Multifunctional Coatings for Biodegradable Biomedical Devices

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Multifunctional Coatings for Biodegradable Biomedical Devices

By

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A thesis presented to
Technological University Dublin for the award of Ph.D

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December 2018
Dedicated to my mother
Abstract
Magnesium (Mg) and its alloys have been widely explored for orthopaedic applications due to its biodegradable nature, biocompatibility and closely related mechanical properties with bone. However, rapid degradation of Mg alloys under physiological conditions is a major challenge for biomedical industries and clinicians. Amongst the various methods employed, deposited coatings have been widely favoured to enhance the corrosion resistance and compatibility of the Mg alloys.

This study focused on developing multifunctional coatings on AZ31 Mg alloy having a corrosion resistant, osteoinductive and antibacterial properties for orthopaedic applications. The aim of this study was achieved through the following objectives:

- Fabrication of corrosion resistant and biocompatible PLGA-silane coatings on AZ31.
- Preparation of Biomimetic Hyaluronic acid functionalized silane coatings on AZ31.
- Fabrication of osteoinductive and antibacterial Hyaluronic acid-lysozyme composite coatings on AZ31.
- Effect of hyaluronic acid molecular weight and Mg2+ on osteoblast functions in vitro.

Overall, this body of work presented:

- Successful assembly of multilayer PLGA coating associated with the amine-terminated MTES-TEOS-APTES silane on the AZ31 Mg alloy.
- PLGA-silane coating improved the corrosion resistance of AZ31 Mg alloy in DMEM by ~2 of magnitude (impedance magnitude and corrosion current density) as confirmed by the electrochemical corrosion studies. Similarly, immersion studies showed ~5-fold increase in the corrosion resistance of PLGA-silane coated AZ31 Mg alloy over uncoated AZ31 equivalents.
- In comparison to the uncoated AZ31 substrate, osteoblasts showed ~2 to 4-fold increase in the proliferation and differentiation of osteoblast cells cultured on the PLGA-silane coated AZ31 substrates for 14 days.
- To further evaluate the corrosion resistant properties of silane coated-AZ31 Mg alloy in the high corrosive electrolyte, the electrochemical corrosion and immersion studies were conducted in HEPES buffered DMEM. The EIS analysis showed an increase in the magnitude of impedance by ~ 2 order of magnitude for MTES-TEOS-APTES modified AZ31 substrate when compared to the uncoated AZ31 equivalents.
To improve the cytocompatibility of AZ31 Mg alloy by providing a biomimetic environment, high molecular weight hyaluronic acid (HA) was coupled to the amine-terminated MTES-TEOS-APTES modified AZ31 substrate. The high molecular weight HA enhanced the proliferation and differentiation of osteoblasts cells by ~ 4 to 6-fold as compared to the uncoated AZ31 Mg alloy.

Furthermore, the effect of high and low molecular weight of HA and Mg$^{2+}$ on osteoblast functions was evaluated. It was observed that the high molecular weight HA and Mg$^{2+}$ synergistically improve the proliferation and differentiation of osteoblast cells by stimulating intracellular calcium ions.

The concerns of bacterial colonisation on Mg-based biodegradable alloy without compromising osteoblast functions was addressed by modifying the amine terminated silane coating with HA-lysozyme (HA-LZ) composite. LZ component of composite significantly reduced the colonisation of *S.aureus* in a concentration-dependent manner by ~ 30 fold when compared to the uncoated AZ31 equivalents. Whereas, HA component of the composite maintained the osteoblasts cellular response in terms of adhesion, proliferation and differentiation.

Hence, the developed multifunctional coatings improved the corrosion resistance, osteoinductive and anti-bacterial properties of the AZ31 Mg alloy.

All work in this thesis has been published.
Declaration

I hereby declare that this thesis which I now submit for examination for the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate studies by the research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any Institute or University.

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Signature

______________________

(Sankalp Agarwal)

11th December 2018
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SANKALP AGARWAL
List of abbreviations

- **AZ31**: Aluminium-Zinc 31
- **MTES (M)**: Methyltriethoxyl silane
- **TEOS (T)**: Tetraethylorthosilicate
- **APTES (A)**: Aminopropyltriethoxy silane
- **PLGA (P)**: Poly(lactide-co-glycolide)
- **DMEM**: Dulbecco’s Modified Eagle’s Medium
- **FBS**: Fetal bovine serum
- **MTT**: 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan
- **DMSO**: Dimethyl sulfoxide
- **EDC**: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
- **NHS**: N-Hydroxysuccinimide
- **MES**: 2-(N-morpholino)ethanesulfonic acid
- **HEPES**: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
- **SEM**: Scanning electron microscopy
- **AFM**: Atomic force microscopy
- **EIS**: Electrochemical impedance spectroscopy
- **ATR-IR**: Attenuation total reflectance-infrared
- **AZ31-MT**: AZ31-Methyltriethoxyl silane-Tetraethylorthosilicate
- **AZ31-MT-A**: AZ31-Methyltriethoxyl silane-Tetraethylorthosilicate-APTES
- **AZ31-MT-A-P**: AZ31-Methyltriethoxyl silane-Tetraethylorthosilicate-APTES-PLGA
- **HA**: Hyaluronic acid:
- **AZ31-MT-A-HA**: AZ31-Methyltriethoxyl silane-Tetraethylorthosilicate-hyaluronic acid
- **ALP**: Alkaline phosphatase
- **LZ**: Lysozyme
- **AZ31-HA-LZ**: AZ31-silane-hyaluronic acid-lysozyme
- **AZ31-LZ**: AZ31-silane-lysozyme
- **AZ31-HA**: AZ31-silane-hyaluronic acid
- **S.aureus**: Staphylococcus aureus
- **l-HA**: low molecular weight hyaluronic acid
- **h-HA**: high molecular weight hyaluronic acid
- 1-HA-AZ31: low molecular weight hyaluronic acid-silane-AZ31
- h-HA-AZ31: high molecular weight hyaluronic acid-silane-AZ31
- l-HA-Ti: low molecular weight hyaluronic acid-silane-Ti
- h-HA-Ti: high molecular weight hyaluronic acid-silane-Ti
- l-/h-HA-AZ31: low and high molecular weight hyaluronic acid-silane-AZ31
- l-/h-HA-Ti: low and high molecular weight hyaluronic acid-silane-Ti
- $[Ca^{2+}]_i$: Intracellular calcium ion concentration
- $[Mg^{2+}]_i$: Intracellular magnesium ion concentration
- $[Mg^{2+}]_e$: Extracellular magnesium ion concentration
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Overview of the thesis

Problem statement

Mg and its alloys have several advantages over conventional orthopaedic implant materials such as stainless steel, titanium and its alloys, ceramics and biodegradable polymer and cobalt-chrome alloys. These advantages include their biocompatibility, similar mechanical properties (Young modulus) to that of natural bone and osteogenic potential of Mg$^{2+}$ ions. However, the major concerns preventing their clinical applications are rapid degradation under physiological conditions, thereby producing an elevated level of hydrogen gas and drastic pH increase in the surrounding tissues. This develops a bio-incompatible environment and hinders the bone regeneration process. Another major problem is the implant associated microbial growth, which causes orthopaedic implant failures.

In order to reduce the rapid degradation of Mg and its alloys, several strategies such as alloying elements and passivation treatments (plasma electrolytic oxidation, anodization and alkaline hydrothermal treatment) have been explored. Alloying elements may improve the corrosion resistance and mechanical properties, but they may cause toxicity to the cells. The passivation treatments are unable to improve the corrosion over a suitable time period and also affect the mechanical properties of Mg alloys. Therefore, surface coatings have been considered as an effective approach to enhance the corrosion resistance as well as biocompatibility of Mg and its alloys.

Aims of the study

The aim of this study is to design multifunctional coatings which not only reduce the corrosion resistance but also show osteogenic and antibacterial properties which enhance the bone regeneration and prevent the implant associated microbial infections.

a) The rapid degradation of Mg alloy is a major challenge, especially during the early period of implantation period (2-3 weeks) which creates toxicity towards the surrounding tissue. Interestingly, Mg$^{2+}$ stimulates the functions of osteoblast cells at an optimum concentration ranging from 5 mM to 15 mM. Therefore, the target of the study is to optimally control the degradation of AZ31 Mg alloy using sol-gel silane coating during early immersion period. It is desired to achieve the stability of the coating for a minimum period of 21 days so that rapid degradation
of the AZ31 Mg alloy could be controlled, thereby providing optimum for the growth of osteoblast cells.

b) The cytocompatibility of the coating plays an important role in osteointegration of the implant material including Mg and its alloys. Therefore, this problem is addressed by functionalizing the corrosion resistant sol-gel coating with biocompatible synthetic aliphatic polyester (e.g. PLGA) or biopolymers (e.g. hyaluronic acid) to improve the adhesion, proliferation and differentiation of osteoblast cells. The aim of functionalising these bioactive polymers is to enhance the response of osteoblast cells during the rapid degradation phase of the AZ31 Mg alloy.

c) The susceptibility of medical implant surfaces to the risk of microbial growth is one of the major causes of orthopaedic implant failure. Therefore, the aim of this study is to develop a bioactive composite coating using biopolymers such as hyaluronic acid and lysozyme, which not only prevents the early colonisation of bacterial cell adhesion on the surface but also improve the osteoblastic activity.

d) Hyaluronic acid has been used to functionalise the implant surface to improve the osteoblastic activity and osteointegration. However, the role of the molecular weight of hyaluronic acid functionalised on the surface and extracellular Mg$^{2+}$ concentration in regulating the osteoblast cell response has not yet been studied. Therefore, the effect of hyaluronic acid of different molecular weight functionalised on sol-gel silane coated AZ31 Mg and Ti alloy on osteoblast functions was evaluated.
Outline of the thesis

a) The first chapter is a literature review, which covers various aspects of corrosion behaviour of Mg and its alloys under physiological conditions and strategies to improve their corrosion resistance and biocompatibility for orthopaedic applications. This chapter has been published: This chapter has been published: S Agarwal, J Curtin, B Duffy, S Jaiswal, Materials Science and Engineering: C, 68, 1, 2016, 948–963.

b) The second chapter describes analytical techniques such as fluorescence spectroscopy, Fourier transforms infrared spectroscopy (FTIR), Inductively coupled plasma optical emission spectroscopy (ICP-OES), Scanning electron microscopy (SEM), Fluorescence microscopy, Atomic force microscopy and Potentiodynamic polarisation, electrical impedance spectroscopy. This chapter also explains the different biochemical assays to assess the cytocompatibility and antimicrobial activity.

c) The third chapter presents the fabrication of multilayer PLGA coating associated with amine terminated MTES-TEOS- APTES organosilane on AZ31 Mg alloys. This chapter includes the systematic investigation of in vitro corrosion protection and biocompatibility of PLGA-silane coating on the AZ31 Mg alloy. In this chapter, PLGA-silane coating improved the corrosion resistance of the AZ31 Mg alloy in DMEM which was confirmed by the electrochemical corrosion and long-term immersion studies. Furthermore, MC3T3E1 osteoblast cells showed improved adhesion, proliferation and differentiation on the PLGA-silane coated AZ31 Mg alloy substrate as compared to the uncoated AZ31 equivalents. This chapter has been published: Agarwal. S., Morshed, M., Labour, M.N., Hoey, D., Duffy, B., Curtin, J. and Jaiswal. S, RSC Advances, 2016 6(115), 113871-113883.

d) The fourth chapter describes the preparation of hyaluronic acid functionalized silane multifunctional coatings on AZ31 Mg alloy. Distinct functions of the coatings have been presented, where silane coating enhances the corrosion resistance and hyaluronic acid improves the cytocompatibility of osteoblast cells. In this chapter, an electrochemical corrosion study showed that sol-gel silane coatings maintained the corrosion protection of AZ31 Mg alloy in a corrosive medium (HEPES-buffered DMEM). The functionalisation of hyaluronic acid
significantly improved the functions of osteoblast cells when compared to the uncoated AZ31 as well as PLGA-silane coated surface. This improved bioactivity on the HA-silane coated substrate confirmed two aspects of the coatings (a) an efficient corrosion protective function of sol-silane coating in HEPES-buffered DMEM and (b) hyaluronic acid provided a biomimetic environment to osteoblast cells.


e) The fifth chapter presents the fabrication of hyaluronic acid-lysozyme composite coating on silane coated AZ31 Mg alloy. The combined antibacterial and osteoinductive activity of hyaluronic acid-lysozyme composite were determined. In this chapter, the lysozyme component of the hyaluronic acid-lysozyme coating showed a concentration-dependent significant reduction of *S.aureus* adhesion on the surface when compared to the respective controls and uncoated AZ31 Mg alloy. Furthermore, composite coated surface significantly enhanced the osteoblast cell adhesion, proliferation and differentiation when compared to the respective control and uncoated AZ31 Mg alloy. Notably, osteoblast functions observed on hyaluronic acid was comparable to that on the hyaluronic acid-lysozyme composite coated surface. These results indicated that lysozyme-composite coating reduced the bacterial cell colonisation without affecting the hyaluronic acid induced osteoinductive activity.

This chapter has been published: Agarwal, S., Riffault, M., Hoey, D., Duffy, B., Curtin, J. and Jaiswal, S. *ACS Biomaterials Science & Engineering*, 2017, 3(12), 3244-3253.

f) The sixth chapter describes the hyaluronic acid molecular weight and Mg$^{2+}$ dependent growth of osteoblasts. In this study, the osteoblastic activity of low and high molecular weight of HA functionalised on silane coated AZ31 Mg (extracellular Mg$^{2+}$ dependent) and Ti alloy (control) was determined. The osteoblast cellular response such as adhesion, proliferation, differentiation and intracellular Mg$^{2+}$ and Ca$^{2+}$ were evaluated. The findings of this study suggested that low molecular weight hyaluronic acid enhanced the proliferation of osteoblast cells when compared to the high-molecular weight hyaluronic acid. On the other
hand, high molecular weight hyaluronic acid and Mg$^{2+}$ synergistically enhanced the differentiation of osteoblast cells when compared to the low-molecular weight hyaluronic acid which is attributed to the upregulated intracellular Ca$^{2+}$ concentration. Therefore, there are sufficient benefits of the use of HA with appropriate molecular weight to modify the surface of biomedical implants for orthopaedic applications. **The work described in this chapter has been published:** Agarwal, S., Duffy, B., Curtin, J. and Jaiswal, S. *ACS Biomater. Sci. Eng.*, 2018, 4 (11), 3874–3884

g) The seventh chapter concludes the present work and overviews future work.

Based on the present study, our research group secured national funding through Science Foundation Ireland and the Department of Business, Enterprise and Innovation
In this chapter, some general and specific aspects of Mg alloys will be introduced and the effect of alloying elements on corrosion behaviour and biocompatibility properties will be summarised. Furthermore, the influence of polymeric deposit coatings, namely sol-gels, synthetic aliphatic polyesters and natural polymers on these properties of Mg and its alloy for orthopaedic applications are presented. Finally, the osteoinductive and antimicrobial activities of Mg-based biomaterials for orthopaedic applications will be presented.

This chapter has been published: S Agarwal, J Curtin, B Duffy, S Jaiswal, Materials Science and Engineering: C, 68, 1, 2016, 948–963.
1.1 Introduction

Metallic orthopaedic implants are used for the replacement and/or regeneration of damaged hard tissues [1]. Metallic implants are preferred for their high mechanical strength and toughness which make them superior to the polymer and polymer-ceramic composites [2]. Orthopaedic metallic implants can be broadly classified into permanent and biodegradable implants [2]. Metals such as stainless steel, titanium and cobalt-chromium alloys have been employed as permanent implants [3]. However, there may be some problems associated with using such permanent metallic implants [4,5]. The first involves the incompatibility of the mechanical properties of metallic alloys and natural bone such as metal alloys having greater elastic modulus to that of bone [6]. Under in vivo conditions, the mechanical mismatch between bone and implants leads to a clinical phenomenon called stress shielding [7]. In stress shielding, the implant carries much of the bulk load and the surrounding bone tissue experiences reduced load stress. This triggers the resorption of surrounding bone tissue [7]. The second problem associated with permanent implants is mechanical wear and corrosion with long-term implantation in the body. This results in the release of toxic metal ions (chromium, nickel, cobalt etc.) in the body which can trigger undesirable immune responses, thereby reducing the biocompatibility of metallic implants [8]. These drawbacks have encouraged researchers and clinicians to look at biodegradable implants, which once used, only remain for an appropriate time to fix the damage.

Biodegradable metals have several advantages when used in orthopaedic temporary implants (screws, pins etc.) [6]. The mechanical properties of Mg and its alloys such as Young’s modulus of elasticity (E= 41-45 GPa) and density (1.74-1.84 g/cm³) are known to be similar that of bone (E= 15-25 GPa and density= 1.8-2.1 g/cm³). This is lower than other biodegradable materials such as Iron-Manganese (Fe-Mn) and Zinc (Zn) based
alloys [2]. Furthermore, the Mg ion is a common metabolite of the body with a daily consumption range of 250-300 mg/day and are naturally stored in bones [9]. Therefore, amongst biodegradable metals, the biocompatibility (low toxicity to the bone-related cells and maintain bone functions) and resemblance of mechanical properties of Mg and its alloy with bone make it suitable for orthopaedic applications.

Ceramics which are inorganic non-metallic materials that have been employed in hard tissue engineering applications are collectively known as Bioceramics [10]. Bioceramics possess desirable properties for biomedical applications such as (i) thermo-chemically stability, (ii) good wear resistance and (iii) are easily mouldable. Additionally, they are biocompatible, non-toxic and non-immunogenic [11,12]. Ceramics have been used commercially in various applications like coatings on implants, maxillofacial reconstruction and drug delivery devices [12,13]. However, bioceramics like hydroxyapatite (HAP) are brittle and possess low tensile strength when compared to Mg-based alloys [12].

Polymeric materials have been employed for tissue engineering applications due to their ductility, biocompatibility and biodegradable properties. Polymers are composed of small repeating monomers which give the polymer its characteristic properties. The degree of cross-linking of monomers determines the physiochemical nature of polymers [14]. In general, polymeric materials are broadly classified into synthetic and natural polymers.

Synthetic polymers such as aliphatic polyester (polylactic acid, polyglycolic acid, poly co-(lactic-co-glycolic acid)) can be synthesized in controlled conditions to regulate properties such as molecular weight and derivatization. These advantages of synthetic aliphatic polyesters enable their use in biomedical applications. Natural polymers such as collagen and protein-based gels, hyaluronic based derivatives, polysaccharide chitosan and heparin-based scaffolds have been successfully used in various tissue engineering
applications [15]. Natural polymers provide biomimetic environment and may encourage expeditious tissue healing by directing cell adhesion and function. Both classes of polymers can be chemically modified to produce tuneable scaffold and biomedical implants with controlled degradation rates [16]. Moreover, several reports showed that the by-products of biodegradable polymers are highly biocompatible [17]. These polymers can be engineered into various shapes and sizes, such as disks, rods, pellets, plates, films and fibres as required. Some applications include biodegradable sutures, bone grafting materials, pins, screws and load-bearing orthopaedic devices [18]. Despite possessing many desirable properties, polymers have low mechanical strength when compared to bioceramics and metal implants, thereby hindering their applications in hard tissue engineering. Typically, yield strength at break should be higher than the yield stress, which can be observed for metals and bioceramics. However, Perego et al showed a deviation from this convention for PLA [34]. Therefore polymers have been largely employed in soft tissue engineering and low-load bearing medical devices [18]. Comparatively, Mg and its alloys have advantages over polymers due to mechanical strength closer to bone.

From the above discussion, it can be observed that Mg-based alloys have mechanical properties (density, yield strength, tensile strength, elongation to break and elastic modulus) similar to that of natural bone as compared to other biodegradable alloys, permanent implants, ceramics and polymers as shown in Table 1. Despite many advantages, the major limitation of Mg-based alloys as biomedical materials is their high corrosion rate [19]. Corrosion results in the formation of H₂ gas; which if rapidly absorbed can lead to a balloon effect (gas pocket formation surrounding the implant) in vivo [20]. Additionally, a shift in alkaline pH in the region surrounding the corroding surface is also a concern for biomedical applications [20].
Table 1. Mechanical properties of Mg and its alloys

<table>
<thead>
<tr>
<th>Tissue/Surgical Implants</th>
<th>Density (g/cm$^3$)</th>
<th>Yield strength (MPa)</th>
<th>Tensile strength (MPa)</th>
<th>Elongation to break (%)</th>
<th>Elastic Modulus (GPa)</th>
<th>Ref</th>
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<tbody>
<tr>
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<td>1.8-2.1</td>
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<td>110-130</td>
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<td>Mg</td>
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<tr>
<td>Pure Mg</td>
<td>1.74-2.0</td>
<td>65-100</td>
<td>90-190</td>
<td>2-10</td>
<td>41-45</td>
<td>[1]</td>
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<td>5 – 11.5</td>
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<td></td>
</tr>
<tr>
<td>PLA-59Kda</td>
<td>1.64</td>
<td>68</td>
<td>58</td>
<td>5.0</td>
<td>3750</td>
<td></td>
</tr>
</tbody>
</table>
There are some strategies to improve the corrosion behaviour and biocompatibility of Mg alloys:

a) Optimising the composition and microstructure, including grain size, crystalline structure phase and texture of the base metal through the development of manufacture process/methods.

b) Apply protective polymer deposit coatings with suitable physiochemical properties.

1.2 Degradation mechanism of Mg and its alloys

The usual degradation of biomedical metals is through the corrosion process. Generally, this process involves electrochemical reactions to produce oxides, hydroxides and H₂ gas species. In physiological conditions, the corrosion reactions of biodegradable metals including Mg and its alloys, involve the following anodic dissolution of metals and cathodic reduction reactions [6].

\[ 
M \rightarrow M^{n+} + ne^- \text{ (anodic reaction)} \quad \ldots \quad \text{Eq 1} 
\]

\[ 
2H_2O + 2e^- \rightarrow H_2 + 2OH^- \text{ (cathodic reaction)} \quad \ldots \quad \text{Eq 2} 
\]

\[ 
M^{n+} + nOH^- \rightarrow M(OH)_n \text{ (overall product formation)} \quad \ldots \quad \text{Eq 3} 
\]

Fig 1-1 illustrates the degradation mechanism of Mg and its alloy under physiological conditions. In general, immediately after contact with moisture/ body fluids, Mg is oxidised to form cations following an anodic reaction (Eq-1). The generated electrons are consumed for reduction of water corresponding to cathodic reactions (Eq-2). These reactions occur randomly over an entire surface, where galvanic couples form due to differences in electrochemical potential between the metal matrix and intermetallic phases or with organic molecules adsorbed on the surface that leads to the dissolution of biodegradable Mg and its alloys (Fig 1-1 (a) and (b)) [35]. Furthermore, the physiological
conditions are a highly corrosive environment to Mg and its alloys due to dissolved oxygen, proteins and electrolyte ions (chloride and hydroxide ions) [35].

Figure 1-1 Schematic diagram for Mg (BM: biomaterial) corrosion in electrolyte/medium [6]

In this environment, pure Mg is susceptible to corrosion due to its high electrochemical potential (E’= +2.37V for Eq-1), which results in the migration of ions from the metal surface to the surrounding fluid. These electrochemical reactions result in the formation of the hydroxide layer (M (OH)ₙ) on the surface of Mg and its alloys (Fig 1-1(c)) (Eq-3). When this metal oxide covers over the entire surface, it acts as a passive layer or kinetic barrier which prevents the further migration of ions or chemical reactions across the metal surface [35]. However, this layer is slightly soluble and susceptible to breakdown, particularly in the presence of chloride ions, that subsequently lead to the pitting corrosion (Eq-4) [36].

\[
\text{Mg(OH)}_2 + 2\text{Cl}^- \rightarrow \text{MgCl}_2 + 2\text{OH}^- \text{................... Eq. 4}
\]
During the degradation process, local pH of the solution increases resulting in the re-passivation of the surface, thereby again depositing $\text{Mg(OH)}_2$ layer on the surface. This results in the deposition of dissolved calcium and phosphates onto the $\text{Mg(OH)}_2$ layer, reducing the degradation rate of Mg alloy. During the implantation period, adherent cells proliferate on the degraded products of the corroded Mg alloy (Fig 1-1d).

Additionally, the corrosion of Mg and its alloys produce H$_2$ gas. Initially, the rapid formation of H$_2$ gas bubbles occurs due to the enriched chloride environment, which can disappear after the initial weeks following surgery [37]. Song et al. postulated that a hydrogen release rate of 0.01 ml.cm$^{-2}$.day$^{-1}$ can be tolerated by the body and does not pose a serious threat [38]. The corrosion in body fluids is influenced by various factors such as pH, concentration and types of ions, protein adsorption on the orthopaedic implant and influence of biochemical activities of surrounding tissues [39–41]. Typical forms of Mg corrosion encountered in physiological conditions are discussed in the following sections.

1.2.1 Galvanic corrosion

Galvanic corrosion takes place when two dissimilar metals with different electrochemical potential come in contact with each other in presence of an electrolyte [1]. The less noble metal acts as an anode which corrodes rapidly producing by-products around the contact site [42]. Galvanic corrosion of Mg is the primary issue for orthopaedic applications. In the galvanic series, Mg is the most active metal, and always an active anode, if it makes contact with other metals acting as cathode [42,43]. Consequently, the Mg alloy implant is preferentially corroded. In addition, an internal galvanic event occurs due to the presence of impurities or intermetallic elements in the Mg matrix.

1.2.2 Pitting corrosion

Pitting is a form of localized corrosion and associated with the breakdown of passivation layers in simulated body fluids (SBF) [42]. It is a serious form of corrosion when
compared to another process since the surface pits are difficult to observe due to the presence of degradation product [44]. The pits are highly corrosive and perforate the metal matrix. In general, after initial nucleation on the surface, impurities in the Mg alloy microstructure assist in further corrosion due to the galvanic differences in materials [45]. Moreover, the combination of physiological and Mg$^+$ ions from anodic dissolution species further accelerate the growth of the pit [45]. Once the pitting initiates, the Mg component can corrode in a very short period of time, which in the case of orthopaedic applications would reduce the load-bearing capacity of implants. Additionally, pitting increases internal localised stresses which can potentially form cracks [1]. The development of stress corrosion cracking (SCC) and metal fatigue cracks in the pits can lead to implant failure under normal loading conditions.

1.3 Factors affecting the corrosion resistance of Mg and its alloy

1.3.1 Buffer systems and Inorganic Ions

In the physiological conditions, several buffers are involved to maintain the neutral pH condition. The commonly used corrosion prototype mediums are SBF and cell culture medium which consist of buffers such as HEPES, Tris–HCl and HCO$_3$/CO$_2$. These chemical components significantly affect the corrosion of Mg and its alloys [41] . The HEPES and Tris-HCl regulate the change in the pH by consuming the OH$^-$ ions and affect the formation of corrosion product, consequently accelerating the dissolution of Mg, as shown in Eq-3. The HCO$_3$/CO$_2$ imparts buffering system in the human body which not only utilise OH$^-$ ions but also induce the precipitation of MgCO$_3$ which contributes to the protection against corrosion by developing the passivation layer on Mg [42]. Furthermore, the presence of inorganic ions in the body fluids influence the degradation of Mg and its alloys primarily by two manners: (a) the abundance of Cl$^-$ ions in
physiological conditions, is aggressive in the removal of passivation layer from the surface and leading to pitting corrosion and (b) the presence of $\text{HPO}_4^{2-}/\text{PO}_4^{3-}$, $\text{HCO}_3^-/\text{CO}_3^{2-}$ anions and Ca$^{2+}$ ions form calcium phosphate and carbonate salt precipitate which protect the erosion of passivation layer on Mg and its alloys, thereby preventing the emergence of pitting corrosion [46].

1.3.2 Mechanical stress

The attractive high strength of biodegradable Mg alloys as compared to biodegradable polymers makes them promising for load-bearing orthopaedic applications. Potential biodegradable biomedical devices would be exposed to a complex stress environment *in vivo*, depending upon the implantation site, and would be expected to function under various mechanical stresses, including fluid shear stress, compression etc.

Furthermore, time-dependent changes in the integrity of the mechanical properties of metal under prolonged mechanical stress is also a crucial factor [47]. Adequate metal implant mechanical strength is required to assist the healing and is important for postoperative recovery. As the degradation of biomaterial proceeds, the degeneration of mechanical integrity is expected. The mechanical loads can accelerate this process because of both corrosion and stress that lead to stress corrosion cracking (SCC) and corrosion fatigue, thereby causing implant failure [48]. The cyclic stress develops the formation of microscopic cracks on the surface and also damages the protective passive layer, while the chloride environment in body fluids further significantly increases crack growth rate [46]. When the loading stress exceeds the threshold of SCC, the crack grows to a critical size resulting in the fracture of the metallic implant. This infers that the environment can significantly reduce the fatigue limit of Mg alloys over an implantation period, producing considerable shorter failure times of implants. However, in case of
coated magnesium alloy, the loss of mechanical properties of the metal occurs at slow rate. Tan et al., showed that Si-coated AZ31 substrate degraded more slowly and retained bending load, >80 % of the initial load after 12 weeks of implantation [108]. There are very few studies on the tribology properties of coated-Mg alloy.

1.3.3 Effects of alloying element on mechanical and corrosion properties

The Mg-based biodegradable substrates can be divided into four major groups: (a) pure Mg, (b) Al-containing alloys (AZ91, AZ31, LAE422, AM60 etc.) (c) Rare earth elements (AE21, WE43 etc.), and (d) Al-free alloys (WE43, MgCa 0.8, MgZn6 etc.). These alloying elements improve the mechanical and physical properties of Mg alloy for orthopaedic applications by (a) optimising grain size, (b) increasing corrosion resistance, (c) providing mechanical strength by the formation of intermetallic states and (d) eases the manufacturing process of Mg alloys [1]. The compositions of some Mg-based alloys are given in Table 2.

Table 2 Compositions of Mg-based alloys

<table>
<thead>
<tr>
<th>Elements (%) wt.</th>
<th>Ca</th>
<th>Al</th>
<th>Mn</th>
<th>Zn</th>
<th>Li</th>
<th>Nd</th>
<th>Zr</th>
<th>Y</th>
<th>Mg</th>
<th>Other trace elements</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg alloys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AZ31</td>
<td>-</td>
<td>2.4</td>
<td>0.4</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>96.7</td>
<td>Cu-0.008,Fe-0.003,</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Be-0.005</td>
<td>[39]</td>
</tr>
<tr>
<td>AZ91</td>
<td>-</td>
<td>9.0</td>
<td>0.13</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>90.37</td>
<td>Cu-0.003,Fe-0.014,</td>
<td>[50]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Be-0.002</td>
<td></td>
</tr>
<tr>
<td>WE43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.4-3.2</td>
<td>0.4</td>
<td>3.7-4.3</td>
<td>92.1-93.5</td>
<td>[51]</td>
</tr>
<tr>
<td>LAE442</td>
<td>-</td>
<td>2.2</td>
<td>0.2</td>
<td>0.2</td>
<td>3.9</td>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>91.5</td>
<td>-</td>
<td>[52]</td>
</tr>
<tr>
<td>AM60</td>
<td>-</td>
<td>6.0</td>
<td>0.13</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>93.77</td>
<td>Cu-0.008,Fe-0.004,</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Be-0.005</td>
<td></td>
</tr>
<tr>
<td>MgCa0.8</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>99.2</td>
<td>-</td>
<td>[52]</td>
</tr>
</tbody>
</table>
The characteristic impurities in Mg alloys are copper (Cu), nickel (Ni), iron (Fe) and beryllium (Be). Typically, Cu is limited to 100-300 ppm, Ni should not exceed 20-50 ppm and Fe and Be are limited to 35-50 ppm and 5 ppm respectively [49]. These impurities should be strictly controlled under toxic limits for biomedical applications.

1.4 Pathophysiology and toxicology of alloying elements used for biodegradable Mg-based orthopaedic implants

The released metallic ions resulting from the corrosion of biodegradable Mg alloys may induce systemic toxicity to human, as well as localised toxicity to the peri-implant cells. Table 3 summarises the pathophysiology and toxicology of Mg and the common alloying elements. Generally, toxic element ions released in the body could be tolerated below a threshold level, while excess release in the body will have adverse effects [53]. As Mg is the most abundant element present in the body, it shows very low toxicity, however, in-depth biological studies are still required. Therefore, the amount of alloying element utilised for the manufacture of Mg-based biomedical implants needs to be optimised with respect to corrosion rates and the physiological environment at implant sites. It is essential that biomedical implants should be designed to control the localised release of metal ions below threshold levels. The concentration of released metal ions into tissues is influenced by various factors like (a) interfacial space between metal and implants, (b) fluid flow shear stresses between the biomedical implant and bone, (c) variation in pH and (d) local blood supply [54].
Table 3 Pathophysiology of alloying elements ([11,55–57])

<table>
<thead>
<tr>
<th>Elements</th>
<th>Effect on the alloying element</th>
<th>The normal amount present in the human body</th>
<th>Pathophysiology</th>
<th>Toxicology</th>
<th>TD50 of Bone-related cells (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg</td>
<td>......</td>
<td>25 g</td>
<td>Normal blood serum level 0.73-1.06 mmol/L. Required for ATP synthesis, activator of many enzymes; co-regulator of protein synthesis, the stabiliser of DNA and RNA</td>
<td>Disorder in magnesium homeostasis leads to nausea, renal failure, impaired breathing.</td>
<td>73× 10⁻³</td>
</tr>
<tr>
<td>Ca</td>
<td>Induce Corrosion resistance in Mg-Ca alloys</td>
<td>1100 g</td>
<td>Normal serum level 0.919-0.993 mg/L. Control muscle contraction, maintain homeostasis of bone, hormones and neurotransmitter release regulator</td>
<td>Dysregulation of calcium levels in the body leads to kidney stones, Hypoparathyroidism, cardiac unrest</td>
<td>&gt;50× 10⁻³</td>
</tr>
<tr>
<td>Al</td>
<td>Acts as a passivating element and improve corrosion resistance</td>
<td>&lt;300 mg</td>
<td>Normal blood serum level 2.1-4.8 µg/L.</td>
<td>Excess amounts lead to neurotoxicity, Alzheimer’s, accumulation in bone leads to decreases osteoclast viability.</td>
<td>&gt;5× 10⁻³</td>
</tr>
<tr>
<td>Zn</td>
<td>Improves compatibility with bone by modifying yield stress and Elastic modulus. Decreases H₂ gas release</td>
<td>2 g</td>
<td>Normal blood serum level 12.4-17.4 µmol/L. Essential elements for the immune system.</td>
<td>Excessive amounts lead neurotoxic, cramps and diarrhoea</td>
<td>9.28 × 10⁻⁵</td>
</tr>
<tr>
<td>Mn</td>
<td>Improve corrosion resistance</td>
<td>12 mg</td>
<td>Normal blood serum level &lt; 0.8 µg/L. Activator of various enzymes</td>
<td>Excessive amounts cause psychiatric and motor disturbances</td>
<td>4.59 × 10⁻⁵</td>
</tr>
<tr>
<td>Li</td>
<td>Improve corrosion resistance</td>
<td>--</td>
<td>Normal blood serum level 2-4 ng/g. Used in the treatment of depressive disorders</td>
<td>Overdose causes impaired kidney function and respiratory disorders</td>
<td>1.32 × 10⁻²</td>
</tr>
<tr>
<td>Cu</td>
<td>Increase the strength of Magnesium cast</td>
<td>--</td>
<td>Normal blood serum level 74-131 µmol/L. Involved in the respiratory chain and enzyme co-factors</td>
<td>Causes hypotension, jaundice, melena etc.</td>
<td>4.15 × 10⁻⁵</td>
</tr>
<tr>
<td>Zr</td>
<td>Improve tensile strength, ductility and corrosion resistance</td>
<td>&lt;250 mg</td>
<td>Low systemic toxicity</td>
<td>Deposited on bone and cationic form cause deposition on bone</td>
<td>1.64 × 10⁻³</td>
</tr>
<tr>
<td>Y</td>
<td>Improve ductility and corrosion</td>
<td>--</td>
<td>Blood serum level &lt; 47 µg</td>
<td>Higher concentration accumulate in liver and gall bladder</td>
<td>2.54 × 10⁻⁴</td>
</tr>
</tbody>
</table>
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The biological effects of released metal ions from biodegradable medical implants affect the cells of the surrounding bone tissue. Table- 3 summarises the toxic dose at 50% cell viability (TD50) of bone-related cells (MC3T3E1 and MG63 cell lines). From Table 3, the alloying elements are divided into mild, moderate and severe toxic elements [58]. The mild toxic elements include Mg, Ca, Li, Al and Zr. However, some reports showed that mild toxic elements like Al (500 nM) at very low concentration induce bone-related cell proliferation[59]. Moderate toxicity elements include Y, whereas Zn, Cu and Mn are severe toxic elements. Thus, moderate or mild toxic elements should only be used to significantly improve the corrosion resistance to make Mg-based alloy orthopaedic implants clinically feasible. Additionally, other strategies such as polymer deposited coatings on Mg-based substrates have also been employed to prevent its rapid degradation, thereby facilitating the controlled exposure of these metal ions into the body [6].

1.5 Effect of polymeric deposit coatings on Mg alloys degradation and biocompatibility

In general, the healing process of bone consists of three phases; inflammatory, reparative and remodelling. In the inflammatory phase, the immune system of the body responds against the foreign body i.e. metallic implant fixed in the body [60]. In the reparative phase, integration of the implant with new bone and regeneration of tissue takes place. Remodelling phase is one of the longest phases of the healing process. For the regeneration of bone, this process takes a minimum of 12 weeks, and unfortunately, most of the Mg-based orthopaedic implants can degrade within this period [6]. Therefore, there is a need to increase the corrosion resistance of Mg-based alloys.
A potential method for enhancing the corrosion resistance of Mg alloy without altering the bulk properties of the material is through protective polymeric coatings. Typically, the bulk properties of Mg regulate the mechanical integrity, but the surface properties play important roles in various physio-chemical processes like the interaction of body fluids, adhesion of biomolecules and cells with biomedical implants, which initiate the corrosion process [6]. Therefore, the coating should be biocompatible, but also degrade at a slower rate than that of Mg and its alloys. Polymeric deposit coatings are attractive since they possess good biocompatibility with the body and have a range of degradation rates. Although, the coatings are susceptible to the delamination during immersion period. The mechanism by which coating retards the onset of Mg alloy corrosion and changes the mode of corrosion is elucidated in Fig 1-2.

Figure 1-2 (a) Schematic diagram of degradation of bare or uncoated and coated Mg alloy, (b) surface condition of coated Mg alloy during initial immersion period and (c) degradation of protective coating [61].

For bare or uncoated alloy, corrosion generally follows the parabolic curve since it is a diffusion-controlled process. When the surface is coated, Mg alloy follows the sigmoidal corrosion law (Fig 1-2a). During the initial immersion period, a low rate of corrosion is
observed due to the protective effect of the coating, which covers over the surface (Fig 1-2b). As the immersion progresses, the protective coating deteriorates (Fig 1-2c) and the reactive surface is exposed to the corrosion medium, thereby accelerating the degradation of the Mg alloy. Later, deposition of corrosion products (self-passivation) acts as a barrier against corrosion. These are two competing processes i.e. break down of the protective layer and self-passivation continues until most of the protective layer is degraded [61].

This section discusses the role of deposited coatings (sol-gel, synthetic aliphatic polyesters and natural polymers) in improving the corrosion resistance and biocompatibility of Mg alloys. Polymers applied as coatings on Mg-based biomaterials for orthopaedic applications are shown in Table 4, accompanied by their degradation products and corrosion behaviour of the polymer coatings.

1.5.1 Sol-gel coatings

The sol-gel process is a technique of creating solid materials from a chemical suspension of colloidal particles (sol) to produce an integrated network (gel) with diverse characteristics, through a series of hydrolysis and condensation polymerization reactions [62]. This processing methodology has facilitated the fabrication of inorganic and hybrid materials with controlled features at the nanoscale. In brief, silicon/metal alkoxides (M(OR)) are used as precursors for a sol-gel synthesis which involves four stages (a) hydrolysis, (b) condensation and polymerisation of monomers to form chains and particles, (c) particle growth and (d) agglomeration of the polymer structures followed by the network formation throughout in liquid medium which increases viscosity to form a gel [63]. In fact, the hydrolysis and condensation reactions start simultaneously, once the hydrolysis reaction has been initiated [64]. These reactions yield by-products such as water and alcohol. These processes are influenced by initial reaction conditions such as pH, the molar ratio of water to precursor solution, temperature and solvent composition.
This wet gel can be dried under various conditions to produce a xerogel with residual porosity. Heating the xerogel further under controlled conditions can produce a glass or ceramic. Before the gelling point, the colloidal solution can be used directly to coat various substrates by classical techniques like dip coating, spin coating and spraying. The schematic of the steps involved in the sol-gel process and their products are shown in Fig 1-3. According to available literature, silane-based sol-gel coatings have been used widely as anti-corrosive and biocompatible coatings on Mg alloy for orthopaedic applications [62]. There are several reasons for using silane as anti-corrosive coatings such as (a) Si-O-Si network (b) lower susceptibility to galvanic reactions with Mg, (c) greater adhesive properties, (d) easily chemically modifiable and (e) less cytotoxic in nature [62]. The use of sol-gel for the protection of Mg-based alloys against corrosion has been well reviewed [36,62].

Figure 1-3 Schematic diagram showing the sol-gel process and its various products [66]
The sol-gel undergoes full hydrolysis and partial condensation occurs before deposition on the substrate seen in Fig 1-4. The sol-gel can be deposited by a number of methods including dip coating, spin coating, roll coating, spraying and electrodeposition. Upon deposition solvent evaporation occurs and the condensation reaction proceeds forming a dense network. Further densification can be accelerated by thermal or UV curing methods. The adhesion of organo-silane molecules to magnesium-based substrates by a metallosiloxane bond (Me-O-Si) has been confirmed [36,62].

Figure 1-4 Sol-gel coating process
1.6 Synthetic aliphatic polyester coatings

In general, polymeric implants for orthopaedic applications have not been found suitable due to poor mechanical properties [1,67]. Therefore, such polymers are generally deposited as anticorrosive coatings on the orthopaedic implant. Usually, these polymeric materials enhance the corrosion resistance of Mg-based metals by isolating the device from the fluidic and corrosive environment of the body [1,14]. The biocompatible properties of polymers are critical because their presence at the interphase of implant and body environment which could elicit the immunological response. Therefore, selecting the appropriate polymer coating is crucial in improving the corrosion resistance and biocompatibility of orthopaedic metals, including Mg and its alloys.

There are several advantages of polymers as they can be easily modified chemically, physically and mechanically, enabling their use in a wide range of biomedical applications [68,69].

Synthetic aliphatic polyesters such as polylactic acid (PLA), poly-co-lactic-glycolic acid (PLGA), polycaprolactone (PCL), polyethyleneimine (PEI) and many other polymers have been used in various biomedical applications [70]. These polymeric materials are attractive coatings on Mg and its alloys to control the initial rate of degradation, as their degradation rate is based on their molecular weight [70]. In particular, lactic and glycolic acid-based polymers such as PLA and PGA, have been studied extensively as corrosion resistant coatings for orthopaedic applications. Similarly, significant work has been carried out on copolymers of lactide and glycolide i.e PLGA [71]. A copolymer of 50% lactide and glycolide degrades faster as compared to the corresponding homopolymer [70]. PCL also degrades slower than PLA [72]. Therefore, using a blend of polymers a suitable system can be designed with a selected corrosion rate. The polymeric deposit
coatings on orthopaedic Mg devices can also enhance the mechanical strength, with nontoxic degradation products enabling osteoinductive and osteogenic environments [68,69,73]. However, many of the currently available polymers do not fulfil the requirement and would require chemical modifications, if they are to be applied for such applications. This section discusses the corrosion resistant and biocompatibility properties of selected and important aliphatic polyester-based polymers coating on Mg and its alloys.

1.6.1 Poly lactic acid

Biodegradable polymers such as polylactic acid (PLA) have been explored extensively for biomedical application. PLA is highly biocompatible, semi-crystalline and hydrophobic in nature [74]. It undergoes hydrolytic degradation and the by-products such as lactic acid is easily metabolised in the body without exhibiting any toxicity. However, a major drawback of PLA as a load bearing implant for orthopaedic applications is that it possesses poor mechanical properties as compared to bone [1]. On the other hand, it can be used as an anti-corrosion coating on Mg alloys to slow down the hydrolytic degradation rate and the evolution of H₂ gas (Table 4) [75]. However, it’s performance is dependent on the concentration of PLA and the application technique [76].

1.6.2 Poly (lactic-co-glycolic) acid

PLGA (Poly(lactic-co-glycolic)acid)) copolymers are FDA approved for clinical applications mainly due to their excellent biocompatibility [74]. They are hydrolytically degraded into glycolic and lactic acid which are easily assimilated by metabolic pathways [77]. The physical, mechanical, chemical and degradation properties of PLGA can be engineered by altering the ratio of the two-co-monomers. Several compositions of copolymers of glycolic acid and lactic acid have been investigated. These copolymers are of usually two types (a) (l) LA/GA (b) (dl) LA/GA. The composition of (l) LA/GA ranges
of 20 to 70% and of 0 to 70% for (dl) LA/GA are amorphous in nature [78]. Reed et al. showed that (l) LA/GA co-polymers are more resistant to hydrolysis [79]. The LA/GA (30/70) has a higher water absorption capacity and more susceptible to hydrolysis, while the LA/GA (50/50) copolymer is most unstable with respect to hydrolysis. These advantages of PLGA have been exploited for important applications such as corrosion protection and drug delivery. The corrosion protection performance of PLGA modified Mg alloys in different electrolytes in terms of corrosion current density and corrosion potential is given in Table 4.

1.6.3 Poly caprolactone

Polycaprolactone (PCL) is the most studied polymer in the family of polylactone [74]. It degrades slower than PLA and is useful for drug delivery as well as anti-corrosive coatings. The homopolymer PCL has a degradation time about 24 to 36 months under in vivo conditions. The rate of hydrolysis can be altered by co-polymerising with other polymers like valerolactones, dl-lactide etc [74]. For example, copolymers with epsilon-caprolactone and dl-lactide have been synthesised to yield a polymeric material with higher degradation rates (e.g. biodegradable sutures). PCL is considered to be a nontoxic biodegradable material and suitable as a protective coating (Table 4) on Mg-based alloy for orthopaedic applications.

1.7 Natural polymers coatings

Natural polymers such as collagen, chitosan, stearic acid and serum albumin have been studied as coatings on the surface of Mg and its alloys for anti-corrosive as well as biocompatible properties [80]. In comparison to sol-gel and synthetic poly-esters, natural polymers exhibit excellent biocompatibility due to their biomimetic nature [81]. For example, extracellular cell matrix (ECM) components possess cell-specific domains such
as RGD (Arg-Gly-Asp) sequence which improve cell attachment [81]. Therefore, natural polymer-based coatings on implanted material enhance the interactions between the implant surface and surrounding tissue matrix, thereby expediting the regeneration of tissues [82]. These polymers are also capable of undergoing surface modifications, hydrogel scaffold synthesis and housing drugs for delivery [83]. Currently, the corrosion resistance of biopolymers is poor when compared to sol-gel and synthetic-polyester based coatings, and an improvement would require in-depth research work. The list of natural polymers coated on Mg and its alloy to study the corrosion resistance is given in Table 4. This section reviews the surface modifications of Mg and its alloys by natural polymers coatings to improve the corrosion resistance and biocompatibility.

1.7.1 Collagen coatings

Collagen is the major component of extracellular materials of bone matrices. Many reports have demonstrated that collagen type-I provides a favourable surface for cell adhesion, functions and cell proliferation of bone related-cells [84]. It is expected that a collagen coating on Mg and its alloys may be suitable for corrosion resistance, although very few reports are available. In previously reported literature, the collagen was applied for the secondary stabilisation of Mg-5Zr and Mg-5Zr-Ca alloys under in vivo conditions [84]. In addition, it has also been explored to develop the hydroxyapatite conversion coating which reduced the release of Mg$^{2+}$ by ~ 7 fold as compared to the uncoated AZ31 equivalents immersed in HBSS over a period of 100 h [85].

1.7.2 Chitosan coatings

Chitosan coatings are generally non-toxic and can play an important function as an adhesive basal matrix for growing cells during the peri-implant healing process while enhancing the corrosion resistance of biodegradable metal [86]. It also showed good adherence to AZ91 Mg alloys and offered protective layer leading to high corrosion
Chapter 1

resistance. Chitosan coating on Mg alloy increased the corrosion resistance with immersion time due to the deposition of a protective passivation layer. In general, surface modifications with natural polymers have shown to improve the corrosion resistance and biocompatibility properties of Mg and its alloys but literature reports for orthopaedic applications are limited. This indicates that the development of these coatings is in its infancy. As natural polymers are abundant, other kinds of natural polymers such as hyaluronic acid, lysozyme, alginate agar, dextran, chitin, chondroitin sulphate etc. offer a wealth of coating opportunity.
Table 4 Corrosion studies of polymers coated on Magnesium alloy.

<table>
<thead>
<tr>
<th>Polymers/Composites</th>
<th>Degradation products</th>
<th>Magnesium alloy Substrate</th>
<th>Icorr (μA/cm², unless Indicated)</th>
<th>Ecorr (V)</th>
<th>Corrosion Medium</th>
<th>Coating Method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synthetic polyesters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poly Lactic acid</td>
<td>Lactic acid</td>
<td>PLA-AZ31</td>
<td>7.72</td>
<td>-1.57</td>
<td>SBF</td>
<td>Dip coating</td>
<td>[75]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLA-MAO-AZ31</td>
<td>1.83</td>
<td>-1.5</td>
<td>--</td>
<td>Dip coating</td>
<td>[87]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mg-1.21Li1.12Ca-1.0Y</td>
<td>1.7</td>
<td>--</td>
<td>Hanks’ buffer</td>
<td>Dip coating</td>
<td></td>
</tr>
<tr>
<td>PLGA</td>
<td>d.l-lactic acid and glycolic acid</td>
<td>Mg-6Zn</td>
<td>0.085</td>
<td>-1.44</td>
<td>0.9% NaCl</td>
<td>Dip coating</td>
<td>[61]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AZ31-PLGA 10 %</td>
<td>5.2</td>
<td>-1.469</td>
<td></td>
<td>Dip coating</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AZ31-PLGA 20 %</td>
<td>6.05</td>
<td>-1.45</td>
<td></td>
<td>Dip coating</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mg4Y-PLGA 10 %</td>
<td>8.09</td>
<td>-1.485</td>
<td>DMEM</td>
<td>Dip coating</td>
<td>[71]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mg4Y-PLGA 20 %</td>
<td>6.85</td>
<td>-1.469</td>
<td></td>
<td>Dip coating</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLGA 50:50</td>
<td>1.12</td>
<td>-1.52</td>
<td></td>
<td>Dip coating</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLGA 75:25</td>
<td>1.56</td>
<td>-1.44</td>
<td>DMEM</td>
<td>Dip coating</td>
<td>[88]</td>
</tr>
<tr>
<td>Poly(capro-lactone)</td>
<td>Caproic acid</td>
<td>MAO–4PCL duplex coated</td>
<td>0.81</td>
<td>-1.72</td>
<td></td>
<td>Dip coating</td>
<td></td>
</tr>
<tr>
<td>Mg MAO–7PCL duplex coated Mg</td>
<td></td>
<td>0.0045</td>
<td>-1.53</td>
<td></td>
<td>Hanks’ buffer</td>
<td>Dip coating</td>
<td>[89]</td>
</tr>
<tr>
<td>Mg- PCL 2.5 wt%</td>
<td></td>
<td>7</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg- PCL 5 wt%</td>
<td></td>
<td>0.1</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg- PCL 7.5 wt%</td>
<td></td>
<td>0.02</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCL-AZ91-LPM</td>
<td>Reduced</td>
<td>1.44</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylene glycol and amine</td>
<td>PCL-AZ91-HPM</td>
<td>1.14</td>
<td>SBF</td>
<td></td>
<td></td>
<td>Spray coating</td>
<td>[33]</td>
</tr>
<tr>
<td>AZ91D-PEI/PSS/8HQ/PSS</td>
<td></td>
<td>24.82</td>
<td>-1.732</td>
<td></td>
<td>m-SBF</td>
<td>Spin coating</td>
<td>[91]</td>
</tr>
</tbody>
</table>
### Sol-gel coatings

<table>
<thead>
<tr>
<th>Coating</th>
<th>Composition</th>
<th>Primer</th>
<th>Dip</th>
<th>Coating Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPETES&amp; BTESPT</td>
<td>-</td>
<td>Mg-6Zn-Ca -1(M)</td>
<td>1.20×10(^{-3})</td>
<td>-1.39</td>
<td>m-SBF</td>
</tr>
<tr>
<td>TiO(_2)</td>
<td>-</td>
<td>Mg-MAO-TiO(_2)</td>
<td>7.59×10(^{-4})</td>
<td>-1.38</td>
<td>Hanks’ solution</td>
</tr>
</tbody>
</table>

### Natural Polymers

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Composition</th>
<th>Primer</th>
<th>Dip</th>
<th>Coating Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitosan</td>
<td>Glucosamine and keto-oligosacchraide</td>
<td>Chi/MAO/Mg-Zn-Ca-120V</td>
<td>14</td>
<td>-1.549</td>
<td>Na(_2)PO(_4)+NaOH</td>
</tr>
<tr>
<td>HA-chitosan</td>
<td>Glucosamine and keto-oligosacchraide</td>
<td>AZ91E-10% chitosan</td>
<td>0.136</td>
<td>-1.535</td>
<td>Synthetic Sweat</td>
</tr>
<tr>
<td>HA-chitosan</td>
<td>Glucosamine and keto-oligosacchraide</td>
<td>HA-5 wt.% chitosan-AZ31</td>
<td>15.1</td>
<td>-1.601</td>
<td>SBF</td>
</tr>
<tr>
<td>Stearic acid and magnesium state</td>
<td>Hydroxylysine/proline/hydroxyproline</td>
<td>AZ31</td>
<td>4</td>
<td>-1.71</td>
<td>Hanks’ solution</td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
<td></td>
<td>0.00223</td>
<td>-1.48</td>
<td>SBF</td>
</tr>
</tbody>
</table>
1.8 Molecular mechanism of Mg$^{2+}$ inducing bone formation

Bone development involves osteogenesis, angiogenesis and osteoclastogenesis [99]. The hierarchy of the bone precursor cells is mesenchymal stem cells, osteoprogenitor cells, pre-osteoblast cells, osteoblast cells and osteocytes (a mature form of osteoblast cells). Mesenchymal stem cells are pluripotent while osteoprogenitor cells are committed to the expansion of the lineage of proliferated osteoblast cells with transitional pre-osteoblast cells. Osteocytes are terminally differentiated osteoblast cells to be embedded within bone lacunae that play a role in phosphate and calcium homeostasis and mechanosensation functional adaptation (Fig 1-6). It is desirable that coated or uncoated Mg alloys should not only possess the corrosion resistance but also induce the osteogenic activity for enhanced healing of the bone injury.

The exact molecular mechanism/cell signalling of Mg$^{2+}$ effect on osteoblast cells activity is not known properly, but a possible mechanism is depicted in Fig 1-5. Yoshizawa et al. reported the stimulation effect of magnesium ions on human bone marrow stem cells (hBMSCs) for osteogenic activity [99]. In this study, the molecular mechanisms of intracellular Mg ions effect on the osteogenic activity of hBMSCs with or without osteoinductive growth factors were evaluated. The [Mg$^{2+}$] ranging from 0 to 10 mM was incubated with hBMSCs for 3 weeks with or without osteoinductive medium and evaluated for osteogenic activity by screening various osteogenic markers (ECM mineralisation and gene expression). The ECM mineralisation and collagen type X expression were increased at 10mM of [Mg$^{2+}$] in the abovementioned conditions. Furthermore, the mechanism of intracellular cell signalling was investigated by analysing the protein production of the hypoxia-inducible factor (HIF)-1α and 2α (transcription factors of COL10A1), vascular endothelial growth factor (VEGF) (activated by HIF-2α)
and peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α (transcription coactivator of VEGF). The results showed that 10mM [Mg^{2+}] ions upregulate the expression of COL10A1 and VEGF via HIF-1α in undifferentiated cells, and osteogenic cells via PGC-1α transcription factor. From this study, it is observed that VEGF plays an important role in the process of defining osteoblastic lineage.

Figure 1-5. Schematic hypothesized intracellular signalling cascade of Mg^{2+} stimulation of bone-related cells.

Another very important process is angiogenesis in the development of new bone. New blood vessels bring oxygen, nutrients and serve as a route for inflammatory cells and bone-like/precursor cells to reach the injury site. Most notably, VEGF produced by inflammatory cells and hBMSC, induce and enhance the growth of blood vessels in fracture bone tissue. VEGF has three isoforms A, B, and C that are homo- and heterodimers which bind to a dimeric receptor complex of 2 receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1) [100].

In the fracture healing process, pro-angiogenic VEGF factor concentrations are elevated at early post-fracture condition [99]. As previously mentioned, VEGF factors are secreted by inflammatory cells recruited at an injury site [99]. Secondly, its expression can be induced by hypoxia conditions, where HIF-1α acts as a transcription factor for VEGF expression [99]. In addition, VEGF activates several other pathways including
intracellular and vascular adhesion molecules (ICAMs and VCAMs), eNOS expression, basal fibroblast glial factors and matrix metalloproteins (Fig 1-6).

Figure 1-6 A schematic showing the dual action of Mg$^{2+}$ on bone forming activity by stimulating osteogenesis and angiogenesis [105].

From the above discussion, it can be concluded that communication between osteoblasts and endothelial cells is essential for bone fracture repair (Fig 1-6). Furthermore, osteoclastogenesis also plays a very important role in bone remodelling. Janning et al., investigated the effect of osteoblast and osteoclast cell proliferation by implanting cylinders of pure Mg(OH)$_2$ into rabbit femur condyles for 2–6 weeks [100]. Results showed an initial increase in bone volume (Bone volume/tissue Volume) in the vicinity of Mg (OH)$_2$ implant surface at 4 weeks, which was equal to 6 weeks of control. It is suggested that osteoclast surface per bone surface ratio decreases significantly in the first four weeks, while increases in the osteoid surface to bone surface ratio was observed. At the sixth week, the osteoclast surface to bone surface ratio reached was similar to that of
the control. Thus, the enhanced bone formation and decreased bone resorption activity resulted in the higher bone mass formation around the Mg(OH)$_2$ implant.

1.9 Anti-biofilm activity of polymers coated on Mg-based biomedical materials

Despite considerable advancement in prevention and treatment of implant associated microbial infections, the risk of implant failure due to the lifelong bacterial colonisation on the implant [101]. Infection related with prosthetics joints (hip or knee replacement) is usually less than 2%, but it increases considerably to 5-40% after revision surgery [105]. Similarly, 5% of fixation implants suffer failure due to the implant-associated infections, whereas the incidence may increase significantly to 30% after fixation of open fractures [105]. In future, it is expected that the incidence of infection rates associated with orthopaedic devices will increase in future because detection method of microbial films formation showed tremendous improvement over a few decades [105-106].

The complex interactions between the pathogenic organisms, implant devices and host immune system are the origin of many devices associated infections. Surface properties including surface hydrophilicity, hydrophobicity and surface charge play important roles in initial adhesion and proliferation of bacteria. Thus, the optimal design for infection resistance is a challenging issue and must satisfy a broad range of requirements like optimal broad-spectrum antimicrobial activity, preventing biofilm formation and biocompatibility. In order to counter this problem, researchers have focussed on different areas of polymer application as its antimicrobial nature, drug releasing agent, composite synthesis with inorganic/organic antimicrobial agents (Metals ions/ metal nanoparticles/polymeric nanoparticles) [102]. However, Mg ions were found to be moderately antimicrobial active, and are more prone to biofilm formation and thus very
few publications have been observed that are pertinent to Mg-based alloys for orthopaedic applications. Robinson et al, studied the antimicrobial activity of Mg$^{2+}$ ions against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* [103]. The Mg$^{2+}$ions induced a change in pH and its effect on antibacterial activity was studied. The change in pH caused by Mg$^{2+}$ was adjusted to neutral to study the antibacterial effect of concentration and pH independently. It was observed that antibacterial activity is independent of concentration, but pH shift to alkali was attributed to the antibacterial activity of Mg$^{2+}$ ions. However, the generation of an alkaline environment caused toxicity to surrounding tissues and thus alone Mg-based materials are not an effective antibacterial agent.

Li et al, studied the drug delivery, antibacterial property and cytocompatibility of gentamycin loaded PLGA nanoparticles on porous Mg scaffold [104]. The prime objective of this work was to achieve sustained drug release of antibiotics at local sites of bone defects. The results showed that the PLGA drug loaded gentamycin showed higher drug releasing efficiency (28-33%) than uncoated gentamycin loaded Mg scaffold (2-3%). The sustained drug release was monitored for more than 14 days, and it inhibited biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis*. The biocompatibility of hBMSC demonstrated the cytocompatibility with PLGA loaded Mg scaffold compared to Mg alone. Hence, this strategy was found to be a promising strategy for sustained/controlled drug delivery systems to prevent orthopaedic implant-associated infections.

Tian et al, investigated the antimicrobial activity and biocompatibility of HA coupled antimicrobial peptide (AMP) (RRWPWWPWRR-NH2) coated Mg alloy (AZ91) [113]. Additionally, the biodegradation of Mg alloy was determined by the change in the weight of the alloy. The loading efficiency of AMP in HA was ~11 µg/cm$^2$ and the release of the
peptide was monitored for 7 days. The antimicrobial activity was efficient against *S. aureus* with 50% inhibition after 4 days and sustained antimicrobial activity for 7 days. Furthermore, it enhanced the proliferation and alkaline phosphatase activity of hBMSC. Under *in vivo* conditions, antimicrobial peptide coated Mg alloy implant in femoral condyle showed new bone formation and anti-inflammatory effects.

**1.10 Summary**

Despite of the immense potential of biodegradable Mg alloys for orthopaedic applications, the major disadvantage is their high corrosion rate, thereby creating a bio-incompatible environment in the surrounding tissues. In order to overcome these drawbacks, strategies such as the effect of alloying elements and polymeric surface modifications of Mg and its alloys have been discussed. It was observed that alloying elements can improve the corrosion resistance of Mg-based alloys. But, the biocompatibility of these alloying elements on bone related cells is an important factor, and the implant should not show cell toxicity.

Recent research on the effect of polymer deposit coatings on corrosion behaviour and biocompatible properties of Mg alloys shows that sol-gel and synthetic polyester based coatings have significantly improved the corrosion resistant. Numerous studies also highlighted the improvement in corrosion properties of natural polymer coatings by incorporating the synthetic polymers. Therefore, novel strategies need to be designed, which could exploit the multifunctional coatings for the development of potential biodegradable orthopaedic Mg based implants.
1.11 References


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Chapter 1


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Chapter 2

Analytical techniques

This chapter overviews the principles of the various spectroscopic, microscopic and electrochemical analytical techniques such as Fluorescence spectroscopy, Fourier transform infrared spectroscopy (FTIR), inductively coupled plasma optical emission spectroscopy (ICP-OES), Scanning electron microscopy (SEM), Atomic force microscopy (AFM), Contact angle measurement. This chapter also discusses the various in vitro biochemical assays to analyse the cytocompatibility of osteoblast cells and antibacterial activities.
2.1 Spectroscopy

Spectroscopy is the study of the interaction between light radiation and matter as a function of wavelength. Spectroscopy refers to the use of a beam of light dispersed according to its wavelength or frequency. The type of spectroscopy depends on the physical quantity measured.

2.1.1 Fluorescence spectroscopy

*Operator: Sankalp Agarwal*

Fluorescence spectroscopy involves the emission of light at higher wavelengths (Emission wavelength) that occurs within nanoseconds after the absorption of shorter wavelength light (Excitation wavelength). The mechanisms by which electronically excited molecules come to ground state are shown in Fig 2-1 [2]. The absorption of a photon takes the molecule from the ground state (singlet state, S0) to either first excited state (singlet state S1) or second excited state (S2). Then the excited molecule relaxes to the lowest energy level (vibrational level) of the first excited state. It can relax from the singlet excited state to the ground state either by emitting a photon or without emitting photons. The emitted fluorescence photons give information about intensity (number of photons released), spectrum, polarization and their time dependence, important parameters that one can use for the characterisation of samples [3].

![Jablonski diagram](image.png)
This technique has become popular for its biochemical and medical applications. Fluorescence spectroscopy has been used to identify cells and tissues based on endogenous or exogenous chromophores [4]. In this study, the bisBenzimide fluorescent dye was used to quantify the DNA of MC3T3E1 osteoblast cells by fluorescence spectroscopy cultured over a period of 21 days in differentiation medium.

### 2.1.2 Fourier transform infrared spectroscopy

*Operator: Sankalp Agarwal*

FTIR spectroscopy is a technique used to detect the presence of functional groups in the sample. It involves the detection in the infrared region and uses the Fourier transform technique for spectral detection and analysis. In this study, sol-gel coating and bio-functionalisation were characterised using FTIR.

Molecular bonds vibrate at different frequencies. Depending on the type of bonds in a molecule, there are several specific frequencies at which it can vibrate. In general, the atoms in a molecule vibrate about a mean position. If there is a net change in the dipole moment of a molecule due to this vibrational motion, then such mode of vibration is said to be infrared (IR) active [5]. The vibrating molecule absorbs energy only from the specific frequency of radiation with which it can coherently interact. Such absorption is known as resonating frequencies which are characteristic of the structure of a particular molecule. The principles of IR can be explained by classical mechanics [5]. The classical model consists of a simple ball and spring model wherein diatomic molecules with two masses, m1 and m2, are connected by a spring. A system of masses joined by springs has a number of fundamental modes of vibration each of which has a particular natural frequency.

*According to Hooke's law when the spring is displaced,*
\[ F = -kx \]  
\text{Eq. 5}

Where, \( F \) = opposing restoring force; \( k \) = force constant; \( x \) = displacement from the equilibrium position. This is a simple harmonic equation (Eq. 5) wherein the frequency \( (\nu) \) of vibration is given by the relation (Eq. 6).

\[ (\nu) = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}} \]  
\text{Eq. 6}

Where \( \mu \) is the reduced mass of atoms present in a compound and \( k \) is the force constant related to the particular vibration using simple laws of mechanics. The characteristic frequencies of some compounds are listed in Table 5.

\textbf{Table 5. Characteristic frequencies of different compound types [5].}

<table>
<thead>
<tr>
<th>Bond</th>
<th>Compound type</th>
<th>Frequency range, cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-H</td>
<td>Alkanes</td>
<td>2960-2850 (s) stretch</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1470-1350 (v) bending</td>
</tr>
<tr>
<td>C=C</td>
<td>Alkenes</td>
<td>1680-1640 stretch</td>
</tr>
<tr>
<td>C≡C</td>
<td>Alkynes</td>
<td>2260-2100 (w, sh) stretch</td>
</tr>
<tr>
<td>C=C</td>
<td>Aromatic ring</td>
<td>1600, 1500 (w) stretch</td>
</tr>
<tr>
<td>C-O</td>
<td>Alcohol, Ether, Carboxylic acids, Esters</td>
<td>1260-1000 (s) stretch</td>
</tr>
<tr>
<td>C=O</td>
<td>Aldehyde, Ketone, Carboxylic acid</td>
<td>1760-1670 (s) stretch</td>
</tr>
<tr>
<td></td>
<td>Monomeric-Alcohols, Phenols</td>
<td>3640-3160 (s, br) stretch</td>
</tr>
<tr>
<td></td>
<td>Hydrogen-bonded-Alcohols, Phenols</td>
<td>3600-3200 (b) stretch</td>
</tr>
<tr>
<td>N-H</td>
<td>Amines</td>
<td>1650-1580 (m) bend</td>
</tr>
<tr>
<td>O-H</td>
<td>Carboxylic acids</td>
<td>3000-2500 (b) stretch</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3500-3300 (m) stretch</td>
</tr>
<tr>
<td>C-N</td>
<td>Amines</td>
<td>1340-1020 (m) stretch</td>
</tr>
<tr>
<td>Si-O-Si</td>
<td>Siloxane</td>
<td>1200, 1075 (v)</td>
</tr>
<tr>
<td>Si-OH</td>
<td>Silanol</td>
<td>960 (v)</td>
</tr>
</tbody>
</table>
Types of vibration modes

The resonating frequencies are also related to bond strength and mass of the associated atom. These frequencies of vibrations for a particular bond type are associated with a number of vibration modes (Fig 2-2). In order to be an IR active vibration mode, a molecule must be associated with the change in dipole moment. A molecule can vibrate in different ways called vibrational mode. For a molecule with N number of atoms, numbers of vibrational modes of linear molecules are given by $3N - 5$ degrees of vibrational modes, where non-linear molecules have $3N - 6$ degrees of vibrational modes.

The vibrational modes are classified as either stretching or bending. Stretching vibrational modes are categorized as being either symmetrical (movement in the same direction) or asymmetrical (movement in opposite directions). Bending vibrations modes are classified as scissoring, rocking, twisting, or wagging [5].

![Different types of vibrational modes](image)

Figure 2-2 Different types of vibrational modes [5]
2.1.3 Inductively coupled plasma optical emission spectroscopy (ICP-OES)

Operator: Sankalp Agarwal

ICP-OES determine trace elements in the different type of samples. In this study, ICP-OES was used to determine the rate of Mg\(^{2+}\) ions release from the uncoated and coated AZ31 substrates in the electrolyte for different test durations.

In ICP-OES, the liquid sample is introduced into the nebulizer chamber along with argon (Ar) gas through the small aperture in the form of tiny droplets that form a mist of μm-sized particles (Fig 2-3). Larger droplets are removed through a drain, while smaller particles travel with the Ar flow and enter the torch. The torch unit of an ICP creates a plasma. At the core of the inductively coupled plasma, the temperature can reach 9000 °C. At this temperature, the sample undergoes evaporation, atomisation, and excitation/ionisation. The plasma-induced excited/ionised atoms to relax and emit photons of characteristic energy for a particular atom or ions. Thus, the wavelength of photons can be used to identify an element and quantified [6].

Figure 2-3 Overview of ICP-OES
Chapter 2

The inductively coupled plasma torch

The torch unit of an ICP produces a sustained plasma. The RF generator is used to set up an electric and magnetic field inside the torch to produce a mixture of cations and electrons through collision. Since the energy adds up to the plasma by RF-induced collision, it is known as inductive coupling and hence the plasma is known as ICP [7]. So, the function of the torch is to (1) evaporate the solvent (water), (2) atomise the atoms (break the ionic bonds and form gaseous state atoms), and (3) excite or ionise the atoms [8].

2.2 Microscopy

A microscope is used to magnify objects by means of lenses that are not visible to the naked eye. In general, microscopy involves the refraction, reflection and diffraction of electromagnetic radiation/electron beam by the specimen and their collection as scattered radiation or signal to create an image. There are different types of microscopes with resolution ranging from $10^{-4}$ m to $10^{-12}$ m [9].

In this study, two different types of microscopes were used. Scanning electron microscope with EDX was used to observe cell morphology, surface morphology and elemental composition of the AZ31 Mg substrates. Fluorescence microscopy was used to observe live and dead osteoblast cells, which are represented by green and red fluorescence respectively.

2.2.1 Scanning electron microscope (SEM)

Operator: Sankalp Agarwal

In scanning electron microscopes electrons are emitted from a metal filament tip and are then accelerated under an applied potential towards the specimen to produce the emission signals. The emission of electrons from the tip can be due to thermionic, field emission
or a combination of both. Modern electron microscopes utilise field emission sources due to the enhanced brightness and emission current stability achievable over thermionic sources. Field emission of electrons from the tip occurs due to the influence of a strong electric field (10⁹ V/m) electrons.

Using a series of lenses and apertures a fine probe of electrons with energies typically up to 30keV is focused on a specimen and scanned along a pattern of parallel lines, Figure 2-4. The generated signals are collected to form an image or to analyse the sample surface. Under vacuum, electrons generated by a field emission source are accelerated in a field gradient. The beam passes through electromagnetic lenses, focussing onto the specimen. As a result of this bombardment, the different types of electrons are emitted from the specimen. A detector catches the emitted electrons and an image of the sample surface is constructed by comparing the intensity of these secondary electrons to the scanning primary electron beam [9]. Finally, the image is displayed on a monitor.

Figure 2-4 SEM instrument (Hitachi SU70) and schematic working of SEM
A major advantage of SEM is that most samples, with the exception of biological samples, can be studied with little preparations. In general, it is important that the sample should be electrically conductive, so that incident electrons are earthed. The accumulation of electron on a non-electrical conductive surface causes a charge build-up, which degrades the SEM image [10]. In order to avoid such problems, conductive materials are used to coat the specimens such as gold, gold/palladium alloy, platinum, iridium, tungsten, chromium and graphite either by low vacuum sputter coating or by high vacuum evaporation [10].

In combination with the Energy Dispersive X-Ray Spectroscopy, elemental quantification of a given sample can be analysed (Fig 2-5). The depth of the electron penetration into the sample will emit a variety of electron/x-rays as shown in Fig 2-5 (a). These, in turn, will depend on the properties of substrates and thus can be used for the characterisation [11].

**Figure 2-5. (a) Interaction of electron beam with specimen and (b) Example of EDX elemental map of sol-gel silane coated Mg alloy.**

EDX works by detecting the X-rays that are produced by the interaction of an electron beam with a sample. Upon interaction, the atoms of the samples are excited by the electron beam, thereby producing X-rays to discharge the excess energy [11]. This energy of X-rays represents emission intensity which is proportional to the concentration of the
elements in the particle and plot the distribution of the identified elements. DOT MAPs can be created from the spectra where a coloured pixel is assigned to the image if a certain element is detected within the segment of the sample. A chemical map can then be constructed for the sample being analysed as seen in Fig 2-5 (b).

2.2.2 Fluorescence microscopy

*Operator: Dr. Marie-Noelle Labour*

Fluorescence microscopy is a form of optical microscopy which uses fluorescence to reveal the structure of fixed and living biological specimens instead of absorption, phase or interference of light [12]. In this microscopy, the specimen is either tagged with a fluorescent probe or itself possesses the property, is illuminated with an excitation wavelength, causing them to emit light of longer wavelengths (emission wavelength). The illuminated light is separated from the emitted light through an emission filter to observe the fluorescent specimens [13].

![Fluorescence microscopy setup](image)

**Figure 2-6 (a)** Typical setup and working of fluorescence microscopy and (b) Fluorescence image of live-dead osteoblast cells on Ti substrate.

Typically, basic components of a fluorescence microscope are a light source (mercury-vapour lamp or xenon arc lamp), the excitation filter, dichroic mirror and emission filters (Fig 2-6(a)). These filters are chosen to match the excitation and emission characteristic
of fluorophore-tagged to the specimen [12]. Multi-colour fluorescent images can be generated by superimposing many single-colour images as presented in Fig 2-6 (b) [12].

2.2.3 Atomic force microscope

Operator: Dr. Luke O’Neil

AFM is a powerful technique used for the topological studies in x, y as well as z-direction at a sub-micro scale. It utilizes a silicon or silicon nitride tip at the end of a cantilever that moves over the sample in a raster scan and bends in response to the force between the tip and the sample (Fig 2-7) [14]. AFM can measure various forces which include mechanical contact force, van der Waals forces, capillary forces, chemical bonding, electrostatic forces, magnetic forces etc [15].

Figure 2-7 Schematic working of AFM [1]

Originally a scanning tunnelling microscope was used to monitor the bending of the cantilever, but now, an optical lever technique is employed for the purpose. As the cantilever bends, light from the laser is reflected onto the split photo-diode. The difference in the signal is used as a measure of the bending of the cantilever. A device made of
piezoelectric ceramic (PZT) in the form of the scanner is used to control the movement of the tip or the sample. The scanner is capable of sub-angstrom-level resolution in the x, y or z-direction [15]. Depending upon the application AFM commonly operated in contact and tapping modes [16]. The most commonly used method is the contact mode AFM where the tip remains in close contact with the sample during the process of scanning. The tapping mode is generally used for the imaging of soft samples which are poorly immobilized. In this mode, the tip oscillates at its resonating frequency and is positioned over the sample so that it contacts the sample for a short time interval during oscillation. AFM has many advantages over electron microscopy that it's a non-destructive method and can be performed in both air and aqueous environment to examine the surface topography at sub-nanometer resolution. As the sample does not need to be in a vacuum for analysis, live bacteria can be viewed in situ.

2.3 In vitro corrosion testing techniques

2.3.1 Potentiodynamic polarisation

This technique is a commonly used method to measure Mg corrosion. In the study, this method was used to study the electrochemical corrosion of AZ31 modified substrates in the electrolyte (cell culture medium). The potentiodynamic polarisation involves sweeping a potential to increase the reaction at the working electrode surface (samples) and record the resulting current [17]. Therefore, results from this technique provide information about the electrochemical behaviour of a metal in a given electrolyte. The potentiodynamic polarisation is representing in a plot of potential versus current density (E vs log i) which can be divided into oxidation (anodic) and reduction (cathodic) reactions, depending upon the polarity of the polarising signal (Fig 2-8). From the co-ordinate of the point where the linear region of each reaction meets, \( E_{corr} \) and \( i_{corr} \) values
can be analysed, and this method is referred to as Tafel-plot analysis. The schematic polarisation curve is shown by Tafel plot (Fig 2-8). The dotted line is the accumulated data and the solid line represents linear approximations, which can be used to estimate current density ($i_{corr}$) and corrosion potential ($E_{corr}$) by Tafel fit analysis. The current density obtained from Tafel fit is proportional to the corrosion rate of the substrate.

Figure 2-8 Polarisation curve with Tafel fit [17].

The potentiodynamic test is performed, once the open circuit potential (OCP) stabilises. The potential at which the current balances the cathodic and anodic reaction is said to be OCP. OCP can be recorded between the reference and working electrodes without passing current through the counter electrode, but a long time may corrode the Mg surface [18, 19].

2.3.2 Electrochemical impedance spectroscopy

The EIS technique is based on the mechanism and properties of the electrical double layer (EDL) [17, 20]. It forms on the structure surface when it is placed in a solution. (Fig 2-9(A)). More specifically in Mg metal, this phenomenon occurs when metal ions diffuse away from the surface under the influence of water molecules and leave the negatively charged electrons behind (Fig 2-9(B)). This negatively charged surface begins to attract
the released ions. These ions along with positively charged ions in the electrolyte are attracted to the negative surface charge. Consequently, an electrolyte layer consisting of metal and electrolyte ion form next to the surface is referred to as EDL (Fig 2-9(C)).

During the experiment, polarisation is applied which forces the ions to move either to the working or counter electrode, in order to maintain electrical neutrality. This perturbs the EDL equilibrium and causes the layer to establish a new equilibrium as the AC potential frequency changes ($10^5$ to $10^{-2}$ Hz). In a corrosion resistant surface, the EDL will be less active, indicating an increase in impedance $|Z|$. The impedance has an equivalent physical meaning as the resistance, which changes with the angular frequency of the applied voltage. EIS data can be expressed in three common types Nyquist plots, Bode magnitude plots and Bode phase plots.

Figure 2-9 Electrical double layer formation- (A) Surface in the electrolyte, (B) release of ions in the electrolyte and (C) positively charged ions attracted to surface
Figure 2-10 (A) Nyquist plot- W is the angular frequency (Hz), Rs is the electrolyte resistance (Ω), REDL and CEDL are the charge transfer resistance and capacitance, and (B) Represents bode plots.

The Nyquist or complex plot presents the impedance magnitude for the real ($\text{Re}(Z)$) and imaginary ($\text{Im}(Z')$) frequency. It is possible to fit an appropriate equivalent circuit to determine the number of time constants, resistance and capacitance (Fig 2-10A). However, this plot does not display the dependence of impedance on the frequency of the signal. To relate the impedance with frequency, it is usually necessary to show Bode plots, in which the magnitude of impedance ($\log |Z|$) and phase is plotted against the log values of corresponding frequencies (Fig 2-10B). From the Bode plot, the corrosion behaviour of metal can be assessed by the value of $|Z|$. Higher values of impedance $|Z|$ imply the substrate is more corrosion resistant [21].

2.3.3 Hydrogen evolution measurement

Hydrogen evolution ($H_2$ gas) testing is an inexpensive and reliable technique, which has been widely adopted to study the corrosion behaviour of Mg and its alloys [22-25]. It involves the volumetric measurement of $H_2$ gas release from a corrosion site. In this method, burette filled with the test electrolyte placed on the top of the sample. The $H_2$ gas generated during corrosion reaction accumulates in the burette and displaces the solution.
inside the burette (Fig 2-11). This provides a direct correlation of the volume of H₂ gas produced. According to Eq-2, 1 mol of corroded Mg will produce in 1 mol of H₂ (g). However, the stoichiometry of the redox equation, producing elemental hydrogen is not fully understood and therefore not 100% efficient. Therefore, the volume of H₂ (g) produced does not directly correlate to the Mg ions. In addition, this method has several disadvantages such as leakage of H₂ due to experiment setup and changing atmospheric pressure.

**Figure 2-11 H₂ gas evolution test setup**

Immersion testing is another method to determine the rate of corrosion derived by the mass loss of Mg substrates [26]. The mass loss is determined by weighing the sample before and after the test (Eq. 7). Alternatively, the release of Mg²⁺ ions released in the electrolyte can be quantified by ICP-OES.

\[
\text{Corrosion rate} = \frac{\text{mass loss}}{(\text{surface area x day})} \quad \text{Eq. 7}
\]
2.4 *In vitro* assessment of cell cytotoxicity

2.4.1 MTT assay

The MTT assay is a colourimetric method for determining the cell metabolic activity [27]. This assay can be used to measure the cell cytotoxicity (loss of viable cells) or cell proliferation (increase number of viable cells) of potential cytotoxic and bioactive agents. The MTT assay involves measuring the activity of NAD(P)H-dependent cellular oxidoreductase enzymes under the defined condition, reflecting the presence of viable cells. These enzymes reduce the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to its insoluble purple coloured formazan crystals. (Fig 2-12). These crystals can be dissolved in dimethyl sulfoxide (DMSO), a solution of sodium dodecyl sulfate in the diluted HCl or an acidified ethanol solution. The absorbance of the coloured solution can be determined by spectrophotometer. In this study, the cytotoxicity of different concentration of Mg²⁺ ions on MC3T3E1 osteoblast cell was evaluated by MTT assay.

![MTT and Formazan](image)

**Figure 2-12** Mitochondrial reduction of MTT to blue Formazan product [27]

2.4.2 DNA quantification by fluorescence assay

There are several methods to quantify the double stranded DNA (dsDNA), however, some have limitations such as interfering absorbance of contaminating molecules nucleotides,
RNA and phenol. The fluorescent bisbenzimide (Hoechst) dye circumvents many of these problems [28-31]. It works with purified and crude DNA extract. However, the quantification of DNA in the crude extract requires high salt concentration. The Hoechst 33258 fluorescent itself in the solution loosely binds to A-T pairs of dsDNA of minor groove, resulting in the increase of fluorescence emission at 460 nm. This assay is sensitive for a concentration of 10 ng/ml to 10 μg/ml of DNA.

2.4.3 Alkaline phosphatase assay (ALP assay)
Alkaline phosphatase plays an important role in the hard tissue formation which is highly expressed in the mineralised cells [32]. It is an early stage marker of MC3T3E1 osteoblast cell differentiation. The mechanism by which this enzyme performs its function is still not completely understood [32]. It appears that this membrane-bound enzyme acts by increasing the localised concentration of inorganic phosphate, a mineralisation promoter, and decreases the extracellular concentration of pyrophosphate, an inhibitor of mineralisation. In this study, the differentiation of osteoblast cells was determined by measuring the intracellular ALP activity using a para-nitrophenyl substrate (p-NPP). ALP enzyme in the crude cell lysate hydrolyses the p-NPP to produce chromogenic para-nitrophenol, which corresponds to the ALP activity of differentiated osteoblast cells (Fig 2-13) [33].

![Reaction of ALP activity assay](image)

**Figure 2-13 Reaction of ALP activity assay**
2.5 *In vitro* assessment of antibacterial activity

2.5.1 Spread plate assay

In this study, antibacterial activity and efficiency of bacterial cell adhesion were determined by spread plate assay. In this method, the surface was exposed to the bacteria for a specific time period and incubated at 37 °C. Then, the adhered or viable bacteria on the surface were dislodged using ultrasound in PBS and serially diluted, before spreading on the agar plate and incubated overnight at 37 °C [34]. The number of colonies represents viable count and expressed as colonies forming unit/ml (Eq. 8) [34]

\[
\text{Viable count} = \frac{\text{Number of colonies}}{\text{volume plated (ml) \times dilution factor}} ....\text{Eq. 8}
\]

2.6 References

Chapter 3

Improved corrosion protection and biocompatibility of PLGA-silane coating on AZ31 Mg alloy for orthopaedic applications

This study reports a multi-step procedure to fabricate novel corrosion resistant and biocompatible PLGA-silane coating on the magnesium (Mg) alloy AZ31. The first step involves alkaline passivation followed by the dip coating in a methyltriethoxysilane (MTES) and tetraethoxysilane (TEOS) mixture to produce a crosslinked siloxane coating. The second step is to impart an amine functionalization to the silane-modified surface by using 3-aminopropyl-triethoxy silane (APTES) for promoting adhesion of the acid terminated poly-(lactic-co-glycolic) acid (PLGA) as a final coating step. Static contact angle measurements, Fourier transform infrared spectroscopy and scanning electron microscopic analysis confirmed the successful assembly of coatings on the AZ31 Mg alloy. An electrochemical corrosion study, measurement of magnesium ion release, pH changes and hydrogen evolution showed enhanced corrosion protection of coated substrate over uncoated AZ31 Mg alloy. An MTT assay, live-dead cells staining, DNA quantification and alkaline phosphatase activity assay were used to measure the biocompatibility, proliferation and differentiation of MC3T3-E1 osteoblast cells. The application of such coatings on the biodegradable Mg alloys enhanced their corrosion resistance and biocompatibility.

3.1 Introduction

Conventional metals such as 316L stainless steel, titanium and cobalt-chromium based medical grade inert alloys are used as permanent orthopaedic implants in order to immobilize fracture and restore damaged bone [1]. Over the past decade, there has been increasing interest to replace these permanent alloys with biodegradable magnesium (Mg) and its alloys which not only degrade under in vivo conditions but also exhibit low toxicity and similar mechanical properties to natural bone [2]. Research to date has shown that Mg and its alloys can be potentially used for orthopaedic fracture fixation such as fixation plates, rods and screws. Such temporary implants can remain for the appropriate time to support the bone fracture recovery before degrading and being absorbed by the body. Biodegradable metallic implants reduce the complications associated with permanent implants such as a potential inflammatory response, physical irritation and implant removal surgery [3]. However, the potential clinical applications of Mg-based alloys have been delayed due to their rapid electrochemical dissolution under physiological conditions, resulting in the elevated release of hydrogen gas and pH changes, which create a bio-incompatible environment to the surrounding tissue [3]. In addition, rapid degradation can lead to the early loss of mechanical stability of Mg-based implants, thereby structural failure before the healing process has progressed sufficiently [2]. Therefore, it is important to have controlled degradation in tandem with the improved biocompatibility of Mg alloys for the orthopaedic implant.

Several strategies have been employed to improve the corrosion resistance and biocompatibility of Mg alloys such as alloying elements, passivation and surface coatings [4]. Variation of the alloying elements may improve the original mechanical properties and enhance the corrosion resistance but are not likely to improve the biocompatibility of Mg alloys. These elements may induce cytotoxicity depending on the locally released
concentration or systemic accumulation of alloying elements [4]. Apart from alloying, surface modifications or passivation of Mg alloys using other routes such as alkaline hydrothermal treatment, micro-arc oxidation (MAO) and ion implantation have been investigated to improve the corrosion resistance [2]. However, these surface modifications alter the mechanical properties and are unable to protect the Mg alloys over extended periods in the corrosive environment [5, 6]. Therefore, surface coatings are now being investigated as an effective approach with the potential to improve the corrosion resistance and biocompatibility of Mg alloys.

Silanes and biodegradable aliphatic polyester coatings have been widely used for enhancing the corrosion resistance and biocompatibility of Mg and its alloys [7, 8]. Organosilanes are materials with the general formula R’ (CH\textsubscript{2})\textsubscript{n} Si (OR)\textsubscript{3}, where R and R’ are hydrolysable alkoxy and organic functional groups respectively [9]. When organosilanes are hydrolysed with water, silanol groups (Si-OH) form facilitating the attachment with hydroxy-activated metal (M-OH) via the formation of thermal and chemically stable Si-O-M bond [8, 10]. The silanol group can also undergo cross-linkage via Si-O-Si siloxane bond, resulting in the formation of a protective layer chemically attached to metal [8]. The utilization of organosilane based coating have been widely explored to enhance the corrosion resistance of Mg-based alloy for biomedical implants applications [11, 12]. However, to date, these coatings have not been shown to improve the biocompatibility of the implant which affects the osseointegration and bone growth. Coatings of biodegradable aliphatic polyesters have previously been used for improving the biocompatibility as well as corrosion resistance of Mg-based alloys [13]. Amongst several aliphatic polyesters, poly(lactide-co-glycolide) (PLGA) coatings are FDA-approved for use on implants. The polymer displays good biocompatibility and is widely used as a delivery vehicle for bioactive molecules as its by-products are metabolically
digestible and provide some corrosion resistance [13]. Therefore, there is an opportunity to combine chemistries as a multifunctional corrosion-resistant biocompatible coating on Mg-based alloys.

In this study, a novel multi-layer PLGA-organosilane coating on AZ31 Mg alloy was developed. To the best of the authors’ knowledge, this is the first report where organosilanes and the aliphatic polyester (PLGA) are used together to develop corrosion resistant and biocompatible coatings on Mg alloys. The organosilanes explored for this study were (MTES-TEOS) and APTES. The MTES-TEOS organosilane corrosion resistant coatings have been previously employed on stainless steel and Mg alloys [14-16][44]. The APTES treatment on MTES-TEOS coated surface provides an amine terminated surface which interacts electrostatically with the acid terminated PLGA coating (Fig 3-1).

**Figure 3-1** A schematic representation is showing the steps involved in PLGA-silane coating on AZ31 Mg alloy. The alkaline passivated AZ31 alloy (step- a) was coated sequentially with MTES-TEOS (step- b), APTES (step- c) and PLGA (step- d) to improve the corrosion resistance and biocompatibility of AZ31 Mg alloy.
The organosilane and PLGA coating enhances the corrosion resistance due to the formation of Si-O-Si bonds and PLGA as a physical barrier which together prevents electrolyte penetration to the AZ31 Mg alloy. Furthermore, improved biocompatibility of the coated substrate over uncoated AZ31 has been observed with the murine osteoblast cells.

3.2 Experimental

3.2.1 Materials

AZ31 alloy sheets were obtained from Shaanxi Taipu Rare Metal Materials Ltd, China and composition are confirmed by SEM EDX (Table 6). Methyltriethoxysilane (MTES) and tetraethoxysilane (TEOS), 3-aminopropyl-triethoxy silane (APTES), poly-(lactic-co-glycolic) acid (PLGA) mol wt 30,000-60,000, phosphate buffer saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Penicillin-streptomycin antibiotics, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich. Milli-Q water was used to prepare solutions.

Table 6 Composition of AZ31 Mg alloy determined by SEM-EDX

<table>
<thead>
<tr>
<th>Elements</th>
<th>Mg</th>
<th>Al</th>
<th>Si</th>
<th>Ca</th>
<th>Mn</th>
<th>Fe</th>
<th>Cu</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>At. Weight%</td>
<td>96.37</td>
<td>2.45</td>
<td>0.1</td>
<td>0.03</td>
<td>0.22</td>
<td>0.05</td>
<td>0.05</td>
<td>0.74</td>
</tr>
</tbody>
</table>

3.2.2 Surface modifications of AZ31 Mg alloy

3.2.2.1 Treatment of AZ31 with NaOH

AZ31 Mg alloy 2 mm thick sheets were cut into 20 x 30 mm or 10 x 20 mm pieces and polished progressively by finer SiC paper from 400 to 1200 grit. The samples were cleaned ultrasonically in acetone for 30 mins and air dried. These polished samples were
immersed in 5 N NaOH for 2 h at 60 °C and then cleaned with deionised water [17]. These hydroxide-treated AZ31 are referred to as AZ31-OH.

3.2.2.2 Preparation of silane and PLGA coatings on AZ31 Mg alloy

The MTES and TEOS sols were prepared in the ethanol solution containing 0.04 N nitric acid as a catalyst [44]. A molar ratio of MTES/TEOS of 2/1 was used with R=2.3 (where, R = [H₂O]/ [MTES+TEOS]). The MTES-TEOS sol was clear and stable having a viscosity of 1.63 mPa at room temperature (Table 7). The APTES sol was prepared using 400 mM APTES in ethanol solution with R = 4.5 (R = [H₂O]/ [APTES]) with a viscosity of 1.82 mPa at room temperature (Table 8). The pre-treated AZ31 substrates (AZ31-OH) were dip-coated in the MTES-TEOS sol, dried with hot air (heat gun) and cured at 120 °C for 1h. Subsequently, MTES-TEOS treated AZ31(AZ31-MT) substrates were dip coated in the APTES sol and cured at 120 °C for 1 h to achieve the amine-terminated AZ31-MT-A substrates. The PLGA coating was prepared by dipping the AZ31-MT-A substrates in 2% and 4 %w/v carboxylic acid-terminated PLGA solution in dichloromethane (Figure 3-1). The resultant substrates were denoted as AZ31-MT-A-P. These two concentrations of PLGA were screened to optimise corrosion protection of AZ31 Mg alloy. To get the maximum coating thickness, the withdrawal speed of the dip coating was fixed at 80 mm/min (max. speed of dip coater)

Table 7 Formulation of MTES-TEOS sol-gel

<table>
<thead>
<tr>
<th>Sol-gel</th>
<th>MTES (ml)</th>
<th>TEOS (ml)</th>
<th>HNO₃ (0.04N) (ml)</th>
<th>Ethanol (ml)</th>
<th>Total volume (ml)</th>
<th>Stirred at room Temperature overnight</th>
<th>R=[H₂O]/[MTES-TEOS]</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTES-TEOS-2:1</td>
<td>16</td>
<td>9</td>
<td>5</td>
<td>20</td>
<td>50</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Molarity</td>
<td>1.6</td>
<td>0.8</td>
<td>5.5</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

69
Table 8 Formulation of APTES sol-gel

<table>
<thead>
<tr>
<th>Sol-gel</th>
<th>APTES (ml)</th>
<th>H₂O (ml)</th>
<th>Ethanol (ml)</th>
<th>Total volume (ml)</th>
<th>Stirred at room Temperature For 1 h</th>
<th>R = [H₂O]/[APTES]</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTES</td>
<td>4.7</td>
<td>1.7</td>
<td>43.6</td>
<td>50</td>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td>Molarity</td>
<td>0.424</td>
<td>1.88</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.3 Characterisation of AZ31 modified samples

All modified (AZ31-OH, AZ31-MT, AZ31-MT-A and AZ31-MT-A-P) and uncoated AZ31 substrates were investigated using attenuated total reflection infrared spectroscopy (ATR-IR). The IR spectra were recorded over a scan range of 4000 to 600 cm⁻¹. The wettability of samples was determined by static water contact angle measurements (FTÅ-200 system) using the sessile drop method (2µl, milli-Q water) [12]. The surface morphology of coatings was studied by scanning electron microscope (Hitachi SU-70 FESEM with EDX and WDX). The chemical compositions of the coatings were studied using energy-dispersive X-ray spectroscopy (EDS, Oxford Instruments). To determine the thickness of AZ31-MT-A-P coatings by SEM, samples were embedded in an epoxy resin which was cured, progressively ground (80-1,200 grit) and finally polished (9, 6 and 1µm) to a smooth surface. Before SEM and EDX analysis, all samples were coated with Au-Pd sputter coating system (Cressington 208HR Sputter Coater. The adhesion of PLGA-silane coating was determined by Crosshatch tape test (ASTM F2452). Firstly, the samples were subjected to cross cut (1 mm lattice) by a sharp blade. The specified tape was placed over crosscut and pressed the tape down onto the surface with sufficient pressure. After 10 to 30 seconds, tape was pulled from the surface and check for the delamination of the coating [46].
3.2.3.1 Electrochemical corrosion measurements

All in vitro degradation tests were performed at 37 ± 0.5 °C in DMEM. Electrochemical measurements were carried out by using 1285 Solartron potentiostat equipped with a 1287 frequency analyser with a standard three-electrode setup comprising of a saturated calomel electrode (SCE) as a reference electrode, a platinum mesh auxiliary electrode, and the test sample (with 1 cm² exposed area) as a working electrode. Potentiodynamic polarization curves were recorded with a scan rate of 5 mv/s. Corrosion current density and corrosion potential were determined using Corrview software. Electrochemical impedance spectra (EIS) of all the samples were conducted over a range of 100 KHz to 0.01 Hz at the measured open circuit potential with an AC amplitude of 10 mV. A stable open circuit was established prior to conducting the electrochemical measurements. This is the maximum frequency range can be applied by the above-mentioned instrument. The high and low frequencies will allow to study the mass and charge transfer electrochemical reactions occur during the corrosion of modified and uncoated AZ31 Mg alloy substrates. The equivalent electrical circuits (EECs) were designed using Zview software. First, values for each parameter of the circuit was determined using standard circuits available in the software.

3.2.3.2 Immersion experiment, pH changes and Hydrogen evolution measurement

In order to determine the magnesium ion (Mg²⁺ ion) concentration and pH of the medium, the coated and uncoated AZ31 substrates were immersed in DMEM with a surface-to-volume ratio of 20 ml. cm⁻² at 37 ± 0.5 °C for 21 days [18]. The Mg²⁺ ion release experiment was conducted as per ASTM G31 standards [18]. The pH value (Thermo-scientific Orion 3 Star pH meter) of DMEM was monitored at different immersion periods. The release of Mg²⁺ ions in DMEM at the different periods was determined using an inductively coupled plasma optical emission spectrometer (Varian Liberty150, ICP-
The immersion medium was refreshed every 2nd day to maintain a stable composition. The changes in the surface morphology and the composition of the coated AZ31-MT-A-P substrate after 7, 14 and 21 days were determined by SEM-EDX and ATR-IR.

The evolution of hydrogen gas was monitored by placing the samples in DMEM at 37 ± 0.5 °C under an inverted funnel connected to graduated burette and measuring the medium level up to 14 days. The ratio of the surface area-to-the volume of DMEM was kept constant at 10 cm². L⁻¹ [19, 20].

3.2.4 Cytocompatibility

3.2.4.1 Indirect cell viability test

The MTT assay was used to determine the cytotoxicity of surface modified AZ31 samples. The test was performed by using an indirect method, where the extracts were used for culturing the cells. Initially, each face of the samples was sterilised by UV radiation for 40 minutes. This sterilisation procedure was followed for all the cell culture experiments conducted in this PhD work. The samples (10 x 20 mm) were placed in serum and antibiotic free DMEM at 37 °C in a 5% CO₂ humidified atmosphere for 72 h with a surface area/extract medium ratio 1.25 ml. cm⁻² in accordance to ISO 10993-12 [21]. The extracted medium was centrifuged (Eppendorf) at a relative centrifugal force (RCF) of 15294 g for 10 minutes and the supernatant was filtered through a 0.22 μm filter (Millipore, Merck). All extracts were stored at 4 °C for further use.

The murine pre-osteoblast cell line (MC3T3-E1) (passage number between 10 to 20) was utilized for all in vitro experiments including cell cytotoxicity study. This cell line has been used as a model system in bone biology. Cells were cultured under 37 °C, 5% CO₂ and 95% relative humidity in DMEM containing 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were seeded in 96 well-plates with the cell density of
10^4 cells.ml⁻¹ and incubated for 24 h. After 24 h, the culture medium was replaced with the extracted medium of different dilutions (50%, 25% and 10%) added with 10% FBS, 1% penicillin and streptomycin (P/S) and incubated for 1, 3 and 5 days. Culture mediums with 10% DMSO and without extracted medium were considered as positive and negative controls respectively. After the incubation, the extracted medium was replaced with fresh culture medium containing MTT dye (100 μl serum-free medium with 25 μl of 5 mg.ml⁻¹ MTT dissolved in PBS) for 4 h to prevent the interference of magnesium ions with the tetrazolium salt [21]. The culture medium with MTT was discarded after the incubation and 100 μl of DMSO was added to each well to dissolve the formazan crystal. The absorbance of the samples was recorded using the Synergy multi-mode microplate reader at a wavelength of 570 nm. To determine the percentage cell viability, the following equation was used:

\[
\text{Percentage cell viability (%) = \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{negative control}}} \times 100}
\]

3.2.4.2 Live/dead cell staining

Cells were seeded (2.5x 10^4 cells.cm⁻²) and maintained as described earlier for 1, 3 and 5 days before the live-dead staining was performed. Calcein AM (eBioscience 65-0853) and propidium iodide (eBioscience 00-6990) were dissolved in DMSO (1mg/ml) and used at a 1:500 ratio in DMEM containing 10% FBS and 1% Penicillin/Streptomycin (P/S). Cells cultured on the experimental substrates were washed with PBS and incubated in a fluorescent dye for 20 minutes at 37 °C. After incubation, the cells were rinsed with PBS and imaged using an Olympus IX83 epifluorescence microscope fitted with a 10X objective lens. For each substrate, images were taken from two to four sites to determine cell adhesion.
3.2.4.3 Cellular morphology imaging by SEM

The morphology of osteoblast cells on coated and uncoated AZ31 Mg alloy substrates was observed after 1, 3- and 5-day(s) culture. Osteoblast cells at a density of 2.5x 10^4 cells.cm^2 were seeded onto the substrates (10 x 20 mm) in complete DMEM medium (1cm^2 = 1.25 ml). The medium was refreshed every day. After incubation, samples were washed with PBS, fixed with formalin and dehydrated in alcohol gradients. The samples were placed on aluminium stubs and sputter coated with Au-Pd and observed in SEM. For each substrate, images were taken from at least three sites on to determine the cell adhesion and morphology.

3.2.4.4 DNA quantification

The DNA quantification of MC3T3E1 osteoblast cells was assessed as a measure of cell proliferation. Cells were cultured onto the coated and uncoated AZ31 substrates at a density of 2.5x 10^4 cells.cm^2 for 3, 7 and 14 days in a differentiation medium. The differentiation medium was prepared by adding 50 µM ascorbic acid, 100 nM dexamethasone and 10 mM β-glycerophosphate in DMEM. After 3, 7 and 14 days of culture, substrates were rinsed with PBS and cells were lysed by using cell lysis buffer (10mM Tris-HCl, 0.5 mM MgCl_2, 0.1% Triton X-100 at pH 7.4). The fluorescent dye, bis-Benzimide H 33258 (Hoechst 33258, Sigma Aldrich) was used to measure the cellular DNA according to manufacturer’s instruction (DNA quantification kit, Fluorescence assay, Sigma Aldrich). Calf thymus DNA was used for the standard curve and fluorescence level was measured using a Spectramax-M3 microplate reader (Molecular Devices).

3.2.4.5 Alkaline phosphatase (ALP) assay

The differentiation of osteoblast cells was determined by measuring intracellular ALP activity using a para-nitrophenyl substrate (p-NPP, Sigma). After 3, 7- and 14-days cells
were cultured in the differentiation medium and then were lysed and incubated with p-NPP substrate for 1 h at 37 °C. The enzymatic reaction was stopped by adding 1N NaOH and the ALP activity was determined by measuring the absorbance of para-nitrophenol (p-NP) at 405 nm wavelength using the microplate reader. A standard curve of different p-NP concentrations was prepared by diluting in 0.02N NaOH. The chromogenic para-nitrophenol product formed was normalised against total protein concentration and the total protein content was measured using the bicinchoninic acid (BCA) protein assay kit (Sigma Aldrich).

3.2.5 Statistical analysis

All the experiments were carried out thrice in triplicates. All data are expressed as mean ± S.D. The differences between the groups were analysed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Statistical significance was considered at **p < 0.01 and *p < 0.05.

3.3 Results and Discussion

3.3.1 Characterisation of surface modified AZ31 Mg alloy

The ATR-IR spectrum of the surface modified AZ31 Mg alloys are shown in Fig 3-2. To facilitate the organosilane coating, the AZ31 Mg alloy was passivated with sodium hydroxide to generate hydroxide layer (AZ31-OH) on the surface which is indicated by the band at 3694 cm⁻¹ [17]. The MTES-TEOS coating on the passivated substrate was evident by IR bands around 1045-1127 cm⁻¹ and 1270 cm⁻¹, corresponding to Si-O asymmetric stretching of Si-O-Si bond [22] and absorption vibration of Si-CH₃ of MTES respectively [14]. Subsequent coating with APTES increases the intensity of siloxane band with an appearance of a new band at 1570 cm⁻¹, which is assigned to the vibrational mode of amine group [22].
Figure 3-2 ATR- IR of uncoated and AZ31 modified substrates.

The bands at 3295 cm\(^{-1}\) and 3368 cm\(^{-1}\) are related to the asymmetric and symmetric stretching modes of NH\(_2\) group respectively [23]. Broadening of the band at 1570 cm\(^{-1}\) was observed when the amine-functionalised silane substrate was coated with PLGA and most of the amine peaks became obscured, indicating the presence of an electrostatic interaction occurred [24, 25]. The presence of the Si-CH\(_3\) band (1270 cm\(^{-1}\)) in all coated substrates indicates the presence of methyl groups which imparts some hydrophobicity to the coating [16, 26].

Fig.3-3 shows the effect of the surface modifications observed by the surface wettability of AZ31 modified substrates.
One way ANOVA is followed by post-hoc Tukey test with a significance level of ** $p < 0.01$ with respect to AZ31 alone.

The bare AZ31 surface presents with a water contact angle of 74.6 ± 0.9°. The treatment with NaOH increases the surface hydrophilicity of bare AZ31 due to the presence of a hydroxyl layer with a water contact angle of 29.1 ± 1.8°. As expected, the MTES-TEOS coating increases the surface hydrophobicity due to the presence of the methyl groups and siloxane bond with a water contact angle of 77.9 ± 1.3° [14, 22]. Subsequent coating with APTES (AZ31-MT-A) decreases the contact angle to 46.4 ± 2.1° which is attributed to the presence of the amine group on the surface [12]. Further modification with PLGA re-establishes the relative hydrophobicity with an increase in water contact angle to 68.2 ± 1.5°. It can be observed that the surfaces are significantly different ($p < 0.01$) at each stage of the functionalization. These results along with ATR-IR analysis confirm the sequential functionalization of the AZ31 alloy coating.

Fig 3-4, shows the SEM images of uncoated and coated substrates (a) AZ31 (polished), (b) AZ31-MT, (c) AZ31-MT-A, (d) AZ31-MT-A-P and (e) cross-section of AZ31-MT-A-P.
Chapter 3

Figure 3-4 SEM images of (a) AZ31, (b) AZ31-MT, (c) AZ31-MT-A, (d) AZ31-MT-A-P and (e) cross section of AZ31-MT-A-P.

The coated substrates (Fig 3-4: (b)-(d)) showed a homogenous and crack-free surface. In addition, delamination of coatings was not observed on the AZ31 coated surface. Fig 3-4 (e) shows the cross-section of the AZ31-MT-A-P substrate with the coating thickness of 1.61 µm.

Furthermore, adhesion of coatings with the AZ31 Mg substrate was determined by the cross-hatch tape test and the photographs of the coated specimen after the tape adhesion shown in Figure 3-5. The silane coating adheres well with the surface without peeling off (Fig 3.5 (b)), which meets the standard for the tape adhesion test (Coating retention > 99%: Met the requirement) [46].

Figure 3-5 Optical images of AZ31-MT-A (a) before and (b) after the cross-hatch test.
Therefore, the combined results of ATR-IR, contact angle measurement and SEM analysis confirm the successful assembly of PLGA-silane coating on the surface of AZ31 substrates.

3.3.2 *In vitro* corrosion and degradation behaviour of AZ31 substrates

3.3.2.1 Electrochemical corrosion measurements

Figure 3-6 shows the potentiodynamic plots for the uncoated and coated AZ31 alloys in DMEM at 37 ºC.

![Potentiodynamic polarization curves](image)

**Figure 3-6** Potentiodynamic polarization curves of AZ31 alone, AZ3-0H, AZ31-MT, AZ31-MT-A and AZ31-MT-A-P substrates.

The corrosion current density ($i_{corr}$) and corrosion potential ($E_{corr}$) obtained from potentiodynamic polarization curves are presented in Table 9. All three coated substrates AZ31-MT, AZ31-MT-A and AZ31-MT-A-P, as well as the AZ31-OH conversion coating, showed lower corrosion current densities along with a progressively more anodic corrosion potential than the uncoated AZ31 alloy. Compared with AZ31, the passivated AZ31-OH inhibits both anodic and cathodic reactions.
Table 9 Corrosion potential and corrosion current density derived from plots for AZ31 alone, AZ3-OH, AZ31-MT, AZ31-MT-A and AZ31-MT-A-P surfaces.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>$E_{corr}$ (V)</th>
<th>$i_{corr}$ (A/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ31</td>
<td>-1.679 ± 0.021</td>
<td>17.9 ± 0.128</td>
</tr>
<tr>
<td>AZ31-OH</td>
<td>-1.652 ± 0.008</td>
<td>15 ± 0.056</td>
</tr>
<tr>
<td>AZ31-MT</td>
<td>-1.677 ± 0.003</td>
<td>0.987 ± 0.039</td>
</tr>
<tr>
<td>AZ31-MT-A</td>
<td>-1.612 ± 0.009</td>
<td>0.26 ± 0.049</td>
</tr>
<tr>
<td>AZ31-MT-A-PLGA-2%</td>
<td>-1.561 ± 0.002</td>
<td>0.214 ± 0.047</td>
</tr>
</tbody>
</table>

However, it has been reported that the alkaline passivated film would be unable to provide any corrosion resistance even for short period due to its instability in the chloride-rich aqueous medium [27]. The maximum improvement can be seen in AZ31-MT-A-P coated substrate, as observed by a decrease in corrosion current density by nearly 84 folds when compared with the uncoated equivalent. Also, the corrosion potential ($E_{corr}$) of AZ31-MT-A-P substrate was ~120 mV nobler as compared to the bare AZ31 alloy, implying less susceptibility to corrosion. Further, the corrosion resistance of PLGA coating at 4% w/v loading has also been performed, which showed lower corrosion resistance when compared to 2% PLGA coated AZ31-MT-A substrates as shown in Fig. 3-7. Similar results were also reported by Li et al., who explained that a highly concentrated loading of PLGA produces thick poor quality coatings [29]. This enhanced corrosion resistance can be attributed to the combined barrier performance of the siloxane network (MT-A) and PLGA coating for electrolyte penetration. This slows the anodic dissolution reactions and blocks the mass transport of Mg$^{2+}$ ions [27]. In addition, the formation of Si-O-Mg bonds at the interface also prevent some anodic reactions [28]. EIS measurements are
used to study the corrosion behaviour of uncoated and coated AZ31 substrates and are represented in the Bode plots in Fig. 3-8.

![Bode plots](image)

**Figure 3-7** Potentiodynamic polarization curves of AZ31-MT-A, AZ31-MT-A-2% PLGA and AZ31-MT-A-4% PLGA

Fig. 3-8 (a) clearly shows that the magnitude of impedance at the lowest frequency ($|Z|_{0.01Hz}$) gradually increases with successive coating applications. In parallel, the phase bode plot also displays an increase in the broad crest wave toward -90°, suggesting improvement in corrosion resistance of AZ31 Mg alloy. Such features have been observed in corrosion resistant coatings on Mg alloys [14, 35, 36]. The $|Z|_{0.01Hz}$ value of passivated AZ31-OH substrate is $\sim 7.4 \times 10^3 \ \Omega.cm^2$, which is slightly higher than the AZ31 alone AZ31 ($\sim 4.8 \times 10^3 \ \Omega.cm^2$). With further modification, the progressive increase in the $|Z|_{0.01Hz}$ value of $\sim 2.2 \times 10^5 \ \Omega.cm^2$ for AZ31-MT to $\sim 5.8 \times 10^5 \ \Omega.cm^2$ and $\sim 1.44 \times 10^6 \ \Omega.cm^2$ for AZ31-MT-A and AZ31-MT-A-P have been observed respectively.
Figure 3-8 (a) Bode plots for AZ31 alone, AZ3-OH, AZ31-MT, AZ31-MT-A and AZ31-MT-A-P surfaces, equivalent circuits used to fit experimental data (b) coated and (c) AZ31 and AZ31-OH substrates.

In addition, the phase angle Bode plots for all coated samples were characterised with two-time constants. One time constant at high frequency refers to the capacitive response or barrier component of the organo-silane (MT and MTA) and PLGA (MT-A-P) coatings, and the second time constant at low frequencies can be assigned to the diffusion process of electrolytes in the coatings.

The AZ31 and AZ31-OH substrates are characterised by two capacitive responses at high and medium frequencies (suppressed response) with additional inductance at a very low frequency (< 0.1Hz). This low-frequency inductance is commonly observed in uncoated Mg alloys, indicating the presence of surface adsorbed species like Mg(OH)⁺ and
Mg(OH)$_2$ [37, 38]. Although the interpretation of EIS data for magnesium alloy is controversial, the current explanation has been generally adopted [35, 38, 39].

The EIS spectra of coated and uncoated substrates were analysed based on the electrical equivalent circuits which fit best with the experimental data. The best fit comprised of two-time constants respectively as shown in Fig.3-8 (b) and (c) respectively. The equivalent circuit consists of $R_s$ (solution resistance), $C_{coat}$ one of the constant phase element (CPE) components, expresses coating capacitance and $R_{coat}$ is the resistance of the coating. $C_{dl}$, another component of CPE, represents the capacitance of the electrical double layer at vulnerable region and a charge transfer resistance $R_{ct}$. For AZ31 and AZ31-OH substrates, all CPEs and resistances are related to the hydroxide film on the surface. The $R_L$ and L represent the inductive resistance and inductance respectively for AZ31 and AZ31-OH substrate. However, data for these two components has not been shown as $C_{coat}$, $R_{coat}$, $C_{dl}$ and $R_{ct}$ are the elements of interest which are comparable to corresponding coated substrates. Moreover, low-frequency inductance has not been observed in any of the coated surfaces. The equivalent circuits were selected based on literature available for corrosion of uncoated and coated Mg alloys [39-41]. The fitting parameters are given in Table 10. It is observed that the $R_{coat}$ and $R_{ct}$ for coated substrates gradually increase with every layer of coating on AZ31 Mg alloy. $R_{ct}$ is a measure of corrosion resistance of alloy samples coated with silane or PLGA-silane. It increased for the AZ31-MT-A-P coated substrate to $5.6467 \times 10^8 \, \Omega \cdot \text{cm}^2$ as compared to AZ31 alloy at $768.5 \, \Omega \cdot \text{cm}^2$. Once exposed to the electrolyte, a thin film of Mg (OH)$_2$ develop on the AZ31 Mg alloy. This can act as an initial passivation layer, but it is susceptible to electrolytic attack, especially chloride ions [25].
Table 10 Parameters of uncoated and coated substrates calculated from the fitting of the experimental impedance spectra (Value ± % error)

<table>
<thead>
<tr>
<th>AZ31 Substrates</th>
<th>AZ31</th>
<th>AZ31-OH</th>
<th>AZ31-MT</th>
<th>AZ31-MT-A</th>
<th>AZ31-MT-A-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rs (Ω.cm²)</td>
<td>150.1±3.05</td>
<td>349±2.565</td>
<td>250.1±14.21</td>
<td>398±12.308</td>
<td>1251±3.083</td>
</tr>
<tr>
<td>Ccoat-T (x 10⁻⁸ F cm⁻²)</td>
<td>1280.2±2.08</td>
<td>386.6±3.39</td>
<td>3.074±2.963</td>
<td>2.845±4.005</td>
<td>1.34±1.659</td>
</tr>
<tr>
<td>Ccoat-P</td>
<td>0.636±5.16</td>
<td>0.725±1.756</td>
<td>0.774±0.351</td>
<td>0.785±0.517</td>
<td>0.833±0.206</td>
</tr>
<tr>
<td>Rout (x 10⁵ Ω.cm²)</td>
<td>0.05679±3.57</td>
<td>0.0755±6.756</td>
<td>1.4694±1.518</td>
<td>3.9657±2.841</td>
<td>9.7297±1.091</td>
</tr>
<tr>
<td>Cdl-T (x 10⁻⁶ F cm⁻²)</td>
<td>290.02±4.27</td>
<td>55.858±5.28</td>
<td>4.476±9.109</td>
<td>1.101±14.25</td>
<td>0.302±5.977</td>
</tr>
<tr>
<td>Cdl-P</td>
<td>0.946±2.94</td>
<td>0.97±5.54</td>
<td>0.650±6.748</td>
<td>0.832±8.73</td>
<td>0.972±2.960</td>
</tr>
<tr>
<td>Rct (Ω.cm²)</td>
<td>768.5±4.39</td>
<td>1371±6.73</td>
<td>82394±5.248</td>
<td>2.453x10⁵±7.36</td>
<td>5.646x10⁸±2.762</td>
</tr>
</tbody>
</table>

The alkaline passivated AZ31-OH showed enhanced corrosion resistance compared to AZ31 alloy (Table 10), but it also susceptible to the degradation in the corrosive medium [25] [42, 43]. The capacitance of a coated substrate is related to the area exposed to the electrolyte. The relatively low capacitance (Ccoat and Cdl) of AZ31-MT-A-P compared to the AZ31 bare alloy (as well as those for other coated substrates), implies that the active area of corrosion with PLGA-silane coating is considerably decreased. As established by potentiodynamic polarization and EIS tests in DMEM, the PLGA–silane coating showed superior corrosion resistance compared to the others tested. This enhanced corrosion resistance offered by silane and PLGA coatings can be attributed to the defect-free coatings and combined physical barrier performance of the siloxane network of MT-A organo-silane and PLGA coating to electrolyte penetration which is in agreement with the SEM images, FTIR spectrum and lower capacitance values [12, 13]. This results in the slowdown of anodic dissolution reactions (Mg → Mg²⁺ + 2e⁻) and charge transfer.
associated with cathodic reactions \(2\text{H}_2\text{O} + 2\text{e}^- \rightarrow \text{H}_2 + 2\text{OH}^-\), which blocks the mass transport of Mg\(^{2+}\) ions and release H\(_2\) gas, as observed from lower \(i_{\text{corr}}\) values and anodic shift of \(E_{\text{corr}}\) [12]. According to previous reports, these electrochemical corrosion studies for Mg-based alloys should be complemented by the other measurements such as Mg\(^{2+}\) ion release and hydrogen gas evolution [19, 38]. Since, the AZ31-MT-A-P coated substrate presents the greatest corrosion resistance, hereafter, further long-term degradation and biocompatibilities studies have been performed with AZ31-MT-A-P substrate and uncoated AZ31 (i.e without any modification) as a control.

### 3.3.2.2 Immersion study

The rate of Mg\(^{2+}\) ions release from the coated and uncoated AZ31 samples in DMEM up to 21 days was analysed by ICP-OES as shown in Fig. 3-9 (a). From the uncoated substrate, the Mg\(^{2+}\) ion release rate up to day 7 increases from 0.068 ± 0.002 mM/h/cm\(^2\) to 0.125 ± 0.003 mM/h/cm\(^2\), thereafter drastically decreases to 0.026 ± 0.003 mM/h/cm\(^2\) at day 21. In contrast, the coated AZ31-MT-A-P substrate had a very slow and steady release of Mg\(^{2+}\) ions ranged from 0.024 ± 0.002 mM/h/cm\(^2\) to 0.009 ± 0.001 mM/h/cm\(^2\) between day 1 and 21 respectively. It can be observed that the average rate of Mg\(^{2+}\) ions release from the coated surface is 0.016 mM/h/cm\(^2\) which is much lower than the release from uncoated surface 0.071 mM/h/cm\(^2\). These results clearly demonstrate that the release rate of Mg\(^{2+}\) ions from the coated substrate was markedly reduced compared to uncoated one. Furthermore, the pH changes of AZ31-MT-A-P and uncoated AZ31 in DMEM over a period 21 day is shown in Fig. 3-9 (b). The pH of both the substrates is alkaline, but the coated AZ31-MT-A-P was lower alkaline when compared to the uncoated AZ31 alloy. The pH of the coated substrate increases gradually then decreases with immersion time, unlike uncoated alloy which showed a rapid increase in pH followed by a gentle decrease.
Figure 3-9 (a) ICP-OES measurement of Mg ions release, (b) pH change and (c) rate of H$_2$ gas release from AZ31 and AZ31-MT-A-P over a period of 21 and 14 days respectively.

The trend of Mg$^{2+}$ release from the coated substrate was consistent with the observed pH change. The rate of hydrogen gas evolution from AZ31 and AZ31-MT-A-P in DMEM has been performed for 14 days as shown in Fig. 3-9 (c). The results confirmed that the rate of H$_2$ gas evolution is much higher for uncoated AZ31 than the coated AZ31-MT-A-P substrate. The uncoated AZ31 alloy showed a characteristically high corrosion rate until day 7 with 0.084 ± 0.004 ml/h/cm$^2$ of H$_2$ gas release. This was followed by a slower
corrosion rate for the remaining period with $0.041 \pm 0.003 \text{ ml/h/cm}^2$ of H$_2$ gas release up to day 14. For AZ31-MT-A-P, a slow gradual decreasing release of H$_2$ gas was observed throughout the period of 14 days dropping from $0.015 \pm 0.007 \text{ ml/h/cm}^2$ to $0.008 \pm 0.001 \text{ ml/h/cm}^2$. Based on the downward trend of H$_2$ release from the uncoated surface, it is expected that the corrosion rate may attain levels of the coated substrate at later period, therefore, we evaluated the evolution of H$_2$ gas release for 14 days instead of 21 days. Moreover, the rapid degradation happens during the early stage of implantation (2-3 weeks) which is also the minimum time required to heal a damaged bone tissue, we limited our study to control the degradation for same period [46, 47]. Therefore, the timelines of 14 and 21 days for evaluating the corrosion resistance and biocompatibility of modified AZ31 Mg alloy were found to be suitable and adopted in this study. Overall, the average rate of H$_2$ gas release over a period 14 day for the coated surface ($0.011 \text{ ml/h/cm}^2$) is much lower when compared to the uncoated surface ($0.062 \text{ ml/h/cm}^2$). These results showed that the PLGA-silane coating controlled the degradation of AZ31 Mg alloy.

Furthermore, the stability of the coating was evaluated by SEM and EDX analysis of corrosion resistant AZ31-MT-A-P substrates for various elements at different times as shown in Fig. 3-10 (a-c). Here, we have not studied the SEM-EDX analysis of the uncoated AZ31 substrate as the dynamics of degradation in physiological conditions has been well explored.

The SEM images showed that with an increase in immersion time from day 7 to 21, the crack formation on the coating surface also increases. The spot EDX analysis and elemental mapping of silicon (Si), phosphorus (P) and calcium (Ca) of the coated substrate were performed to assess the corrosion products formed on the sample and the presence of coating component (Si). The spot EDX elemental analysis around the cracks
of the coated surface, as shown within the table in Fig 3-10 are rich in Ca, P, Mg and O. Here, a higher content of Ca-P deposition with low Si content near the cracks were observed with the immersion time of 7, 14 and 21 days.

Figure 3-10 Surface morphology and EDX mapping of AZ31-MT-A-P immersed in DMEM for days (a) 7, (b) 14 and (c) 21 respectively. EDX was performed near cracks as denoted by arrows.

The EDX elemental mapping of day 7, 14 and 21 indicated the presence of Si, P and Ca. From the EDX maps, a weak signal of Si element has been observed near the cracks (as shown by arrows), whereas at the same site strong signal of P (indicated by arrows) can be observed on day 14 and 21. The spot EDX analysis and elemental mapping indicate the degradation of coating and the formation of the protective layer, as observed from the weak Si signal (a component of silane coating) and Ca-P deposition respectively.

Furthermore, the degradation of PLGA coating was determined by IR analysis of –C=O band at 1760 cm⁻¹ (Fig 3-11). On day 21, the –C=O band was not detectable, indicating
the hydrolysis of the PLGA coating. Previous reports showed that PLGA is susceptible to hydrolytic degradation, thereby failing to offer long-term corrosion resistance alone [13, 29].

![Figure 3-11 ATR-IR of the AZ31-MT-A-P substrates aged in DMEM for 7, 14, and 21 days.](image)

The possible reasons for the increased development of cracks in the coated AZ31 alloy with immersion time are due to the electrolyte (DMEM) penetration into the coating and subsequent corrosion of underlying alkaline treated-Mg alloy with the release of Mg²⁺ ions at the site of corrosion. This leads to the degradation of the surface coating as observed from weak Si signals, which is a component of organosilane coating from EDX analysis and disappearance of PLGA IR band, indicating the degradation of PLGA–organosilane coating over time. However, the delamination of the coating has not been observed, confirming the stability of the coating. It is well known that the hydroxide layer on the AZ31 does not provide long-term corrosion resistance [17]. Therefore, it is expected that efflux of Mg²⁺ ions will primarily occur at the site of cracks. This leads to the localized increase in pH, thereby forming the protective layer arising from Ca-P
deposition, reducing the corrosion rate of Mg alloy [30]. This was observed from the SEM-EDX analysis of coated substrate (Fig. 3-10 a-c). This prevents the further chemical reaction across the metal surface and reduces the corrosion of Mg alloy, which is also evident by the reduced rate of magnesium ions release, H₂ gas evolution and pH change with an increase in immersion time for both coated and uncoated surfaces (Fig. 3-9). Previous reports also showed that degradation layer during the corrosion process which consists of calcium phosphate precipitates and other corrosion products reduced the degradation rate of the Mg-based alloy in vitro with time [30, 31]. Since the organosilane coating on the AZ31 Mg alloy has not been completely degraded, this controlled degradation of the coated AZ31 substrate could be attributed to the stability of coating as well as in situ formations of such protective layers.

3.3.3 Cytocompatibility

The indirect cytotoxicity of the uncoated and coated AZ31 alloy was performed for the different duration (day 1, 3 and 5) using MTT assay as shown in Fig 3-12.

Figure 3-12 Cell viability of MC3T3E1 osteoblast cells cultured in the extracts of AZ31 and AZ31-MT-A-P substrates over 5 days of incubation period. Arrows are indicating the % extracts (v/v) which corresponds to its logarithmic values on the x-axis.
A non-linear regression analysis of cell viability of neat or diluted extracts of coated and uncoated substrates was plotted. Based on corrosion studies, rapid degradation of Mg alloy can be observed in the first week of the immersion period. Therefore, cell cytotoxic assays was evaluated over a period of 5 days. The cell viability results for the coated AZ31-MT-A-P substrate using 100% of the extract (Mg\(^{2+}\) ions concentration is 17.69 ± 0.12 mM, pH 8.03 ± 0.04) is significantly higher than the uncoated AZ31 alloy (Mg\(^{2+}\) ion concentration is 58.01 ± 0.26 mM, pH 8.76 ± 0.09) and with further dilution of extracts. It was observed that the cell viability is markedly higher for all days with 100% extract of the coated AZ31 substrate as compared to the uncoated AZ31 alloy. In addition, no reduction in cell viability was observed with the diluted extracts from 25% to 10% of the coated substrate for all days. On the contrary, the uncoated AZ31 alloy extracts showed higher cytotoxicity with all extracts. It is known that cells viability is very sensitive to environmental fluctuation, especially changes in pH due to the faster degradation of bare AZ31 alloy [2]. This increased the pH of the bare AZ31 extraction medium, detrimentally affected the cells. Several reports have shown that elevated levels of Mg\(^{2+}\) ions at high pH can cause osmotic shock to osteoblast cells that lead to cell death [19, 32]. Hence, it can be concluded that the coated AZ31 substrates demonstrate controlled degradation and provided a biocompatible environment for osteoblast cell proliferation.

Fig. 3-13 shows the pre-osteoblasts directly cultured on uncoated AZ31, coated AZ31-MT-A-P substrates for 1, 3 and 5 days, and then stained with calcein AM and propidium iodide. From day 1 to 5, the coated substrate (Fig 3-13 (d-f)) demonstrated a marked increase in live cell density (green fluorescence) when compared to uncoated AZ31 alloy Fig 3-13 (a-c), indicating that cells are proliferating faster. Very few dead cells (red fluorescence) were observed on the coated surface over uncoated AZ31 alloy, indicating the enhanced biocompatibility of the AZ31-MT-A-P surface.
Figure 3-13 Fluorescence microscopic images of live-dead MC3T3E1 cells attached to AZ31 (a-c) and AZ31-MT-A-P (d-f) for day 1, 3 and 5 respectively. (Scale bar is 100 μm)

SEM images of the fixed osteoblast cells on uncoated AZ31 alloy and coated surface at day 1, 3 and 5 post seeding are shown in Fig 3-14 (a-f). The cells were grown on the AZ31 alloy surface on day 1 (Fig 3-14 a) have attached (indicated by red arrows) while dead or distorted shaped (yellow arrows) osteoblast cells were also observed. This observation is similar to the live/dead staining of osteoblasts adhered AZ31 substrate on day 1. With increased duration of culture exposure, greater localised attachment of osteoblast cells can be observed on the AZ31 surface (Fig 3-14 b and c). In contrast, the attachment of osteoblast cells is delocalised on the AZ31-MT-A-P substrate following as with the increase in the incubation period from day 1 to 5 (Fig 3-14 (d-f)). A distinct morphology of osteoblast cells cannot be observed on day 5 (Fig 3-14 f), because of the dense coverage of confluent osteoblast cells which intercalate with each other. These results indicate that the PLGA component of the coated surface facilitates the greater
osteoblast cells adhesion and proliferation, thereby enhancing the biocompatibility of the coating. Several reports have shown that the application of PLGA as a biocompatible coating on Mg-based alloy enables the osteoblast cell attachment and proliferation [13, 29]. In comparison to other studies [13, 29], the present study showed better cell attachment and morphology, greater cell-to-cell interactions and improved proliferation without any signs of localised cell growth on the surface due to the evolution of H$_2$ gas bubbles, which affects the adhesion of the cells. These observations are supported by the results of live-dead fluorescent images of osteoblast cells cultured on these substrates under the same conditions. Hence, it can be concluded that the coated AZ31-MT-A-P substrate is more biocompatible than uncoated AZ31 alloy.

Figure 3-14 SEM images of fixed MC3T3E1 osteoblast cells cultured on AZ31 (a-c) and AZ31-MT-A-P (d-f) substrates after incubation of 1, 3 and 5 day(s). (Red and yellow arrows are indicating attached cells and dead or distorted shaped osteoblast cells respectively.)
Furthermore, the proliferation and differentiation of the osteoblast cell were determined for 14 days. The reasons for adopting this timeline include (a) evaluating the osteoblastic activity during the corrosion period (as studied above), (b) ALP is an early stage marker, which express during day 3 to 7, thereafter intracellular level decreases and (c) the timelines for evaluating the total DNA was also adopted in accordance with the expression levels of intracellular ALP.

The DNA content of osteoblast cells cultured on the uncoated AZ31 and AZ31-MT-A-P substrates for day 3, 7 and 14 was measured by fluorescence assay as shown in Fig 3-15.

![Figure 3-15](image-url)

**Figure 3-15** Quantification of total DNA content and ALP activity of osteoblast cultured on uncoated AZ31 and coated AZ31-MT-A-P substrates for 3, 7 and 14 days. One way ANOVA with post-hoc Tukey test with significance level of **p < 0.01** between the samples showing folds of DNA content and ΔΔp < 0.01 signifies ALP activity.

The concentration of DNA is the direct measure of cells that attach and proliferate on a substrate surface. The initial cell attachment on the coated AZ31-MT-A-P substrate was nearly 4 fold higher relative to AZ31 equivalent, whereas it decreased between day 7 and
14. This is due to the increase in DNA concentration for AZ31 substrates at the later stages of the culture period. It is reported that the formation of a passivation layer slows down the corrosion rate of Mg alloy with the incubation time, thereby supporting the growth of cells [30, 33]. This DNA quantification study is consistent with the cell viability assay and live/dead staining qualitative results which indicated that the cell viability and proliferation are higher on the coated surface when compared to the uncoated AZ31 surface.

Osteoblasts express ALP in the early stages of osteoblastic commitment and are actively involved in the mineralisation of the extracellular matrix through the generation of phosphate ions from the hydrolysis of organic substrates [34]. The differentiation of osteoblast cells on the coated and uncoated AZ31-MT-A-P substrate was evaluated up to 14 days using ALP as an early stage marker. The ALP activity of osteoblast cells cultured on the uncoated AZ31 and coated AZ31-MT-A-P substrates is presented in Fig 3-15. The osteoblasts that adhered to the coated surface showed significant higher ALP expression (p < 0.01) when compared to the uncoated AZ31 alloy over a culture duration of 14 days. The enhanced proliferation of cells is the likely reason for greater cell differentiation on the coated AZ31-MT-A-P surface as PLGA does not have an intrinsic property to stimulate the osteoinductive activity [17, 34]. Hence, the multilayer PLGA-silane coating is a promising way to improve the osteointegration of Mg-based implants.

3.4 Conclusion

A novel multi-layered PLGA-silane coating on a biodegradable Mg alloy has been developed. The corrosion resistance of AZ31 alloy was considerably improved with every step of coating. The AZ31-MT-A-P coated substrate showed enhanced and long-term corrosion resistance over uncoated AZ31 alloy while keeping the conditions necessary
for biocompatibility. The stability of the coatings as well as in situ formations of a protective layer played an important role in the controlled degradation of the coated AZ31 alloy. The coated AZ31 Mg alloy showed good osteoblastic cytocompatibility, allowing cell adhesion, proliferation and differentiation when compared to the uncoated equivalent. The cytocompatibility results indicate that the PLGA coating facilitates better cell adhesion and proliferation, thereby enhancing the biocompatibility of the coating. These results indicate that the proposed coating for Mg alloys is a promising strategy to improve the corrosion resistance and cellular integration with the surrounding tissue. It can also potentially serve to deliver desired drugs, growth factors and other bio-active molecules at the site of implantation during the coating degradation process (such as PLGA), expediting the bone healing process. In addition, the amine group of the sol gel coating can also be used to functionalise biopolymers having a carboxylic acid group to improve the cytocompatibility of the biodegradable implants for orthopaedic applications.
3.5 References


Chapter 4

Enhanced corrosion resistance and cytocompatibility of biomimetic hyaluronic acid functionalised silane coating on AZ31 Mg alloy for orthopaedic applications

This work reports the corrosion resistant and cytocompatible properties of the hyaluronic acid-silane coating on AZ31 Mg alloy. In this study, the osteoinductive properties of high molecular weight hyaluronic acid (HA, 1-4 MDa) and the corrosion protection of silane coatings were incorporated as a composite coating on biodegradable AZ31 Mg alloy for orthopaedic applications. The multi-step fabrication of coatings first involved dip coating of a passivated AZ31 Mg alloy with a methyltriethoxysilane-tetraethoxysilane sol-gel (AZ31-MT), followed by 3-aminopropyl-triethoxy silane (AZ31-MT-A) to create an amine-functionalised AZ31-MT-A surface. The amine functionalised surface facilitated the immobilisation of HA via EDC-NHS coupling reactions at two different concentrations i.e 1 mg.ml⁻¹ (AZ31-MT-A-HA1) and 2 mg.ml⁻¹ (AZ31-MT-A-HA2). The corrosion resistance and cytocompatibility of these coated surfaces were evaluated. The corrosion studies showed an enhanced corrosion resistance of HA functionalised silane coated AZ31 substrate over the uncoated equivalent alloy. Furthermore, the cytocompatibility studies showed HA concentration-dependent improvement of osteoblast cellular response.

4.1 Introduction

In chapter 3, the fabrication of PLGA-silane coatings on AZ31 Mg and their effectiveness in enhancing the corrosion resistance and biocompatibility of Mg alloy was studied. The amine group of the silane (AZ31-MT-A) coating was used to facilitate the fabrication of stable PLGA coating on its surface. The corrosion resistance properties of the silane coating observed in DMEM (non-buffered), controlled the degradation of Mg alloy which provided the appropriate condition for the growth of osteoblasts, whereas PLGA provided a bioactive surface for improved osteoblasts’ adhesion, proliferation and differentiation. Since the biological fluids are stabilised by a buffer system, it is important to study the corrosion resistance properties of the silane coating in similar conditions. In addition, osteoblast functions can be further improved by providing biomimetic conditions (e.g. use of extracellular matrix components including hyaluronic acid for the fabrication of bioactive coatings).

In general, the effectiveness of an orthopaedic implant in restoring damaged bone is determined by the bone-implant interaction which is characterised by the tight bond between the implant and bone surfaces, thereby minimising the risk of an implant loosening over time [1]. In order to enhance the biological acceptance of orthopaedic implants, functionalisation of the extracellular matrix (ECM) components has been widely employed. Hyaluronic acid (HA) is one of the major components of the extracellular matrix which is involved in cell adhesion, proliferation and differentiation. In previous studies, HA immobilised on the implant substrates (e.g. titanium alloy) has not been reported as an effective promoter of osteoblastic activity [27,28]. Moreover, the rationality of employing HA of different molecular weights for surface modification has not yet been considered. According to Zhao et al, the osteoinductive activity of bone-related cells increases in response to the high molecular weight of HA [3]. Therefore,
is important to consider the molecular weight of HA to modify the surface for different applications.

In this study, the aim is to develop a coating system where HA is bound via a silane modified surface to deliver a corrosion resistance and cytocompatible coating on AZ31 Mg alloy for orthopaedic applications. Therefore, the functionalisation of high molecular weight HA with silanes coated on the biodegradable AZ31 Mg alloy is proposed for the first time in the present work. The silanes involve an initial MTES-TEOS treatment, followed by APTES to deliver an amine terminated surface which can be coupled to HA through EDC-NHS coupling reactions. The corrosion resistance of the coated surface was evaluated in HEPES buffered DMEM. Furthermore, the effect of HA on the osteoblast cellular response was evaluated.

4.2 Experimental

4.2.1 Materials

AZ31 alloy sheets were obtained from Shaanxi Taipu Rare Metal Materials Ltd, China. Methyltriethoxysilane, tetraethoxysilane, 3-aminopropyl-triethoxy silane, Hyaluronic acid from rooster comb (1-4 MDa), phosphate buffer saline (PBS), Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F-12), fetal bovine serum (FBS), Penicillin-streptomycin antibiotics, phosphatase substrate and bisBenzimide H 33342 trihydrochloride, NHS (N-hydroxysuccinimide), EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and MES (2-(N-Morpholino)ethanesulfonic acid) buffer were purchased from Sigma Aldrich.

4.2.2 Surface modification of AZ31 Mg alloy

4.2.2.1 Treatment of AZ31 with NaOH

As mentioned previously in section 3.2.2.1
4.2.2.2 Preparation of hyaluronic acid functionalised organosilane coating on AZ31 Mg alloy

4.2.2.3 Preparation of organosilane coating (AZ31-MT-A) on AZ31 Mg alloy

Preparation of organo-silane coating is mentioned in section 3.2.2.2

4.2.2.4 HA conjugation to organo-silane coating

High molecular weight HA was functionalised onto AZ31-MT-A by a carbodiimide-mediated coupling reaction as detailed in a previous study [4]. The final concentrations of HA used to functionalise the AZ31-MT-A samples are 1 mg.ml\(^{-1}\) and 2 mg.ml\(^{-1}\) with the resultant samples denoted as AZ31-MT-A-HA1 and AZ31-MT-A-HA2 respectively (Fig 4-1). It is not possible to coat hyaluronic acid beyond 2 mg.ml\(^{-1}\) concentration because, at higher concentrations, high molecular weight HA becomes a gel-like solution. This also prevents the complete solubilisation of HA. Moreover, due to the high viscosity, it was difficult to prepare a reproducible and uniform coating of HA.

Figure 4-1 Schematic representation is showing the steps involved in the hyaluronic acid-silane coating on AZ31 Mg alloy. The alkaline passivated AZ31 alloy (step- a)
was coated sequentially with MTES-TEOS (step-b), APTES (step-c) and hyaluronic acid (step-d) to improve the corrosion resistance and cytocompatibility of AZ31 Mg alloy.

The effective concentration of HA immobilised on the surface was determined by the Morgan-Elson fluorometric enzyme assay [5]. The AZ31-MT-A-HA1 and AZ31-MT-A-HA2 substrates were exposed to the hyaluronidase enzyme for an appropriate time at 37.5 ºC. Then, the enzyme solution was collected and incubated in a boiling water bath for 5 minutes to inactivate the enzyme. After cooling to room temperature, 25 µl of tetraborate reagent (0.8 M K₂B₄O₇·4H₂O) was added and incubated for 3 minutes in a boiling water bath to start the Morgan-Elson fluorometric reaction. After cooling to room temperature, 0.75 ml of 10 %w/v DMAB (p-Dimethylaminobenzaldehyde) reagent was added and incubated for 20 minutes at 37.5 ºC. The release of N-acetyl glucosamine was measured by fluorescence (Ex/Em: 545/604 nm) while the concentration of HA functionalised was determined by using a standard curve of HA.

4.2.3 Characterisation of AZ31 modified samples

In addition to the characterisation techniques mentioned in the section 3.2.3, Atomic force microscopy (Asylum MFP-3D-BIO, USA) was used to determine surface roughness with a scan area of 20 x 20 µm. For each substrate, images were taken from at least three sites on two independent samples to determine the surface roughness.

4.2.3.1 Electrochemical corrosion studies

All in vitro Mg alloy degradation tests were performed at 37 ± 0.5 ºC in HEPES-buffered DMEM (15 mM HEPES). Electrochemical impedance spectroscopy measurements were conducted as detailed in section 3.2.3.1
4.2.3.2 Immersion experiment, pH changes and H₂ gas evolution measurement

All parameters and method employed to conduct immersion experiment and pH measurements are mentioned in section 3.2.3.2. The immersion medium employed for this study is HEPES-buffered DMEM.

The rate of H₂ gas release was monitored by placing the samples in HEPES buffered DMEM at 37 ± 0.5 °C under an inverted funnel connected to graduated burette and measuring the medium level within the burette for up to 10 days. The ratio of the surface area-to-volume of DMEM was kept constant at 10 cm². L⁻¹ [6].

4.2.4 Cytocompatibility

4.2.4.1 Cellular morphology imaging by SEM

Cellular morphology imaging by SEM was performed according to the methods described in sections 3.2.4.3

4.2.4.2 Live/dead cell staining

Live/dead cell staining was performed according to the methods described in section 3.2.4.2

4.2.4.3 DNA quantification

The total DNA content was determined according to the methods described in section 3.2.4.4

4.2.4.4 Alkaline phosphatase (ALP) assay

ALP activity was determined as per methods described in the section 3.2.4.5

4.2.5 Statistical analysis

All the experiments were carried out thrice in triplicates. All data are expressed as mean ± S.D. The differences between the groups were analysed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Statistically significance was considered at **p < 0.01 and *p < 0.05.
4.3 Results and discussion

4.3.1 Characterisation of AZ31 modified surface

In order to bond with the silane coating, the AZ31 surface was passivated with NaOH to produce surface hydroxy groups. The AZ31-OH was sequentially treated with hydrolysed MTES-TEOS and APTES sols to prepare a two-layer silane coating through siloxane bond formation. Following exposure of the MTES-TEOS sol, the ATR-IR spectrum (Fig. 4-2) shows bands around 3694 cm\(^{-1}\), 1045-1127 cm\(^{-1}\) and 1270 cm\(^{-1}\) which are attributed to -OH groups (Fig. 4-2b), Si-O asymmetric stretching of -Si-O-Si- and Si-CH\(_3\) (Fig. 4-2c) of MTES respectively [7].

![Figure 4-2 ATR-IR of spectra (a) AZ31 alone, (b) AZ31-OH, (c) AZ31-MT, (d) AZ31-MT-A, (e) AZ31-MT-A-HA1, (f) AZ31-MT-A-HA2, (g) HA alone](image-url)
Subsequent coating with APTES increases the intensity of -Si-O-Si- band with the emergence of new bands at 1570 cm\(^{-1}\), 3295 cm\(^{-1}\) and 3368 cm\(^{-1}\) (Fig. 4-2d) which are associated with asymmetric and symmetric stretching modes of the NH\(_2\) group respectively [7] [8]. The amino silane surface was then functionalised with HA through EDC-NHS coupling reactions at increasing concentrations. The effective concentrations of the bound HA obtained from Morgan-Elson assay for AZ31-MT-A-HA1 and AZ31-MT-A-HA2 substrates were found to be 45.45 ± 0.05 µg.cm\(^{-2}\) and 66.77 ± 0.25 µg.cm\(^{-2}\) respectively.

The amide I (a shoulder band), amide-II band and stretching vibrations of carboxylate C=O of HA are indicated at 1670 cm\(^{-1}\) (Fig. 4-2-g3), 1540 cm\(^{-1}\) (Fig. 4-2-g2) and 1604 cm\(^{-1}\) (Fig. 4-2-g1) respectively [9]. With the HA functionalisation, the bands of amide I (Fig. 4-2-f3 and 4-2-e3) and amide II bands (Fig. 4-2-f2, 4-2-e2) can be observed prominently (showed in the magnified inset.). Moreover, the carbonyl HA band (Fig. 4-2-g1) and APTES amine band (Fig. 4-2-d4) cannot be observed in the resultant spectra indicating successful coupling at the surface.

Furthermore, AFM was used to observe the topography of surface modifications as shown in Fig. 4-3. The surface roughness (\(R_s\)) of the AZ31 and AZ31-OH substrates was 74.5 ± 8.2 nm and 62.2 ± 3.9 nm respectively. The \(R_s\) values of AZ31-MT and AZ31-MT-A coated surface were found to be 43.1 ± 0.5 nm and 45.7 ± 0.82 nm respectively. The \(R_s\) values then decreased considerably for AZ31-MT-A-HA1 and AZ31-MT-A-HA2 substrates to 4.86 ± 0.07 nm and 7.84 ± 0.39 nm respectively, significantly lower than the bare AZ31 alloy alone. The fibrils pattern of HA can be observed which most likely results from drying induced aggregation of HA chains [10].
Figure 4-3 Surface morphology of uncoated and coated substrates was determined using AFM for (a) AZ31, (b) AZ31-OH, (c) AZ31-MT-A, (d) AZ31-MT-A-HA1 and (e) AZ31-MT-A-HA2. (Arrows are indicating aggregation of HA chains)

The effect of surface modifications also changes the wettability of the surfaces as shown in Fig. 4-4.

Figure 4-4 Contact angle of AZ31 modified substrates. One-way ANOVA with post-hoc Tukey test with significance level of ** $p < 0.01$ at every step of modification except for HA modification. For AZ31-MT-HA1 and AZ31-MT-HA2 test of significance was performed with AZ31 alone.
Compared to the AZ31 alloy, the water contact angle showed significant changes after every step of modification (passivation treatment and silane). After HA functionalisation, the AZ31-MT-A-HA1 and AZ31-MT-A-HA2 showed a significant decrease in contact angle having values 43.4° ± 1.4 and 41.3° ± 6.3 respectively when compared to the AZ31 alone.

4.3.2 In vitro corrosion and degradation studies

4.3.2.1 Electrochemical impedance spectroscopy

The EIS data of uncoated and coated AZ31 substrates are shown in Bode impedance plots in Fig. 4-5(a).

Figure 4-5 Bode plots of (a) AZ31 alone, AZ3-OH, AZ31-MT, AZ31-MT-A, AZ31-MT-A-HA1 and AZ31-MT-A-HA2 surfaces, and (b) equivalent circuits used to fit experimental data; coated and uncoated (AZ31 and AZ31-OH substrates)
The impedance (|\(Z_{0.01Hz}\)|) increases gradually with every step of the silane coating. When compared to the AZ31 alloy, the impedance of AZ31-MT-A increased by nearly two orders of magnitude, indicating effective protection of AZ31 alloy. Although |\(Z_{0.01 Hz}\)| decreases for AZ31-MT-A-HA1 and AZ31-MT-A-HA2 substrates, it is still higher than the AZ31 substrate. The analysis of the EIS spectra was performed based on the proposed electrical equivalent circuits (EEC) depicted in Fig. 4-5(b) which fit best with the experimental data and parameters are given in Table 11.

Table 11 Parameters of uncoated and coated substrates calculated from the fitting of the experimental impedance spectra (Values ± % error)

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<tr>
<td>(R_s) (Ω.cm(^2))</td>
<td>430 ± 2.01</td>
<td>412 ± 8.92</td>
<td>421.1 ± 11.12</td>
<td>460 ± 9.83</td>
<td>453 ± 10.02</td>
<td>430 ± 7.71</td>
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<tr>
<td>(\text{CPE}_{\text{coat}-T}) (x 10(^{-8}) F cm(^{-2}))</td>
<td>700.98 ± 1.97</td>
<td>605.49 ± 1.27</td>
<td>22.92 ± 3.01</td>
<td>4.385 ± 5.001</td>
<td>13.263 ± 3.12</td>
<td>6.422 ± 2.403</td>
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<tr>
<td>(\text{CPE}_{\text{coat}-P})</td>
<td>0.739 ± 3.631</td>
<td>0.740 ± 2.02</td>
<td>0.667 ± 0.386</td>
<td>0.739 ± 0.646</td>
<td>0.632 ± 2.16</td>
<td>0.751 ± 0.269</td>
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<tr>
<td>(R_{\text{coat}}) (x 10(^5) Ω.cm(^2))</td>
<td>0.01435 ± 6.721</td>
<td>0.0181 ± 4.926</td>
<td>0.5191 ± 8.50</td>
<td>1.5591 ± 6.12</td>
<td>1.191 ± 4.961</td>
<td>0.5638 ± 5.903</td>
</tr>
<tr>
<td>(\text{CPE}_{d\text{l}-T}) (x 10(^{-6}) F cm(^{-2}))</td>
<td>124.07 ± 2.06</td>
<td>71.72 ± 2.73</td>
<td>7.289 ± 2.21</td>
<td>4.571 ± 1.90</td>
<td>3.187 ± 8.896</td>
<td>7.136 ± 16.789</td>
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<tr>
<td>(\text{CPE}_{d\text{l}-P})</td>
<td>0.805 ± 5.110</td>
<td>0.878 ± 4.18</td>
<td>0.679 ± 8.512</td>
<td>0.778 ± 11.856</td>
<td>0.948 ± 3.535</td>
<td>0.879 ± 9.302</td>
</tr>
<tr>
<td>(R_{\text{cl}}) (Ω.cm(^2))</td>
<td>575 ± 6.27</td>
<td>633 ± 7.76</td>
<td>24775 ± 3.43</td>
<td>57543 ± 4.91</td>
<td>52195 ± 2.36</td>
<td>30999 ± 6.718</td>
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Bodes phase angle plots for all coated substrates are characterised by high and low frequencies time constants which correspond to the response of charge transfer and mass diffusion-controlled reactions respectively. The AZ31 and AZ31-OH substrates are also characterised by two capacitive responses at higher and medium frequencies with an additional inductance at low frequency (< 0.1Hz). The inductance is related to adsorbed intermediate species during corrosion [11]. The EEC consists of a solution resistance \(R_s\), a constant phase element of electrolyte/coating layer (\(\text{CPE}_{\text{coat}}\) (\(Q_{\text{coat}}\))) and a double
electrical layer presence in the metal/coating (CPE\textsubscript{dl}(Q\textsubscript{dl})) interfaces (as seen under non-ideal conditions). R\textsubscript{coat} and R\textsubscript{ct} are the coating and charge transfer resistances respectively. The EECs were selected based on the available literature for the corrosion of uncoated and coated Mg alloys [24, 27].

As shown in Table 11, R\textsubscript{coat} and R\textsubscript{ct} gradually increase for AZ31-MT and AZ31-MT-A substrates before decreasing for the HA modified surfaces. R\textsubscript{ct} which is a measure of corrosion resistance of coated AZ31 substrates suggests a considerable increase in corrosion resistance for AZ31-MT-A substrate (57 KΩ.cm\textsuperscript{2}) as compared to other modified substrates and bare AZ31 alloy (575 Ω.cm\textsuperscript{2}). Similarly, the coating capacitance (CPE\textsubscript{coat} and CPE\textsubscript{dl}) for the AZ31-MT-A substrate is lowest amongst all those tested, implying that the corrosion activity of the AZ31-MT-A substrate is substantially decreased.

This improved corrosion resistance of AZ31-MT-A substrate in buffered DMEM can be attributed to physical barrier performance of the siloxane coating network to electrolyte penetration [13]. This results in retarding of the anodic dissolution reactions and preventing the mass transport of Mg\textsuperscript{2+} ions and H\textsubscript{2} gas release [7] [13]. In a previous report, γ-APS-BTSE treated AZ31 Mg alloy showed lower corrosion resistance than the present AZ31-MT-A model [14]. However, coating with increasing concentrations of HA was unable to enhance the AZ31 Mg alloy corrosion resistance. This may be due to the silane coating saturated with the electrolyte (MES buffer, pH 5.5) during the covalent coupling reaction [14]. This hinders the physical barrier function to resist electrolyte penetration. Moreover, it is known that HA modifications have been used to impart the surface hydrophilicity due to its excellent water absorption properties [15]. From the electrochemistry results, HA functionalisation on AZ31-MT-A substrate does not offer any additional corrosion resistance. Therefore, further degradation studies have been
performed with the corrosion resistant component (AZ31-MT-A) of the HA-silane coating.

4.3.2.2 Immersion experiment, pH changes, and H₂ evolution studies

Fig. 4-6 (a) shows the Mg²⁺ ion release rate, pH change and evolution of H₂ gas from the coated AZ31-MT-A and uncoated AZ31 substrates aged in HEPES buffered DMEM for 21 and 10 days respectively. The explanations regarding the timelines adopted for these experiments and exclusion of AZ31 as a control in Fig 4-6 (b) are mentioned in Chapter 3 (section 3.3.2).

Figure 4-6 (a) ICP-OES measurement of Mg ions release rate, pH change and rate of H₂ gas release from AZ31 and AZ31-MT-A over a period of 21 and 10 days respectively, and (b) Electron micrograph and EDX mapping of AZ31-MT-A substrate immersed in HEPES buffered DMEM for 7, 14 and 21 days.
The coated AZ31-MT-A substrate showed a very slow and steady release of Mg\(^{2+}\) ions which was \sim 8\) fold (0.0015 mM/hr) lower when compared to uncoated AZ31 alloy (0.013 mM/hr) until day 9. For the remaining immersion period, the Mg\(^{2+}\) ion concentration decreases thereafter to \sim 2.5\) fold (0.001 mM/hr) lower than the uncoated AZ31 equivalents (0.003 mM/hr). This occurs mainly due to the decrease in the Mg\(^{2+}\) ion release rate from the uncoated AZ31 alloy. This result would confirm that the controlled degradation of coated AZ31 Mg alloy, especially during the initial period, plays an important role in maintaining physiological conditions required for bone healing.

Furthermore, the pH changes of HEPES buffered DMEM containing uncoated and AZ31-MT-A coated substrates for 21 days showed that the AZ31-MT-A substrate was less alkaline as compared to the uncoated AZ31 alloy. The pH of the AZ31-MT-A substrate increases gradually until day 9 (pH 8.19 ± 0.03) and remains unchanged for the remaining immersion time (pH 8.23 ± 0.05), whereas AZ31 alloy exhibits a sudden increase in pH until day 9 (pH 8.43± 0.04) and then stabilises for the remaining period (pH 8.49 ± 0.05).

The rate of hydrogen gas evolution was measured from AZ31 and AZ31-MT-A substrates in HEPES buffered DMEM for 10 days. The coated substrate released less H\(_2\) gas over 10 days (from 0.045 ml/h to 0.019 ml/h) when compared to uncoated AZ31 (from 0.22 ml/h to 0.055 ml/h). Therefore, the coated AZ31-MT-A surface displayed superior performance (in terms of pH change, H\(_2\) and Mg\(^{2+}\) ions release) when compared to the uncoated AZ31 substrate. The difference was especially apparent during the early period of immersion.

Furthermore, the surface profile of the AZ31-MT-A substrate in buffered DMEM for different times was evaluated by SEM-EDX as illustrated in Fig. 4-6 (b). The coated substrate shows increasing crack incidence over time and evidence of corrosion debris. The EDX mapping and elements quantification (inset tables) of the aged coated substrate
are rich in carbon (C), calcium (Ca), oxygen (O) and phosphate (P). A protective layer containing a relatively high content of O, C, Ca and P deposits were observed on the coated substrate from day 7 to 21. In addition, a weak signal of Si observed on day 21, which may indicate the degradation of the silane coating.

The possible reasons for the crack formation and increased deposition of corrosion by-products on the coated AZ31 alloy with the immersion time include electrolyte penetration into the coating and subsequent corrosion of the underlying passivated AZ31 alloy with the release of Mg$^{2+}$ ions at the site of corrosion [16]. This results in the degradation of the silane coating over a period of time. However, the complete degradation of the silane coating has not been observed (Si EDX mapping). Generally, hydroxide passivated AZ31 surface is unable to provide long-term corrosion resistance in the chloride-rich environment [17]. This leads to the efflux of Mg$^{2+}$ ions from the cracks which react with water to form an alkaline environment and produces Mg (OH)$_2$ and H$_2$ gas [18]. The overall (anodic and cathodic) reaction as given below:

$$\text{Mg} \rightarrow \text{Mg}^{2+} + 2e^- \quad \text{(anodic reaction)} \quad \text{Eq-1}$$

$$2\text{H}_2\text{O} + 2e^- \rightarrow \text{H}_2 + 2\text{OH}^- \quad \text{(cathodic reaction)} \quad \text{Eq-2}$$

$$\text{Mg} + 2\text{H}_2\text{O} \rightarrow \text{H}_2 + \text{Mg(OH)}_2 \quad \text{(overall reaction)} \quad \text{Eq-3}$$

The evolution of H$_2$ gas reduces the coating stability, which enhances electrolyte penetration through the coating, promoting further corrosion. However, increases in pH of the medium induce the formation of a protective precipitate of Ca-P and Mg(OH)$_2$ at the alloy surface, thereby reducing the degradation of Mg alloy [13]. This results in the reduced Mg$^{2+}$ ion release rate, H$_2$ gas release and pH change especially during a later stage of immersion period as observed for both coated and uncoated substrates (Fig. 4-6(a)). Previous reports also showed that the degradation layer formed during the corrosion process reduced the degradation rate of Mg alloys in vitro [19]. This controlled
degradation of coated AZ31-MT-A substrate could be attributed to the coating stability and \textit{in situ} formation of protective degradation layers, helping maintain conditions required for osteoblast cell growth.

4.3.3 Cytocompatibility

For cytocompatibility studies, HA was functionalised onto the AZ31-MT-A substrate to develop a biocompatible and osteoinductive surface. Fig. 4-7 (a) shows the live-dead staining of MC3T3-E1 cells cultured on uncoated AZ31, AZ31-MT-A-HA1 and AZ31-MT-A-HA2 coated substrates for 1 and 3 days. For both time points, the AZ31-MT-A-HA1 and AZ31-MT-A-HA2 substrates showed the HA concentration-dependent increase in live cell density (green fluorescence) with very few dead cells (red fluorescence) as compared to uncoated AZ31 alloy, indicating an enhanced cell adhesion and proliferation of osteoblast cells on HA coated AZ31-MT-A substrates.

Figure 4-7 (a) Fluorescence microscopy images of live-dead MC3T3E1 cells and (b) SEM morphology of fixed MC3T3E1 osteoblast cells attached to AZ31, AZ31-MT-A-HA1, and AZ31-MT-A-HA2 for day 1 and 3. White arrows are indicating osteoblast cells.
Fig. 4-7 (b) shows the SEM images of the fixed osteoblast cells on HA coated AZ31-MT-A and uncoated AZ31 substrates on day 1 and 3. The cells attached to uncoated AZ31 alloy on day 1 and 3 are sparse and circular in shape (indicated by white arrows), indicating very poor adhesion to the substrate. On the other hand, osteoblast cells on AZ31-MT-A-HA1 and AZ31-MT-A-HA2 substrates displayed more flattened spread morphology, indicating good osteoblast cell attachment. These results suggested that HA coated surfaces facilitate greater adhesion of osteoblast cells.

Fig. 4-8 shows the quantification of the total DNA content of osteoblast cells, which is considered as a measure of cell proliferation. A significant increase in the folds of DNA content was observed for the HA-coated surfaces on day 3, indicating that such a coating favours osteoblast cell attachment.

Figure 4-8 Quantification of total DNA content and ALP activity of osteoblast cultured on uncoated AZ31 and coated AZ31-MT-A-P substrates for 3, 7 and 14 days. One-way ANOVA is followed by post-hoc Tukey test having a significance level of ** p < 0.01 between the samples.
On HA coated surfaces, adhered cells showed nearly 5 folds increase in proliferation between 7 and 14 days relative to AZ31 equivalents. These results were also consistent with the live-dead images of osteoblast cells. The ALP activity of osteoblast cells grown over 14 days on the AZ31, AZ31-MT-A-HA1 and AZ31-MT-A-HA2 substrates are presented in Fig. 4-8. Osteoblasts cultured on HA functionalised substrates displayed significantly higher ALP expression (p < 0.01) when relating to the uncoated AZ31 alloy over a culture duration of 14 days. ALP is one of the most widely used early stage markers of osteoblast differentiation, which participates in ECM mineralisation [20]. HA is one of the major ECM component of many cells including osteoblast cells which regulates cell adhesion, proliferation and expression of osteoinductive factors including ALP [21]. However, very few reports evaluated cell adhesion, proliferation and differentiation ability of HA functionalisation on various metal substrates. Chua et al. showed the poor adhesion of osteoblasts on HA modified Ti samples as compared to the chitosan/HA or chitosan/HA/RGD peptides functionalised Ti surface [2]. Similarly, Hu et al. also reported poor adhesion and proliferation of osteoblasts on hyaluronic acid-catechol functionalised on Ti surface (Ti-HAC) as compared to the Ti-HAC-VEGF and Ti-CMC-VEGF [22]. On the contrary, the present study demonstrates remarkable osteoblasts’ adhesion, proliferation and differentiation on HA functionalised AZ31 substrates. Zhao et al. studied the hyaluronic acid molecular weight dependent differentiation of rBMSCs [3]. It has been reported that high molecular weight HA with higher concentration promoted cell proliferation and differentiation. The enhanced osteogenic differentiation of osteoblast cells on HA1/HA2 coated AZ31 substrate could be attributed to the immobilisation of high molecular weight HA.
4.4 Conclusion

In the present work, a multilayered hyaluronic acid functionalised silane coating on AZ31 Mg alloy was fabricated successfully. Electrochemical corrosion and immersion studies demonstrated that the silane component of the coating significantly improved the corrosion resistance of the AZ31-MT-A-HA1/HA2 substrates as compared to the uncoated AZ31 Mg alloy. Furthermore, immersion studies showed that stability of the silane coating as well as in situ formations of passivation layer played an important role in the controlled degradation of the coated AZ31 Mg alloy. In comparison to the PLGA-silane coating (chapter 3), the silane component of the HA-silane coating showed effective corrosion protection in a highly corrosive HEPES-modified DMEM. Based on electrochemical and immersion studies, the corrosion resistance has decreased considerably in this electrolyte in comparison to the PLGA-silane coated surface (DMEM was used as an electrolyte), but overall increase when compared to the uncoated surface is similar. Moreover, an increase in the degradation of HA-coated AZ31 Mg alloy (observed from the electrochemical corrosion study) did not affect the viability of the adhered osteoblast cells on its surface. In contrast, osteoblast cells showed a considerable increase in cell viability, adhesion, proliferation and differentiation on HA-coated AZ31 when compared to PLGA-silane, coated substrates. These results showed (a) silane component of the coating effectively controlled the degradation of Mg alloy without affecting the biocompatibility of the substrate and (b) HA functionalisation on silane-modified AZ31 substrate provided a biomimetic environment which is evident from an improved osteoblast cellular response. Collectively, these results demonstrated that the proposed strategy to develop a multifunctional coating which can improve the corrosion resistance and cytocompatibility of AZ31 Mg alloy would be highly desirable for orthopaedic applications.
4.5 References


[16] M. Jamesh, S. Kumar, T.S.N.S. Narayanan, Electrodeposition of hydroxyapatite


Chapter 5

Biomimetic hyaluronic acid-lysozyme composite coating on AZ31 Mg alloy with combined antibacterial and osteoinductive activities

This study presents the covalent grafting of a hyaluronic acid-lysozyme (HA-LZ) composite onto corrosion resistant silane coated AZ31 Mg alloy via EDC-NHS coupling reactions. The HA-LZ composite coatings created a smooth and hydrophilic surface with the increased concentration of functional lysozyme complexed to the hyaluronic acid group. This was confirmed by the measurement of AFM, water contact angle, and quantification of hyaluronic acid and lysozyme. The colonisation of S.aureus on HA-LZ composite coated substrates was significantly reduced as compared to the hyaluronic acid, lysozyme coated and uncoated AZ31 controls. This is due to the enhanced antibacterial activity of the lysozyme component as observed from the spread plate assay, propidium iodide staining and scanning electron microscopy. Furthermore, morphology of the osteoblast cells, alkaline phosphatase activity and DNA quantification studies demonstrated the improved biocompatibility and osteoinductive properties of HA-LZ coated substrates.

The work described in this chapter has been published: Agarwal, S., Riffault, M., Hoey, D., Duffy, B., Curtin, J. and Jaiswal, S. Biomimetic hyaluronic acid-lysozyme composite coating on AZ31 Mg alloy with combined antibacterial and osteoinductive activities. ACS Biomaterials Science & Engineering, 2017, 3(12), 3244-3253.
5.1 Introduction

In chapter 4, the corrosion resistance and osteoanabolic properties of hyaluronic acid modified silane coated AZ31 Mg alloy were evaluated. The silane coating enhanced the corrosion resistance performance in HEPES buffered DMEM and controlled the degradation of AZ31 Mg alloy, thereby providing a suitable environment for osteoblasts survival. The osteoblasts showed improved functions on the hyaluronic acid modified-silane coated AZ31 surface. However, concerns have been raised over the implant associated microbial infections, which is a major cause of device failure. Therefore, it is important to develop antimicrobial coatings which can prevent bacterial colonisation on the implant surface without affecting osteoblast functions. Previous studies reported that the corrosion of Mg-based alloys causes an increase in the pH of the surrounding medium, thereby inhibiting the growth of bacteria [1]. However, biological fluids are stabilised by buffer systems that maintain physiological processes. Therefore, highly alkaline environments that arise due to corrosion of Mg alloys are buffered under in vivo conditions, thereby limiting the antimicrobial activity of the implant [2]. Previous studies have also demonstrated that some bare and corrosion-resistant Mg-based alloys are highly susceptible to microbial growth on the surface [2–4]. Furthermore, it is important that the orthopaedic implant should expedite the healing of any defective bone. The orthopaedic implant efficiency in restoring damaged bone is determined by its osteointegration. This is characterised by the tight bond between the bone and implant surface, thereby minimising the risk of implant loosening over time [5].

To improve the biological acceptance of orthopaedic implants in terms of osteoinductivity and inhibiting bacterial colonisation on the surface, bio-macromolecules such as hyaluronic acid and lysozyme can be employed on the corrosion-resistant Mg-based alloys. The combination of hyaluronic acid and lysozyme have been explored previously
on ocular lenses [6]. Hyaluronic acid is a polyanionic multifunctional polymer present as a component of extracellular matrix which plays an important role in promoting osteoblast cell motility and proliferation [6,7]. Additionally, its anionic and hydrophilic nature also reduces bacterial cell adhesion [6,8]. However, the application of HA onto an implant surface cannot completely avoid infections. Lysozyme is a lytic enzyme present in egg white, human tears as well as in other secretions [6]. Its antimicrobial properties are associated with the hydrolysis of the peptidoglycan layer in the bacterial cell wall, thereby perturbing the membrane functions. Moreover, as a part of a natural defence system, lysozyme is environmentally friendly and acts with more specificity than conventional biocides, such as quaternary ammonium salts and antibiotics, thereby preventing the risk of developing multidrug-resistant bacteria. Lysozyme has been utilised to modify the surfaces of biomedical devices to prevent bacterial growth [6,9].

Previously, adsorption of lysozyme on hyaluronic acid functionalised mesoporous SBA-15 particles was studied as a therapeutic agent [10]. Therefore, the synergistic immobilisation of hyaluronic acid and lysozyme on a corrosion resistant silane coated AZ31 Mg alloy surface is proposed for the first time in this work to confer simultaneous antimicrobial and osteoinductive properties for challenging biodegradable orthopaedic applications.

In the present study, the biomimetic hyaluronic acid-lysozyme (HA-LZ) composite coating was developed on the AZ31 Mg alloy and the combined antibacterial and osteoinductive activities were evaluated. The covalent binding of HA-LZ composite with the amine-terminated surface AZ31-MTES-TEOS-APTES (AZ31-MT-A) was achieved via EDC-NHS coupling reactions. The antimicrobial activity of HA-LZ immobilised on AZ31-MT-A coated Mg alloy was evaluated against *S. aureus* bacteria. Further, the
improved biocompatibility of HA-LZ coated surface has been demonstrated with murine osteoblasts.

5.2 Experimental

5.2.1 Materials

AZ31 alloy sheets were purchased from Shaanxi Taipu Rare Metal Materials Ltd, China. Methyltriethoxysilane and tetraethoxysilane, 3-aminopropyl-triethoxy silane, Hyaluronic acid (HA) from rooster comb (1-4 million Daltons), lysozyme from chicken egg white, phosphate buffer saline (PBS), Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F-12), fetal bovine serum (FBS), Penicillin-streptomycin antibiotics, phosphatase substrate and bisBenzimide H 33342 trihydrochloride, NHS (N-Hydroxysuccinimide), EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) and MES (2-(N-Morpholino) ethane sulfonic acid) buffer were purchased from Sigma Aldrich. Mueller-Hinton agar and nutrient broth were purchased from Lab M.

5.2.2 Surface modification with hyaluronic acid-lysozyme composite on AZ31 Mg alloy

5.2.2.1 Treatment of AZ31 with NaOH

The surface treatment was performed according to the method described in section 3.2.2.1

5.2.2.2 Functionalisation of hyaluronic acid-lysozyme functionalisation on silane coated AZ31 Mg alloy

Preparation of organo-silane coating is mentioned in section 3.2.2.2. Furthermore, the hyaluronic acid-lysozyme composite was functionalised to amine terminated AZ31-MT-A substrates. In this process, lysozyme (LZ) (10% and 20 % w/w of HA), HA (1 mg.ml⁻¹), NHS (0.1M) and EDC (0.4 M) were sequentially added into a MES buffer (0.01 M, pH 5.5) and stirred for 2h [6]. Similar reactions were also performed with respective LZ (10% and 20 % w/v) and HA (1 mg.ml⁻¹) concentrations which were considered as
controls. Here, we have used the above-mentioned LZ concentrations because at a higher concentration flocculation with white precipitate was observed in the solution. The AZ31-MT-A coating was immersed in the above solution for 4 h. After incubation, substrates were rinsed with MES buffer to remove physically adsorbed HA and LZ. The resultant HA-LZ (10% and 20%), LZ (10% and 20%) and HA-coated AZ31-MT-A surfaces were denoted as AZ31-HA-LZ-10%, AZ31-HA-LZ-20%, AZ31-LZ-10%, AZ31-LZ-20% and AZ31-HA respectively.

5.2.3 Characterisation of AZ31 modified substrates

5.2.3.1 Surface morphology and wettability of the substrate
The surface morphology of all modified (AZ31-HA-LZ-10%, AZ31-HA-LZ-20%, AZ31-LZ-10%, AZ31-LZ-20% and AZ31-HA) and uncoated AZ31 substrates were characterised by atomic force microscopy in tapping mode (AFM, Asylum MFP-3D-BIO, USA). The wettability of the samples was determined by static water contact angle measurements (FTÅ-200 system) using a sessile drop method (2µl, Milli-Q water).

5.2.3.2 Quantification of immobilised hyaluronic acid and lysozyme on the AZ31 surface
Quantification of hyaluronic acid was performed according to the method described in section 4.2.3.2.
The lysozyme activity of HA-LZ and LZ coatings was determined using 4-methylumbelliferonyl β-D-N,N’, N”-triacetylchitotrioside (4-MU-GlcNAc3) as a fluorimetric enzyme substrate [12,13]. The substrate was dissolved at a concentration of 100 µM in 50 mM sodium acetate buffer of pH 5 and incubated with the HA-LZ coated samples at 37 °C for 30-60 mins. The lysozyme activity was stopped by using a glycine-carbonate buffer at a pH of 10.5 and the fluorescence of 4-methyl umbelliferone was measured at Ex/Em =360/445 nm. The amount of 4-MU produced during the enzymatic
reaction was determined using its standard curve. One unit of the lysozyme activity for 4-MU-GlcNAc$_3$ hydrolysis was defined as the amount of enzyme that generates 1 µM of 4-MU per minute at 37 °C at pH 5.

5.2.4 Cytocompatibility

5.2.4.1 DNA quantification

The experimental section of DNA quantification is mentioned in section 3.2.4.4

5.2.4.2 Alkaline phosphatase (ALP) assay

The experiment was carried out according to the section 3.2.4.5

5.2.4.3 Actin Phalloidin and DAPI staining

The morphology of actin cytoskeleton of osteoblast cells was investigated 24 h after seeding. Briefly, cells were washed with PBS, fixed with 10% formalin, and permeabilised with 0.1% Triton (Sigma) in PBS. Actin cytoskeleton was stained with Phalloidin (1/40 in PBS, AlexaFluor488-Phalloidin, Life Technologies) for 20 minutes, and nuclei were stained with DAPI (1/2000 in PBS, Sigma). All cells were imaged using an Olympus IX83 epifluorescence microscope fitted with a 10x objective.

5.2.4.4 Cell morphology imaging by SEM

This section is similar to that of mentioned in section 3.2.4.3

5.2.5 Antibacterial activity

5.2.5.1 Spread plate assay

The antibacterial activity of uncoated and coated AZ31 substrates was determined using *S. aureus* (ATCC 25923), the most common pathogen associated with orthopaedic implant infections. For all the experiments, the substrates were sterilised by exposing UV radiations for 40 mins on each side. The substrates were immersed in sterile plastic tubes with 20 ml (1 cm$^2$ = 20 ml) of 1 x 10$^6$ cells.ml$^{-1}$ of initial bacterial suspension in PBS and
incubated at 37 °C with constant shaking at 200 rpm. The substrates were removed from the tubes after 2, 4 and 12 h to evaluate the antibacterial activity.

The substrates were gently washed thrice with PBS and sonicated in PBS for 5 mins to dislodge the cells from the surface. The 0.2 ml of bacterial suspension in PBS was plated onto the triplicate agar plate and incubated at 37 °C for 24 h. This timeline will allow for evaluation of the initial bacterial cell attachment and colonisation on the coated and uncoated AZ31 substrates. The colonies were counted, and results were expressed as a mean of colony forming units (CFU)/ml/cm² [14].

5.2.5.2 Propidium iodide (PI) exclusion assay

The coated and uncoated substrates (Size: 1 cm²) were incubated with *S.aureus* bacteria (10⁶ cells/ml) in PBS for 2, 4 and 12 h. After the incubation, the substrates were treated with PI at a concentration of 60 µM for 30 minutes in the dark. The substrates were washed with PBS twice and placed in the black bottom 24 well plates to measure the fluorescence (Ex /Em: 535/617 nm) using Spectramax-M3 microplate reader.

5.2.5.3 Bacterial cell morphology by SEM

All the coated and uncoated substrates were incubated for 4 h with bacteria and gently washed with the PBS. The bacterial cells were fixed in 10% formalin and dehydrated in alcohol gradients before being sputter coated with Au/Pd and imaged using SEM.

5.2.6 Statistical analysis

All of the experiments were conducted in triplicate. All data are expressed as mean ± S.D (n=3). The differences between the groups were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test. Statistically significance was considered at **p < 0.01 and *p < 0.05.
Chapter 5

5.3 Results and discussion

5.3.1 Characterisation of AZ31 modified substrates

Prior to application of HA and LZ coatings, the AZ31 surface was treated with the sequential steps of MTES-TEOS and APTES silanes to achieve amine functionalised coating. In previous studies, it has been demonstrated that the amino group of such coatings can be functionalised with various biomolecules such as hyaluronic acid, bovine serum albumin, RGD peptides etc., through amide bonds by EDC-NHS chemistry [15–17]. The functionalisation of HA-LZ coating onto the amine group of APTES coatings was confirmed by AFM, contact angle measurement as well as quantification of HA and LZ by enzymatic methods as shown in Fig 5-1 and Table 12 respectively.

**Table 12 Summary of characterization parameters of different substrates**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AZ31</th>
<th>AZ31-MT-A</th>
<th>AZ31-HA</th>
<th>AZ31-HA-LZ-10%</th>
<th>AZ31-HA-LZ-20%</th>
<th>AZ31-LZ-10%</th>
<th>AZ31-LZ-20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Roughness ((R_a), nm)</td>
<td>74.5 ± 8.2</td>
<td>45.7 ± 0.82</td>
<td>4.86 ± 0.07</td>
<td>17.1 ± 1.84</td>
<td>21.14 ± 6.45</td>
<td>24.3 ± 3.15</td>
<td>25.7 ± 7.3</td>
</tr>
<tr>
<td>Contact angle (degrees)</td>
<td>74.57 ± 0.92</td>
<td>46.4 ± 2.1</td>
<td>41.24 ± 2.66</td>
<td>48.85 ± 1.07</td>
<td>51.20 ± 3.36</td>
<td>53.37 ± 5.29</td>
<td>55.67 ± 0.92</td>
</tr>
<tr>
<td>HA (ng/ml/cm²)</td>
<td>---</td>
<td>---</td>
<td>0.82 ± 0.05</td>
<td>48.22 ± 0.10</td>
<td>47.53 ± 0.05</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Lysozyme activity (U/ml/cm²)</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>0.534 ± 0.042</td>
<td>0.588 ± 0.021</td>
<td>0.465 ± 0.03</td>
<td>0.513 ± 0.027</td>
</tr>
</tbody>
</table>

The surface roughness (\(R_a\)) of the AZ31 and AZ31-MT-A are 74.5 ± 8.2 nm and 45.7 ± 0.82 nm respectively. The roughness reduced considerably after coating with HA-LZ composite and LZ coatings on AZ31-MT-A substrate ranging from 17.1 ± 1.84 nm to 25.7 ± 7.3 nm. However, the increase in surface roughness for the composite coatings as compared to the AZ31-HA substrate with \(R_a\) of 4.86 ± 0.07 nm could be attributed to the increased content of LZ. Static water contact angle measurement was used to determine the hydrophilic changes of modified AZ31 substrates (Table 12). When compared to the
AZ31 alloy, the AZ31-MT-A showed a lower contact angle of 46.4 ± 2.1°, indicating the presence of polar amine groups on the surface. The wettability of the HA-LZ and LZ modified surfaces decreased ranging from 48.85 ± 1.07° to 55.67 ± 0.92° when compared to the AZ31-HA surface with a contact angle of 41.24 ± 2.66°. However, these coated substrates showed a considerable increase in wettability when compared to the AZ31 substrate (74.5 ± 8.2°). The wettability of the substrates is strongly related to the surface roughness and can play an important role in bacteria cell adhesion [19].

Figure 5-1 Surface morphology of uncoated and coated substrates was determined using AFM for (a) AZ31, (b) AZ31-HA, (c) AZ31-HA-LZ-10%, (d) AZ31-HA-LZ-20%, (e) AZ31-LZ-10% and (f) AZ31-LZ-20% substrates.

Furthermore, the concentration of immobilised HA and lysozyme activity of the coatings were quantified (Table 12). It was found that the concentration of HA immobilised on AZ31-HA, AZ31- HA-LZ-10% and AZ31-HA-LZ-20% substrates were very similar and found to be ~ 48 µg/ml/cm². The lysozyme activity of AZ31- HA-LZ-10% and AZ31-HA-LZ-20% substrates were higher and found to be 0.534 ± 0.042 and 0.588 ± 0.021 U/ml/cm² respectively when compared to the activity of AZ31-LZ-10% and AZ31-LZ-
20% substrates at 0.465 ± 0.03 U/ml/cm\(^2\) and 0.513 ± 0.027 U/ml/cm\(^2\) respectively. Previous studies have shown that hyaluronic acid can interact electrostatically with lysozyme without affecting the activity of lysozyme in the HA-LZ composite [20,21]. Therefore, these characterisation studies confirmed the surface modifications of AZ31 Mg alloy with HA-LZ composite.

### 5.3.2 Antibacterial properties

Results from the analysis of bacterial colonisation on HA, HA-LZ, LZ coated and uncoated AZ31 substrates were determined using a spread plate assay and shown in Fig. 5-2.

![Figure 5-2](image)

Figure 5-2 Viability of adhered *S. aureus* on different substrates was measured using CFU assay 2 h, 4 h and 12 h after immersion in bacterial cultures. One way ANOVA with posthoc Tukey test with significance level of ** p < 0.01, * p < 0.05."

The results demonstrated that bacterial cells adhered to the AZ31 and AZ31-HA surfaces markedly increased by 144.6\% and 110.6\% respectively over the 12 h period. However, the number of bacteria on the AZ31-HA surface is significantly less than the AZ31 alloy. In contrast, the AZ31-HA-LZ-10\% and AZ31-HA-LZ-20\% substrates showed a
significant reduction in bacteria cell adhesion as compared to the AZ31 alone during the 12 h incubation period. Similarly, these substrates also showed a significant decrease in bacterial cell adhesion as compared to the AZ31-LZ-10% and AZ31-LZ-20% controls. The overall decrease in the bacterial colonies of the HA-LZ composite coatings is an indication of the lysozyme concentration-dependent anti-adhesive and/or anti-bacterial properties over uncoated AZ31 and other modified substrates.

Furthermore, the bactericidal activity of the coated and uncoated AZ31 substrates was determined fluorometrically using PI staining or exclusion of the bacteria adhered to the substrates as shown in Fig. 5-3.

![Figure 5-3 S. aureus viability was measured by fluorescence using the PI exclusion assay following incubation on different substrates. One way ANOVA is followed with a posthoc Tukey test with significance level of ** p < 0.01, * p < 0.05.](image)

The uncoated AZ31 substrate demonstrated antibacterial activity in the initial period of incubation but it decreases in the remaining period, whereas AZ31-HA-LZ-10% and AZ31-HA-LZ-20% showed a significant increase in antibacterial activity as compared to
the bare AZ31, AZ31-LZ-10% and AZ31-LZ-20% substrates over 12 h of incubation. The AZ31-HA substrate showed minimal antibacterial activity, as there are no bactericidal functional groups present in HA.

Fig. 5-4 shows the morphology of the bacteria on the uncoated and coated substrates after 4 h of incubation in bacterial suspension. From Fig. 5-4((a)-(b)), the bacteria adhered to the AZ31 and AZ31-HA surfaces maintained good shape indicating cytocompatibility of the bacteria. On the HA-LZ composite (Fig. 5-4(c-d)) and LZ coated (Fig.5-4(e-f)) substrates, only a few bacteria maintained their native shape and morphology (indicated by green arrows). In addition, cell debris (indicated by yellow arrows) and affected cells presenting distorted morphology (indicated by red arrows) are observed on HA-LZ composite and LZ coated substrates.

Figure 5-4 Representative SEM images of *S.aureus* on (a) AZ31, (b) AZ31-HA, (c) AZ31-HA-LZ-10%, (d) AZ31-HA-LZ-20%, (e) AZ31-LZ-10% and (f) AZ31-LZ-20% substrates after 4h of incubation (Red, yellow and green arrows are indicating affected cells, cell debris and unaffected cells respectively)
Many studies have reported that antibacterial activity of Mg-based alloys being directly related to the increased pH of the medium [1,22]. However, recent studies showed that in buffered environments, Mg-based alloys are susceptible to bacterial colonisation under *in vitro* and *in vivo* conditions [2,3,23]. In this study, an increased bacteria cell adhesion or reduced antibacterial activity of uncoated AZ31 substrates during the incubation period in bacterial suspension in PBS was observed. This is likely due to the insufficient increase in the pH of the medium (8.16 ± 0.15), in addition to the greater roughness and contact angle of the surfaces (Table 12). In contrast, the enhanced antibacterial activity of AZ31-HA-LZ-10% and AZ31-HA-LZ-20% substrates over respective controls AZ31-LZ-10% and AZ31-LZ-20% was observed as demonstrated in Fig.5-2 and Fig. 5-4, which could be attributed to the higher content of lysozyme immobilisation with hyaluronic acid (Table 12). The reduced bacterial cell adhesion on the hyaluronic acid coated AZ31-HA surface can be attributed to the bacterial anti-adhesive characteristics of HA polymer due to the anionic nature and lubricity of the coating as well as low surface roughness [8].

As stated previously, the most common pathogen associated with the orthopaedic implants is *S.aureus* [8]. Several studies reported the bactericidal activity of lysozyme against *S.aureus* [24,25]. The antibacterial activity of lysozyme involves its disruptive interaction with the bacterial membrane. Lysozyme disrupts the cell wall by catalysing the hydrolysis of the β 1-4 linkages between the N-acetylmuramic acid and N-acetyl-D-glucosamine residues in chitodextrins, thereby increasing the permeability of bacterial cell wall and causing the cells to rupture. According to the contact killing mechanism of lysozyme, bacteria first adhere to the surface leading to the breakdown of the bacterial cell wall [6]. The previous study reported the efficient antibacterial activity of the HA-LZ coated intraocular lenses against *S.aureus* for preventing endophthalmitis [6]. However, there is limited information available about the efficacy of HA-LZ as a
Chapter 5

composite coating to prevent the bacterial colonisation for orthopaedic applications and their effect on the osteoblast cell response. Having demonstrated the antimicrobial properties of HA-LZ against *S. aureus*, the most common pathogen associated with orthopaedic implants, we next investigated the cytocompatibility of HA-LZ with osteoblast cultures.

### 5.3.3 Cytocompatibility

Fig. 5-5 shows the morphology of osteoblast cells on the uncoated and coated AZ31 substrates at day 1. The osteoblast cells that attached to the uncoated AZ31 substrate are sparse and circular in shape as shown in SEM image Fig. 5-5 I(a), demonstrating the poor adhesion to the substrate. The AZ31-HA (Fig. 5-5(b)), AZ31-HA-LZ-10% (Fig. 5-5(c)) and AZ31-HA-LZ-20% (Fig.5-5(d)) surfaces demonstrate flattened, extended morphology and greater cell-to-cell interaction of adhered osteoblasts. In contrast, AZ31-LZ-10% (Fig. 5-5(e)) and AZ31-LZ-20% (Fig.5-5(f)) coated substrates displayed an elongated morphology.

Figure 5-5 (I) Representative SEM and (II) fluorescent images of the actin cytoskeleton of osteoblasts cells cultured on AZ31 (a-a’), AZ31-HA (b-b’), AZ31-
HA-LZ-10% (c-c′), AZ31-HA-LZ-20% (d-d′), AZ31-LZ-10% (e-e′) and AZ31-LZ-20% (f-f′) substrates for day 1 respectively. Actin cytoskeleton was stained with Phalloidin (green) and nuclei were stained with DAPI (blue). (Scale bar of SEM images is 50 µm)

Fig. 5-5 (II) series show the actin staining of osteoblast cells on different substrates after 24 h of incubation. The cellular morphology of osteoblast cells is circular and poorly defined on the uncoated AZ31 substrate (Fig 5-5(a′)), without strong actin filament staining, indicating the cells adhered poorly and did not develop a filamentous actin cytoskeleton on the AZ31 substrate. Similar osteoblast morphology on the uncoated AZ31 substrate can be observed from SEM image (Fig. 5-5(a)). On the AZ31-HA, AZ31-HA-LZ-10% and AZ31-HA-LZ-20% substrates, osteoblast cells appear cuboidal in morphology, showing strong actin filament organisation extending in different directions as shown in Fig. 5-5(b′, c′ and d′) respectively, indicative of an osteoblastic phenotype. On the AZ31-LZ-10% (Fig. 5e′) and AZ31-LZ-20% (Fig. 5f ′) substrates, cells displayed somewhat elongated actin filaments. Overall, these results indicate that HA-LZ composite coated substrates facilitate improved adhesion and morphological phenotype of osteoblast cells as compared to the uncoated substrates.

Fig. 5-6 shows the DNA quantification of osteoblast cells cultured on the uncoated and coated AZ31 substrates which are considered to be a direct measure of cell proliferation over a period of 14 days. The coated substrates showed 3 to 6 folds increase in the total DNA content as compared to the AZ31 control. In comparison to the AZ31-LZ-10% and AZ31-LZ-20% substrates, a significant increase in the folds of DNA content was observed for AZ31-HA-LZ-10% and AZ31-HA-LZ-20% substrates over a period of 14 days. This indicated the improved osteoblast adhesion and proliferation on the HA-LZ coated substrates.
Figure 5-6 DNA content was measured following culture of osteoblast cells on AZ31, (b) AZ31-HA, (c) AZ31-HA-LZ-10%, (d) AZ31-HA-LZ-20%, (e) AZ31-LZ-10% and (f) AZ31-LZ-20% substrates for 3, 7 and 14 days respectively. One way ANOVA with posthoc Tukey test with significance level of ** p < 0.01, * p < 0.05.

These results showed that even though the osteoblast adhered to AZ31-LZ coatings (Fig. 5-5), the proliferation of osteoblast cells was not comparable to the AZ31-HA and AZ31-HA-LZ substrates. Moreover, the change in DNA concentration for the AZ31-HA and AZ31-HA-LZ coated substrate is insignificant, thereby indicating that the osteoblast cell proliferation was not affected by lysozyme in the HA-LZ composite coatings.

Furthermore, the ALP activity of osteoblast cells cultured on the uncoated and coated AZ31 substrate for 14 days is presented in Fig. 5-7. The ALP activity of osteoblast cells cultured on HA coated substrates (i.e. AZ31-HA, AZ31-HA-LZ-10% and AZ31-HA-LZ-20%) demonstrated significantly higher activity as compared to AZ31, AZ31-LZ-10% and AZ31-LZ-20% substrates. However, the ALP activity of AZ31-HA coated surface was significantly higher when compared to the HA-LZ coated equivalents particularly on day 7. The decrease in the ALP activity of the HA-LZ coated surface is
likely due to the presence of lysozyme itself as the similar trend was also observed for AZ31-LZ-10% and AZ31-LZ-20% coated substrates

Figure 5-7 Intracellular ALP activity was used to measure the differentiation of osteoblast cells cultured on AZ31, (b) AZ31-HA, (c) AZ31-HA-LZ-10%, (d) AZ31-HA-LZ-20%, (e) AZ31-LZ-10% and AZ31-LZ-20% substrates for 3, 7 and 14 days respectively, One way ANOVA with posthoc Tukey test with significance level of ** p < 0.01, * p < 0.05.

This would indicate that the level of ALP activity is affected by the presence of lysozyme. However, at a later stage of differentiation (day 14), the ALP activity of HA-LZ coated substrate is comparable to the HA-coated equivalent and significantly higher when compared to AZ31 Mg alloy alone. Bone regeneration is a complex process which involves the early stages of cell adhesion and proliferation, and later stages of cell differentiation and function [26]. From this study, it can be observed that HA-LZ coated surface showed comparable and enhanced cell adhesion and proliferation when compared to HA-coated and uncoated AZ31 equivalents respectively (Fig. 5-5 and 5-6). Osteoblast cells express ALP in the early stage of osteoblast differentiation and participate in the mineralisation of the extracellular matrix through the generation of phosphate ions from
Chapter 5

the hydrolysis of organic substrates [27]. HA is one of the major components of the extracellular matrix (ECM) of many cells including osteoblasts which not only plays a structural role in the bone matrix but regulates cell adhesion, proliferation, migration, wound healing and expression of osteoinductive factors including ALP. It also possesses the receptors for CD 44 (cell surface glycoprotein) and RHAMM (receptor for HA-mediated motility) which promote the differentiation of osteoblast cells [26,28]. Therefore, the decrease in the ALP activity on HA-LZ composite coated substrate in the early stage of differentiation is likely due to the interference in the above-mentioned interactions of osteoblast cells with the HA due to the lysozyme component. However, an in-depth study will be required to elucidate the mechanisms regulating the differentiation of osteoblast cells on such composite coatings.

5.4 Conclusion

In this study, the HA-LZ composite was functionalised onto a silane-based corrosion resistant coating on the AZ31 Mg alloy. Among all coated substrates, the AZ31-HA-LZ-20% substrate showed a significant higher bactericidal activity against S. aureus bacteria without greatly compromising the cytocompatibility of osteoblasts. Improved cell adhesion with flattened cuboidal morphology, proliferation and differentiation of osteoblast cells was observed on the AZ31-HA-LZ composite coated substrates relative to the uncoated AZ31 and AZ31-LZ substrates. At the later stages of osteoblast differentiation, the HA-LZ and HA functionalised AZ31 substrates showed a similar level of ALP activity. Collectively, these results indicate that such composite coatings are of great interest and may prevent bacterial colonisation whilst enhancing the cytocompatibility of AZ31 Mg alloy for orthopaedic implant applications.
5.5 References


Chapter 6

The effect of high and low molecular weight hyaluronic acid functionalised-AZ31 Mg and Ti alloys on proliferation and differentiation of osteoblast cells

This study evaluated osteoblast functions such as adhesion, proliferation and differentiation in response to high and low molecular weight HA (denoted as h-HA and l-HA respectively) functionalised on Ti alloy (h-HA-Ti and l-HA-Ti substrates respectively) and corrosion-resistant silane coated-AZ31 Mg alloys (h-HA-AZ31 and l-HA-AZ31 respectively). The DNA quantification study showed that adhesion and proliferation of osteoblast cells were significantly decreased by h-HA immobilised on Ti alloy (Ti) or AZ31 Mg alloy substrates when compared to low molecular weight counterpart over a period of 14 days. Notably, h-HA significantly increased the osteogenic differentiation of osteoblast when compared to the l-HA, as confirmed by the enhanced expression of ALP, total collagen and mineralisation of the extracellular matrix. In particular, the h-HA-AZ31 substrates greatly enhanced the osteoblasts differentiation amongst tested samples (l-HA-AZ31, l-HA-Ti, h-HA-Ti and Ti alone), which is ascribed to the osteoinductive activity of h-HA, relatively up-regulated intracellular Ca$^{2+}$ ([Ca$^\text{2+}$]) and Mg$^{2+}$ ([Mg$^{2+}$]) concentrations as well as the alkalisation of the surrounding cell culture medium.

The work described in this chapter has been published: Agarwal, S., Duffy, B., Curtin, J. and Jaiswal, S, Effect of high and low molecular weight hyaluronic acid functionalised-AZ31 Mg and Ti alloys on proliferation and differentiation of osteoblast cells, *ACS Biomater. Sci. Eng.*, 2018, 4 (11), 3874–3884
6.1 Introduction

In chapter 4 and 5, hyaluronic acid showed enhanced osteoblasts’ adhesion, proliferation and differentiation. In addition, Mg\(^{2+}\) in the cell culture medium further enhanced the osteoblastic activity (as observed in chapter 3). Therefore, it is important to explore the effect of AZ31 Mg substrate and hyaluronic acid combination on osteoblasts adhesion, proliferation and differentiation.

Generally, the most distributed form of HA in the normal tissue is high molecular weight HA (h-HA) (>10\(^3\) kDa) [1]. During pathological conditions, including tissue repair, h-HA undergoes either hyaluronidase mediated degradation or oxidative hydrolysis to produce low molecular weight HA (l-HA) (<10\(^3\) kDa) [2]. Both forms (h-HA and l-HA) regulate different cellular activities such as cell proliferation and differentiation at different stages of bone repair or remodelling [3]. During tissue injury, l-HA stimulates cell motility and proliferation, exhibiting pro-angiogenic and pro-inflammatory responses [4]. In contrast to l-HA, h-HA has been shown to improve the differentiation and enhance or maintain the cell-cell communication [5]. However, the exact role of the h-HA during tissue healing has not been assessed conclusively. Based on previous studies, h-HA is thought to play an important role during later stages of bone remodelling per se by enhancing the differentiation of osteoprogenitor cells or osteoblast lineage cells, thereby aiding to achieve an original biological state of the tissue [3,6,7]. Since there is a clear distinction in the biological activity of HA-based on molecular weight, it is important to quantify the effect. This would play a significant role when it is functionalised onto metal implants for different applications. Importantly, many reported studies functionalised HA on Ti alloys without considering the biological properties of HA [8,9]. This shortcoming may have prevented the exploitation of untapped bone forming potential of hyaluronic acid for orthopaedic applications. Magnesium-based alloys have been subjected to
various surface modifications to enhance their corrosion resistance and biocompatibility [10]. In addition, low levels Mg\(^{2+}\) released in the surrounding medium through due to controlled degradation of Mg-based alloys has been shown to improve the osteoblastic activity [11–13]. However, rapid degradation of Mg alloys leads to alkalisation of the surrounding medium (pH > 9) causing toxicity to the bone-related cells [14,15]. Previous studies employed various strategies to improve the cytocompatibility and osseointegration of Mg-based alloys using surface functionalisation of biomacromolecules such as collagen, albumin, heparin, chitosan or their composites [10,16].

Our previous study showed that hyaluronic acid coatings on treated AZ31 substrates enhanced the osteoblastic activity as compared to the bare alloy [17]. This improved cytocompatibility is attributed to the osteoinductive property of HA and controlled degradation of the AZ31-Mg alloy. However, the assessment of osteoblasts’ functions and the mechanisms involved in response to different molecular weight HA and extracellular Mg\(^{2+}\) have not been well explored to date.

In this study, low and high molecular weight HA (denoted as l-/h-HA) was grafted onto silane coated AZ31 (l-/h-HA-AZ31) and Ti (l-/h-HA-Ti). The fabrication of the coatings on AZ31 Mg alloy involves an initial MTES-TEOS sol-gel treatment, followed by APTES to deliver an amine terminated surface (as described previously [15]) which can be covalently coated with l-HA and h-HA via EDC-NHS coupling reactions. Our previous studies showed that the multi-functionality of this assembly of silane coating not only improves the corrosion protection of AZ31 Mg alloy but also facilitates the functionalisation of bioactive biomacromolecules. The effect of l-/h-HA functionalised Ti and AZ31 Mg alloys on osteoblast cell proliferation, differentiation and functions were evaluated. In addition, the cellular mechanisms by which h-HA and l-HA along with Mg\(^{2+}\) conditioned medium affecting the differentiation of osteoblasts were also studied.
6.2 Experimental

6.2.1 Material

AZ31 alloy and Ti alloy (Ti-6Al-4V) sheets were obtained from Shaanxi Taipu Rare Metal Materials Ltd, China and Good Fellow Ltd, USA respectively. Methyltriethoxysilane, tetraethoxysilane, 3-aminopropyl-triethoxy silane, high molecular weight Hyaluronic acid from rooster comb (1-4 MDa), low molecular weight hyaluronic acid from *Streptococcus equi. spp.* (30-50 kDa) phosphate buffer saline (PBS), Dulbecco's Modified Eagle's Medium/Nutrient F-12 Ham (DMEM/F-12), fetal bovine serum (FBS), Penicillin-streptomycin antibiotics, phosphatase substrate and bisBenzimide H 33342 trihydrochloride, NHS (N-Hydroxysuccinimide), EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and MES (2-(N-Morpholino)ethanesulfonic acid) buffer were purchased from Sigma Aldrich.

6.3 Surface modifications of AZ31 Mg and Ti alloy

6.3.1 Treatment of AZ31 and Ti with NaOH

As mentioned previously in section 3.2.2.1

6.3.2 Preparation of the hyaluronic acid functionalised silane coating on AZ31 Mg and Ti alloy

Preparation of organo-silane coating is mentioned in section 3.2.2.2. Furthermore, 1 mg/ml of high and low molecular weight HA solutions were used to functionalise AZ31-MT-A and Ti-MT-A substrates by a carbodiimide-mediated coupling reaction as detailed in the previous study [18][17,19]. The resultant high and low molecular HA functionalised AZ31-MT-A or Ti-MT-A substrates are denoted as h-HA-AZ31, l-HA-AZ31, h-HA-Ti and l-HA-Ti substrates respectively. Here, we used 1 mg/ml of HA because of two issues:
a) At a higher concentration, high and low molecular weight HA become a gel-like solution. This also prevents the complete solubilisation of HA.

b) Due to the high viscosity, it is difficult to prepare a reproducible and uniform coating of HA.

6.4 Cytocompatibility

6.4.1 Imaging of osteoblasts morphology by SEM

This section is similar to that of mentioned in section 3.2.4.3

6.4.2 DNA quantification

The experimental section of DNA quantification is mentioned in section 3.2.4.4

6.4.3 Alkaline phosphatase (ALP) assay

The experiment was carried out according to the section 3.2.4.5

6.4.4 Quantification of magnesium and calcium ions

After 3, 7 and 14 days of cell culture under differentiation conditions, osteoblasts were scraped and lysed using cell lysis buffer [20]. The Mg$^{2+}$ and Ca$^{2+}$ in the cell lysate were determined using respective magnesium and calcium assays kit (Sigma Aldrich). The intracellular Mg$^{2+}$ and Ca$^{2+}$ concentrations were measured by absorbance at 450 nm and 575 nm respectively. Standards curves were prepared as per kit instructions. The Mg$^{2+}$ concentration in the culture medium was also determined using a magnesium assay kit (Sigma Aldrich).

6.4.5 Total collagen content

The total collagen content of all the samples was determined using a hydroxyproline assay kit (Sigma Aldrich). Osteoblasts cultured on different substrates for 3, 7 and 14 days under differentiation conditions were scraped and subjected to acid hydrolysis using 12 N HCl for 12 hrs incubated at 100 ºC in a pressure-tight polypropylene vial with PTFE-lined cap [21]. Thereafter, following the instruction given in the kit, the absorbance was measured.
at 560 nm. The total hydroxyproline content was calculated using standards provided in the kit (Sigma Aldrich).

6.4.6 Assessment of osteoblasts mineralisation using SEM-EDX

After 14 days of culturing, osteoblasts adhered samples were washed thrice with PBS and fixed with formalin. The cell-fixed samples were dehydrated in alcohol gradients. After the sputter coating with Au-Pd, the elemental content of the adhered cells on different substrates was determined using SEM-EDX [20].

6.5 Statistical analysis

All of the experiments were conducted in triplicate. All data are expressed as mean ± S.D. The differences between the groups were analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey test.

6.6 Results

6.6.1 Characterisation of modified AZ31 and Ti substrates

The functionalisation of l-HA and h-HA on AZ31 and Ti substrates was confirmed using AFM, static water contact angle and HA quantification as shown in Table 13. The surface roughness ($Ra$) of uncoated AZ31 and Ti substrates were found to be 47.43 ± 4.11 nm and 14.5 ± 2.11 nm respectively. The l-HA functionalised on AZ31 and Ti substrates showed slightly reduced surface roughness as compared to h-HA functionalised AZ31/Ti substrates.

Table 13 Summary of characterisation parameters for different substrates

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Roughness (nm)</th>
<th>Contact angle (degrees)</th>
<th>HA ($\mu g/cm^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZ31</td>
<td>47.43 ± 4.11</td>
<td>59.67 ± 6.99</td>
<td>…..</td>
</tr>
<tr>
<td>h-HA-AZ31</td>
<td>11.29 ± 0.21</td>
<td>44.22 ± 3.58</td>
<td>47.62 ± 1.05</td>
</tr>
<tr>
<td>l-HA-AZ31</td>
<td>7.67 ± 0.17</td>
<td>36.06 ± 2.03</td>
<td>41.58 ± 2.05</td>
</tr>
<tr>
<td>h-HA-Ti</td>
<td>8.66 ± 0.58</td>
<td>40.54 ± 3.03</td>
<td>48.51 ± 0.02</td>
</tr>
<tr>
<td>l-HA-Ti</td>
<td>7.08 ± 0.71</td>
<td>26.62 ± 4.46</td>
<td>40.6 ± 1.49</td>
</tr>
<tr>
<td>Ti</td>
<td>14.5 ± 2.11</td>
<td>45.31 ± 5.92</td>
<td>…..</td>
</tr>
</tbody>
</table>
The AFM images of these substrates are given in Figure 6-1. Overall, the roughness of substrates reduced for HA-functionalised AZ31/Ti substrates when compared to respective uncoated substrates.

The static water contact angle was determined to confirm the wettability of the uncoated and coated substrates as shown in Table 13. The l-HA coated AZ31 (36.06° ± 2.03°) and Ti (26.62° ± 4.46°) substrates showed relatively higher hydrophilic surface as compared to h-HA functionalised AZ31 (44.22° ± 3.58°) and Ti (40.54° ± 3.03°) substrates. The HA-coated substrates showed a considerable increase in the wettability of surface when compared to the uncoated AZ31 (59.67° ± 6.99°) and Ti (45.31° ± 5.92°) substrates. Furthermore, the concentration of HA immobilised on the surface was quantified as given in Table 14. The concentration of h-HA immobilised on AZ31 and Ti substrate were found to be similar and found to be ~48 µg/cm², whereas l-HA showed the lower concentration of ~41 µg/cm².

Figure 6-1 AFM images for uncoated and coated AZ31-Mg and Ti alloys substrates.
6.6.2 Osteoblast cells Adhesion

Representative SEM images of osteoblasts adhered to AZ31-Mg and Ti alloys treated with or without l-HA and h-HA are shown in Fig 6-2 (I). After 24 h of incubation, osteoblasts are sparse and showed circular morphology on the uncoated AZ31 Mg alloy (Fig 6-2 I(a)), demonstrating poor adhesion to the substrate. The h-AZ31-HA (Fig 6-2 I(b)) and l-AZ31-HA (Fig 6-2 I(c)) surfaces showed flattened, cuboidal shaped and extended morphology of adhered osteoblast cells with greater cell-to-cell interaction. On the other hand, h-HA-Ti (Fig 6-2 I(d)) and l-HA-Ti (Fig 6-2 I(e)) surface showed triangular or irregular shaped morphology of attached osteoblast cells. The Ti alloy substrate control showed a greater number of adhered osteoblasts with spindle-shaped morphologies. Fig 6-2 (II) shows the quantification of total DNA content on these substrates which is considered a measure of osteoblasts adhered to the surface. The DNA content of osteoblasts on the AZ31 Mg alloy was found to be significantly lower when compared to all substrates. Poor adherence of osteoblasts to the HA-coated AZ31 surfaces was observed when compared to HA-coated Ti or Ti alone substrates. Interestingly, l-HA-coated Ti substrate showed significantly improved cell adhesion over h-HA-Ti, h-HA-AZ31 and l-HA-AZ31 substrates.
Figure 6-2 (I) SEM and (II) total DNA content of osteoblast cells cultured on different substrates for 24 h: (a) AZ31, (b) h-HA-AZ31, (c) l-HA-AZ31, (d) h-HA-Ti, (e) l-HA-Ti and (f) Ti. The scale of SEM images is 100 µm. Statistical analysis: Values are expressed as mean ± SD. One-way ANOVA with posthoc Tukey test with significance level of **P < 0.01 versus AZ31 control; #P < 0.05 indicates the comparison of l-/h-HA-AZ31 with the l/h-HA-Ti group; ΔP < 0.05 and ΔΔP < 0.01 indicate the comparison of Ti with the l-/h-HA-Ti group.
6.6.3 Osteoblast cell proliferation

Fig 6-3 shows the quantification of total DNA content of osteoblasts cultured on different substrates over a period of 14 days. The quantification of total DNA content was considered as a measure of cell proliferation. Since bare AZ31 substrate showed high cytotoxicity with very few live cells as observed from Fig 6-2, it was considered futile to use this as a control for further studies. From Fig 6-3, it can be noted that osteoblast proliferation on l-HA-AZ31 and l-HA-Ti were found to be significantly higher as compared to the h-HA-AZ31 and h-HA-Ti respectively over 14 days of cell culture. These results agree with SEM images and cell adhesion studies, indicating that l-HA promotes osteoblast cell proliferation over h-HA. Amongst all substrates, bare Ti alloy showed significantly enhanced osteoblast cell proliferation.

![Graph showing DNA concentration over time for different substrates](image)

Figure 6-3 Total DNA content was determined following osteoblast cultured on l-HA-AZ31, h-HA-AZ31, l-HA-Ti, h-HA-Ti and Ti substrates for 3, 7 and 14 days. Statistical analysis: Values are expressed as mean ± SD. One-way ANOVA with posthoc Tukey test with significance level of *P < 0.05, **P < 0.01 indicating the comparison between l-/h-HA-AZ31 and l-/h-HA-Ti substrates ; ##P < 0.01 indicates comparison between samples (l-/h-HA-AZ31 and l-/h-HA-Ti substrates versus Ti); ∆P < 0.05 and ∆∆ P < 0.01 indicate the pairwise comparison of samples.
6.6.4 Osteogenic differentiation of osteoblasts

Differentiation markers of osteoblasts cultured on HA coated substrates under osteogenic condition were screened as shown in Fig 6-4. The ALP activity of osteoblasts cultured on l-/h-HA-AZ31/Ti coated substrates determined for 14 days presented in Fig 6-4(a). Osteoblasts cultured on l-HA-AZ31 and l-HA-Ti showed significantly low ALP activity as compared to h-HA-AZ31 and h-HA-Ti respectively, over a period of 14 days. Overall, osteoblasts cultured on HA functionalised AZ31 Mg surface exhibited reduced intracellular ALP levels as compared to the HA-Ti substrates.

Figure 6-4 Evaluation of osteogenic differentiation of osteoblasts cultured on l-HA-AZ31, h-HA-AZ31, l-HA-Ti, h-HA-Ti and Ti substrates for 3, 7 and 14 days using (a) Intracellular ALP activity and (b) Hydroxyproline content. Statistical analysis:
Values are expressed as mean ± SD. One-way ANOVA with posthoc Tukey test with significance level of *P < 0.05, **P < 0.01 indicating the comparison between 1-/h-HA-AZ31 and 1-/h-HA-Ti substrates; #P <0.05, ##P < 0.01 indicate comparison between samples (1-/h-HA-AZ31 and 1-/h-HA-Ti substrates versus Ti); ∆P < 0.05 and ∆∆ P < 0.01 indicate the pairwise comparison of samples.

Furthermore, ALP activity was normalised to total DNA content of respective samples (Fig 6-4(a)). This result shows that h-HA coated AZ31/Ti substrates significantly enhanced the ALP activity of osteoblasts when compared to the l-HA coated AZ31/Ti substrates, whereas the Ti substrate showed lowest ALP activity during 14 days of the culture period. The order of ALP expression showed by the coated substrates is h-HA-AZ31 > l-HA-AZ31 > h-HA-Ti > l-HA-Ti ≥ Ti. These results indicate that the magnesium alloy substrate and h-HA synergistically induce the intracellular ALP activity of osteoblast cells.

Furthermore, the expression of total collagen by osteoblasts in response to l-/h-HA coated on AZ31/Ti substrates was evaluated using a hydroxyproline assay. The total collagen content was normalised to DNA content as shown in Fig 6-4(b). The h-HA-AZ31 surface (P<0.01) significantly stimulated the collagen synthesis when compared to l-HA-AZ31, h-HA-Ti, l-HA-Ti and Ti equivalents throughout the culture period. Furthermore, h-HA-Ti also showed a significant increase in total collagen content as compared to l-HA-Ti and bare Ti substrates. The expression level of collagen was found to be in the order of h-HA-AZ31 > l-HA-AZ31 > h-HA-Ti > l-HA-Ti > Ti. These results are in agreement with the intracellular ALP enzyme activity, thereby indicating that h-HA and AZ31 Mg alloy induced an enhanced differentiation as compared to the l-/h-HA-coated Ti alloy.

Fig 6-5 shows the representative SEM images of mineralisation of osteoblasts cultured under the osteogenic condition for 14 days.
Figure 6-5 Representative SEM images of osteoblasts cultured on (a) l-HA-AZ31, (b) h-HA-AZ31, (c) l-HA-Ti, (d) h-HA-Ti and (e) Ti substrates for 14 days in osteogenic CCM. The scale of SEM images is 50 µm. Red arrows are indicating Ca-P deposits.

Compared with l/h-HA-Ti (Fig 6-5(c) and d)), deposits of calcium (Ca) and phosphate (P) mineral nodules can be observed around the osteoblasts cultured on l-/h-HA-AZ31 surfaces. On the other hand, uniformly distributed Ca-P mineral particles observed on and around the osteoblasts adhered to l-/h-HA-Ti and Ti only substrates. Furthermore, elemental analysis of osteoblasts cultured on different substrates for 14 days is presented in Table 14. From Table 14, Ca and P contents (% At. wt.) detected on l-HA-Ti and h-HA-Ti were found to be higher than l-HA-AZ31 and h-HA-AZ31 respectively. Notably, AZ31-HA, Ti-HA and Ti without cells also showed Ca and P deposition. Since minor differences in element deposition on l-HA-AZ31/Ti and h-HA-AZ31/Ti controls were observed, an average of their elemental analysis is represented as AZ31-HA (w/o cells) and Ti-HA (w/o cells) samples. This indicated a very high content of non-specific Ca and P deposits on Ti (w/o cells) and Ti-HA (w/o cells) substrates which makes up ~ 83% of
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Ca and ~76-80% of P deposition of the corresponding substrates (l-HA-Ti, h-HA-Ti and Ti) with osteoblasts.

Table 14 Elemental contents detected by EDX of osteoblasts cultured on different substrates for 14 days. Statistical analysis: Values are expressed as mean ± SD.

<table>
<thead>
<tr>
<th>Elements</th>
<th>l-HA-AZ31</th>
<th>h-HA-AZ31</th>
<th>l-HA-Ti</th>
<th>h-HA-Ti</th>
<th>Ti alone</th>
<th>Ti-HA-w/o cells</th>
<th>AZ31-HA-w/o cells</th>
<th>Ti-w/o cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>C K</td>
<td>14.02 ± 0.86</td>
<td>8.61 ± 0.88</td>
<td>5.71 ± 0.19</td>
<td>5.56 ± 0.36</td>
<td>13.88 ± 1.60</td>
<td>8.98 ± 1.59</td>
<td>14.08 ± 0.2</td>
<td>16.13 ± 0.72</td>
</tr>
<tr>
<td>Mg K</td>
<td>1.51 ± 0.11</td>
<td>2.86 ± 0.33</td>
<td>0.57 ± 0.05</td>
<td>0.53 ± 0.03</td>
<td>0.26 ± 0.07</td>
<td>0.37 ± 0.07</td>
<td>0.47 ± 0.16</td>
<td>0.64 ± 0.127</td>
</tr>
<tr>
<td>Si K</td>
<td>9.62 ± 3.34</td>
<td>6.84 ± 4.04</td>
<td>-0.07 ± 0.015</td>
<td>-0.1 ± 0.2</td>
<td>0.30 ±0.57</td>
<td>0.47 ± 0.65</td>
<td>43.67 ± 0.25</td>
<td>1.295 ± 0.176</td>
</tr>
<tr>
<td>P K</td>
<td>5.97 ± 1.02</td>
<td>11.66 ± 1.83</td>
<td>16.38 ± 0.13</td>
<td>18.32 ± 0.24</td>
<td>9.53 ± 4.91</td>
<td>13.10 ± 2.3</td>
<td>0.9 ± 0.07</td>
<td>6.92 ± 1.18</td>
</tr>
<tr>
<td>Ca K</td>
<td>8.46 ± 1.31</td>
<td>15.96 ± 1.45</td>
<td>29.11 ± 0.8</td>
<td>30.68 ± 1.25</td>
<td>15.77 ± 2.81</td>
<td>25.33 ± 1.18</td>
<td>0.36 ± 0.014</td>
<td>12.725 ± 0.53</td>
</tr>
<tr>
<td>O</td>
<td>60.41 ± 3.25</td>
<td>54.06 ± 1.69</td>
<td>50.29 ± 0.41</td>
<td>45.99 ± 0.7</td>
<td>60.85 ± 0.55</td>
<td>51.74 ± 1.39</td>
<td>40.52 ± 1.5</td>
<td>62.29 ± 1.42</td>
</tr>
</tbody>
</table>

| Change with respect to controls (Ti-HA-w/o cells, AZ31-HA-w/o cells and Ti-w/o cells) |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| l-HA-AZ31       | h-HA-AZ31       | l-HA-Ti         | h-HA-Ti         | Ti              |
| P K             | 5.07            | 12.56           | 3.28            | 5.22            | 2.61            |
| Ca K            | 8.1             | 15.6            | 3.78            | 5.35            | 3.02            |

On the other hand, a considerable low non-specific minerals deposition was observed for AZ31-HA (w/o cells) samples, with deposits of ~2-4% of Ca and ~7%-15% of P on the l-HA-AZ31 and h-HA-AZ31 substrates respectively. Therefore, osteoblasts cultured on l/h-HA-AZ31 substrates showed a greater degree of mineralisation, when compared to l/h-HA-Ti equivalent as shown in Table 14.

Furthermore, Mg-based alloys degrade rapidly under physiological conditions, thereby creating an alkaline environment which interferes with the cell physiology [20]. The degradation of HA-coated AZ31 Mg alloy (with and without osteoblasts) and change in pH of the medium during a culture period of 14 days are exhibited in Fig 6-6. Hereafter, the substrates with cells are denoted as l-HA-AZ31, h-HA-AZ31, l-HA-Ti, h-HA-Ti and Ti.
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Figure 6-6 (a) Mg$^{2+}$ release and (b) pH change of CCM containing l-HA-AZ31, h-HA-AZ31, l-HA-Ti, h-HA-Ti and Ti substrates for 3, 7 and 14 days. Statistical analysis: Values are expressed as mean ± SD. One-way ANOVA with posthoc Tukey test with significance level of *P < 0.05, **P < 0.01 indicate the comparison of l-/h-HA-AZ31 substrates versus l-/h-HA-Ti, Ti and culture medium ; #P < 0.05 comparison of different samples versus culture medium; ∆P < 0.05 and ∆∆ P < 0.01 indicate comparison of different samples versus AZ31-HA-w/o cells.
The concentration of Mg\(^{2+}\) released from the AZ31-HA substrate (w/o cells) was found to be significantly higher than l-HA-AZ31, h-HA-AZ31 and cell culture medium (CCM) samples over a period of 14 days, as shown in Fig 6-6(a). Moreover, the release of Mg\(^{2+}\) from l/h-HA-AZ31 and AZ31-HA (w/o cells) decreased steadily with the culture period. The concentration of Mg\(^{2+}\) in CCM containing l/h-HA-Ti and Ti substrates is lower than the CCM control, indicating that osteoblasts are using Mg\(^{2+}\) present in the culture medium for the normal physiological process. Furthermore, change in the pH of CCM affects the osteoblasts functions. Fig 6-6 (b) shows the bulk change in pH of the CCM containing different substrates (with and without osteoblast cells) over a period of 14 days. The pH of the CCM for the Ti-based substrates remains constant around 7.4. On the other hand, the AZ31-based substrates showed alkaline behaviour which decreased gradually over 14 days of the culture period. It can be seen that l/h-HA-AZ31 (w/o cells) showed relatively higher alkaline pH (~8.1) when compared to l-HA-AZ31 (with cells, pH~7.7) and h-HA-AZ31 substrates (with cells, pH ~7.8) after 14 days of incubation. These results indicated that osteoblasts monolayer functions as a corrosion inhibitor of AZ31 Mg alloy and/or using Mg\(^{2+}\) for cellular growth and development.

Previous studies showed that extracellular Mg\(^{2+}\) (here, Mg\(^{2+}\) concentration in CCM is denoted as [Mg\(^{2+}\)]\(_{e}\)) affects the cell behaviour by regulating intracellular Mg\(^{2+}\) and Ca\(^{2+}\) concentrations (denoted as [Mg\(^{2+}\)]\(_{i}\) and [Ca\(^{2+}\)]\(_{i}\), respectively). To understand the effect of molecular weight of HA and [Mg\(^{2+}\)]\(_{e}\), the [Mg\(^{2+}\)]\(_{i}\) and [Ca\(^{2+}\)]\(_{i}\) of osteoblasts cultured on l-/h-HA-AZ31, l-/h-HA-Ti and Ti substrates for 3, 7 and 14 days were examined, as shown in Fig 6-7. Firstly, it can be seen that at each time point, l-/h-HA-AZ31 showed a significantly higher [Mg\(^{2+}\)]\(_{i}\) as compared to l-/h-HA-Ti and Ti substrates.
Figure 6-7 (a) [Mg$^{2+}$]$_i$ and (b) [Ca$^{2+}$]$_i$ of osteoblasts cultured on l-HA-AZ31, h-HA-AZ31, l-HA-Ti, h-HA-Ti and Ti substrates for 3, 7 and 14 days. Statistical analysis: Values are expressed as mean ± SD. One-way ANOVA with posthoc Tukey test with significance level of *P < 0.05, **P < 0.01 indicate the comparison between l-/h-HA-AZ31 and l-/h-HA-Ti substrates; #P < 0.05, ##P <0.01 indicate comparison of different samples (l-/h-HA-AZ31 and l-/h-HA-Ti substrates vs Ti); ∆P < 0.05 and ∆∆ P < 0.01 indicate the pairwise comparison of samples.
Secondly, l-/h-HA showed a higher level of \([\text{Ca}^{2+}]\), as compared to l-/h-HA-Ti and Ti substrate (Fig 6-7(b)), which is inversely proportional to \([\text{Mg}^{2+}]\), of l-/h-HA-AZ31 substrates (Fig 6-7(a)) over 14 days of the culture period. Notably, without much change in \([\text{Mg}^{2+}]\), h-HA-Ti showed a significant increase in \([\text{Ca}^{2+}]\), when compared to the l-HA-Ti, whereas Ti alone showed the least increase in \([\text{Ca}^{2+}]\). These results indicated that \([\text{Mg}^{2+}]\) acts as a pro-osteogenic agent irrespective of the type of HA employed on the surface, whereas h-HA enhanced the pro-osteogenic differentiation as compared to l-HA (Fig 6-7(b)).

6.7 Discussion

The biological acceptance of the orthopaedic implants in terms of osteoblasts’ functions plays a vital role in the expediting bone tissue healing surrounding the implants (Ti and Mg-based implants). In order to improve the cytocompatibility of the implants, various biopolymers including HA have been functionalised onto metal implant surfaces [16,21,22]. HA is present in various body tissues including synovial fluid, the vitreous body of eye, brain and cartilage [23]. It also plays an important role in regulating various biological processes including osteoblasts’ cell adhesion, proliferation and differentiation [1]. In previous studies, hyaluronic acid was functionalised with Ti surfaces, to improve osteoblastic activity [8, 18, 21]. However, these were fundamental studies and molecular weight of HA was not considered while employing it as an osteoinductive agent. Furthermore, deposit coatings improve the corrosion resistance and cytocompatibility of the biodegradable Mg-based alloys which are widely used for orthopaedic applications [10]. In this work, we used previously reported corrosion resistant sol-gel coating to develop l-/h-HA functionalised AZ31 and Ti substrates [4, 14,15]. According to previous reports, \(\text{Mg}^{2+}\) supplements induce the proliferation and differentiation of osteoblast cells [11,24,25].
6.7.1 Effect of molecular weight of hyaluronic acid-functionalised-AZ31 and-Ti substrates on osteoblast cell adhesion and proliferation.

In addition, reports also demonstrated that h-HA stimulate the differentiation as compared to l-HA [3,7]. However, these studies didn’t consider the properties of HA to be used for the functionalisation of Ti [8,18,21]. This is the first report evaluating the osteoblast cellular response to (a) l/h-HA functionalised on Ti and corrosion resistant AZ31 substrate and (b) Mg$^{2+}$ stimulated CCM of h/l-HA-AZ31 substrates (produced due to the corrosion of AZ31 Mg alloy). Characterisation of l-/h-HA coated AZ31-Mg and Ti alloys using AFM, contact angle measurements and quantification of hyaluronic acid confirmed the HA-functionalisation on the silane coated AZ31 and Ti substrates.

Furthermore, we found that osteoblasts’ adhesion decreases on h-HA coated AZ31 and Ti alloy as compared to l-HA coated substrates, whereas osteoblasts showed enhanced cell adhesion on bare Ti surface amongst all other substrates. Previous reports showed that the resistance to cell adhesion on h-HA surfaces over l-HA is probably due to the greater repulsion effect of the negatively charged proteoglycan of the cell with the h-HA [21]. Previous reports also showed that the wettability of the surface affects the adhesion of the osteoblast cells [26]. Therefore, improved osteoblasts cell adhesion on l-HA over h-HA coated substrate may be also attributed to the relatively greater hydrophilicity of the former substrate. The cell shape of osteoblasts was found to be flattened, extended and interconnected on HA coated substrates. However, osteoblasts adhered to bare AZ31 Mg alloy were circularly shaped and sparsely viable, which is likely due to its rapid degradation in HEPES DMEM [14,15,17]. Furthermore, cell adhesion and proliferation of osteoblasts on different substrates were determined in terms of increase in DNA concentration during the culture period. It was seen that cell adhesion density decreased on l-/h-HA coated AZ31 substrates as compared to the l/h-HA coated or uncoated Ti
substrates. A similar trend was observed in the proliferation of osteoblasts monitored over a period of 14 days. The cell proliferation on l-HA-AZ31/Ti was significantly increased when compared to h-HA-AZ31/Ti substrates. Kim et al. showed that in comparison to high molecular weight HA (i.e. 200 kDa), low molecular (50 kDa) counterparts may provide favourable conditions for osteoblast proliferation [27]. Likewise, Zhao et al. also demonstrated the higher osteo-proliferative activity on low molecular weight HA (≤ 110 kDa) as compared to high molecular weight HA (≤ 2500 kDa) [3]. The findings of the present study are in agreement with those of the previous studies and suggest that osteoblasts can actively proliferate on low molecular weight HA functionalised AZ31 and Ti substrates. However, the proliferation of osteoblasts on l-/h-HA-AZ31 was decreased significantly when compared to l-/h-HA-Ti equivalents. This could be attributed to the biodegradable nature of the AZ31 substrate producing corrosion by-products (evolution of H₂ gas and pH change), thereby affecting cell viability and adhesion. In addition, functionalisation of hyaluronic acid on biodegradable Mg alloy is not just to support the growth of the bone-related cells but also the degradable nature of Mg and its alloy is beneficial for orthopaedic implant applications.

6.7.2 Effect of molecular weight of hyaluronic acid-functionalised-AZ31 and-Ti substrates on the osteogenic differentiation of osteoblast cells.

The effect of HA molecular weight functionalised on Ti and AZ31 substrates on the osteogenic differentiation of osteoblast cells was evaluated by ALP activity, expression of collagen and formation of mineral nodules. Contrary to the trend of osteoblasts proliferation, h-HA-AZ31 and l-HA-AZ31 showed enhanced differentiation efficiency over l-HA-Ti and h-HA-Ti substrates respectively, whereas the Ti substrate showed the least ALP activity. The results indicated that there are two factors stimulating the intracellular ALP activity, namely (a) functionalisation of the substrate with h-HA and
(b) higher concentration of \([\text{Mg}^{2+}]_e\) in CCM of AZ31 substrates (Fig 6-8). Osteoblasts express the ALP enzyme in the early stages of osteogenic differentiation, which participates in the mineralisation of the extracellular matrix (ECM) [28]. Previous studies showed that h-HA enhanced the differentiation of osteoblasts by upregulating the ALP activity as compared to the l-HA [3]. In addition, it has been reported that \([\text{Mg}^{2+}]_e (5\text{mM}-10\text{mM})\) in CCM stimulate the intracellular ALP activity [12].

![Diagram showing the effect of h-HA, l-HA, and Mg²⁺ on osteoblast differentiation markers](image)

**Figure 6-8** Effect of l-HA, h-HA and \([\text{Mg}^{2+}]_e\) on the differentiation markers of osteoblast cells. In comparison to l-HA, h-HA enhanced \([\text{Ca}^{2+}]_i\) and other differentiation markers. Presence of \([\text{Mg}^{2+}]_e(\text{AZ31})\) augmented the \([\text{Ca}^{2+}]_i\) and differentiation of osteoblasts.

Furthermore, the synthesis of collagen during osteogenic differentiation of osteoblasts on different substrates was evaluated by hydroxyproline assay. Collagen is the most abundant extracellular matrix synthesised by osteoblast cells and essential for ECM mineralisation [29]. Generally, the synthesis of collagen increases throughout the culture period and is a strong indicator of osteoblasts differentiation. Osteoblasts cultured on h-HA-AZ31 and l-HA-AZ31 showed significantly higher collagen synthesis as compared to the h-HA-Ti and l-HA-Ti substrates respectively, whereas uncoated Ti substrate showed the lowest hydroxyproline content amongst other substrates. Previously Zhao and Lai *et al.* reported that in comparison to l-HA, cells in response to h-HA induced a
greater expression of collagen [3, 21]. In addition, the Mg$^{2+}$ ions conditioned CCM has also been reported to enhance the collagen synthesis [12, 20]. The presented results are in agreement with the previous studies, which indicated that h-HA and extracellular Mg$^{2+}$ synergistically enhanced the differentiation of osteoblasts. In an ordered sequence of events observed during osteoblast differentiation, the increased ALP activity in the early stage is followed by the increased synthesis of collagen, which participates in the mineralisation of ECM [20]. The effect of the molecular weight of hyaluronic acid functionalised AZ31 and Ti substrates on the mineralisation of ECM after 14 days of cell culture was evaluated by SEM-EDX analysis. It was observed that the deposition of Ca and P minerals were higher on l/h-HA-AZ31 substrates as compared to l/h-HA-Ti substrate (Fig 6-8). The enhanced matrix mineralisation activity of h-HA in presence of extracellular Mg$^{2+}$ (h-HA-AZ31 substrate) was accompanied by greater expression of intracellular ALP and collagen during various stages of the differentiation process (Fig 6-8). These results support the enhanced differentiation of osteoblast cells cultured on the h-HA-AZ31 substrate.

Furthermore, various studies have been conducted to evaluate the effect of l-/h-HA concentration on osteoblasts differentiation [3, 4, 7]. In this study, the grafting amount of l-HA functionalised on AZ31 and Ti substrate is less than the h-HA counterpart. Since we have used an enzymatic method to quantify HA, it is expected to get a relatively higher grafted amount of high molecular weight HA due to long chain length when compared to the low molecular weight HA surface. According to previously reported literature, a given level of osteoblast differentiation induced by h-HA observed at a significantly lower concentration as compared to l-HA [3, 4, 7]. Interestingly these studies also demonstrated that improved osteoblast functions induced by h-HA over l-HA have been maintained; even if the concentration for l-HA is at a higher level to that of h-HA. Thus, an improved
osteoblast function in response to h-HA functionalised on AZ31 and Ti surface as compared to l-HA counterparts can be attributed to the effect of molecular weight instead of grafting levels of l-/h-HA. The abovementioned differentiation results indicate that irrespective of the AZ31 or Ti metal substrate studied, h-HA significantly enhanced the differentiation of osteoblasts over l-HA. It is well known that hyaluronic acid interacts with receptors expressed by bone-related cells, such as CD44 (cell membrane-tethered glycoprotein) and RHAMM (receptor for HA-mediated motility), which are expressed by osteoblast lineage cells [30]. Hyaluronic acid is a principal ligand for the CD44 receptor [31]. Previous studies reported that h-HA can enhance the differentiation markers of the osteoblast cells through the stimulation of CD44 [4]. Similarly, Chen et al, demonstrated the reduced mineralisation of CD44 knocked out dental pulp cells in response to h-HA, thereby emphasising the role of the CD44 receptor in the differentiation of osteoblasts [31]. Another study reported that several CD44 receptor binding sites are proportional to the molecular weight of hyaluronic acid, thereby increasing CD44 receptor density, and more differentiation can be achieved. RHAMM is another prominent receptor which could make up the loss of CD44 [4]. Therefore, the enhanced differentiation activity of h-HA functionalised surface as compared to low molecular weight counterpart can be attributed the CD44-related cell signalling. The results also show that osteoblasts cultured on l/h-HA AZ31 substrates further enhanced the HA-induced differentiation. Generally, Mg-based alloys in the chloride-rich aqueous environment can rapidly degrade to release Mg$^{2+}$ ions, thereby increasing the solution alkalinity and inducing toxicity in the surrounding cell/tissues [10]. Therefore, it is important to control the degradation of Mg alloys under physiological conditions for potential biodegradable orthopaedic implants.
6.7.2.1 Role of the molecular weight of hyaluronic acid and extracellular Mg$^{2+}$ on intracellular Ca$^{2+}$ and Mg$^{2+}$ in the osteogenic differentiation of osteoblast cells

Many reports demonstrated enhanced differentiation of osteoblasts cultured in Mg$^{2+}$ ions conditioned CCM with alkaline pH (7.6-8.5) [20,32]. In this study, we determined the pH and Mg$^{2+}$ released in DMEM from l/h-HA coated-AZ31 and l/h-HA coated-Ti substrates seeded with and without osteoblasts for 3, 7 and 14 days. It was observed that $[\text{Mg}^{2+}]_{e}$ for all of the AZ3-based substrates decreased steadily at each of the time points, however, the concentration was still higher than CCM of Ti-based substrates and CCM alone. The decreasing release rate of Mg$^{2+}$ ions with the incubation time can be attributed to Mg alloy forming a protective surface passivation layer [33]. Interestingly, the immersion medium of l/h-HA-AZ31 seeded with osteoblasts showed a significant decrease in $[\text{Mg}^{2+}]_{e}$ when compared to AZ31-HA substrate without cells at each time point. Similarly, the pH decreased significantly for l/h-HA-AZ31 substrates with osteoblasts as compared AZ31-HA substrate without osteoblasts. These results indicate that the corrosion of l/h-HA-AZ31 with osteoblasts reduced significantly as compared to the counterpart without cells. There are few explanations for such enhanced corrosion protection of l/h-HA-AZ31 (with osteoblasts). Firstly, mono-layer of osteoblast cells can act as a corrosion protection layer by depositing Ca-P minerals on the surface through the process of ECM mineralisation and/or formation of passivation layer due to the degradation of the AZ31-Mg alloy as observed from EDX elemental analysis [15,34,35]. Secondly, osteoblasts use Mg$^{2+}$ for various metabolic activities. Previous studies demonstrated the influence of living cells in preventing the degradation of Mg alloy through the enhanced metabolic activity of cells (reducing the pH of the medium) and deposition of corrosion products on the surface [34,35]. These previous studies conducted experiments in non-osteogenic medium condition, whereas the present study showed the
influence of ECM mineralisation on the corrosion protection of AZ31 Mg alloy in the differentiation CCM. It has been suggested that moderate $[\text{Mg}^{2+}]_e$ can be tolerated by bone-related cells and give rise to the higher expression of differentiation markers [12,24]. However, excessive release of Mg$^{2+}$ ions resulting from rapid degradation of Mg alloy is a concern and can cause cell damage jeopardising the bone healing process [36–38]. Regarding pH values, it has been reported that differentiation of osteoblasts significantly enhances with the pH increase up to 8.5 [20,32]. Previous work described that the excessive corrosion of AZ31 Mg alloy lead to the alkalisation of CCM and can cause the pH to increase above 9.0, which is detrimental to cell adhesion, proliferation and differentiation [36–38]. The results presented in this study showed the controlled degradation of Mg alloy, which provided favourable conditions such as appropriate pH ($< 8.5$) and optimum $[\text{Mg}^{2+}]_e$ for enhanced osteoblast differentiation when compared to the Ti-based samples.

In order to understand the effect of l-/h-HA and/or $[\text{Mg}^{2+}]_e$ on the mineralisation of osteoblasts, we determined the $[\text{Mg}^{2+}]_i$ and $[\text{Ca}^{2+}]_i$ of osteoblasts at different time periods. The results showed that h-HA significantly stimulate $[\text{Ca}^{2+}]_i$ as compared to l-HA samples during 14 days of cell culture (Fig 6-8). Previous studies reported that increased CD44 receptor density on h-HA illicit greater mineralisation of osteoblasts [3,4]. Boonrungsiman et al, explained not only the genesis and role of intracellular Ca$^{2+}$ in propagating the nucleation of mineralised nodules within the ECM but also identified higher $[\text{Ca}^{2+}]_i$ during osteogenic differentiation of osteoblast cells [39]. Therefore, in comparison to l-HA coated surfaces, enhanced differentiation of osteoblasts in response to h-HA can be attributed to increased CD44 receptor density on h-HA-coated Ti/AZ31 substrates. The influence of $[\text{Mg}^{2+}]_e$ on osteogenic differentiation of osteoblast by regulating $[\text{Mg}^{2+}]_i$ and $[\text{Ca}^{2+}]_i$ has been explained by previous studies [11,20,23].
However, reports showed different $[\text{Mg}^{2+}]_e$ improving the differentiation of osteoblast ranging from $<1$ to $10\text{mM}$ [11,12]. In this study, we found that decreasing $[\text{Mg}^{2+}]_e$ ($\sim12\text{mM (day 3) to 5mM (day 14)}$) throughout the culture period also match this trend for $[\text{Mg}^{2+}]_i$, but reveal contrary for $[\text{Ca}^{2+}]_i$. This indicates that (a) $[\text{Ca}^{2+}]_i$ is regulated by $[\text{Mg}^{2+}]_e$ and (b) lower $[\text{Mg}^{2+}]_i$ stimulates the $[\text{Ca}^{2+}]_i$. This also showed that at an optimum concentration of $[\text{Mg}^{2+}]_e$ ions enhanced the differentiation of osteoblasts as observed in the case of AZ31 substrates (Figure 6-5 and 6-7). Our findings are in agreement with the previous studies which reported the $[\text{Mg}^{2+}]_e$ dependent increase of $[\text{Mg}^{2+}]_i$ and $[\text{Ca}^{2+}]_i$. [11,20,23]. Deficient $[\text{Mg}^{2+}]_i$ was reported to reduce the osteoblastic activity and impair bone remodelling [13]. However, an overdose of $[\text{Mg}^{2+}]_i$ is detrimental to the viability of cells and retarding the ECM mineralisation of bone-related cells [11,40]. Therefore, enhanced mineralisation showed by the h-HA-AZ31 substrate can be attributed to the Mg$^{2+}$ released from the AZ31 substrate and positive effects of h-HA.

In chapter 3, the enhanced proliferation of osteoblasts on PLGA-silane coating was considered as the likely reason of higher intracellular levels because PLGA does not have an intrinsic property to stimulate the cell differentiation as compared to other bioactive polymers including hyaluronic acid. On the other hand, we have shown the comparison of the differentiation and proliferation activities of h-HA and l-HA functionalised-AZ31 and Ti substrates. In comparison to l-HA, h-HA enhanced the differentiation of osteoblast cells and vice-versa for proliferative activities. As discussed in the introduction section, based on the molecular weight, HA has unique functionalities and role during the bone regeneration process. Moreover, l-HA and h-HA also have a varying effect on the osteoblasts’ response in terms of $[\text{Ca}^{2+}]_i$ and $[\text{Mg}^{2+}]_i$, regulating the osteogenic differentiation of osteoblast cells as explained in the discussion. These are the few reasons
which explain the deviation in the osteoblasts’ response on l-h-PA coated AZ31/Ti substrates to that of PLGA-silane coated AZ31 substrates.

6.8 Conclusion

This study illustrated the proliferation and differentiation of osteoblast cells in response to the hyaluronic acid of different molecular weight immobilised on silane coated AZ31 Mg and Ti alloy substrates. The proliferation of osteoblasts on l-HA coated AZ31/Ti substrates significantly enhanced when compared to the h-HA counterpart. However, the overall proliferation of osteoblasts was significantly low on HA-coated (low and high molecular weight HA) AZ31 Mg substrates when compared to HA-coated Ti substrates, which is attributed to the corrosion of AZ31 Mg alloy in the culture medium. On the contrary, osteogenic differentiation was enhanced in response to h-HA over l-HA modified AZ31 and Ti substrates. The steady decrease of extracellular Mg$^{2+}$ over 14 days regulated the concomitant increase in [Ca$^{2+}$], with the corresponding decrease in [Mg$^{2+}$]. However, the latter showed a significant increase for HA-coated AZ31 substrates (h-HA-AZ31 and l-HA-AZ31) when compared to the Ti-based substrates (h-HA-Ti, l-HA-Ti and uncoated Ti). Particularly, h-HA coated AZ31 substrates (h-HA-AZ31) greatly enhanced the osteoblasts differentiation and mineralisation of ECM, which can be ascribed to the osteoinductive activity of h-HA, alkaline cell culture medium as well as upregulated [Ca$^{2+}$]. These findings are critical to understanding the role of hyaluronic acid molecular weight functionalised on different metal substrates affecting bone healing.
6.9 References


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Chapter 7

Summary, general conclusions and future work

7.1 Summary of the PhD work

Previous studies showed that Mg-based alloys have been widely explored for several advantages over non-degradable metal implants such as (a) their biodegradable nature (b) mechanical properties closer to that of bone and (c) osteopromotive activity. However, Mg-based alloys rapid degrade in chloride-rich physiological conditions, especially during early implantation period (2-3 weeks). Corrosion results in the formation of H$_2$ gas and alkalinity of the surrounding medium which together promote toxicity towards the surrounding tissue. In this PhD work, we designed bio-functionalised (PLGA and hyaluronic acid) corrosion-resistant sol-gel coatings on AZ31-Mg alloy to optimally control the degradation during early immersion period which could improve the osteoblasts’ functions, instead of becoming detrimental to the cell growth. Furthermore, previous studies demonstrated the susceptibility of Mg-based biodegradable alloy to bacterial colonisation in vivo. On the contrary, many studies reported that corrosion of Mg alloy causes an increase in the pH of the medium, thereby inhibiting the bacterial growth. However, this increase in the pH can affect the viability of the osteoblast cells. Therefore, this problem is addressed by functionalising the corrosion resistant coating with hyaluronic acid-lysozyme composite to prevent the initial bacterial cell adhesion and colonisation on the surface.

This improvement was achieved through a series of investigations as now summarised:
7.1.1 Corrosion resistant properties of PLGA and HA-modified sol-gel silane coated AZ31 Mg alloy for orthopaedic implants applications (Chapter 3 and Chapter 4)

To enhance the corrosion resistance of AZ31 Mg alloy, a layer-by-layer coating of MTES-TEOS and APTES sol-gel was employed and characterised using contact angle measurements and FTIR and AFM to confirm the successful assembly of silane coating. Furthermore, the corrosion resistant properties of the biofunctionalised-sol gel-silane coating were evaluated using electrochemical corrosion study in DMEM and HEPES-buffered DMEM. In the case of PLGA-silane coated surface (chapter 3), electrochemical corrosion analysis (EIS and PDS) showed a progressive increase in the corrosion resistance (AZ31-MT-A-P > AZ31-MT-A > AZ31-MT >AZ31) in DMEM electrolyte. For the HA-silane coated AZ31 substrate (chapter 4), the change in the corrosion resistance of AZ31 Mg alloy showed a trend AZ31-MT-A > AZ31-MT-A-HA1 > AZ31-MT-A-HA2 >AZ31-MT > AZ31. Coatings with increasing concentration of hyaluronic acid were unable to provide additional corrosion resistance. This is due to the hydrophilic nature of the hyaluronic acid which failed to prevent the electrolyte penetration into the coating. Overall, the corrosion resistance decreased in HEPES modified DMEM electrolyte when compared to the DMEM. However, the silane component of HA-silane coatings (AZ31-MT-A) showed a ~2 order of magnitude increase in the corrosion resistance when compared to the uncoated AZ31 alone in both electrolytes. Based on the electrochemical corrosion study, AZ31-MT-A coated substrates showing the greatest corrosion resistance were selected for the immersion experiment.

In comparison to the uncoated AZ31 substrate, the PLGA-silane coating on AZ31 reduced the release of Mg$^{2+}$ and H$_2$ gas by 4 to 5-fold in DMEM during the immersion period of 21 and 14 days respectively. In contrast, the Mg$^{2+}$ and H$_2$ gas release from AZ31-MT-A substrate in HEPES-modified DMEM showed a considerable increase when
compared to the PLGA-silane coated substrate in DMEM over a period 21 and 10 days respectively. This increase in the degradation of AZ31 Mg alloy is due to the corrosive nature of the former electrolyte. However, the fold increase in corrosion resistance when compared to the uncoated substrate is similar to that PLGA-silane coated substrate. Furthermore, the alkalinity induced by the PLGA-silane and AZ31-MT-A substrates was found to be ~pH 8.1 on day 21 in the respective electrolytes. The resistance to change in pH with the increase in Mg$^{2+}$ release is due to the buffering nature of HEPES. In addition, osteoblast cells prefer a slightly alkaline pH for optimal growth. Furthermore, SEM-EDX analysis was used to evaluate the morphology and passivation level of corroded samples. Both PLGA-silane and AZ31-MT-A substrates immersed in the respective electrolytes exhibited occurrence in crack formation in the coating during the immersion period. However, a passivation layer containing a relatively high content of Ca-P were observed on the coated substrate which prevented the degradation of AZ31 during the later immersion period, which is also evident from the immersion studies. Based on the corrosion studies, it can be observed that the silane component of the coating optimally controlled the degradation alloy which could support the growth of the osteoblast cells.

7.1.2 Biocompatible properties of PLGA, HA and HA-LZ functionalised-sol gel silane coated AZ31 substrates (Chapter 3 to 6)

Furthermore, the cytocompatibility of biofunctionalized sol-gel silane corrosion resistant coated AZ31 Mg alloy was evaluated during the degradation phase of Mg alloy. In comparison to the PLGA-silane coated substrate (chapter 3), osteoblast cells showed more flattened morphology, better cell attachment and ~ 3 folds increase in proliferation and differentiation on the HA-coated substrate (chapter 4). Moreover, both PLGA-silane and HA-coated AZ31 substrates showed a significant improvement in osteoblast cells’ functions when compared to the uncoated AZ31 equivalents. Based on these results,
following observations can be made (a) degradation of AZ31 alloy did not affect the osteoblastic activity over a period of 14 days and (b) hyaluronic acid provided a biomimetic environment for enhanced the cytocompatibility of AZ31 Mg alloy.

To further evaluate the osteoblastic activity of hyaluronic acid, the effect of hyaluronic acid-molecular weight and Mg\(^{2+}\) was studied (chapter 6). It was observed that l-HA and h-HA enhanced cell proliferation and differentiation respectively. Particularly, h-HA functionalised on HA coated substrate showed the highest differentiation activity in terms of ALP and collagen expression during 14 days of cell culture. This upregulated differentiation response of osteoblast cells was attributed to upregulated intracellular Ca\(^{2+}\) by (a) h-HA and (b) optimum extracellular Mg\(^{2+}\) (or release from the AZ31 surface) and pH (~8.1). These studies showed that corrosion resistant coating optimally degrades the Mg alloy during a period of 2-weeks which represent the early implantation period and bioactive coatings along with Mg\(^{2+}\) improved the functions of osteoblast cells.

The problem associated with bacterial colonisation on the implant surface was addressed by functionalising hyaluronic acid-lysozyme composite on the silane coated AZ31 substrate (chapter 5). The lysozyme component of the composite coating acted as an antimicrobial agent and exhibited concentration-dependent antimicrobial activity when compared to the uncoated, HA and lysozyme alone controls over a period of 12 h as confirmed by PI exclusion assay, spread plate assay and SEM. Furthermore, the HA-LZ composite coating showed ~2 to 4-fold increase on the proliferation and differentiation of osteoblast cells when compared to the lysozyme control only. However, the osteoblastic activity on the composite coated substrates was found to be similar to that of HA-coated substrates. Based on these results, it can be observed that the lysozyme component of the coating reduced the bacterial adhesion without showing a detrimental effect on the osteoblastic activity.
In a nutshell, bio functionalised sol-gel silane coatings optimally controlled the degradation of AZ31 Mg alloy, improved the osteoblasts’ functions and prevented the bacterial colonisation on the surface. These coatings overcome the drawbacks associated with the biodegradable Mg alloy (H$_2$ gas release, alkalinity, cell cytotoxicity and implant associated bacterial infection), especially during early implantation period which deterred their clinical applications.

However, there are practical limitations of this work which would be require further investigation improve the corrosion resistant efficiency and biocompatibility of the sol-gel silane coating on AZ31 Mg alloy. Firstly, the degradation of Mg alloy undergoes rapid degradation under a cyclic load. Therefore, the corrosion resistance of the Mg alloy needs to be evaluated under a cyclic mechanical load to check the efficiency of the coating in preventing the implant failure. Secondly, the mechanical properties (Hardness, elastic modulus, adhesion energy, residual stress) of the coatings should be tested because it plays an important role in the reliability of the product. In the case of coating failure, its function may be affected. Thirdly, immune response to a foreign body (coatings and metal implant) affects the functionality of the implants in vivo. In this study, we have modified the surface by biomolecules (hyaluronic and lysozyme) to enhance the osseointegration and reduce the inflammatory response. However, the degradation products of a biodegradable metal implant can induce several types of immune response and it is important to evaluate this aspect of biocompatibility. All these limitations of the present study are recommended to be explored in and the methodology to achieve these objectives are given in the future work.
7.2 General conclusions

The quality of patient care has increased dramatically in recent years due to the development of lightweight orthopaedic metal implants. The purpose of this study is to improve the corrosion resistance and biocompatibility of the AZ31 Mg alloy by developing multi-functional coatings on AZ31 Mg alloy.

General conclusions drawn from this study are given below:

- The degradation of AZ31 Mg alloy can be controlled by fabricating a multilayer MTES-TEOS and APTES sol-gel silane coating.
- The cytocompatibility of AZ31 Mg alloy can be improved by fabricating bioactive polymer (PLGA, hyaluronic acid and hyaluronic acid-lysozyme composite) coatings on it.
- These coatings were characterised using SEM, AFM, FTIR and contact angle studies.
- The coating thickness and morphology of PLGA-silane on AZ31 Mg alloy were determined using SEM. The formation of the siloxane network in sol-gel silane coating and an amide bond between APTES and hyaluronic acid was confirmed by FTIR studies.
- The low surface roughness of hyaluronic acid and hyaluronic acid-lysozyme composite coated AZ31 Mg alloy over uncoated AZ31 Mg alloy was confirmed by AFM measurements. Furthermore, the hydrophobicity and hydrophilicity of sol-gel silane coating and bioactive polymer coating (PLGA, hyaluronic acid and hyaluronic acid-lysozyme composite) were determined by contact angle measurements.
• The silane coated AZ31 Mg alloy improved the corrosion resistance in DMEM and HEPES modified DMEM over a period of 14 to 21 days as observed from electrochemical corrosion studies and immersion experiments.

• The reduced rate of Mg$^{2+}$ release in the cell culture medium and slightly alkaline pH (< 8.5) in case of silane coated AZ31 Mg substrates improved proliferation and differentiation of osteoblast cells.

• The high degradation rate of uncoated AZ31 Mg alloy creates toxicity to the osteoblast cells owing to the increase in the pH of the cell culture medium (pH > 8.5).

• PLGA-modified silane coated AZ31 substrate showed enhanced osteoblast cell adhesion, proliferation and differentiation when compared to the uncoated AZ31 Mg alloy equivalents.

• Biomimetic hyaluronic acid (HA)-silane coated AZ31 Mg alloy enhanced the osteoblast cell adhesion with more flattened morphology and greater cell to cell interactions.

• The level of cell adhesion and proliferation of osteoblasts increased with the concentration of hyaluronic acid employed for the modification of silane coated AZ31 Mg alloy.

• Similarly, hyaluronic acid enhanced the expression of intracellular levels of ALP activity as compared to the uncoated AZ31 Mg alloy.

• Susceptibility of the hyaluronic acid-silane coated AZ31 surface to the bacterial colonisation was reduced by the incorporation of antimicrobial lysozyme protein, which formed antimicrobial hyaluronic acid-lysozyme composite coated surface.

• *S.aureus* adhesion on uncoated AZ31 surface increased with the incubation period.
• Antimicrobial activity against *S. aureus* increased with the concentration of immobilised lysozyme (10% and 20% w/w of HA).

• Hyaluronic acid-lysozyme composites showed enhanced antimicrobial activity as compared to respective lysozyme and uncoated AZ31 controls. This is attributed to the greater affinity of lysozyme in the presence of hyaluronic acid-silane coated AZ31 surface when compared to the lysozyme control.

• Hyaluronic acid-lysozyme composite showed osteoinductive activity comparable to the hyaluronic acid-silane coated AZ31 surface.

• This showed that the lysozyme component of the coating did not affect the osteoinductive properties of hyaluronic acid while it was acting as an antimicrobial coating and preventing the bacterial colonisation on the AZ31 surface.

• Hyaluronic acid and Mg$^{2+}$-dependent osteoblastic activity was studied in-detail by functionalising high and low molecular weight hyaluronic acid onto AZ31 Mg and Ti alloys.

• High molecular weight-hyaluronic acid reduced the proliferation of osteoblast cells as compared to low molecular weight hyaluronic acid functionalised surfaces.

• Overall, hyaluronic acid (low and high molecular weight) functionalised AZ31 substrate showed lower osteoblast proliferation as compared to HA functionalised-Ti surface. This reduction in osteoblast proliferation is attributed to the degradation of AZ31 Mg alloy during the culture period.

• On the contrary, high molecular weight HA-functionalised surface showed improved osteogenic differentiation of osteoblast cells as compared to the low molecular weight counterpart.
• Overall, HA-coated (low and high molecular weight) functionalised AZ31 surface showed enhanced osteoinductive activity when compared HA-coated Ti surface.

• Osteoblasts cultured on high molecular weight HA showed significantly higher intracellular Ca$^{2+}$ concentration as compared to the low molecular weight HA.

• Overall, osteoblast cultured on HA coated AZ31 substrate showed a significant higher intracellular Ca$^{2+}$ and Mg$^{2+}$ concentrations when compared to the HA-coated Ti surface.

• In addition, the pH of the cell culture medium containing HA-functionalised AZ31 substrate was found to be alkaline (< 8.5).

• This showed that enhanced osteoblasts functions observed on high molecular weight HA-AZ31 substrates which can be ascribed to the osteoinductive activity of high molecular weight HA, high intracellular Ca$^{2+}$ concentration and alkaline cell culture medium.

7.3 Future work

As explained in section 7.1, the aim of enhancing the corrosion resistance and cytocompatibility of Mg alloy using multifunctional silane-based coatings has been achieved under in vitro conditions. However, it is important to validate the functionality of these corrosion-resistant AZ31 substrates in vivo in terms of the mechanical properties of the coatings as well as corroded implants and immunological response against the coated-AZ31 substrates.

7.3.1 Corrosion fatigue behaviour of the surface modified AZ31 Mg alloy in cell culture medium

The corrosion resistant properties of the coated and uncoated AZ31 Mg alloy have been demonstrated in chapter 3 and 4. In general, the failure of orthopaedic implants is
Chapter 7

associated with corrosion fatigue, which is the synergistic effect of cyclic mechanical loading and corrosion [1]. Therefore, it is important to evaluate the corrosion fatigue of the modified substrates employed in vitro. Such tests should be carried out as per ASTM-F2477-07 [2]. This test will allow simulating the load that an orthopaedic implant will experience in vivo.

7.3.1.1 Preparation of samples

Corrosion fatigue test can be performed on the specimen with a circular cross-section 5 mm in diameter and 10 mm gauge length as per ASTM E466 [2]. The surface of these samples can be modified with bioactive and sol-gel silane coating as per the method described in previous chapters.

7.3.1.2 Corrosion fatigue test

For this test, it is recommended to prefer an electrolyte which will be used for cell culture studies. In the present work, HEPES-modified DMEM was preferred because it has provided a buffered condition which an implant will experience in vivo. The setup required to conduct this test can be adopted from Gu et al [2]. The release of Mg$^{2+}$ during the corrosion fatigue test and the surface of the fatigue fractured samples can be determined by ICP-OES and SEM respectively.

7.3.2 Evaluation of mechanical properties of the deposit coatings on AZ31 Mg alloy.

The mechanical properties of the bio-functionalised (PLGA, HA and HA-LZ) sol-gel silane coated AZ31 Mg alloy (Elastic modulus, the hardness of the coatings, fracture toughness and residual stress) can be determined using nanoindentation technique.

7.3.3 Immunological response to the biofunctionalised sol-gel silane coated AZ31 Mg alloy under in vitro and in vivo conditions

In previous chapters, the biocompatibility in terms of osteoblasts proliferation and differentiation of bio-functionalised (PLGA, HA and HA-LZ) sol-gel silane coated AZ31
Mg alloy was demonstrated. However, it is known that degradation products of Mg-based alloys can induce various immunological response which includes immune suppression through the apoptosis of immune cells, hypersensitive reactions and foreign-body reactions. It is a pre-requisite to achieve desired biocompatibility with minimum foreign body response to biodegradable Mg-based alloy for orthopaedic implants applications.

7.3.3.1 Evaluation of immune response to the bioactive polymer (PLGA, hyaluronic acid and hyaluronic acid-lysozyme) functionalised sol-gel silane coated AZ31 Mg alloy substrates in vitro conditions

a) **Cytocompatibility of surface modified AZ31 Mg alloy substrates:** Two cell lines THP-1 cells and THP-1 macrophages can be used to evaluate the effect of Mg extracts of these substrates on cell viability, differentiation, cell cycle and cell migration. In addition, these cells can also be cultured directly on the coated surface to perform this experiment.

b) **The inflammatory response to surface modified AZ31 Mg alloy substrates:** Inflammatory cytokines such as TNF-alpha can be quantified in the cell cultured medium upon exposure of magnesium extracts to these cells.

7.3.3.2 In vivo evaluation of immunological response to the bioactive polymer (PLGA, hyaluronic acid and hyaluronic acid-lysozyme) functionalised sol-gel silane coated AZ31 Mg alloy substrates

a) **Bone remodelling and corrosion analysis:** micro-CT can be used to determine new bone formation, loss of implant volume and gas formation in vivo.

b) **Evaluation of inflammatory markers in blood serum:** Several inflammatory markers such as TNF- alpha IL-1beta and IL-6 can be quantified using ELISA. In addition, other markers such as TGF-beta and PDGF can also be quantified which have an influence on the initiation of bone healing [3].
c) **Phagocytic activity**: The high phagocytic activity of neutrophils is very important for appropriate bone healing because it helps both in clearing the dead cells and recruitment of progenitor cells to maintain the homeostasis. This test is based on ingesting GFP-tagged *E. coli* by neutrophils, thereby generating fluorescence signal which can be quantified by flow cytometry. The details of this experiment are given in Pichler *et al* [4].

**7.3.4 Evaluation of PLGA-silane and hyaluronic acid coated AZ31 Mg alloy in vivo**

From chapters 3, 4 and 6, the PLGA and hyaluronic acid–silane coated AZ31 substrates showed the controlled degradation as well as stimulated the osteoblast differentiation and functions. Therefore, the corrosion properties and bone-forming activity of these substrates need to be evaluated in rat or other animal models.

**7.3.4.1 The bone-forming activity of PLGA or Hyaluronic acid-silane coated AZ31 alloy under in vivo conditions**

Generally, biodegradable implants are temporary fixtures such as screw, plates or pins. These fixtures can be coated and implanted in either proximal metaphyseal region of the tibia or distal femur epiphysis to study the bone-fracture healing. The following experiments can be performed to evaluate the bone-forming activity of corrosion resistant AZ31 substrates against uncoated AZ31 Mg alloy.

a) **Evaluation of H$_2$ gas release**: The formation of gas during the implantation period can be detected by x-ray imaging and micro-CT.

b) **Bone remodelling studies**: Remodelling of bone and new bone formation can be evaluated by analysis of bone morphometric parameters using micro-CT.

c) **Surface bioactivity of coated samples**: Pathological examinations using hematoxylin and eosin staining of slice tissue and immunohistochemistry stainings (BMP2, TGF-β1, PDGF and TRAP)
7.3.5 Susceptibility to biofilm formation and bone-forming activity of hyaluronic acid-lysozyme composite-silane coated AZ31 Mg alloy

In chapter 5, the hyaluronic acid-lysozyme composite coating on AZ31 Mg alloy showed a significant reduction in \textit{S.aureus} colonisation on the surface without affecting osteoblasts functions. The efficacy of the composite coating can be further validated in an \textit{in vivo} model. The following methods can be used to testify the antimicrobial and osteoinductive properties of composite coating \textit{in vivo}:

a) **Implant preparation:** The hyaluronic acid-lysozyme coated AZ31 Mg alloy device could be treated with bioluminescently labelled bacterial strains (\textit{S.aureus}) for the appropriate time. The coated implant can be inserted into the long bones or beneath the skin folds.

b) **Determination of antibacterial activity:** The antibacterial activity or bacterial infection surrounding the implant can be determined using Bioimagers. The luminosity associated with the bacterial growth levels can be recorded during the implantation period. In addition, implants can be carefully removed after euthanization of animals and subject to the quantification of bacterial growth using spread plate assay, live-dead bacteria staining or SEM.

c) **Surface bioactivity of coated samples:** Pathological examinations using hematoxylin and eosin staining of slice tissue and immunohistochemistry staining (BMP2, TGF-\(\beta\)1, PDGF, TRAP etc.)

d) **Bone remodelling studies:** Remodelling of bone and new bone formation can be evaluated by analysis of bone morphometric parameters using micro-CT.

e) **Evaluation of H\(_2\) gas release:** The formation of gas pockets during the implantation period can be detected by x-ray imaging and micro-CT.
7.4 References


Appendix I

Calibration curves

S1: Bovine serum Albumin (BSA)

\[ y = 0.0007x \]
\[ R^2 = 0.9791 \]

S2: para-nitro phenol (p-nP)

\[ y = 0.0026x \]
\[ R^2 = 0.9984 \]
S3: Hyaluronic acid (HA)

![Graph of fluorescence vs. hyaluronic acid concentration](image)

\[ y = 0.0254x \]

\[ R^2 = 0.9831 \]

S4: Calf thymus DNA

![Graph of fluorescence intensity vs. calf thymus DNA concentration](image)

\[ y = 0.387x \]

\[ R^2 = 0.9889 \]
S5: Magnesium ion quantification (colorimetric)

\[ y = 0.0843x \]
\[ R^2 = 0.976 \]

S6: Calcium ion quantification (colorimetric)

\[ y = 0.3196x \]
\[ R^2 = 0.9905 \]

S7: Hydroxyproline (Collagen quantification)

\[ y = 0.6275x \]
\[ R^2 = 0.9961 \]
S8: 4-Methyl umbelliferon (4-MU) (Lysozyme activity determination)

\[ y = 51.225x \]

\[ R^2 = 0.9996 \]

**Concentration of 4-MU (µM)**

**Fluorescence (Ex/Em(nm): 360/445)**
Appendix II

List of peer reviewed journal articles


Conference Abstract

Appendix III

Oral and poster presentations

1. S Agarwal, J Curtin, B Duffy and S Jaiswal, Hyaluronic acid functionalized multifunctional silane coatings on AZ31 Mg alloy for orthopaedic applications, 9th Biometal symposium in Italy, September 2017. (Oral and poster presentation)

2. S Agarwal, J Curtin, B Duffy and S Jaiswal, Silane coating for surface modification of Mg alloy, 7th Biometal symposium in Italy, August 2015. (Received best poster award) (Oral and poster)