss from the nHA-coated samples after 21 days of immersion was 31.80 mg, much less than the non-coated samples of a mass loss of 45.95 mg, thereby showing that the nHA-coating protected the surface and improved the degradation.

3.3.3 Media pH and Mg ion concentration after immersion in r-SBF

The pH of r-SBF in nHA-coated and non-coated Mg samples after each prescribed immersion period is shown in Fig. 11. The pH of r-SBF cultured with the non-coated Mg samples increased significantly more than that of the nHA-coated Mg samples after 2

days of degradation. A similar trend was observed in the Mg ion concentration in the r-SBF media, dissolved from the nHA-coated and non-coated Mg samples after each prescribed immersion period (Fig. 12). The concentration of Mg ions in r-SBF dissolved from the non-coated Mg samples increased significantly more than that of the nHA-coated Mg samples after 3 days of degradation.

3.3.4 Discussion of the degradation of nHA-coated and non-coated Mg in r-SBF

The results showed significantly improved degradation performance in the nHA-coated Mg samples, compared to the non-coated Mg samples in r-SBF (Fig. 8, 9, 10). It is important to note that, prior to degradation, the nHA-coating on the coated Mg samples were not entirely coated; the corners of the Mg sample remained exposed, as shown in Fig. 8, at 0 hrs. Over time, it can be observed that the nHA-coated Mg sample maintained its structural integrity, while the corners degraded in r-SBF, suggesting further that the coated surface protected the Mg sample from a quicker degradation (Fig. 8). Most of the nHA-coated samples' loss in mass can be attributed to the degradation of the exposed Mg surface on the corners of the samples.

The initial increase of mass (Fig. 10) and initial decrease of media pH (Fig. 11) and Mg ion concentration (Fig. 12), are due to the salt precipitation from the r-SBF solution. This accumulation of precipitates may have initially slowed down the degradation, indicating that the samples became somewhat passivated due to the accumulation.

The pH and Mg ion concentration of the r-SBF media improved when cultured with the nHA-coated Mg samples. The coating slowed the diffusion of water into the Mg sample, thereby slowing the dissolution of the Mg. Mg dissociates by a reaction with

water to produce $\text{Mg}(\text{OH})_2$; therefore the coating protected Mg from direct contact with water to form OH^- , thereby reducing pH. Moreover, the increase in Mg dissolution observed from the non-coated Mg samples contributed to the increase in pH due to the increasing OH^- production.

After 5 and 7 days, the pH and Mg ion concentration began to drop (Fig. 11, 12), which are due to the reduction in the samples' mass. However, the pH and Mg ion concentration of the r-SBF media cultured with the non-coated samples continued to be significantly higher than that of the nHA-coated samples until 21 days, where a significant decrease is observed due to the non-coated samples' almost complete degradation.

The human body contains 35g per 70kg of Mg. The body needs 375 mg/day. Hypomagnesemia (deficient Mg) can cause cardiac and skeletal muscle weakness which can be treated with supplemental Mg fluids.⁴⁹ However, hypermagnesemia (excess Mg) can be life threatening. Excess Mg can cause muscular paralysis, hypotension, respiratory distress, and even cardiac arrest if levels are extremely high.⁴⁹ However, due to the body's efficient Mg excretion through the urine, hypomagnesemia rarely occurs.⁴⁹

3.3.5 Surface characterization after immersion in r-SBF

After immersion in r-SBF, SEM image of the nHA-coated Mg sample after 17 days of degradation (Fig. 13A) showed particulate features similar to the initial nHA-coating, before degradation (Fig. 2). To note, after 17 days, the nHA-coated Mg maintained its overall structural integrity; most of the sample degraded from the corners, where the Mg surface was exposed. The EDS analysis suggested the surface composed of Mg, O, C, Ca,

and P with a Ca/P ratio of 1.56 (Fig. 13B, Table 3). The Ca/P ratio remained close to the initial Ca/P ratio 1.60. Again, the O is from the chemical composition of HA and the Mg degradation reaction with the r-SBF solution. Ca and P came from the HA coating and precipitation from the r-SBF solution. The C may have been due to the precipitation products of the r-SBF solution and/or the carbon dioxide that may have dissolved in the corrosion solution and incorporated in the degradation layer during incubation.

It is obvious that the non-coated sample degraded significantly. After 17 days of immersion in r-SBF, SEM image of the non-coated Mg showed cracks on the surface with obvious overlaying precipitates (Fig. 14A) and the EDS analysis suggested the surface composed of O, Na, C, Ca, and P and a large amount of Mg (Fig. 14B, Table 3) in comparison to the nHA-coated Mg sample after 17 days of degradation. The Na, P, and Ca precipitated from the r-SBF solution during incubation. The calculated Ca/P ratio on the surface is 0.92, according to the EDS analysis. SEM image of the non-coated Mg sample after 21 days of degradation in r-SBF, showed similar surface features and precipitates in micrometer scale (Fig. 15A). To note, after 21 days, the non-coated Mg sample was almost completely degraded (less than 1 mg left). The calculated Ca/P ratio is 1.05 according to the EDS analysis (Fig. 15B, Table 3).

SEM image of the nHA-coated Mg sample after 31 days of degradation (Fig. 16A), showed particulate features similar to the nHA-coating, before degradation (Fig. 2A) and after 17 days of degradation (Fig 13A). After 31 days, the sample was almost completely degraded (less than 4 mg left). The EDS analysis suggested the surface composed of Mg O, Na, C, Ca, P with a Ca/P ratio of 1.44 (Fig. 16B, Table 3). SEM image and EDS

analysis of another region closer to the edge of the same sample showed large cracks in the Mg surface and some overlaying precipitates (Fig. 16C), with O, Na, Ca, P, trace amounts Cl, and a larger amount of Mg on the surface (Fig. 16D, Table 3) compared to Fig 16A and B. The calculated Ca/P ratio is 1.12.

3.3.6 Discussion of nHA-coated and non-coated surface characterization after immersion in r-SBF

The Ca/P ratios, before and after immersion in r-SBF, are summarized in Table 3. The Ca/P ratio of the nHA-coated Mg after 17 days of degradation in r-SBF remained considerably high (1.56) (Fig. 13B, Table 3), close to that of natural HA and the initial HA of 1.60 (Fig. 2B, Table 3). The sample also showed particulate features similar to the initial nHA-coating, before degradation (Fig. 2). Therefore, the nHA-coating still maintained its integrity and adhesion to the sample after 17 days of degradation. XRD would need to be performed in order to determine the phase of this calcium-phosphate coating.

Ca and P were also observed on the non-coated Mg sample after 17 and 21 days of degradation in r-SBF. This is due to the Ca and P precipitating from the r-SBF solution, and accumulating on the surface. This demonstrates how the deposition of calcium-phosphate from the degradation medium is a dynamic process involving partial degradation of the inner layer and the deposition of Ca and P.

It's important to note a large amount of Mg was observed on the non-coated Mg samples after 17 and 21 days of degradation, in comparison to nHA coated Mg sample after 17 days of degradation. To note, after 17 and 21 days of degradation, the non-coated

Mg almost completely degraded, while the nHA-coated Mg maintained its structural integrity. Therefore, more of the Mg was exposed to Cl attack, which sped up degradation.

After the nHA-coated Mg samples were almost completely degraded at 31 days of degradation, Ca/P ratio was 1.44 at a region at the center of the sample, and 1.12 at a region near to the edge of the sample. It is important to note that the region with the Ca/P ratio of 1.14 was on the degraded edge of the sample, where the Mg surface is exposed to Cl attack; this explains the large cracks and obvious degradation observed on the surface (Fig. 16C).

3.4 Discussion of the factors affecting degradation

3.4.1 Comparison of degradation behavior in PBS and r-SBF

The unique composition of PBS and r-SBF and the frequency of media change affected the rate and process of Mg dissociation. The atomic percentage (atomic %) of surface element compositions of the nHA-coated and non-coated Mg samples before and after degradation in PBS and r-SBF, are summarized in Table 3. Trace amounts of Cl were only observed on the nHA-coated Mg surface at 31 days of degradation in r-SBF (Fig. 16D, Table 3), when the sample was almost completely degraded. The Cl was observed on the edges of the sample, in areas with cracked surfaces. However, a much larger amount of Cl was detected on the surfaces of the samples immersed in PBS after 28 days of degradation (Fig. 7B, Table 3). Given the fact that MgCl_2 is a highly soluble compound, it is therefore likely that most of the Cl remained soluble in the r-SBF solution due to the frequent media change. The Mg samples immersed in r-SBF were

refilled with fresh solution after each immersion period, different from the procedure compared with that for the samples immersed in PBS, where the samples were immersed continuously without frequent media change until the PBS dried out at 28 days. Therefore, the Mg samples were not immersed in the r-SBF solution long enough for the Cl to accumulate on the surface layer.

Moreover, as mentioned before, no Ca was detected in the nHA-coated Mg sample, and very little P was present after 28 days of degradation in PBS. However, large amounts of Ca and P were observed on the nHA-coated and non-coated Mg sample after degradation in r-SBF. Therefore, the nHA coating dissolved from the Mg substrate while immersed in PBS for 28 days. However, again, the nHA-coated and non-coated Mg samples were immersed continuously in PBS for 28 days, without frequent media change. Moreover, r-SBF contains both Ca and P, where PBS contains only P. Rettig et al. reported that phosphate presence in the immersion media is essential for calcium deposition in their study; moreover, Ca was discovered to only precipitate onto Mg in the form of calcium-phosphate when immersed in SBF.⁴⁸ The lack of Ca in the PBS solution may have been the reason for the lack of Ca on the nHA coated Mg sample after 28 days of degradation. Phosphate, on the other hand, can precipitate with other ions such as Mg, due to their low solubility.⁴⁸ However, the continuous culture in PBS without media change may have contributed to the small amount of P present on the surface. The samples immersed in PBS were exposed to a concentration of phosphate ions at one time for a 28 day period, where as the samples immersed in r-SBF were exposed to a new concentration of phosphate ions after every immersion period. Since the samples in PBS

were exposed to one concentration of phosphate ions and since there was a small amount of phosphate residual left on the Mg degradation layer after 28 days of culture, calcium could not remain stable on the surface.

These degradation results suggested that nHA coatings positively mediated Mg degradation in both PBS and r-SBF.

3.4.2 The effects of fluid flow on degradation

The body's fluid is constantly circulating, and is continually being refreshed. The speed at which the body's fluid changes depends on its anatomical location. Fluid change is quick in the vascular regions of the body, whereas it slows around the tissue fat. Therefore observing the degradation using frequent media change gives more ideal degradation behavior, *in vivo*, compared to less frequent media change. The frequent media change performed on the nHA-coated and non-coated Mg samples immersed in r-SBF is more similar to the *in vivo* body conditions, compared to the lack of media change performed on the Mg samples immersed in PBS.

3.4.3 The effects of nHA-coating on Mg degradation

Rapid degradation of an implant material can lead to shortening of an implant lifetime. It can be seen that the protectiveness provided by the coating is initially strong, but decreases with time. Still, the coated samples show more improvement than the non-coated samples. This protection behavior is desirable of an implant because degradation should initially be slow in order for the implant to maintain its mechanical integrity during the period of healing. Once the new bone is regenerated, the implant is expected to degrade after the initial period. However, the initial protection provided by the coating

must be prolonged to 12 to 14 weeks, the period necessary for bone tissue to heal.

Therefore, for a more practical measure of the degradation rate of a Mg implant, the immersion test should be performed on an actual bone implant. If degradation studies were performed on a larger nHA-coated Mg substrate, such as a Mg interference screw that is used to fix fractures, for example, would give a better demonstration of the time scale of degradation of an nHA-coated Mg implant.

3.5 BMSC Adhesion

3.5.1 Results of BMSC adhesion on and surrounding nHA-coated and non-coated Mg

After 24-hr culture in the supplemented DMEM, surface oxidation was observed on the surface of the nHA-coated and non-coated Mg samples (Fig. 17C, D). No change in media color was observed surrounding the nHA-coated and non-coated Mg samples with only 0.09 difference in pH, after 24 hr culture (Fig. 18). The cell studies were conducted on one sample of each nHA-coated and non-coated Mg sample.

Fluorescence images revealed obvious MSCs on the control PSTC surface, as evident by their spindle-shaped morphology (Fig. 19C, C'). The cells on and surrounding the nHA-coated and non-coated Mg samples appeared rounded in shape, which made it difficult to determine cell type (Fig 19A', B'). Future short term and long term cell studies, with larger number of samples and specific biomarkers, are still needed in order to achieve better cell analysis. The calculated adhesion density of BMSC (Fig. 21) demonstrated that cell adhesion on the surrounding nHA-coated or non-coated Mg surfaces were significantly less than that on the control PSTC surface, after 24-hr culture.

No significant difference in the cell adhesion density on the nHA-coated and non-coated Mg surfaces were detected compared to the control (Fig. 20, 21). Significantly more cells adhered on the nHA-coated and non-coated Mg surfaces than that surrounding the samples (Fig. 19,20, 21). This indicates that the nHA-coated and non-coated Mg samples promoted more cell activity on the surface. Cell adhesion density was divided by seeding density (10,000 cells/cm²) to obtain the percentage of adhered cells per cm². The calculated percentage of BMSC's that were adhered on and surrounding the samples, and on control, are labeled on the top of the respective bar graph (Fig. 21). Results showed 44.7% and 48.6% BMSC's adhered on the nHA-coated and non-coated Mg samples, respectively. However, no significant difference in the cell adhesion density was detected on the nHA-coated and non-coated Mg samples after 24 hr-culture. Results showed 13.0% and 8.9% BMSC's adhered on the surface surrounding the nHA-coated and non-coated Mg samples, respectively. Again, no significant difference was detected. The control PSTC had 153.5% cells adhered. More than 100% cell adhesion on the PSTC indicates the proliferation of the BMSCs.

3.5.2 Discussion of factors affecting BMSC adhesion

A drop in pH of the media cultured with cells alone was observed, compared to the pH of the DMEM media alone. This is due to the buildup of lactic acid produced from the by-products of cellular metabolism. An increase in pH was observed when cultured with the nHA-coated and non-coated Mg samples compared to the controls. This is due to the Mg reaction with water that produced OH⁻, which consequently increased pH. This Mg degradation that resulted in the increase of pH may have affected the cell attachment to

the PSTC plate surrounding the nHA-coated and non-coated Mg samples and, therefore, contributed to the observed cell results. However, a small difference in pH between the media cultured with the nHA-coated and non-coated samples was observed. This can justify the fact that cell studies showed similar cell response between both the nHA-coated and non-coated Mg samples. However, the cell studies indicated significantly improved BMSC adhesion on the surfaces of the nHA-coated and non-coated Mg samples, in comparison to the cells surrounding the Mg samples. This demonstrates that both the nHA-coated and non-coated Mg samples promoted more cell activity on the surface.

Consequently, experiments need to be repeated on a larger number of samples and with specific biomarkers to allow for more conclusive cell analyses.

3.5.3 The effect of HA coated implants on bone regeneration

In vivo studies of calcium-phosphate coated Mg alloy implants have exhibited increased osteoconductivity and osteogenesis compared to Mg alloys without calcium-phosphate coating.¹⁷ Additionally, when placed in a rat model, nHA-coated Ti implants were found to enhance new bone growth and bone bonding strength with the implant, indicating enhanced implant osteointegration⁵¹ Therefore, these *in vivo* studies have shown improved bone formation around HA coated implants, however the results obtained from this *in vitro* BMSC adhesion study are still inconclusive. It is impractical to draw conclusions at this stage because of the small sample size used for this study. Also, as noted before, the nHA-coating on the coated Mg samples were not entirely coated prior to studies; the corners of the Mg samples remained exposed; moreover,

although OCP has shown cytocompatibility,⁵² further studies need to be conducted on pure HA-coated Mg coatings. Additionally, improved cell results are expected with nHA-coating consisting of particle size in the range of natural bone. Further studies must be conducted on nHA coatings with smaller particle size. It is also important to note that although the nHA-coated and non-coated Mg samples showed different surface morphology and elemental compositions before and after the degradation studies in r-SBF, no statistically significant difference in the cell adhesion density was detected on the nHA-coated and non-coated Mg samples after 24 hrs of culture. Moreover, the degradation of nHA-coated and non-coated Mg samples in r-SBF was very similar in terms of weight change, pH, and Mg ion concentration at the 24 hr time point. All these factors may have contributed to the observed cell adhesion results. Further short term and long term studies must be conducted in order understand the full picture of how nHA-coated Mg implants influence cell interactions.

4. Conclusion

In comparison to the non-coated Mg samples, the nHA-coated Mg samples significantly decreased degradation rate indicating that the nHA-coating was protecting the Mg samples from rapid degradation. Therefore, nHA-coatings are promising for controlling the biodegradation of Mg-based orthopedic implants and devices. As the cell studies indicated, BMSC adhesion improved on the surface of the nHA-coated and non-coated Mg samples in comparison to the cells surrounding the Mg samples. These findings indicate that the nHA-coated and non-coated Mg samples promoted more cell activity on the surface. However, the cell experiments need to be repeated on a larger

number of samples with specific biomarkers in order to draw more meaningful conclusions. Further short term and long term cell studies, should also be conducted to achieve better cell adhesion analysis.

5. Future work

Bone implants undergo various stresses, *in vivo*, which can affect its overall degradation and bioactivity.⁵³ *In vivo*, dynamic physiological fluid flow also contributes to shear stress applied to the implants. Therefore, dynamic fluid flow should also be accounted for, due to its effect on degradation,⁵⁴ as well as its effect on the surrounding Mg concentration and pH, and therefore, cell response. Performing degradation and cell studies *in vivo* or in simulated *in vivo* conditions will give a more ideal demonstration of Mg behavior in physiological environment.

Further studies are needed to optimize nanostructured coatings to control Mg degradation, as mentioned previously. Further work will investigate the cytocompatibility and bioactivity such as proliferation and differentiation of the nHA-coated and non-coated Mg, *in vitro*. Mechanical tests are also needed to determine the nHA bond strength to the Mg, and the overall coating mechanical strength with the Mg.

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FIGURES

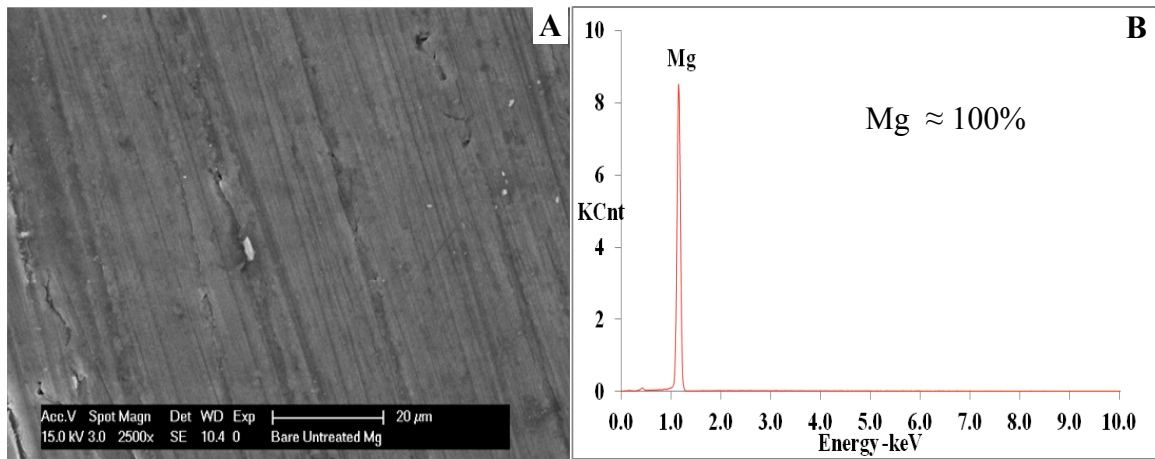


Figure 1: (A) SEM image and (B) EDS analysis of polished Mg sample before surface coating. Scale bar = 20 μm.

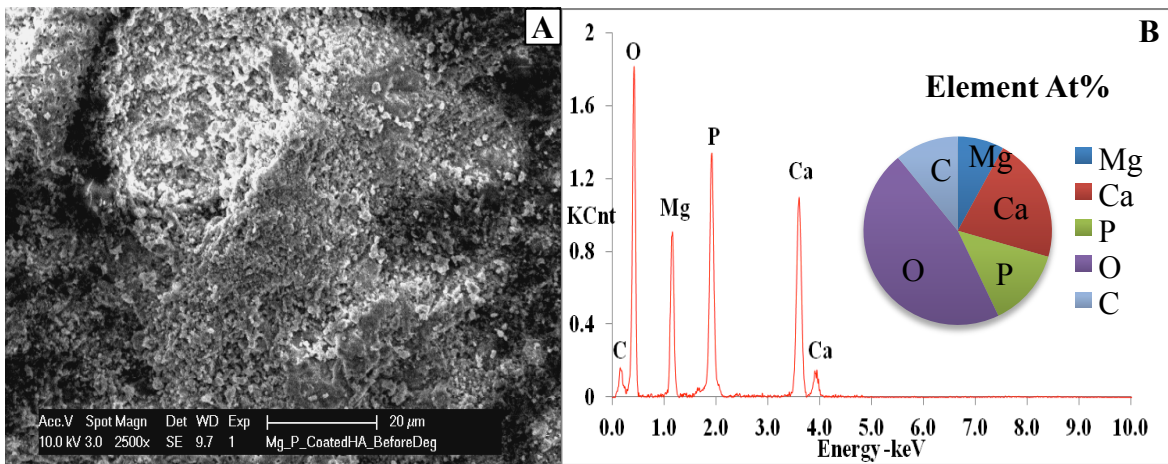
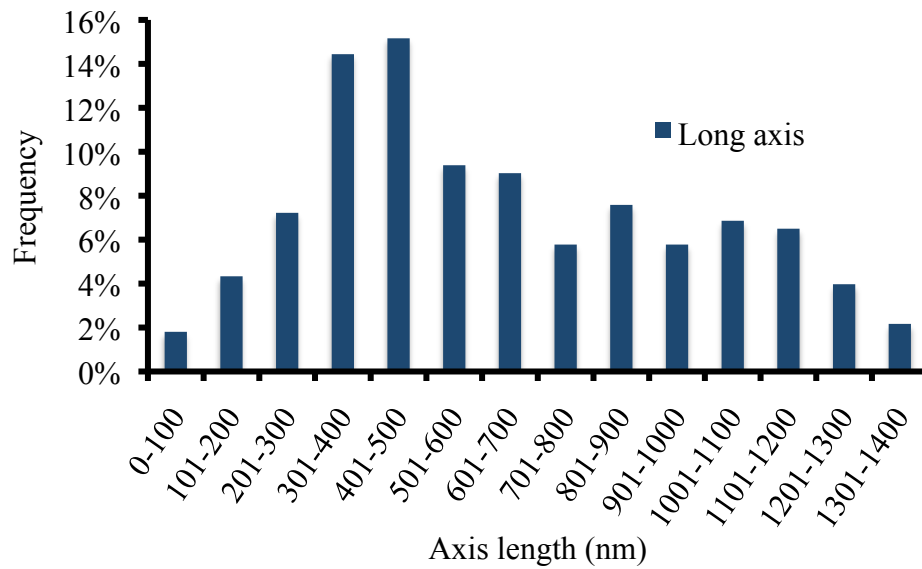
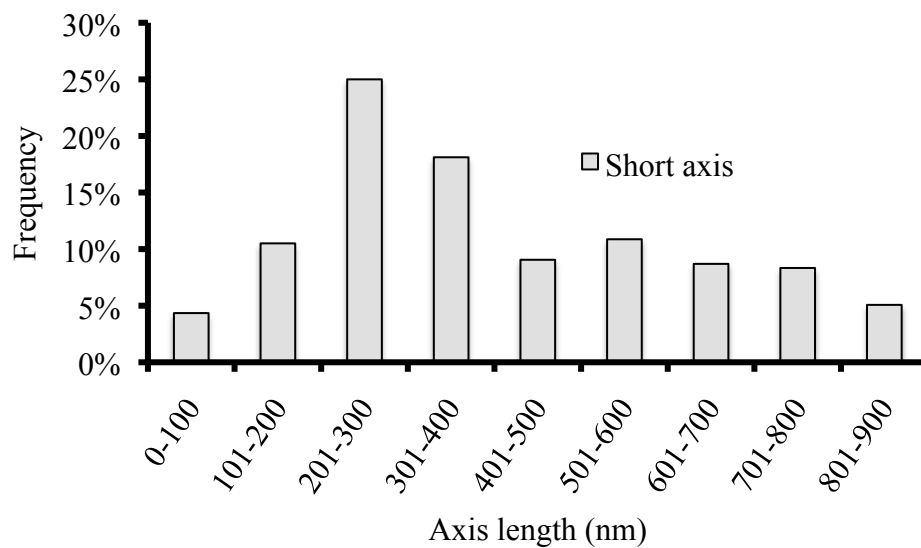


Figure 2: (A) SEM image and (B) EDS analysis of nHA-coated Mg sample. The Ca/P ratio is 1.60, close to the natural HA Ca/P ratio of 1.66. Scale bar = 20 μm.



(A) Frequency of nHA-coating particle size in long axis



(B) Frequency of nHA-coating particle size in short axis

Figure 3: Frequency of nHA particle size in long axis and short axis. (A) The long axis particle size ranged from 50-1400 nm, with the most frequent particle size between 300-500 nm. (B) The short axis particle size ranged from 35-900 nm with the most frequent short-axis particle size between 200-300 nm

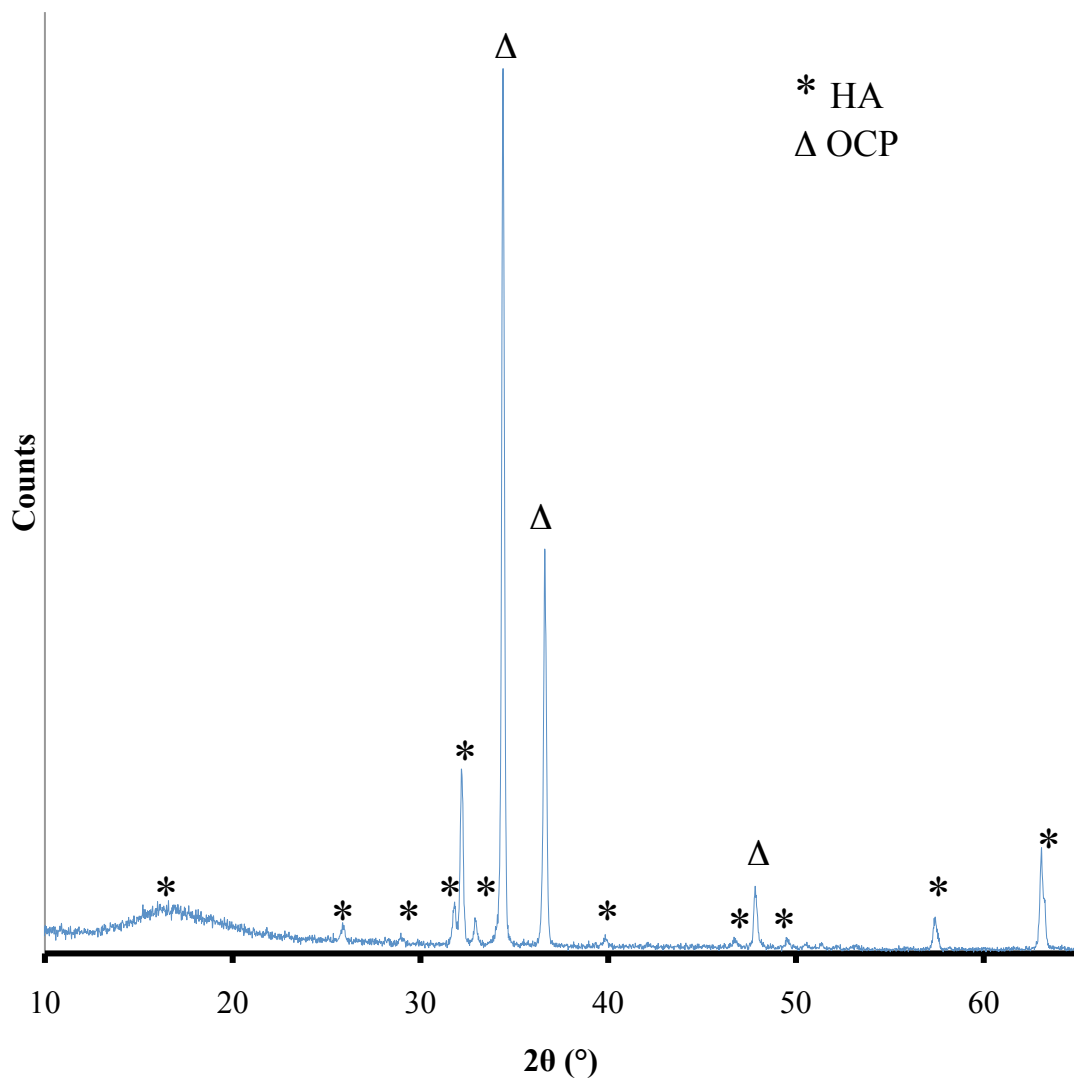


Figure 4: XRD analysis of nHA-coated Mg sample. The presence of HA, OCP and amorphous phases was detected.

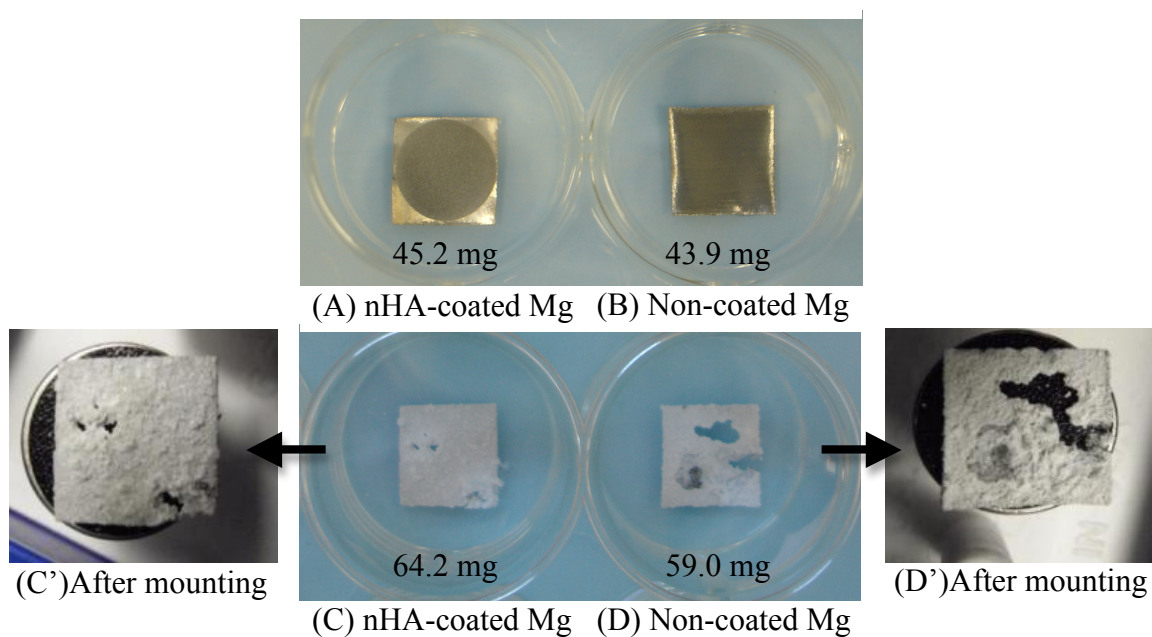


Figure 5: Photographs of (A) nHA-coated and (B) non-coated Mg samples as compared with (C) nHA-coated and (D) non-coated Mg samples after 28 days of degradation in PBS.

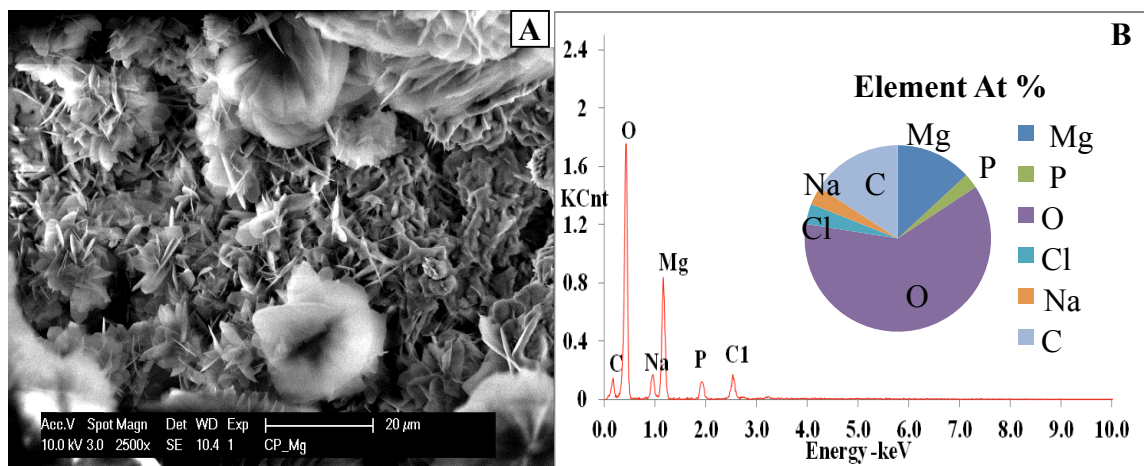


Figure 6: (A) SEM image and (B) EDS analysis of nHA-coated Mg sample after 28 days degradation in PBS. Scale bar = 20 μ m.

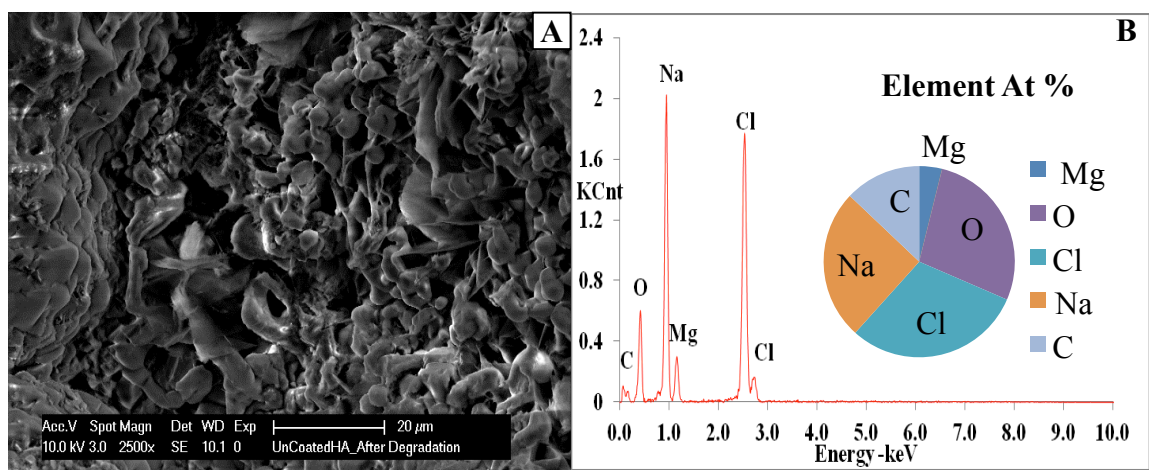


Figure 7: (A) SEM image and (B) EDS analysis of non-coated Mg sample after 28 days of degradation in PBS. Scale bar = 20 μm.

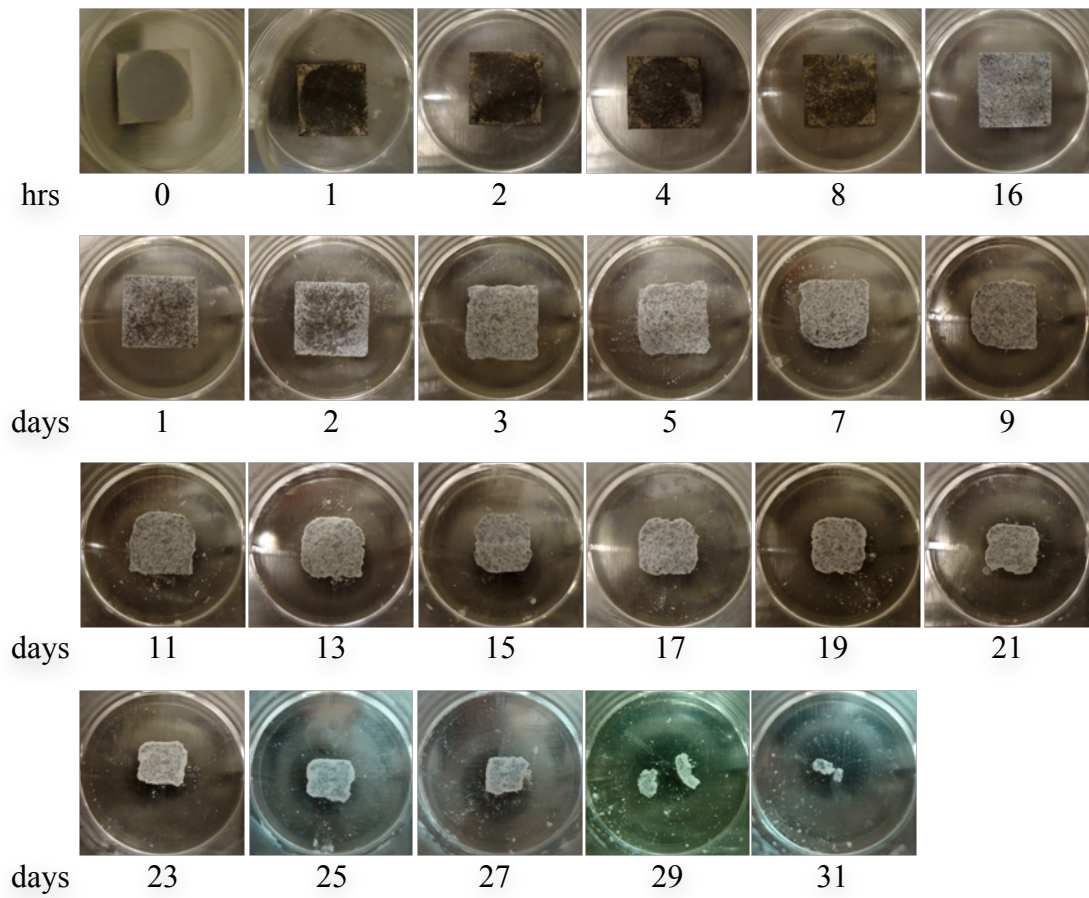


Figure 8: Photographs of degradation appearance of nHA-coated Mg sample in r-SBF after each prescribed immersion period.

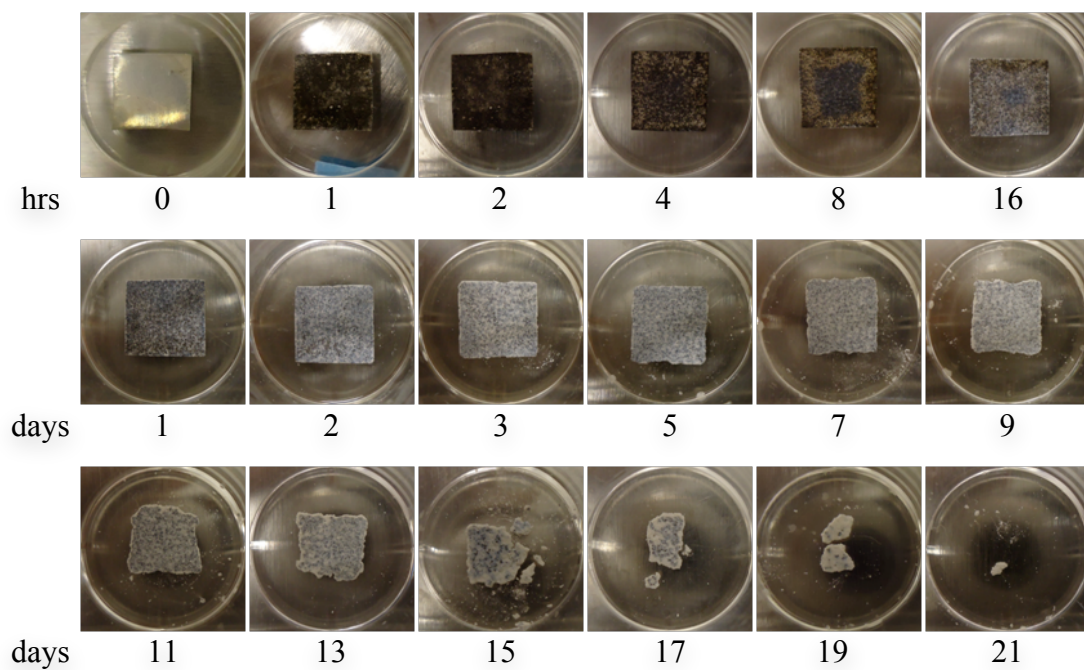


Figure 9: Photographs of degradation appearance of non-coated Mg sample in r-SBF after each prescribed immersion period.

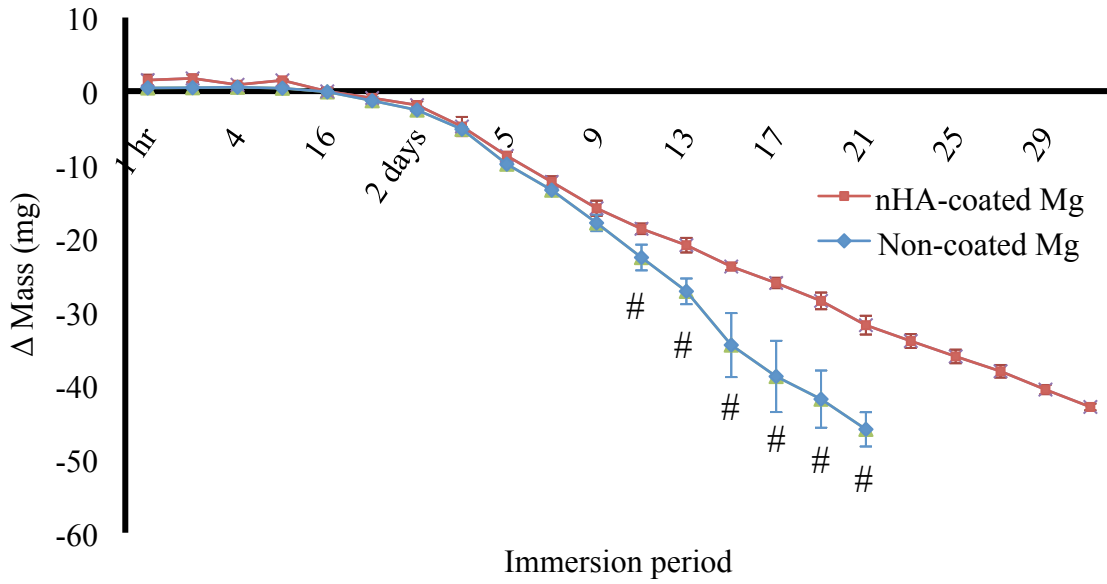


Figure 10: Mass change (ΔM , $\Delta M = M_{\text{final}} - M_{\text{initial}}$) of nHA-coated and non-coated Mg samples in r-SBF after each prescribed immersion period. Over time, the non-coated Mg samples showed more mass loss than the nHA-coated Mg samples, indicating a faster degradation. This difference in mass loss was statistically significant at the later stage of immersion in r-SBF, as indicated by $\# \alpha = 0.05$ (compared to nHA-coated Mg samples).

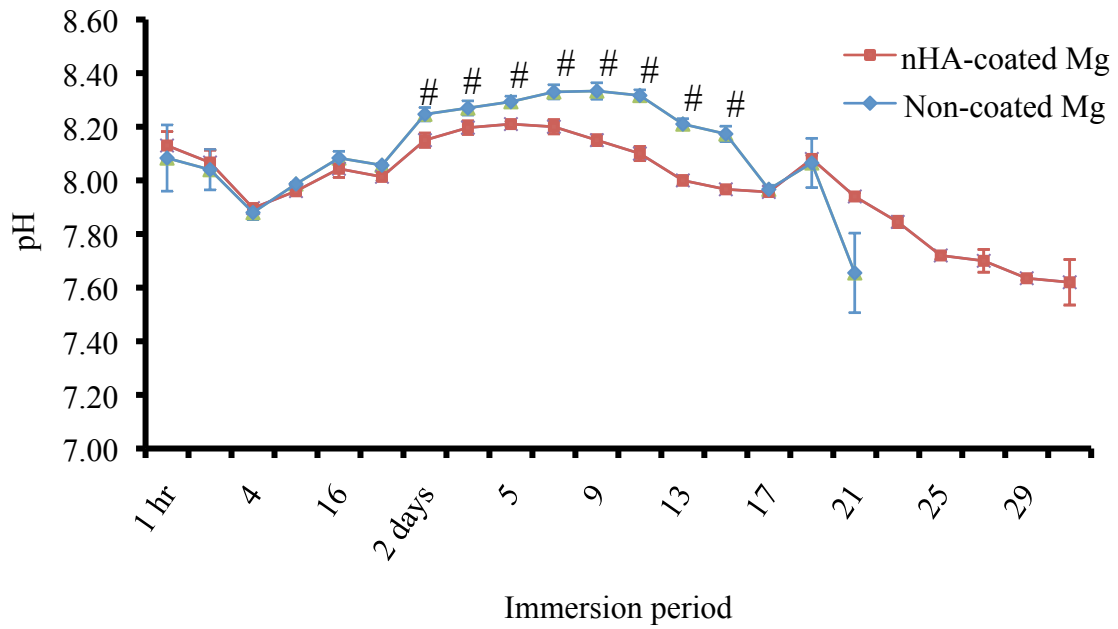


Figure 11: The pH of r-SBF after immersion of the nHA-coated and non-coated Mg samples for each prescribed period. The pH of the r-SBF media cultured with the non-coated Mg samples increased significantly more than that of the nHA coated Mg samples after 2 days of degradation. This difference in pH was statistically significant from 2 to 15 days of immersion in r-SBF, as indicated by $^{\#}\alpha = 0.05$ (compared to nHA-coated Mg samples). The final drop in the pH of the r-SBF media cultured with the non-coated Mg samples is due to the significant reduction of Mg sample mass.

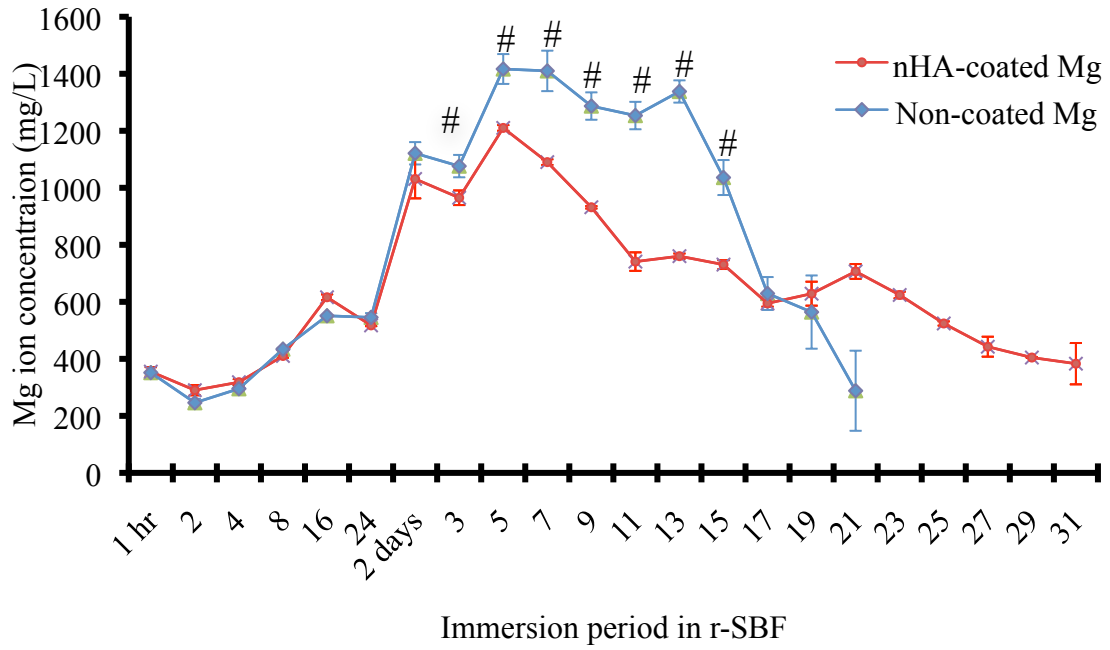


Figure 12: The concentration of Mg ions in r-SBF, dissolved from the nHA-coated and non-coated Mg samples after each prescribed immersion period. The concentration of Mg ions in r-SBF dissolved from the non-coated Mg samples increased significantly more than that of the nHA coated Mg samples after 3 days of degradation. This difference in Mg ion concentration was statistically significant from 3 to 15 days of immersion in r-SBF, as indicated by $^{\#}\alpha = 0.05$ (compared to nHA-coated Mg samples). The final decrease in the concentration of Mg ions dissolved from the non-coated samples is due to the significant reduction in Mg sample mass.

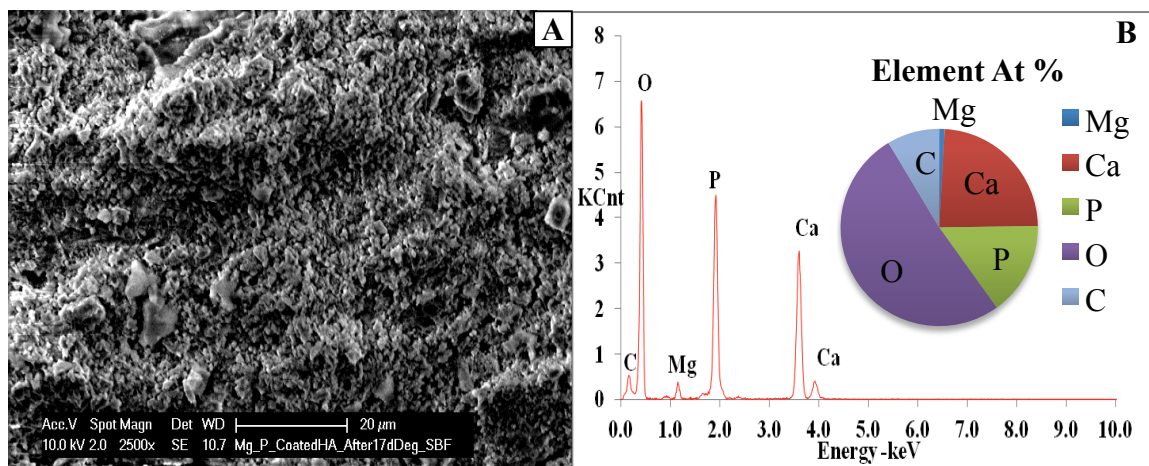


Figure 13: (A) SEM image and (B) EDS analysis of nHA-coated Mg sample after 17 days of degradation in r-SBF. Scale bar = 20 μm.

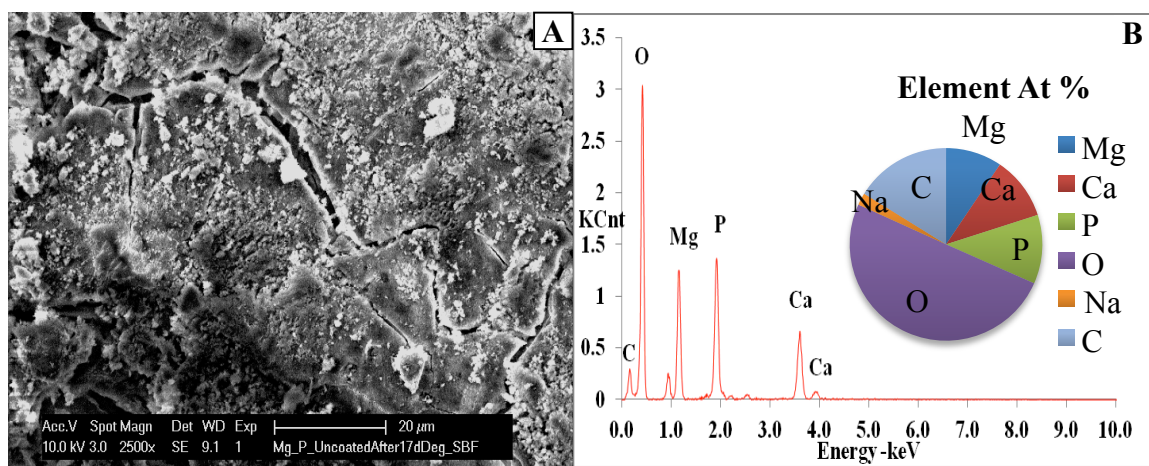


Figure 14: (A) SEM image and (B) EDS analysis of non-coated Mg sample after 17 days of degradation in r-SBF.

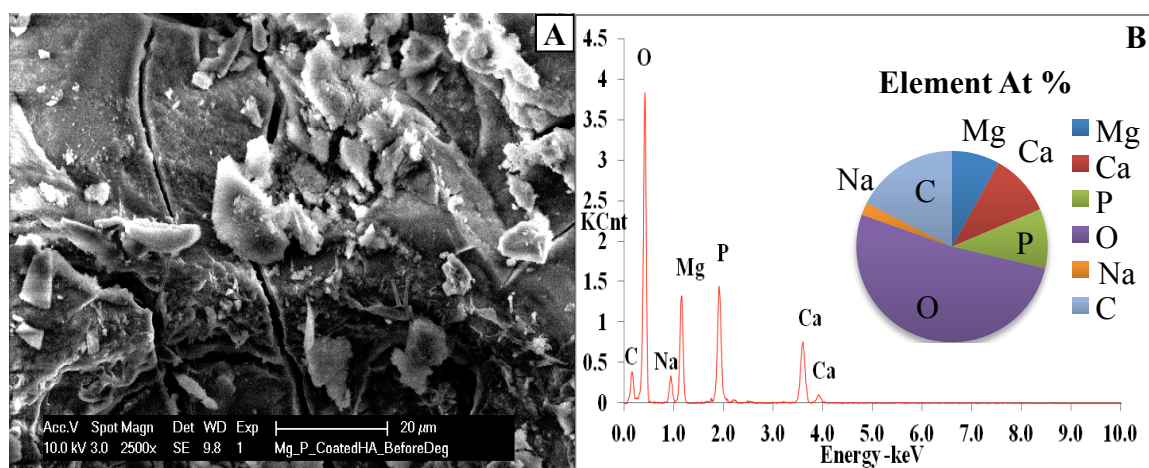


Figure 15: (A) SEM image and (B) EDS analysis of non-coated Mg sample after 21 days of degradation in r-SBF.

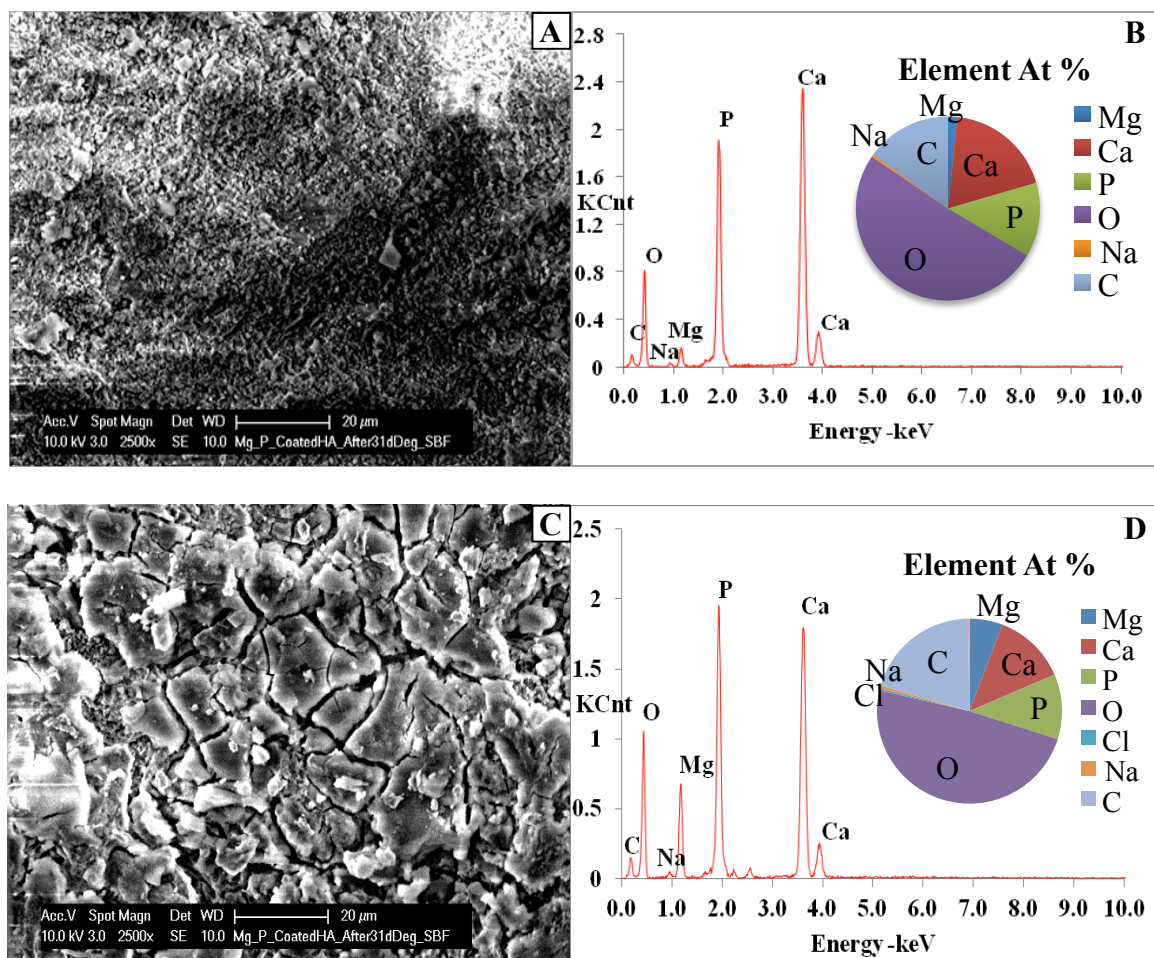


Figure 16: (A, C) SEM images and (B, D) EDS analyses of nHA-coated Mg sample after 31 days of degradation in r-SBF. (A) and (C) images were taken from the center and edge of the same nHA-coated Mg sample, respectively. (B) and (D) EDS analyses were based on the images of (A) and (C), respectively. Scale bar = 20 μm.

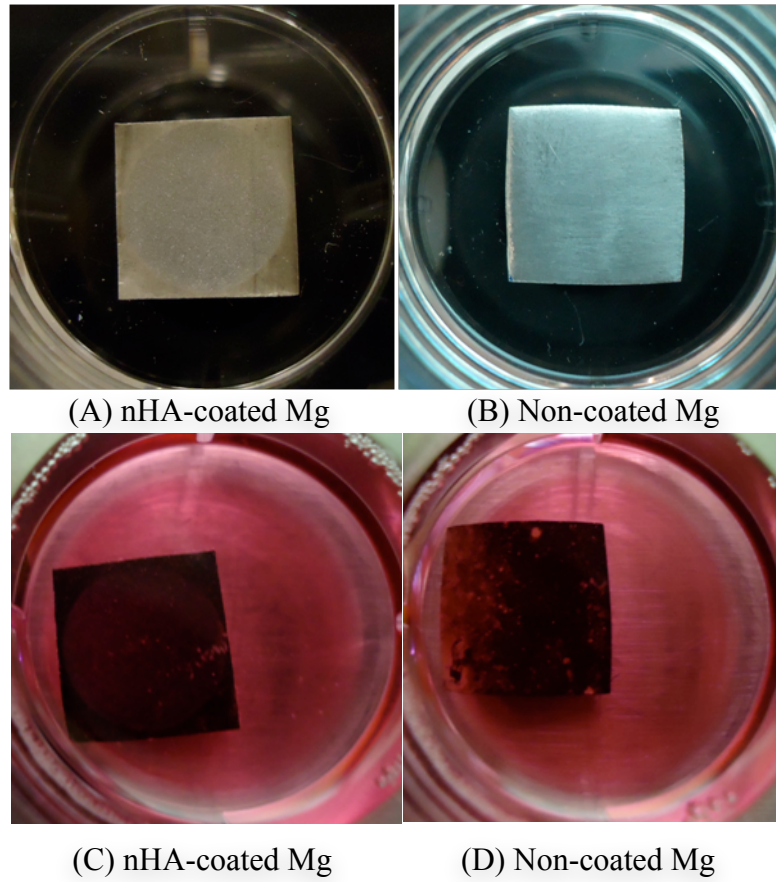


Figure 17: Photographs of the (A) nHA-coated and (B) non-coated Mg samples as compared with (C) nHA-coated and (D) non-coated Mg samples after 24 hr BMSC culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (P/S).

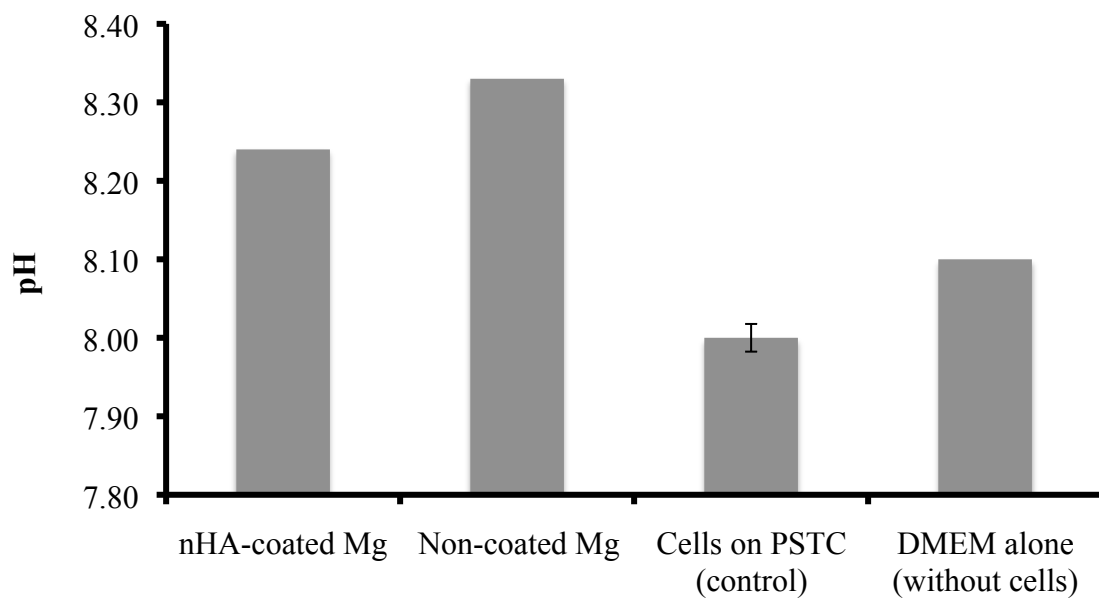
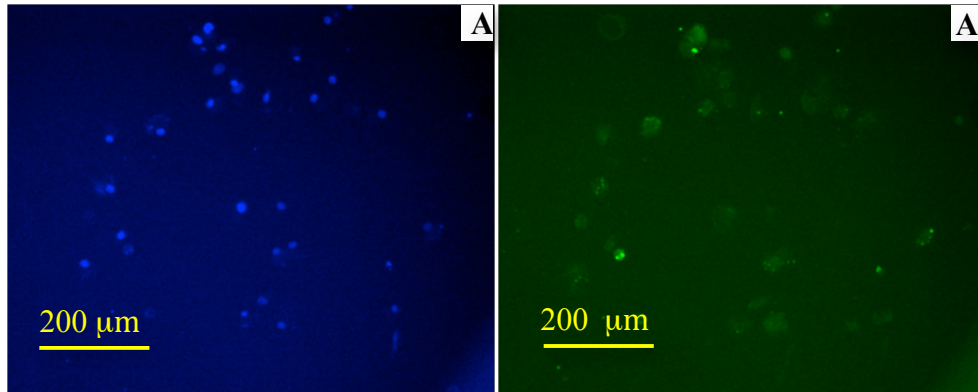
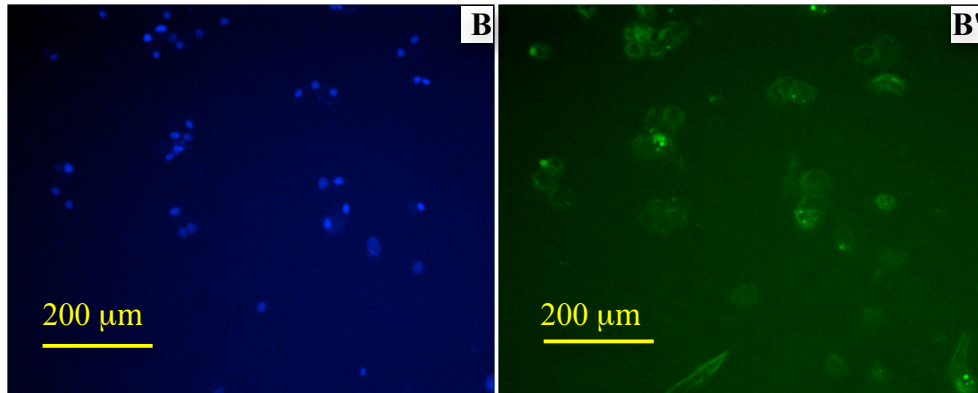


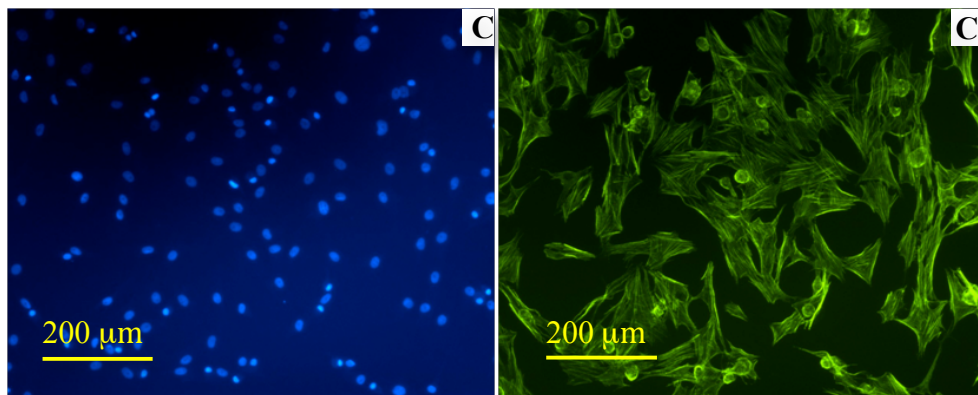
Figure 18: The pH of the cell culture media after 24 hr incubation with the nHA-coated and non-coated Mg samples in comparison to the controls.



Cells surrounding nHA-coated Mg

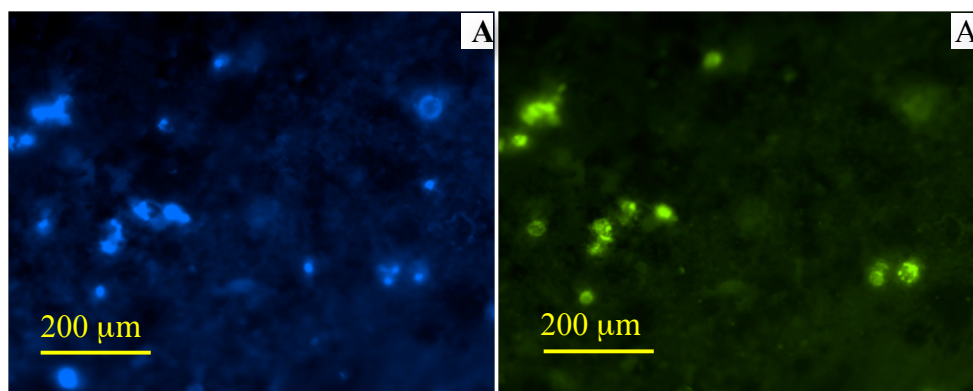


Cells surrounding non-coated Mg

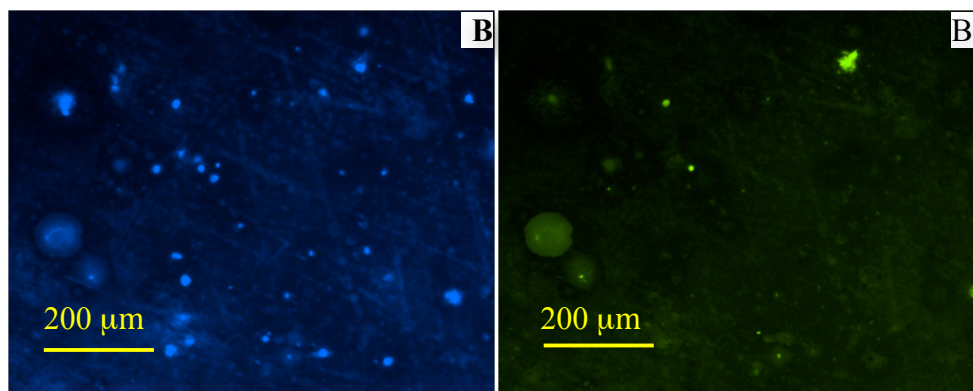


Cells on PSTC (control)

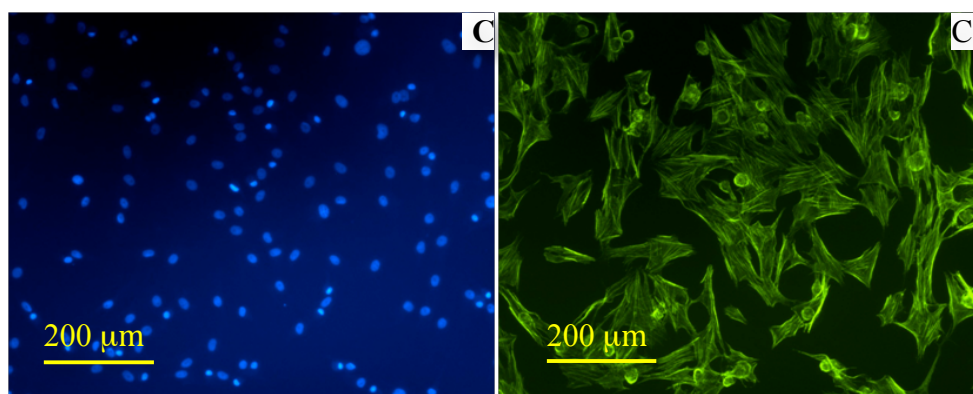
Figure 19: Fluorescent images of BMSC adhesion surrounding (A, A') nHA-coated and (B, B') non-coated Mg samples and (C, C') on the PSTC plate (control), after 24-hr culture. (A-C) DAPI nuclei and (A'-C') Alexa Fluor® 488 cytoskeleton stained BMSCs surrounding nHA-coated and non-coated Mg samples, and on PSTC plate. Scale bar = 200 μm .



Cells on nHA-coated Mg



Cells on non-coated Mg



Cells on PSTC (control)

Figure 20: Fluorescent images of BMSC adhesion on (A, A') nHA-coated and (B, B') non-coated Mg samples and (C, C') on PSTC plate (control), after 24 hr culture. (A-C) DAPI nuclei and (A'-C') Alexa Fluor® 488 cytoskeleton stained BMSCs on nHA-coated and non-coated Mg samples, and on PSTC plate. Scale bar = 200 μm .

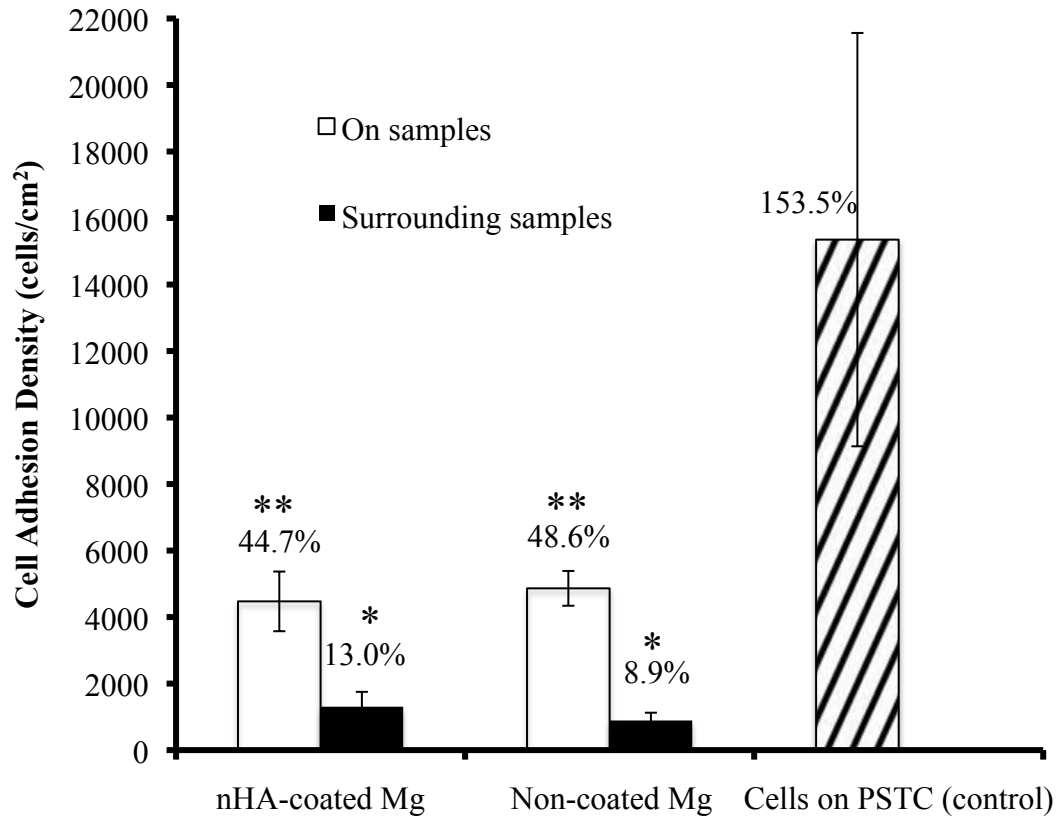


Figure 21: BMSC adhesion on and surrounding the nHA-coated and non-coated Mg samples and control, after 24 hr culture. The numbers labeled on top of each bar graph indicate the percentage of BMSCs that adhered on and surrounding the samples and control. * $p < 0.05$ (compared to control). ** $p < 0.05$ (compared to cells surrounding nHA-coated and non-coated Mg, respectively).

TABLES

Table 1: The concentration of ions in PBS.

Ion	PBS [mM]
Na ⁺	139.6
K ⁺	4.4
Cl ⁻	146.4
PO ₄ ³⁻	6.5

Table 2: The concentration of ions in r-SBF in comparison to human blood plasma.

Ion	r-SBF [mM]	Blood Plasma [mM]
Na ⁺	142.0	142.0
K ⁺	5.0	5.0
Mg ²⁺	1.5	1.5
Ca ²⁺	2.5	2.5
Cl ⁻	103	103
HCO ₃ ⁻	27	27
HPO ₄ ²⁻	1.0	1.0
SO ₄ ²⁻	0.5	0.5

Table 3: The surface elemental compositions of nHA-coated and non-coated Mg samples in atomic percent (atomic %) before and after degradation in PBS and r-SBF.

<i>Element</i>	<i>Before degradation</i>		<i>Degradation in PBS</i>		<i>Degradation in r-SBF</i>				
			<i>After 28 days</i>		<i>After 17 days</i>		<i>After 21 days</i>	<i>After 31 days</i>	
	<i>nHA Coated Mg (Fig. 2B)</i>	<i>Non-coated Mg (Fig. 1B)</i>	<i>nHA Coated Mg (Fig. 6B)</i>	<i>Non-coated Mg (Fig. 7B)</i>	<i>nHA Coated Mg (Fig. 13B)</i>	<i>Non-coated Mg (Fig. 14B)</i>	<i>Non-coated Mg (Fig. 15B)</i>	<i>nHA Coated Mg (Fig. 16B)</i>	<i>nHA Coated Mg (Fig. 16D)</i>
Mg	8	100	13	4	1	9	8	2	6
Ca	22	-	-	-	24	11	11	19	13
P	14	-	3	-	15	12	10	13	11
O	47	trace	62	28	51	50	52	51	49
Cl	-	-	4	30	-	-	-	-	1
Na	-	-	3	25	-	2	2	1	1
C	11	-	16	13	9	16	17	15	20
Ca/P	1.6	-	no Ca	no Ca	1.56	0.92	1.05	1.44	1.12