Final Draft
of the original manuscript:

Hou, R.-Q.; Scharnagl, N.; Willumeit-Roemer, R.; Feyerabend, F.:
Different effects of single protein vs. protein mixtures on magnesium degradation under cell culture conditions.
In: Acta Biomaterialia. Vol. 98 (2019) 256 - 268.
First published online by Elsevier: 14.02.2019

https://dx.doi.org/10.1016/j.actbio.2019.02.013

Different effects of single protein vs. protein mixtures on magnesium degradation under cell culture conditions

Rui-Qing Hou [a*], Nico Scharnagl [a], Regine Willumeit-Römer [a], Frank Feyerabend [a*]

[a] Helmholtz-Zentrum Geesthacht, Institute of Material Research, Max-Planck-Str. 1, 21502 Geesthacht, Germany

[*] Corresponding author:

Rui-Qing Hou, Phone: +49 (0)4152 871941, Fax: +49 (0)4152 871920,

E-mail: *ruiqing.hou@hzg.de*

Dr. Frank Feyerabend, Phone: +49 (0)4152 871259, Fax: +49 (0)4152 872595,

E-mail: *frank.feyerabend@hzg.de*

Abstract:

Bovine serum albumin (BSA) or fetal bovine serum (FBS), as the protein component, is usually added into solution to study the influence of proteins on Mg degradation. However, the specific character of proteins used and the interaction between organic molecules in FBS do not draw enough attention. This study investigated the influence of BSA, fibrinogen (Fib) and FBS on Mg degradation in Hanks' balanced salt solution without (HBSS) or with calcium (HBSSCa) and Dulbecco's modified eagle medium Glutamax-I (DMEM). The results reveal that the effect of BSA, Fib and FBS on the degradation rate of Mg is time- and media-dependent, as a result of the overlap of protein adsorption, binding/chelating to ions and interaction between organic molecules. The binding/chelating of proteins and/or the possible effect of proteins on the kinetics of products formation lead to the formation of different degradation precipitates on Mg surface in HBSS. The interaction between proteins and Ca^{2+}/PO_4^{3-} accelerates the formation of Ca-P salts in HBSSCa and DMEM, thereby impeding the degradation of Mg. Moreover, the interplay between organic molecules and the specific character of proteins are highlighted by the cooperative (in media + FBS) or competitive (in DMEM + BSA + Fib) effect of proteins in the presence of more kinds of proteins and the different effect of BSA and Fib on the degradation of Mg. Therefore, the addition of proteins to testing medium is necessary for *in vitr*o tests and DMEM + 10% FBS is recommended as the *in vitro* testing medium to present an *in vivo*-like degradation for Mg.

Keywords: BSA; Fibrinogen; FBS; immersion; EDX

1. Introduction

A full understanding of the *in vivo* degradation of Mg alloys can pave the way for tailoring the degradation and biological response of Mg implants and can aid at establishing *in vitro* evaluation standards for Mg degradation. To achieve this goal, the degradation of Mg alloys has been investigated under different simulated physiological conditions [1, 2]. Proteins, as an important component of *in vivo* conditions, have been investigated to elucidate their roles in the

degradation of Mg. Bovine serum albumin (BSA, ~66 kDa), the main protein ingredient of fetal bovine serum (FBS) and the most abundant blood protein in cattle, is used in numerous biochemical applications due to its stability and lack of interference within biological reactions. To determine the effect of proteins on Mg degradation, it is normally added as simplified protein component to testing medium [3-7]. On the other hand, FBS is also used for *in vitro* investigations, especially as supplement for cell culture media [8-13]. Although the major components of FBS are proteins, its composition is fairly complex. Besides albumin (BSA), FBS contains various inorganic ions, for example Ca^{2+}, and some other important trace compounds, such as antibodies, growth factors, etc.. These compounds can affect the degradation of Mg under certain conditions. For instance, Ca^{2+} reduces the degradation rate of Mg, when hydrocarbonate and phosphate ions are present [14, 15]. Small organic molecules can promote the degradation of Mg after long-term immersion [16]. Therefore, BSA and FBS show different influence on Mg degradation, even under the same conditions. For example, Kirkland et. al [10] reported that the addition of 10% FBS to minimum essential medium (MEM) decreased the degradation rate of MgCa alloys, while Walker et.al. [3] reported that BSA promoted the mass loss of Mg-0.8Ca alloy in MEM.

To compare the results from different literature, the corrosion inhibition efficiency (η) of proteins for Mg degradation was calculated by the following equation [17]:

$$\eta = \frac{\mathrm{DR}_{np} - DR_p}{\mathrm{DR}_{np}} = \frac{i_{np} - i_p}{i_{np}} = \frac{R_p - R_{np}}{R_p} \qquad (1)$$

where DR_p and DR_{np}, i_p and i_{np}, R_p and R_{np} are degradation rate, corrosion current density, polarization resistance of Mg in medium with and without proteins, respectively.

The inhibition efficiency of proteins for pure Mg is summarized in Fig. 1 [3, 4, 8, 11, 18-20]. It shows positive efficiencies when FBS is added to testing media, indicating a decrease of the degradation rate of Mg. However, except the coated Mg (PEO coating or Ca-P salts coating),

negative efficiencies are found for the addition of BSA to testing media, suggesting the acceleration of Mg degradation. There are also some reports, showing the decreased degradation rate for pure Mg induced by BSA [5, 21], which are not summarized here due to the unavailability of the degradation rate. The difference from various reports is ascribed to not only the complex composition of FBS, but also the differences of protein concentration, test duration, medium composition and conditions used [22, 23].

As stated by various authors [3, 8, 9, 11, 12, 24], the effect of proteins on Mg degradation is mainly ascribed to the adsorption and binding/chelating of proteins. The adsorption of protein on surface is determined by the external parameters (temperature, pH, ionic strength), protein properties (size, structural stability, composition) and surface properties (surface energy, polarity, charge, morphology) [25]. The adsorption of proteins on Mg surface is believed to enhance the compactness of degradation layer on Mg surface, subsequently decreasing the degradation of Mg [8]. Nevertheless, the binding/chelating of Mg^{2+} to proteins can promote the Mg dissolution reaction [26], which is largely correlated to some parameters, such as temperature, pH, protein concentration, Mg^{2+} concentration and protein structure [27, 28]. Therefore, it is not surprising that the effect of proteins on Mg degradation is correlated to the specific properties of proteins (molecular weight, conformation, affinity to Mg^{2+}, charge, etc.).

Due to the great complexity of proteins and the very wide range of proteins in the human body (e.g. albumin, fibrinogen, globulins), the adsorption of proteins in physiological environments is rather complex. In protein mixtures, the protein adsorption behavior is often a result of an overlap of adsorption, exchange, desorption and repulsion processes. Clearly there will be a competitive adsorption between proteins (known as “Vroman Effect”), which is related to the mobility and affinity of proteins, the surface and solvent properties [29]. A typical example is that fibrinogen (Fib) absorbed on a biopolymer surface is replaced by high molecular weight kininogen [30]. The co-existence of these proteins can affect the influence of single protein on

Mg degradation, therefore also affect the degradation rate of Mg and/or the degradation products on Mg surface. However, when the effect of FBS on Mg degradation was discussed, the complexity of FBS and the interplay between organic molecules did not get enough considerations.

Furthermore, there is an ongoing process to pursue comparable physiological conditions to *in vivo*. This has led to the examination of the influence of proteins on Mg degradation in different media as shown in Fig. 1. The circumstance (pH, osmolality, Mg^{2+} concentration) near Mg surface is definitely variable in different media. All such media-triggered changes of circumstance can affect protein performance during immersion, thereby affecting the degradation rate and/or degradation mechanism of Mg. Therefore, it is of great importance to choose an appropriate medium to analyze the effects of proteins on Mg degradation.

The aims of this study are: (1) to investigate the influence of protein on Mg degradation in different media in order to elucidate the role of media in the protein-containing degradation process; (2) to analyze the difference between the influence of BSA and FBS on Mg degradation; (3) to study the comprehensive effect of proteins on Mg degradation when more kinds of proteins are present in medium (medium + BSA + Fib or medium + FBS) under cell culture conditions.

2. Materials and Methods

2.1. Materials

Pure Mg specimens were cut out of the ingots (Magnesium Elektron, Manchester, UK, chemical composition is shown in Table 1) with the dimension of 10 mm × 10 mm × 4 mm. Prior to use, the specimens were wet ground with 800 to 2500 grit SiC abrasive paper (Schmitz-Metallographie GmbH, Herzogenrath, Germany) to remove the surface impurities. Then the samples were ultrasonically cleaned for 20 min in N-hexane, 20 min in acetone and 20 min in

100% ethanol (Merck KGaA, Darmstadt, Germany). Finally, the samples were dried in 12-wells cell culture plates (Greiner Bio-One, Frickenhausen, Germany) under sterile conditions.

2.2. Immersion tests

The base immersion media are Hank's balanced salt solution without (HBSS) or with calcium (HBSSCa, 1.8 mM $CaCl_2$) and Dulbecco's modified eagle medium Glutamax-I (DMEM) (Life Technologies, Darmstadt, Germany), the detailed compositions is already published in literature [13, 31]. 1.8 mM $CaCl_2$ was chose to prepare HBSSCa because it is same concentration in DMEM and other simulated body solution and it is close to the Ca^{2+} concentration in plasma [32]. 10% FBS (PAA laboratories, Linz, Austria) was added in HBSS, HBSSCa and DMEM, respectively, to prepare the media HBSS + 10% FBS, HBSSCa + 10% FBS and DMEM + 10% FBS. The concentration of BSA (Carl ROTH GmbH, Karlsruhe, Germany) was set at a similar concentration (3.4 mg/mL) as 10% FBS to avoid the difference from proteins concentration as much as possible. The concentration of Fib (1.6-4.0 g/L in plasma, Sigma-Aldrich, Saint Louis, USA) was set to 0.34 mg/mL due to the ten times lower concentration than BSA in plasma (35-40 g/L in plasma) [33, 34]. Finally, the media were sterile filtered (0.2 µm pore size, Thermo Fisher, Karlsruhe, Germany).

The samples were weighed before immersion tests (Waagen-Schmitt GmbH, Hamburg, Germany). Six replicate samples for each time point were performed in media at a ratio of 0.2 g/L (sample/medium) under cell culture conditions (37 °C, 5% CO_2, 20% O_2, 95% rel. humidity). The immersion media were changed every 2 to 3 days to simulate a semi static condition. At each medium change, changes in pH (Sentron Argus X pH-meter, Fisher Scientific, Schwerte, Germany) and osmolality (Osmomat 030, Gonotec, Berlin, Germany) were measured. pH and osmolality of the media incubated without samples were determined as control.

After immersion for 3, 7 and 14 days, samples were cleaned with sterile distilled water and dried at 50 °C in air. The degradation products were removed by immersing in chromic acid (180 g/L chromium (VI) oxide in distilled water, VWR International, Darmstadt, Germany) for 20 min at room temperature. Subsequently, samples were cleaned with distilled water and 100% ethanol. After drying, the weights of samples were determined again to obtain the mass loss during the immersion. The mean degradation depth (h) of samples in μm was calculated using the following equation:

$$h = \frac{10000 \cdot \Delta m}{A \cdot \rho} = \frac{DR \cdot t}{8.76} \qquad (2)$$

where A is the surface area in cm^2, ρ is the density of pure Mg (1.74 g/cm^3), t is the immersion time in hours, Δm is the observed mass loss in gram and DR is the degradation rate in mm/year. The mean degradation depth can be transformed into degradation rate (mm/year, presented in table s2) by using equation (2). The rationale of mean degradation depth used has been discussed in literature [35]. The effect of chromic acid on the mass loss of pure Mg without immersion was checked by immersing samples in chromic acid for 20 min. No changes in weight of samples before and after immersion suggest no dissolution of the bulk material during immersion in chromic acid.

2.3. Protein concentration

The changes of protein concentration in HBSS and HBSSCa were determined by bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, USA). Simultaneously, the media without samples were checked as control. 10 μL solutions of media + BSA, media + BSA + Fib and media + FBS were taken out from the plates after certain immersion time, then diluted to 25 μL with distilled water in 96-well plates. 25 μL solution of media + Fib also was taken out after certain immersion time in a 96-well plate. Subsequently, 200 μL working reagent was added to each well and the plates were mixed thoroughly on a plate shaker for 30 s. Afterwards, the plates

were incubated at 37 ˚C for 30 min. Finally, the absorbance at 570 nm was measured on a microplate reader (Tecan Sunrise TECAN Deutschland GmbH, Crailsheim, Germany). The concentration of proteins in solution can be deduced from the standard curves (0-2000 mg/L). Due to the interference of phenol red and some amino acids, the protein concentration in DMEM cannot be determined by BCA assay.

2.4. Surface characterizations

After immersion, to investigate the surface morphologies and identify the products on the surfaces, samples after drying were directly examined using scanning electron microscopy (SEM, Phenom-World, Eindhoven, Netherlands), X-ray diffraction (XRD, Bruker D8 Advance, Karlsruhe, Germany) and infrared spectrometer (Bruker Hyperion 2000, Ettlingen, Germany). SEM in backscattering mode with an accelerating voltage of 15 kV was used to analyze the morphologies. XRD was performed in grazing incidence geometry at an incident angle of 3° between $2\theta = 10° - 75°$ at 0.01° intervals and with 0.5 s counting time per point. The generator was set for 40 kV and 40 mA. The BrukerEVA software (PDF-2 Release 2015 RDB) was used to analyze the data. The infrared spectra were recorded with a resolution of 2 cm^{-1}, taking 512 scans by using infrared spectroscopy in reflectance mode (Bruker Hyperion 2000, Ettlingen, Germany). The results were evaluated by Bruker OPUS software version 7.5.18.

2.5. Composition of the degradation layer

The cross sections of samples were prepared by embedding samples vertically into resin (Demotec 30, Nidderau, Germany), then successively wet ground with 800 to 2500 grit SiC abrasive paper, subsequently polished with colloidal silica suspension (Cloeren Technology GmbH, Wegberg, Germany). The cross sections of degradation layers were taken by SEM (Tescan Vega3 SB, Brno, Czech Republic) in backscattered electron (BSE) mode to measure the thickness of the degradation layer. At least 80 different sites were examined for each sample. The

distribution of elements were analyzed by energy dispersive X-ray spectroscopy (EDX) under an accelerating voltage of 15 kV with a resolution of 256 pixels. Each pixel was acquired for 80 ms. The diameter of the beam was about 300 nm according to the used beam intensity. The weight percentages of phosphorus and calcium in degradation products were obtained from the mapping results by using Iridium Ultra software (Eumex Instrumentebau GmbH, Heidenrod, Germany). The thickness of Ca/P-rich layer was measured from the mapping results using Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose, USA) and at least 10 measurements for each sample were performed.

2.6. Statistical analysis

The statistical analysis was done by using the software Origin 9.0 (Originlab Corporation, Wellesley Hills, USA). One-way analysis of variance (ANOVA) on ranks with Dunn's multiple comparison post hoc tests was used.

3. Results

3.1. Mean degradation depth, pH, osmolality

As shown in Fig. 2, the mean degradation depths of pure Mg were calculated after 3, 7 and 14 days of immersion in HBSS, HBSSCa and DMEM with or without BSA, Fib or FBS. As expected, the addition of Ca ions to HBSS decreased the degradation of Mg after 3 days of immersion and Mg degraded significantly faster in HBSS and HBSSCa than in DMEM. In HBSSCa, proteins significantly reduced the degradation of Mg, especially when Fib was present. The addition of BSA / Fib / FBS to HBSS or DMEM also led to a lower degradation depth after 3 and 7 days of exposure, suggesting lower degradation rates of Mg when BSA / Fib / FBS was present during the short-term immersion. After 14 days of immersion, BSA exhibited little effect on Mg degradation in both HBSS and DMEM. However, FBS promoted the degradation of Mg in HBSS, but showed an adverse result in DMEM. More importantly, when BSA and Fib were

simultaneously present in HBSS or DMEM, the mean degradation depth increased compared with that in HBSS or DMEM with BSA or Fib alone.

The changes in pH and osmolality of media are depicted in Fig. 3. In HBSS-based media, changes in pH slightly increased with the immersion time, and the addition of proteins led to a higher pH compared with the control (HBSS) after 3 days of immersion. In HBSSCa-based media, changes of pH for the control (HBSSCa) remained stable, while the addition of proteins resulted in a decrease of pH with the immersion time. A similar decreasing trend of pH could also be found in DMEM-based media. Moreover, the addition of proteins to DMEM resulted in a lower pH during immersion except in DMEM + BSA + Fib. The changes in osmolality of media decreased with the immersion time irrespective of media compositions. The addition of proteins resulted in a higher osmolality in HBSS, while they had an adverse effect on the osmolality in HBSSCa and DMEM except in DMEM + BSA + Fib.

3.2. Protein concentration during immersion

To examine the adsorption of proteins during immersion, the concentration of proteins in HBSS-based and HBSSCa-based media were monitored during 3 days of immersion. As shown in Fig. 4, the initial concentrations of proteins were similar in HBSS + BSA and HBSS + FBS (3.3-3.7 mg/mL). Compared with the controls (media without sample), the concentration of proteins gradually decreased with the immersion time in HBSS-based media, HBSSCa + BSA + Fib and HBSSCa + FBS, while no significant decreases of protein concentration were observed in HBSS + BSA / Fib, indicating that proteins behaved differently in HBSS and HBSSCa, which is in agreement with our previous result [24]. The decrease of protein concentration demonstrated the participation of proteins in the Mg degradation process by adsorption or other ways.

3.3. Surface morphology

The surface morphologies of samples after immersion in different media are shown in Fig. 5a and b. In HBSS, macroscopic white precipitates were formed on surface as a conglomerate of very thin platelets. The addition of BSA led to the formation of needle-like precipitates and both of these two kinds of precipitates (conglomerate of thin platelets and needle-like precipitates) were formed on Mg surface in HBSS + Fib. In comparison, only conglomerates of thin platelets were formed in HBSS + BSA + Fib and there were only few precipitates on the surface in HBSS + FBS. Moreover, the precipitates changed with the exposure time, especially for the needle-like precipitates. In HBSSCa-based media (Fig. 5b), the flocculent precipitates were formed in HBSSCa after 7 days of immersion, while granular precipitates were visible on Mg surface in HBSSCa + BSA and HBSSCa + Fib. No obvious precipitates were observed on the sample surfaces formed in HBSSCa + BSA + Fib and HBSSCa + FBS. Similar results were also gained in DMEM-based media (Fig. s1), indicating little morphology difference induced by the addition of proteins and little changes with the immersion time, so SEM images of samples immersed in DMEM-based media are not shown here. Samples showed uniform degradation (no localized degradation) in all the media due to the high purity of Mg used, which indicated by the morphology of sample after the removal of degradation product (data not shown).

3.4. Degradation products

XRD results (Fig. 6a) revealed that the conglomerates of very thin platelets formed in HBSS-based media and the flocculent precipitates formed in HBSSCa were mainly composed of hydromagnesite (reference card 00-070-1177) and dypingite (reference card 00-29-0857 and 00-23-1218), while needle-like precipitates formed in HBSS + BSA were well-formed nesquehonite (reference card 00-020-0669). The precipitates formed in HBSS + BSA + Fib contained not only nesquehonite, but also hydromagnesite and dypingite. However, the XRD patterns of samples in the rest of media only gave the Mg diffraction peaks as shown the spectrum obtained from DMEM, indicating amorphous or nanocrystaline products on these sample surfaces.

IR reflection spectra were conducted for the samples immersed in HBSS + FBS, HBSSCa + protein and DMEM-based media. Only the range from 2200 cm^{-1} to 550 cm^{-1} is depicted in Fig. 6b and c, as the major information is located in this range. The band near 1646 cm^{-1} is attributed to the OH-bending of absorbed water and/or the amide I of organic molecules absorbed on Mg surface [36]. The broad band from 1600 cm^{-1} to 1300 cm^{-1} is a result of the overlap between the anti-symmetrical CO_3^{2-} stretching and the band II/III/VI stretching of organic molecules [13]. Compared with the spectra from BSA and Fib, the bands from proteins can be observed for protein-containing media, indicating the adsorption of proteins on Mg surface. The bands from the asymmetric stretching of phosphate obviously shifted from 1182 cm^{-1} and 1093 cm^{-1} in DMEM to around 1134 cm^{-1} in DMEM with proteins [37], and a similar shift of bands from phosphate could also be observed in HBSSCa-based media, indicating the possible different conformation or compositions of Ca/Mg-PO_4 when protein is present. The band at 861 cm^{-1} is assigned to the CO_3^{2-} bending vibration [38]. Therefore, it can be concluded that the degradation products on Mg surface are mainly composed of Mg/Ca-PO_4, Ca/$MgCO_3$ and absorbed organic components in HBSSCa and DMEM. As previously proposed [39], $Mg(OH)_2$ and MgO also are the possible products on Mg, but they are not detected herein. This could be due to the fact that only the outmost surface was detected and the signal to noise ratio is high in the low wavenumber range.

3.5. Degradation layer analysis

To analyze the variations of element distribution in degradation products, chemical element mappings were performed for samples after different immersion time in different media. The detection of Na, Cl and S were negligible for all samples. Typical examples are given in Fig. 7 for the distributions of Mg, O, C, P and Ca in the degradation layer (the distribution of elements for all degradation layer formed in different media are provided in Fig. s2-4). Negligible Ca (< 0.1 wt.%) was present in the degradation layer formed in HBSS and HBSS + BSA / Fib / BSA +

Fib due to the high purity of BSA and Fib used and the absence of Ca in HBSS. As expected, O mainly distributed in degradation layer, while C existed mainly in the resin. The obvious differences came from the distributions of P and Ca. In HBSS and HBSS + BSA / Fib / BSA + Fib, the existence of P in degradation layer indicated the formation of Mg-PO_4 during immersion. A Ca/P-rich layer could be observed for the degradation layer formed in HBSSCa-based and DMEM-based media. Furthermore, the addition of proteins generally resulted in the thickening of Ca/P-rich outmost layer. The consistent distribution of Ca and P in degradation layer suggested the formation of Ca-P salts on the surface. However, when BSA and Fib were simultaneously present in DMEM, the formation of Ca-P salts in the degradation layer was largely inhibited.

The thicknesses of degradation layers are displayed in Fig. 8. It showed the thickness of degradation layer increased with the immersion time except for HBSS + BSA. Generally, the degradation layer formed in HBSS-based media was much thicker than that formed in HBSSCa-based and DMEM-based media, especially during the first 7 days of immersion. The addition of proteins to media resulted in a thinner degradation layer except in DMEM + BSA + Fib.

The weight percentages of Ca and P in degradation layer were calculated according to the chemical mapping results. As depicted in Fig. 9, the *wt. %* of Ca and P increased with the immersion time irrespective of media composition. Generally, higher contents of Ca and P existed in degradation layer formed in DMEM and HBSSCa than in HBSS, which resulted from the higher concentration of PO_4^{3-} or Ca^{2+} in HBSSCa and DMEM. Moreover, the addition of proteins also evidently promoted the increase of the *wt. %* of Ca and P in degradation layer. The presence of FBS generally resulted in a higher content of Ca and P in degradation layer than BSA, especially during the initial immersion time. The *wt.%* of Ca and P formed in HBSSCa + BSA + Fib was higher than that in HBSSCa + BSA, but comparable to that in HBSSCa + Fib. However,

the content of Ca and P was much lower in degradation layer formed in DMEM + BSA + Fib than in DMEM + BSA and DMEM + Fib.

Furthermore, the thickness of Ca/P-rich layer in the top of degradation layer formed in HBSSCa-based and DMEM-based media were examined as shown in Fig. 10. Generally, the thickness of this Ca/P-rich layer increased when proteins were present in media. In HBSSCa, co-existence of BSA and Fib resulted in a comparable thickness of the Ca/P-rich layer to that formed in HBSSCa + BSA or Fib, while in DMEM the thickness of this Ca/P-rich layer significantly decreased when both BSA and Fib were present compared with that in DMEM + BSA or Fib.

4. Discussion

HBSS has been supported to be the simplest medium compared with SBF and DMEM [31, 39]. In this study, 1.8 mM $CaCl_2$ was added to HBSS to prepare the HBSSCa medium to investigate the synergistic effect of proteins and Ca^{2+} for Mg degradation. Generally, cell culture medium, DMEM, is less corrosive and more complex for Mg than HBSS due to the existence of Ca^{2+} and Mg^{2+}, and high HCO_3^- and PO_4^{3-} concentration in DMEM [31]. Therefore, in this study, HBSS, HBSSCa and DMEM were chosen as base media to study the influence of proteins on the degradation of Mg in different testing solutions under cell culture conditions.

Degradation products in HBSS-based media

In HBSS, $Mg(OH)_2$, $MgCO_3$ and $Mg_3(PO_4)_2$ as degradation products have been found in the degradation layer of Mg in previous report [16]. High mean degradation depth, large changes in pH and osmolality of media indicate the fast degradation of Mg in HBSS. The resulting supersaturation of Mg^{2+} in HBSS leads to the formation of crystalline precipitates (hydromagnesite or nesquehonite) on the sample surface as described by equations (4-7). When proteins were added to HBSS, either different precipitates (nesquehonite in HBSS + BSA), a mixture of all (in HBSS + Fib), or nearly no detectable precipitates (in HBSS + FBS) were

formed on the Mg surface, which is in agreement with the previous result that the organic molecules can affect the formation of crystalline precipitates on Mg surface [16].

$$Mg^{2+} + CO_3^{2-} \rightarrow MgCO_3 \quad (3)$$

$$5\ Mg^{2+} + 5\ OH^- + 4\ CO_3^{2-} + 4\ H_2O \rightarrow Mg_5(CO_3)_4(OH)_2 \cdot 4H_2O \quad (4)$$

$$5\ MgCO_3 + 5H_2O \rightarrow Mg_5(CO_3)_4(OH)_2 \cdot 4H_2O + CO_2 \quad (5)$$

$$Mg^{2+} + CO_3^{2-} + 3H_2O \rightarrow MgCO_3 \cdot 3H_2O \quad (6)$$

$$MgCO_3 + 3\ H_2O \rightarrow MgCO_3 \cdot 3H_2O \quad (7)$$

Because CO_3^{2-} can be continuously supplied by CO_2 under cell culture conditions, from the thermodynamic view (as shown in supplementary), the pH of the media and the free Mg^{2+} concentration become the two dominant parameters for the formation of crystalline precipitates. There is no evident difference between pH in HBSS-based media during the immersion, while higher osmolality in HBSS + proteins indicates a higher total Mg^{2+} concentration [40]. When proteins are present in media, a part of Mg^{2+} is bound to proteins as shown in equation (8).

$$C\ (\text{total } Mg^{2+}) = C\ (\text{bound } Mg^{2+}) + C\ (\text{free } Mg^{2+}) \quad (8)$$

At least three binding sites of albumin for Ca^{2+}/Mg^{2+} have been identified and they possess variable affinity and binding capacity [28, 41]. Mg^{2+} competes with Ca^{2+} for the binding sites in serum albumin, but with a lower binding affinity ($K_a(Ca^{2+}) = 1.5\times10^3\ M^{-1}$, $K_a(Mg^{2+}) = 1\times10^2\ M^{-1}$) [28, 42]. Moreover, the binding of Ca^{2+}/Mg^{2+} to albumin is getting much higher with increasing ion concentration and increasing pH above the physiological level [27, 41, 43, 44]. Therefore, the binding of Mg^{2+} to proteins could be one reason for the effect of proteins on the formation of different precipitates in HBSS-based media.

On the other hand, in view of thermodynamics (Fig. s5), hydromagnesite should be formed before the formation of nesquehonite as Mg^{2+} concentration increases in the range of pH 8-9.

However, only nesquehonite is observed in HBSS + BSA. The possible explanation is that the presence of proteins may affect the nucleation of products or the kinetics of products formation process [45].

Degradation products in HBSSCa-based media

When $CaCl_2$ was added to HBSS (HBSSCa), $CaCO_3$ and Ca-P salts also could be possibly formed on Mg surface as following:

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \qquad (9)$$

$$x\ Mg^{2+} + y\ Ca^{2+} + z\ PO_4^{3-} \rightarrow Mg_xCa_y(PO_4)_z \qquad (10)$$

$$x\ Mg^{2+} + y\ Ca^{2+} + z\ PO_4^{3-} + n\ CO_3^{2-} \rightarrow Mg_xCa_y(PO_4)_z(CO_3)_n \qquad (11)$$

According to the thermodynamical solubility calculations (as shown in supplementary) [31], $CaCO_3$, Ca-P salts could be formed on Mg surface before the formation of $MgCO_3$ and crystalline precipitates, which is in accordance with the result that a Ca/P-rich layer formed in the top of degradation layer. This Ca/P-rich layer is believed to significantly slow down the degradation rate of Mg [46]. However, as layer thickness and osmolality indicated, this layer is still water/ion permeable, which induces a continuous degradation of Mg, eventually leading to the formation of crystalline precipitates on Mg surface after 7 days of immersion in HBSSCa. The presence of proteins in HBSSCa plainly inhibit the formation of crystalline precipitates on Mg surface. It could be ascribed to several reasons: the low degradation rate, the binding of Mg^{2+}/Ca^{2+} to proteins and the promoted formation of Ca/P-rich layer in the top of degradation layer (including the granular Ca-P precipitates). The increase of Ca/P content and Ca/P-rich layer thickness compared with the control (HBSSCa) (Fig. 9 and 10) demonstrate that proteins largely promote the formation of Ca-P salts on Mg surface, which is believed to be one of the reasons for the protein-induced decrease of Mg degradation.

Degradation products in DMEM-based media

In DMEM, due to the higher concentration of PO_4^{3-} and HCO_3^-, the degradation of Mg is much slower than in HBSS and HBSSCa. The lower increase of pH is ascribed to the stronger buffering capacity (high concentration of HCO_3^- and organic molecules) and slower degradation. None of crystalline precipitates are detectable, indicating that free Mg^{2+} concentration in DMEM did not reach the saturation during immersion. Organic molecules, amorphous carbonate and phosphate (Ca/Mg-CO_3, Ca/Mg-PO_4) are proven to be the main degradation products in the top of Mg surface, which is in agreement with the reported results that outer Ca/P-rich layer always forms separately from the inner degradation layer ($Mg(OH)_2$, (Mg,Ca)-CO_3) [46-49]. Proteins show little influence on the surface morphology in DMEM (Fig. s1). The obvious feature caused by the presence of proteins is the thickening of the outmost Ca/P-rich layers in the top of degradation surfaces, which shows a similar trend to that in HBSSCa but no granular Ca-P precipitates are visible in DMEM-based media. As reflected in Fig.11, the increasing content of Ca-P salts in the top of layer and its thickness generally corresponds to the lower degradation rate of Mg. It shows a similar tendency for samples in HBSSCa-based and DMEM-based media. This result reveals that the promoted formation of Ca-P products in the top of degradation layer could be one of the possible reasons for the reduced degradation rate by protein.

The promoted formation of Ca-P salts in HBSSCa and DMEM is related to the complex biomineralization process. Generally, collagen fibrils provide a framework known as extracellular matrix, a set of negatively charged phosphorylated non-collagenous proteins attract Ca^{2+} and PO_4^{3-} ions through their charged amino acid residues and increase the local supersaturation to form nuclei of a critical size, which can develop into hydroxyapatite (HA) crystals [45, 50, 51]. Therefore, the electrostatic interaction between proteins and Ca^{2+}/PO_4^{3-} could be the reason for the promoted Ca-P salt formation on Mg surface, indicating the importance of the interaction between Ca^{2+}, PO_4^{3+} ions and proteins to HA precipitation. However, other adverse results were also reported that FBS led to a low content of P and Ca in

the degradation products on Mg surfaces [8, 9]. This discrepancy may derive from the different detection methods or the different testing environment used.

Effect of proteins on Mg degradation

When comparing the effect of proteins on Mg degradation in these three different media, it can be found that Ca^{2+} largely enhance the corrosion inhibition effect of proteins on Mg degradation, indicating the synergistic effect of proteins and Ca^{2+} on Mg degradation, especially for Fib and FBS. This may be related to the high affinity of protein to Ca^{2+} [42, 43, 52]. Moreover, the addition of Ca^{2+} can change the zeta potential of degradation products, thereby affecting the electrostatic-dominated interaction between proteins and degradation products [53].

Two main influences induced by proteins on degradation layer of Mg are the thickening of the outmost Ca/P-rich layer and the adsorption of proteins. They can slow down the degradation of Mg, however, Mg degradation is still ongoing in media. With the dissolved Mg^{2+} concentration increasing in media, the binding/chelating of Mg^{2+} to proteins increases [41], which promotes the dissolution of Mg in media. Moreover, protein adsorption is believed to be much faster than binding/chelation during the initial stage and the binding/chelating effect can provide long-lasting gradual influence on Mg degradation [6]. Therefore, the combined effect of adsorption and binding/chelation of proteins results in the time/medium-dependent effect of proteins on Mg degradation. Similar results also have been found for other Mg alloys, such as Mg-Mn alloy [6], AZ31 and AZ91 [12].

In this study, Fib and BSA always show different influence on Mg degradation. This is related to not only the large molecule weight of Fib, but also the different properties of Fib and BSA. Our previous result shows a higher adsorption of Fib compared to BSA on possible Mg degradation products [24], indicating a different affinity of proteins to the same surface. Moreover, Fib has a stronger effect on the of solution fluidity [54] and the surface tension [55, 56] compared with

other proteins. These may be the reasons for the observed phenomenon that bubbles always were limited to the Mg surface in media + Fib during immersion (results not shown), indicating the decrease of mass transfer process in the media. This could be another reason for the different influence of Fib and BSA on Mg degradation.

The organic molecules may interact with each other in the presence of more kinds of organic molecules, which exhibits cooperative or competitive interaction under different conditions. Typical examples are the increase of degradation rate when both BSA and Fib are present in DMEM and the slower degradation of Mg in media with FBS than in media with BSA. Another already discovered fact caused by the interaction between Fib and serum proteins is that the effect of Fib in decreasing the fluidity of water is appreciably less in the presence of serum proteins than in purified solutions of Fib [54]. The organic molecules in FBS own different physiochemical characteristics, which can largely affect their adsorption or binding/chelating performance. For example, albumin is a heart-like shaped protein and its isoelectric point (pI) is about 4.8 [28, 57], while Fib (~340 kDa) is a large rod-like shaped glycoprotein (pI = ~5.8) [57, 58], indicating different electrostatic interaction, hydrogen bonding and hydrophobic/hydrophilic interaction between proteins and Mg surface. The competitive adsorption between organic molecules also has been widely reported as ‘Vroman Effect’ [29, 59-61]. Furthermore, the different structure and conformation result in different binding affinity of proteins to various kinds of ions, for example, iron (Fe) ion dissolved from impurity. Transferrins (~80 kDa, pI: ~5.2-6.4), existent in FBS, are iron-binding blood plasma glycoproteins with two specific high-affinity sites for reversible Fe ions binding [62-64]. The binding of Fe ions to transferrin may decrease Mg degradation because the limitation of the Fe re-deposition on Mg surface can efficiently lower the degradation rate of Mg [65, 66]. Therefore, the effect of proteins on Mg degradation is the result of an overlap of protein adsorption, binding/chelating to different ions

(Mg^{2+}, Fe^{3+}, etc.) and interactions between organic molecules. These basic mechanisms are depicted in Fig. 12.

The degradation layer formed in plasma, blood or under *in vivo* conditions all shows an outer rim rich of P and Ca for pure Mg [47, 67, 68]. A similar result also can be obtained in HBSSCa + proteins and DMEM-based media in this study, indicating a comparable degradation process or mechanism to *in vivo* conditions. Furthermore, the degradation rate of pure Mg *in vivo* is in the range of 0.10-0.40 mm/year [14, 47, 69], while in this study the degradation rates of Mg are 0.25-0.50 mm/year in HBSSCa + Fib / BSA + Fib / FBS (1.65-1.95 mm/year in HBSSCa) and 0.20-0.45 mm/year in DMEM + BSA / Fib / FBS (0.30-0.60 mm/year in DMEM). Therefore, the addition of proteins to media generally enables the degradation of Mg to be more comparable to *in vivo* conditions and DMEM + 10% FBS is recommended to present more physiological conditions for Mg samples compared to other media. To fully understand the roles of proteins in the degradation of Mg, more physiological conditions (dynamic conditions, etc.) and further detailed investigations about the interaction between different organic molecules are still required.

5. Conclusions

In this study, the effect of proteins (BSA, Fib, FBS) on Mg degradation was evaluated by analyzing the degradation rate and degradation products. The results indicate that the effect of proteins on Mg degradation should be studied under more physiological media (DMEM) due to the different effect of proteins on the Mg degradation in different media. The enhancement of outmost Ca/P-rich layer in protein-containing media indicates the relationship between proteins and surface mineralization process, further emphasizes the necessity of the addition of proteins to *in vitro* testing media. DMEM + 10% FBS is recommended to test the *in vitro* Mg degradation due to the more comparable degradation to *in vivo*. The different performance between single protein and protein mixtures for Mg degradation reveals that the addition of a single protein to

testing media cannot represent the effect of proteins on degradation of Mg under *in vivo* conditions, and the interaction between organic molecules in protein mixtures should be taken into account. Moreover, the comprehensive influence of protein mixtures on Mg degradation depends on the conditions used and the characteristics of proteins. The further insights should be gained for the interaction between organic molecules to fully understand the role of proteins in Mg degradation.

Acknowledgements

RQH thanks the scholarship support from China Scholarship Council (CSC). This work is supported by the Helmholtz Virtual Institute "In vivo studies of biodegradable magnesium based implant materials (MetBioMat)" (grant number: VH-VI-523). The invaluable technical supports and discussions from the colleagues at the Division of Metallic Biomaterials (HZG) is acknowledged.

Disclosures:

The authors have no proprietary or commercial interests in any product discussed here.

Funding: This work was supported by the Helmholtz Virtual Institute "In vivo studies of biodegradable magnesium based implant materials (MetBioMat)" (grant number: VH-VI-523). China Scholarship Council (CSC) provides financial support for RQH (201509350010). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References:

[1] A. Doepke, J. Kuhlmann, X. Guo, R.T. Voorhees, W.R. Heineman, A system for characterizing Mg corrosion in aqueous solutions using electrochemical sensors and impedance spectroscopy, Acta Biomater. 9 (2013) 9211-9219.

[2] G.F. Yang, Y.C. Kim, H.S. Han, G.C. Lee, H.K. Seok, J.C. Lee, In vitro dynamic degradation behavior of new magnesium alloy for orthopedic applications, J. Biomed. Mater. Res. B Appl. Biomater. 103 (2015) 807-815.

[3] J. Walker, S. Shadanbaz, N.T. Kirkland, E. Stace, T. Woodfield, M.P. Staiger, G.J. Dias, Magnesium alloys: predicting in vivo corrosion with in vitro immersion testing, J. Biomed. Mater. Res. B Appl. Biomater. 100 (2012) 1134-1141.

[4] S. Shadanbaz, J. Walker, T.B. Woodfield, M.P. Staiger, G.J. Dias, Monetite and brushite coated magnesium: in vivo and in vitro models for degradation analysis, J. Mater. Sci. Mater. Med. 25 (2014) 173-183.

[5] C.-L. Liu, Y. Zhang, C.-Y. Zhang, W. Wang, W.-J. Huang, P.K. Chu, Synergistic effect of chloride ion and albumin on the corrosion of pure magnesium, Front. Mater. Sci. 8 (2014) 244-255.

[6] Y. Wang, C.S. Lim, C.V. Lim, M.S. Yong, E.K. Teo, L.N. Moh, In vitro degradation behavior of M1A magnesium alloy in protein-containing simulated body fluid, Mat. Sci. Eng. C-Mater. 31 (2011) 579-587.

[7] C.L. Liu, Y.J. Wang, R.C. Zeng, X.M. Zhang, W.J. Huang, P.K. Chu, In vitro corrosion degradation behaviour of Mg–Ca alloy in the presence of albumin, Corros. Sci. 52 (2010) 3341-3347.

[8] V. Wagener, S. Virtanen, Protective layer formation on magnesium in cell culture medium, Mat. Sci. Eng. C-Mater. 63 (2016) 341-351.

[9] J. Zhang, N. Kong, Y. Shi, J. Niu, L. Mao, H. Li, M. Xiong, G. Yuan, Influence of proteins and cells on in vitro corrosion of Mg–Nd–Zn–Zr alloy, Corros. Sci. 85 (2014) 477-481.

[10] N.T. Kirkland, N. Birbilis, J. Walker, T. Woodfield, G.J. Dias, M.P. Staiger, In-vitro dissolution of magnesium–calcium binary alloys: Clarifying the unique role of calcium additions in bioresorbable magnesium implant alloys, J. Biomed. Mater. Res. B Appl. Biomater. 95 (2010) 91-100.

[11] A. Yamamoto, S. Hiromoto, Effect of inorganic salts, amino acids and proteins on the degradation of pure magnesium in vitro, Mat. Sci. Eng. C-Mater. 29 (2009) 1559-1568.

[12] X. Gu, Y. Zheng, L. Chen, Influence of artificial biological fluid composition on the biocorrosion of potential orthopedic Mg–Ca, AZ31, AZ91 alloys, Biomed. Mater. 4 (2009) 065011.

[13] R. Willumeit, J. Fischer, F. Feyerabend, N. Hort, U. Bismayer, S. Heidrich, B. Mihailova, Chemical surface alteration of biodegradable magnesium exposed to corrosion media, Acta Biomater. 7 (2011) 2704-2715.

[14] N.A. Agha, F. Feyerabend, B. Mihailova, S. Heidrich, U. Bismayer, R. Willumeit-Romer, Magnesium degradation influenced by buffering salts in concentrations typical of in vitro and in vivo models, Mat. Sci. Eng. C-Mater. 58 (2016) 817-825.

[15] D. Mei, S.V. Lamaka, J. Gonzalez, F. Feyerabend, R. Willumeit-Römer, M.L. Zheludkevich, The role of individual components of simulated body fluid on the corrosion behavior of commercially pure Mg, Corros. Sci. 147 (2018) 81-93.

[16] R.-Q. Hou, N. Scharnagl, F. Feyerabend, R. Willumeit-Römer, Exploring the effects of organic molecules on the degradation of magnesium under cell culture conditions, Corros. Sci. 132 (2018) 35-45.

[17] K. Tebbji, A. Aouniti, A. Attayibat, B. Hammouti, H. Oudda, M. Benkaddour, S. Radi, A. Nahle, Inhibition efficiency of two bipyrazole derivatives on steel corrosion in hydrochloric acid media, Indian J. Chem. Techn. 18 (2011) 244-253.

[18] P. Wan, X. Lin, L. Tan, L. Li, W. Li, K. Yang, Influence of albumin and inorganic ions on electrochemical corrosion behavior of plasma electrolytic oxidation coated magnesium for surgical implants, Appl. Surf. Sci. 282 (2013) 186-194.

[19] N.T. Kirkland, J. Waterman, N. Birbilis, G. Dias, T.B. Woodfield, R.M. Hartshorn, M.P. Staiger, Buffer-regulated biocorrosion of pure magnesium, J. Mater. Sci. Mater. Med. 23 (2012) 283-291.

[20] W.D. Mueller, M. Fernández Lorenzo de Mele, M.L. Nascimento, M. Zeddies, Degradation of magnesium and its alloys: dependence on the composition of the synthetic biological media, J. Biomed. Mater. Res. A 90 (2009) 487-495.

[21] V. Wagener, A.-S. Faltz, M.S. Killian, P. Schmuki, S. Virtanen, Protein interactions with corroding metal surfaces: comparison of Mg and Fe, Faraday. Discuss. 180 (2015) 347-360.
[22] F.E.-T. Heakal, A.M. Bakry, Serum albumin can influence magnesium alloy degradation in simulated blood plasma for cardiovascular stenting, Mater. Chem. Phys. 220 (2018) 35-49.
[23] S. Höhn, S. Virtanen, A.R. Boccaccini, Protein Adsorption on Magnesium and its alloys: A review, Appl. Surf. Sci. 464 (2018) 212-219.
[24] R. Hou, R. Willumeit-Römer, V.M. Garamus, M. Frant, J. Koll, F. Feyerabend, Adsorption of Proteins on Degradable Magnesium - Which Factors are Relevant?, ACS Appl. Mater. Inter. 10 (2018) 42175-42185.
[25] M. Rabe, D. Verdes, S. Seeger, Understanding protein adsorption phenomena at solid surfaces, Adv. Colloid Interfac 162 (2011) 87-106.
[26] I. Johnson, W. Jiang, H. Liu, The effects of serum proteins on magnesium alloy degradation in vitro, Sci. Rep.-UK 7 (2017) 14335.
[27] U. Kragh-Hansen, H. Vorum, Quantitative analyses of the interaction between calcium ions and human serum albumin, Clin. Chem. 39 (1993) 202-208.
[28] K.A. Majorek, P.J. Porebski, A. Dayal, M.D. Zimmerman, K. Jablonska, A.J. Stewart, M. Chruszcz, W. Minor, Structural and immunologic characterization of bovine, horse, and rabbit serum albumins, Mol. Immunol. 52 (2012) 174-182.
[29] J. Andrade, V. Hlady, Protein adsorption and materials biocompatibility: a tutorial review and suggested hypotheses, Biopolymers/Non-Exclusion HPLC, Springer1986, pp. 1-63.
[30] L. Vroman, A. Adams, G. Fischer, P. Munoz, Interaction of high molecular weight kininogen, factor XII, and fibrinogen in plasma at interfaces, Blood 55 (1980) 156-159.
[31] M. Kieke, F. Feyerabend, J. Lemaitre, P. Behrens, R. Willumeit-Römer, Degradation rates and products of pure magnesium exposed to different aqueous media under physiological conditions, BioNanoMaterials 17 (2016) 131-143.
[32] J. Gonzalez, R.Q. Hou, E.P. Nidadavolu, R. Willumeit-Römer, F. Feyerabend, Magnesium degradation under physiological conditions–Best practice, Bioactive Materials 3 (2018) 174-185.
[33] S. Whiting, Pathology First Biochemistry Reference Intervals, in: F. Akinlade (Ed.) PF BIOCHEMISTRY REFERENCE INTERVALS, 2017.
[34] R. Asselta, S. Duga, M. Tenchini, The molecular basis of quantitative fibrinogen disorders, J. Thromb. Haemostas. 4 (2006) 2115-2129.
[35] E.P.S. Nidadavolu, F. Feyerabend, T. Ebel, R. Willumeit-Römer, M. Dahms, On the Determination of Magnesium Degradation Rates under Physiological Conditions, Materials 9 (2016) 627-737.
[36] Y. Xin, K. Huo, H. Tao, G. Tang, P.K. Chu, Influence of aggressive ions on the degradation behavior of biomedical magnesium alloy in physiological environment, Acta Biomater. 4 (2008) 2008-2015.
[37] W. Jastrzębski, M. Sitarz, M. Rokita, K. Bułat, Infrared spectroscopy of different phosphates structures, Spectrochim. Acta A 79 (2011) 722-727.
[38] M. Jönsson, D. Persson, D. Thierry, Corrosion product formation during NaCl induced atmospheric corrosion of magnesium alloy AZ91D, Corros. Sci. 49 (2007) 1540-1558.
[39] D. Tie, F. Feyerabend, N. Hort, R. Willumeit, D. Hoeche, XPS studies of magnesium surfaces after exposure to Dulbecco's Modified Eagle Medium, Hank's buffered salt solution, and simulated body fluid, Adv. Eng. Mater. 12 (2010) B699-B704.
[40] F. Feyerabend, H. Drucker, D. Laipple, C. Vogt, M. Stekker, N. Hort, R. Willumeit, Ion release from magnesium materials in physiological solutions under different oxygen tensions, J. Mater. Sci. Mater. Med. 23 (2012) 9-24.
[41] A. Besarab, A. DeGuzman, J.W. Swanson, Effect of albumin and free calcium concentrations on calcium binding in vitro, J. Clin. Pathol. 34 (1981) 1361-1367.
[42] K. Pedersen, Binding of calcium to serum albumin III. Influence of ionic strength and ionic medium, Scand. J. Clin. Lab. Inv. 29 (1972) 427-432.
[43] R. Frye, H. Lees, G. Rechnitz, Magnesium-albumin binding measurements using ion-selective membrane electrodes, Clin. Biochem. 7 (1974) 258-270.
[44] Y.C. Guillaume, C. Guinchard, A. Berthelot, Affinity chromatography study of magnesium and calcium binding to human serum albumin: pH and temperature variations, Talanta 53 (2000) 561-569.

[45] M. Tavafoghi, M. Cerruti, The role of amino acids in hydroxyapatite mineralization, J. R. Soc. Interface 13 (2016) 20160462.
[46] I. Marco, F. Feyerabend, R. Willumeit-Römer, O. Van der Biest, Degradation testing of Mg alloys in Dulbecco's modified eagle medium: Influence of medium sterilization, Mat. Sci. Eng. C-Mater. 62 (2016) 68-78.
[47] I. Marco, A. Myrissa, E. Martinelli, F. Feyerabend, R. Willumeit-Römer, A. Weinberg, O. Van der Biest, In vivo and in vitro degradation comparison of pure Mg, Mg-10Gd and Mg-2Ag: a short term study, Eur. Cells Mater. 33 (2017) 90-104.
[48] L. Yang, E. Zhang, Biocorrosion behavior of magnesium alloy in different simulated fluids for biomedical application, Mat. Sci. Eng. C-Mater. 29 (2009) 1691-1696.
[49] Y. Zong, G. Yuan, X. Zhang, L. Mao, J. Niu, W. Ding, Comparison of biodegradable behaviors of AZ31 and Mg–Nd–Zn–Zr alloys in Hank's physiological solution, Mater. Sci. Eng. B-Solid 177 (2012) 395-401.
[50] A. George, A. Veis, Phosphorylated proteins and control over apatite nucleation, crystal growth, and inhibition, Chem. Rev. 108 (2008) 4670-4693.
[51] W.J. Landis, R. Jacquet, Association of calcium and phosphate ions with collagen in the mineralization of vertebrate tissues, Calcified. Tissue Int 93 (2013) 329-337.
[52] G. Marguerie, G. Chagniel, M. Suscillon, The binding of calcium to bovine fibrinogen, Biochimica et Biophysica Acta (BBA)-Protein Structure 490 (1977) 94-103.
[53] G. Yin, Z. Liu, J. Zhan, F. Ding, N. Yuan, Impacts of the surface charge property on protein adsorption on hydroxyapatite, Chem. Eng. J. 87 (2002) 181-186.
[54] E.C. Bingham, R.R. Roepke, The rheology of the blood. II. The effect of fibrinogen on the fluidity of blood plasma, J. Am. Chem. Soc. 64 (1942) 1204-1206.
[55] E.M. Hernández, E.I. Franses, Adsorption and surface tension of fibrinogen at the air/water interface, Colloid Surface. A 214 (2003) 249-262.
[56] N. Hassan, J. Maldonado-Valderrama, A.P. Gunning, V.J. Morris, J.M. Ruso, Surface characterization and AFM Imaging of mixed fibrinogen− surfactant films, J. Phys. Chem. B 115 (2011) 6304-6311.
[57] A. Marucco, I. Fenoglio, F. Turci, B. Fubini, Interaction of fibrinogen and albumin with titanium dioxide nanoparticles of different crystalline phases, Journal of Physics: Conference Series, IOP Publishing, 2013, p. 012014.
[58] A. Bratek-Skicki, P. Żeliszewska, J.M. Ruso, Fibrinogen: a journey into biotechnology, Soft matter 12 (2016) 8639-8653.
[59] J. Brash, P. Ten Hove, Effect of plasma dilution on adsorption of fibrinogen to solid surfaces, Thromb. Haemostasis. 51 (1984) 326-330.
[60] J. Huang, Y. Yue, C. Zheng, Vroman effect of plasma protein adsorption to biomaterials surfaces, J Biomed. Eng. 16 (1999) 371-376.
[61] P. Soman, C.A. Siedlecki, Effects of protein solution composition on the time-dependent functional activity of fibrinogen on surfaces, Langmuir. 27 (2011) 10814-10819.
[62] D.G. Makey, U.S. Seal, The detection of four molecular forms of human transferrin during the iron binding process, Biochimica et Biophysica Acta (BBA)-Protein Structure 453 (1976) 250-256.
[63] E. Betgovargez, V. Knudson, M.H. Simonian, Characterization of proteins in the human serum proteome, J. Biomol. Techques 16 (2005) 306-310.
[64] A.G. Hovanessian, Z.L. Awdeh, Gel Isoelectric Focusing of Human-Serum Transferrin, The FEBS Journal 68 (1976) 333-338.
[65] S.V. Lamaka, D. Höche, R.P. Petrauskas, C. Blawert, M.L. Zheludkevich, A new concept for corrosion inhibition of magnesium: Suppression of iron re-deposition, Electrochem. Commun. 62 (2016) 5-8.
[66] D. Höche, C. Blawert, S.V. Lamaka, N. Scharnagl, C. Mendis, M.L. Zheludkevich, The effect of iron re-deposition on the corrosion of impurity-containing magnesium, Phys. Chem. Chem. Phys. 18 (2016) 1279-1291.
[67] P.K. Bowen, J. Drelich, J. Goldman, Magnesium in the murine artery: Probing the products of corrosion, Acta Biomater. 10 (2014) 1475-1483.

[68] F. Feyerabend, H.P. Wendel, B. Mihailova, S. Heidrich, N.A. Agha, U. Bismayer, R. Willumeit-Romer, Blood compatibility of magnesium and its alloys, Acta Biomater. 25 (2015) 384-394.
[69] A.H. Martinez Sanchez, B.J. Luthringer, F. Feyerabend, R. Willumeit, Mg and Mg alloys: how comparable are in vitro and in vivo corrosion rates? A review, Acta Biomater. 13 (2015) 16-31.