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Exploring the effects of organic molecules on the degradation of magnesium under cell culture conditions

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ABSTRACT

Several typical organic components, L-ascorbic acid (L-AA), L-glutamine (L-Gln), L-alanyl-L-glutamine (L-Ala-L-Gln) and fetal bovine serum (FBS) were chosen to elucidate the effects of organic components on the degradation of pure Mg under cell culture conditions. The results revealed that the influence of organic components on the degradation of pure Mg is time-dependent and they play an important role in the formation of the degradation layer. The addition of organic components favors the precipitation of nesquehonite rather than hydromagnesite in the "outer" layer, while in the "inner" layer the organic components accelerates the formation of phosphate ($Mg\text{-}PO_4$, Ca-P salts) during immersion.

1. Introduction

Magnesium (Mg) alloys have become more and more favorable in medical applications due to their excellent biodegradability and bioresorbability [1–3] and clinical applications are already reported [4]. However, a too high, in some cases unpredictable, degradation rate is still a problem for their applications because it can lead to the formation of gas pockets, high ionic concentrations and pH which result in the failure of implant in the early service [5–7]. Therefore, the establishment of a reliable evaluation standard is a prerequisite for their application as absorbable metallic implants with controllable degradation speed.

For the successful establishment of this reliable evaluation standard, it is necessary to evaluate the effect of physiological parameters on the degradation of Mg-based biomaterials. Many degradation studies have been conducted in simple to complex salt solutions, such as Hank's solution and simulated body fluid (SBF), to investigate the influence of inorganic ions on the degradation of Mg alloy [8–13]. The impurities of materials (for example Fe) is also very important for Mg degradation due to the enhancement of galvanic corrosion [14]. Additionally, the pH buffering of media and the temperature used for *in vitro* tests always affect the Mg degradation [15]. Chloride ions (Cl^-), as the most important part, largely accelerate the degradation of Mg implants by breaking the integrity of the surface film [[8–13],10,16], phosphates decrease the degradation rate due to the formation of dense phosphate layer [8], and an appropriate amount of carbonate ions can induce rapid surface passivation because of the precipitation of magnesium carbonate [[8–13],9]. The monovalent cations (K^+, Na^+) in solution are more prone to accelerate the degradation of Mg than divalent cations (Ca^{2+}, Mg^{2+}) [12]. However, these simple salt solutions under normal conditions are far away from the real physiological environment which comprises a very complex mixture of organic and inorganic molecules and a different concentration of O_2/CO_2 at different implant sites, which results in the difference between *in vitro* and *in vivo* degradation [17,18].

Consequently, the inclusion of organic components in simulated solutions is the next step towards a closer simulation of *in vivo* conditions and a better understanding of the degradation of Mg alloys. Therefore, cell culture media containing amino acids and vitamins, such as Dulbecco's modified Eagles' medium (DMEM) and minimum essential medium (MEM), have already been used for the degradation tests under cell culture conditions. These organic components significantly altered the degradation behavior of Mg due to the chelating/binding effect and the adsorption of organic molecules on Mg surface [15,19–23]. Furthermore, the adsorption of proteins has been used to develop protective films for the controllable degradation of Mg alloys [24–26]. On the other hand, organic components also affect the formation of the degradation layer during immersion. For example, proteins can adsorb on the Mg surface, thereby leading to the variation of the surface layer compactness or thickness [27,28]. Some conditions also result in the severe deposition of precipitates on the surface, resulting in two sections of the degradation layer: loosely "outer" crystalline layer and "inner" amorphous layer [29]. In different media, the organic components also show different influence on the formation of degradation products. In HBSS, the addition of fetal bovine serum (FBS) postponed the formation of "outer" crystalline products, while the

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formation of Ca-P salts was promoted by FBS in simulated body fluid (SBF) [30]. However, the influence of organic components on the degradation of Mg had not reached a consensus due to the complicated system when organic components were added and their complexity in chemical reactions with the environment. Moreover, reliable *in vitro* evaluation standards for absorbable Mg have been neither established nor compared with *in vivo* tests. Therefore, it is of great significance to have insight into the effects of organic components on the degradation of Mg.

The aim of this study is to examine the effects of several typical organic components used in cell culture media on the degradation of Mg. A simple salt solution, Hank's balanced salt solution (HBSS) without calcium and magnesium, was used as the base solution. L-ascorbic acid (L-AA), L-glutamine (L-Gln), L-alanyl-L-glutamine (L-Ala-L-Gln) and fetal bovine serum (FBS) were chosen to elucidate the influence of organic components on the degradation process. L-AA (vitamin C) is one of the most important vitamins in human body, which enhances the immune system and prevents various oxidative stress-related diseases, such as cancer and cardiovascular diseases [31,32]. L-Gln is the most abundant free amino acid in human body with the largest storage area in skeletal muscle [33]. It is involved in many physiological functions including cellular proliferation, acid-base balance, antioxidant synthesis and protein synthesis in the muscle [34–38]. However, L-Gln is unstable in solution during heat sterilization and prolonged storage, thereby deaminating into ammonia which has deleterious effects on cells [39]. Therefore, L-Ala-L-Gln as a dipeptide is used as a substitute for L-Gln in cell culture media. It is less ammoniagenic than L-Gln, which contributes to its advantages as a media component [40]. By using these four organic components, the effects of organic components on the degradation of pure Mg were investigated by analyzing the changes of media during immersion and the degradation layers formed in different media.

2. Materials and methods

2.1. Materials

Pure Mg (99.94%, chemical composition is shown in Table 1) used in this study was purchased from Magnesium Elektron (Manchester, UK). Rectangular specimens with dimensions of 10 mm × 10 mm × 4 mm were cut out of the ingots via arc erosion (AMS tech., Taiwan). Before use, the specimens were successively wet ground with SiC abrasive paper (Schmitz-Metallographie GmbH, Herzogenrath, Germany) from 800 to 2500 grit, then ultrasonically cleaned for 20 min in N-hexane, 5 min in acetone and 20 min in ethanol (Merck KGaA, Darmstadt, Germany). Finally, the samples were dried in 12-wells cell culture plates (Greiner Bio-One, Frickenhausen, Germany) for at least 30 min in air under sterile conditions. The homogeneity of materials has been investigated by characterizing the microstructure and surface roughness before the immersion tests, the representative results were supplied in Supplementary files (Fig. S1).

2.2. Immersion media

Hank's balanced salt solution (HBSS, Order-No. 14715, Life Technologies, Darmstadt, Germany) without calcium and magnesium addition (the composition is shown in Table 2), was used as a base solution for all immersion media. L-AA (Sigma-Aldrich Chemie, Steinheim, Germany), L-Gln (Thermo Fisher, Karlsruhe, Germany), L-Ala-L-Gln (Biowest, Darmstadt, Germany) and 10% FBS (PAA laboratories, Linz, Austria) as a protein component, were added in a concentration comparable to that in two cell culture media, Dulbecco's modified Eagle's medium (DMEM) and Minimum Essential Media Alpha (α-MEM). After these components except FBS were added into HBSS, the media were sterile filtered by bottle top-filters with a pore size of 0.2 µm (Thermo Fisher, Karlsruhe, Germany). Thus, eight immersion media were prepared as following:

A: Base solution (HBSS)
B: HBSS + 50 mg/L L-AA
C: HBSS + 292 mg/L L-Gln
D: HBSS + 862 mg/L L-Ala-L-Gln
E: HBSS + 50 mg/L L-AA + 292 mg/L L-Gln
F: HBSS + 50 mg/L L-AA + 862 mg/L L-Ala-L-Gln
G: HBSS + 50 mg/L L-AA + 292 mg/L L-Gln + 10% FBS
H: HBSS + 50 mg/L L-AA + 862 mg/L L-Ala-L-Gln + 10% FBS

Table 1
Chemical composition of pure Mg.

Composition (*Wt.%*)	Ca	Zn	Fe	Cu	Si	Mn	Mg
Weight percentage	0.0004	0.018	0.0044	0.0089	0.0014	0.0021	balance

Table 2
The composition of Hanks' balanced salt solution (HBSS).

Ingredient	mg/L	mM
Potassium chloride (KCl)	400	5.33
Potassium Phosphate monobasic (KH_2PO_4)	60	0.441
Sodium bicarbonate ($NaHCO_3$)	350	4.17
Sodium chloride (NaCl)	8000	137.93
Sodium phosphate dibasic ($Na_2HPO_4{\cdot}2H_2O$) anhydrous	48	0.338
D-Glucose (Dextrose)	1000	5.56

2.3. Immersion test

Before sterilization, the initial weights of samples as well as the initial pH (SENTRON ARGUS X pH-meter, Fisher Scientific, Schwerte, Germany) and osmolality (Osmomat 030, Gonotec, Berlin, Germany) for the eight immersion media were recorded. Eighteen samples were immersed in media as a sample weight to medium volume ratio of 0.2 g/mL under cell culture conditions (37 °C, 5% CO_2, 20% O_2, 95% rel. humidity) in an incubator (Heraeus BBD 6620, Thermo Fisher Scientific, Schwerte, Germany). The immersion media were changed every 2 or 3 days to present a semi-static immersion test. pH and osmolality were determined after each change of the media. Media without samples were incubated in parallel as controls.

A 24-well Sensor Dish with integrated sensor spots at the bottom of each well (PreSens Precision Sensing GmbH, Regensburg Germany) was used to monitor the change of pH with the immersion time. The concentration of L-Gln in media was determined by using the glutamine colorimetric assay kit (BioVision, Inc., Zürich, Schweiz). The standard curve was prepared by using Gln standard solutions with a successive concentration from 0 µM to 250 µM. At each time point, 10 µL of media with or without samples (three replicates) were taken out from well plates, then centrifuged at 10000*g* for 5 min at 4 °C. 2 µL of the supernatant was added to 96-well clear plates with flat bottom. The volume was adjusted to 40 µL/well with ddH_2O. 10 µL hydrolysis mix solution was added to each well, then the plates were incubated for 30 min at 37 °C. Subsequently, 50 µL reaction mix solution was added to each well. The plates were incubated for 60 min at 37 °C. Finally, the absorbance at 450 nm was measured with a reference absorbance at 620 nm in Tescan A-5082 microplate reader (Sunrise Romote, Austria).

2.4. Degradation rate

Six samples were taken out of the respective media at the time points of 3 days, 7 days and 14 days, then cleaned twice with double distilled water and dried at 50 °C in air. Two samples were left to analyze the surface product and prepare the cross section. Degradation