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Magnesium metal—A potential biomaterial with antibone cancer properties

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Abstract: Reactive oxygen plays an important role in the pathogenesis of many serious illnesses, including bony cancer. Recently, it has been suggested that hydrogen (H_2), a selective antioxidant, can effectively scavenge free radicals. Biomedical magnesium (Mg) used for bone defect repair in the surgery of bony cancer could release H_2 because of the degradation, so Mg might have the potential to prevent bony cancer from metastasis and recurrence. In this study, alkali-heat treatment method was employed to modify the surface structure of Mg metal, so as to control the degradation of Mg metal and the H_2 releasing rate. Then the released H_2 was introduced to the Fenton Reaction system to detect its effect on scavenging free radicals. The modified Mg metal was employed as the substrate for bone cancer cell culture to study the effect of the H_2 releasing on scavenging free radicals in the cells. It is found that the H_2 released from the Mg degradation could scavenge free radicals both in the Fenton Reaction system and bone cancer cells. The effect on the scavenging free radical is proportional to the rate of H_2 releasing. It suggested that Mg might be a potential material with anti-bone cancer properties. It is hopeful to both repair the bone defect and prevent bony cancer from metastasis and recurrence for the bony cancer patients by biomedical Mg metal. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2013.

Key Words: magnesium metal, biodegradation, H_2 releasing, free radicals scavenging, anti-bone cancer

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INTRODUCTION

Bone cancer has become a terrible killer for human health. According to a statistic report released by American Cancer Society, the fatality rate because of bone cancer is up to 4%%% of the total population, and the male patient is two times as the female patient.[1] In order to extend the lives and improve the life quality of the people fighting the bone cancer, the cancer is always resected and some biomaterials are employed to fill the defects. The bone cancer must be removed as thoroughly as possible in surgery to avoid its metastasis and recurrence.

Titanium and alloys, autogenous bone, hydroxyapatite bioceramics, and bioglass are widely used in clinic as bone repair materials. After being implanted in the bone defects, they could bond with the bone tissues around defects by osseointegration or bioactive bonding. Therefore, the bone defects are always well repaired. But these materials are in lack of properties to inhibit bone cancer. Once the bone cancer cells metastasize or spread in the body, the people suffering bone cancer cannot get rid of death, and the fatality rate after five years is up to 70%.[1] Thus, there is considerable interest to develop an effective bone repair material with anti-bone cancer properties.

In the past decades, it has been found that oxidative stress is a feature of cancers. The oxidative stress is caused by excessive production of free radicals, mainly including reactive oxygen species (ROS).[2] Excessive ROS play an important role in the occurrence and metastasis of cancer.[3–8] Therefore, scavenging excess free radicals has become an important means to inhibit cancer. Some medicines, such as folic acid, tea polyphenol, and ginsenoside, could inhibit the growth of cancer cells. This phenomenon may depend on the antioxidant activity of these medicines.[9–11]

Recently, it is reported that hydrogen (H_2) has the properties of antioxidation, anticancer, and anti-inflammatory.[12–15] It is generally accepted that H_2 is a gas with reducing reactive free radicals properties. In 2007, Ohsawa[16] reported that H_2 could selectively reduce hydroxyl radicals ($^{\bullet}OH$) and peroxynitrite ($ONOO^-$) in vitro, but not affect

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physiological ROS and suggested H_2 protested cells and tissues against strong oxidative stress by scavenging $^{\bullet}OH$ (1):

$$H_2 + {}^{\bullet}OH \rightarrow H_2O + H^{\bullet} \quad (1)$$

Mg and its alloys are biodegradable in the body. After being implanted in the bone, the Mg and its alloys will be absorbed by the body and be replaced by the new bone. In recent years, some reports showed that the degradation of Mg and its alloy could be controlled by surface modification to make the rate of the bone growth match to the degradation rate of the metal.[17–20] Thus, Mg and its alloys have a good application prospect in the field of bone tissue repair. The following reaction is the degradation reaction of Mg in the body fluid:

$$Mg + 2H_2O \rightarrow Mg(OH)_2 + H_2 \uparrow \quad (2)$$

After Mg and its alloy being implanted in the bone defects, H_2 will be released during the metal degradation Eq. (2). It is possible to control the H_2 releasing rate by the surface modification. The released H_2 can scavenge excess free radicals around the bone defects, so as to prevent cancer cells from metastasis and recurrence.

This article provided for the first time a potential effect of biodegradable Mg metal as the bone repair materials on the scavenging of free radicals. The Mg metal might be useful for inhibiting bone cancer from metastasis and recurrence. Alkali-heat treatment surface modification method was employed to control the degradation of the metal.

MATERIALS AND METHODS

Preparation of specimens

Commercial pure Mg (99.95%) was used as substrate in this study. Mg metal (P-Mg) was cut into plates 10 × 10 × 1 mm^3 in size, mechanically polished with 400#, 800#, and 1000#, then ultrasonically cleaned successively by acetone, absolute ethanol, and distilled water three times individually in turn. The resulting Mg samples were subjected to alkaline treatment for 24 h at 60°C in 5 mL 1*M*-$NaHCO_3$ solution to get A-Mg, followed by 10-h heat treatment at 773 K in air, and the received plates were AH-Mg. All the received plates were rinsed with ultrapure water and dried in air before further characterization.

Surface characterization

X-ray diffraction (XRD, DX-1000 X-ray diffract meter) and scanning electron microscopy (SEM, HITACHI-S4800, JAPAN) were used to examine the chemical and physical changes occurred on the surfaces. XRD patterns are carried out with Cu Kα radiation working with 40 kV and 25 mA. The 2θ range is from 10° to 70° at a scan rate of 0.02°/s. The changes in surface morphology and microstructures of the samples were observed by SEM with an accelerating voltage of 30 kV.

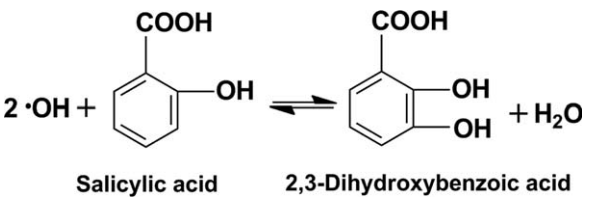
SBF immersion test

Immersion test was carried out in stimulate body fluid (SBF), containing NaCl 8.035 g/L, $NaHCO_3$ 0.355 g/L, KCl 0.225 g/L, $K_2HPO_4{\cdot}3H_2O$ 0.231 g/L, $MgCl_2{\cdot}6H_2O$ 0.311 g/L, 1*M* HCl, $CaCl_2{\cdot}2H_2O$ 0.388 g/L, Na_2SO_4 0.072 g/L, Tris ($C_4H_{11}NO_3$) 6.118 g/L. The SBF solution was prepared according to the procedure of Ref. 21, and restored in the fridge (4°C). The surface composition and morphology of the samples after soaking in SBF (10 mL) for 24 h were observed by X-ray diffraction and scanning electron microscopy. The pH changes of solution and H_2 release volumes were monitored after the metal was soaked in SBF. An average of three measurements was obtained for each time.

Hydroxyl radicals scavenging by H_2 in Fenton reaction system

In order to determine the effect of H_2 on hydroxyl radicals scavenging, hydroxyl radicals were produced by Fenton reaction Eq. (3), and different amount of H_2 from Mg degradation in sulfuric acid solution was introduced into the reaction. Salicylic acid method Eq. (5) was used to detect the concentration changes of hydroxyl radicals in the Fenton reaction system with or without H_2. When the Fenton reaction was initiated by adding H_2O_2 (0.3%, 0.1 mL) into $FeSO_4$ (1.2 m*M*, 3 mL) solution, 0, 35, or 70 mL/min H_2 was introduced into the system for 5 min. Then salicylic acid (1.8 m*M* in ethanol, 1.5 mL) was added into the Fenton reaction system to catch hydroxyl radicals. After catching the hydroxyl radicals, the salicylic acid transferred to the 2, 3-dihydroxybenzoic acid (3), which could be characterized with microplate reader. The measurements were taken at 510 nm by a microplate reader (Thermo Multiskan MK3, Therom Fiaher Scientific, USA).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-\bullet} \quad (3)$$



(4)

Free radicals scavenging in bony cancer cells

Cell culture. Osteosarcoma cell line, MG63, was chosen for this study. MG63 was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% new born calf serum and 1% antibiotic under a 5% CO_2 atmosphere at 37°C. The P-Mg and AH-Mg after radiation sterilization were soaked in serum cell culture medium for 24 h. During the immersion period, the medium was changed every 2 h before culturing cells in order to make the condition of the metal substrates suitable for cell growth. The soaked P-Mg and AH-Mg were put into 24-well plates, and 1.5 mL of floating cell (about 5 × 10^5 cells mL^{-1}) was added into each well to be cultured for 4, 6, and 8 h in a sterile environment. The medium was changed every 2 h. The pH in serum cell culture medium was measured every 2 h before and after culturing cells. At 4, 6, and 8 h, the cell-cultured samples were taken out from the culture plate

and washed with PBS two times. The cells were stained with 5 μg mL^{-1} fluorescein diacetate. The CLSM (Leica.SP5, Germany) was used to analyze the cell attachment and growth. SEM was used to identify the morphologies of the cells on the sample surfaces at 4, 6, and 8 h. Before the SEM observation, the cell-cultured samples were fixed in 2.5% glutaraldehyde PBS overnight at 4°C, and then washed with PBS to remove the residual glutaraldehyde. The fixed samples were subsequently dehydrated with a graded series of ethanol, freeze-dried, and finally coated with gold for SEM observation.

Free radicals detection. ROS Assay Kit (Beyotime, CHINA) was used to detect the levels of free radicals in MG63 cultured on metal surfaces and culture plates as a control group for 4, 6, and 8 h. At 4, 6, and 8 h, the culture medium in 24-well plates was removed, and dichlorofluorescein diacetate (DCFH-DA) diluted to 1000 times with serum-free medium was put into 24-well plates for 20 min incubation at 37°C. DCFH-DA is an uncharged, cell permeant fluorescent probe. Inside the cells DCFH-DA is cleaved by nonspecific esterases forming DCFH, which is the nonfluorescent form and is oxidized to the fluorescent compound 2',7'-dichlorofluorescein (DCF) in the presence of ROS. The cell-cultured samples were taken out from the culture plate and washed with serum-free medium three times to remove the residual DCFH-DA. Then, MG63 was dissociated from metal surfaces and washed with PBS two times to remove the residual serum culture medium. Finally, the absorbance of free radicals was measured at 525 nm by a microplate reader. The cell counting chamber was used to count cells. The average OD value of single cell was calculated by the division of the absorbance value and cell counts.

Statistical analysis

The results were statistically studied with SPSS11.0 software and the corresponding *p*-values were considered to be significant at values less than 0.05. All the statistical analysis was performed with at least three samples for each single experiment.

RESULTS

Characterization of alkaline heat treated Mg metal

Figure 1 presented the XRD patterns of the P-Mg, A-Mg and AH-Mg samples. It showed that there were Mg peaks on the surfaces of P-Mg, and A-Mg. The peaks of $MgCO_3{\cdot}3H_2O$ were

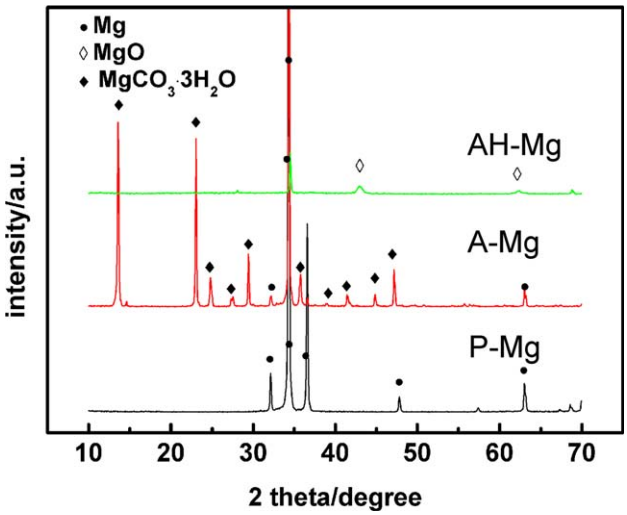


FIGURE 1. XRD patterns of P-Mg, A-Mg, and AH-Mg samples (P-Mg: untreated Mg; A-Mg: $NaHCO_3$ treated Mg; AH-Mg: $NaHCO_3$-heat treated Mg metal). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

recognized after alkaline treatment. MgO peaks could be seen on AH-Mg, whereas no $MgCO_3{\cdot}3H_2O$ peaks could be seen after the alkaline heat treatment.

Figure 2 showed SEM images of surface morphologies of P-Mg, A-Mg, and AH-Mg samples. The flat surface was seen on the untreated sample. After alkaline treatment, the flat surface was destroyed and the surface was covered with scattered columnar shape crystals. During the alkaline treatment, a great deal of bulbs appeared on the substrate surface and white precipitate was observed by naked eyes. Puffy nano-sized granular shape crystals were seen on the surface after further heat treatment.

Immersion behavior of alkaline heat treated and untreated Mg metals in SBF

Figure 3 presented the XRD patterns of the P-Mg and AH-Mg samples after immersion in SBF at 37°C for 24 h. It showed that $Ca_2P_2O_7$ was formed on the surface of AH-Mg sample. But no $Ca_2P_2O_7$ appeared on the surface of P-Mg sample and Mg $(OH)_2$ was the resulting surface corrosion products for P-Mg sample after immersion in SBF for 24 h. It is reported that the calcium pyrophosphate is one of the intermediate products in biomineralization process. It had the potential to regulate the onset of calcification and might

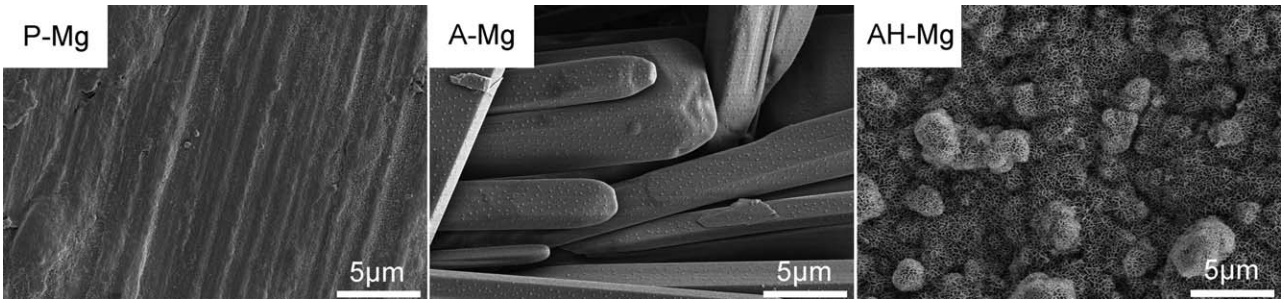


FIGURE 2. SEM images of the surface morphologies of P-Mg, A-Mg, and AH-Mg samples.

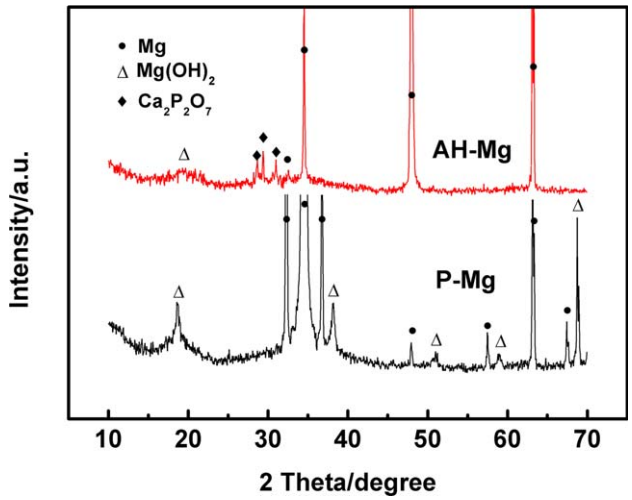


FIGURE 3. XRD patterns of P-Mg and AH-Mg samples immersed in SBF for 24 h. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

act as a trigger to promote mineralization in the body.[22] The calcium pyrophosphate, but not apatite product of the SBF immersion might be because of the effects of Mg ions. It is well accepted that Mg ions could inhibit apatite crystal growth in the biological system,[23] which might make the intermediate product of calcium pyrophosphate form on the metal surface.

Figure 4 showed the surface micrographs of P-Mg and AH-Mg samples immersed in SBF for 24 h. After immersion, the surface of P-Mg sample became loose and porous, whereas the surface of AH-Mg sample was covered with a layer of dense deposits. The result indicated that the surface of P-Mg sample corroded more seriously than that of AH-Mg.

Figure 5 showed the change of the pH value of SBF solution as a function of immersion time. It could be seen that the pH change of the solution corresponding to AH-Mg sample increased more slowly than P-Mg sample did. Figure 6 indicated the hydrogen release volumes as a function of immersion. The hydrogen release volume of AH-Mg sample in SBF was much smaller than that of P-Mg sample. The hydrogen release reflected a similar degradation behavior to the change in pH value. These results indicated the degradation of AH-Mg was much slower than that of P-Mg. The alkaline heat treatment could reduce the degradation of Mg metal.

Hydroxyl radicals scavenging by H_2 in Fenton reaction system

Figure 7 showed the OD values of hydroxyl radical concentration in different systems. It could be seen that the OD values decreased with the addition of H_2 into the Fenton reaction system. It was obvious that the higher H_2 volume added into the system, the lower the OD values for the free radical groups. The difference between the systems added with different H_2 volumes was significant ($p < 0.05$). Figure 7 indicated that H_2 could effectively scavenge hydroxyl radicals, and the different amount of hydrogen gas had the different ability to scavenge hydroxyl radicals.

According to the calculation formula of free radicals scavenging rate: SR= (Ac − As)/ (Ac − A), the scavenging rates for H_2 gases with rates of 35 or 70 mL/min were 22.10% and 57.90%, respectively, where SR means scavenging rate, Ac is the OD value of without H_2, As is the OD value of with H_2, and A is the OD value of without H_2O_2. The results showed that the amount of hydrogen gas and the scavenging rate were positively correlated.

Cell culture on the surfaces of alkaline-heated and untreated Mg metals in MG63

Figure 8 showed the adhesion of MG63 cells at 4, 6, and 8 h cultured on the surfaces of P-Mg and AH-Mg, which were pretreated in serum cell culture medium for 24 h. At 4 h, the P-Mg groups had better cell response than the AH-Mg groups. The cells on the surfaces of P-Mg exhibited spreading better with characteristic spindle-like morphology, and the cells on the surfaces of AH-Mg exhibited sphere-like characteristic. At 6 h, the cells on the surfaces of P-Mg exhibited large spreading, and the cell density on the surfaces of AH-Mg became lower. At 8 h, the cells on the surfaces of P-Mg extended completely. Compared to cells cultured for 4 and 6 h, the cells on the surfaces of AH-Mg exhibited larger spreading with sphere-like characteristic at 8 h. The results showed that the surface of P-Mg had relatively

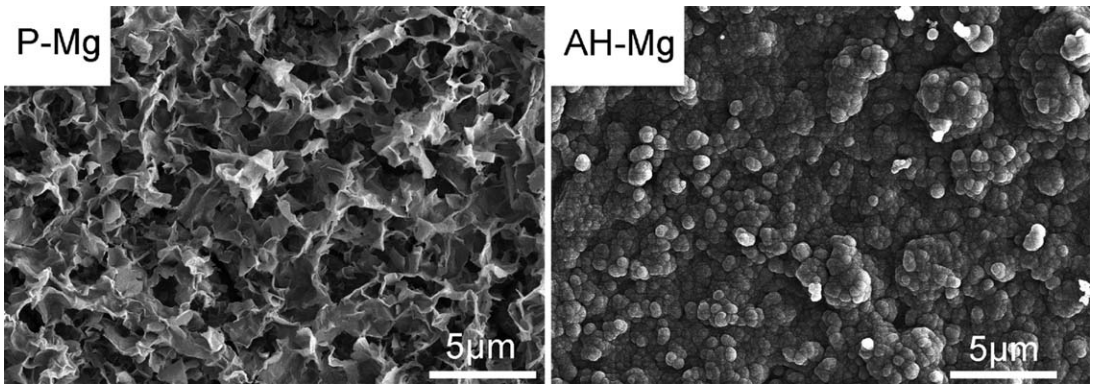


FIGURE 4. SEM micrographs of P-Mg and AH-Mg samples immersed in SBF for 24 h.

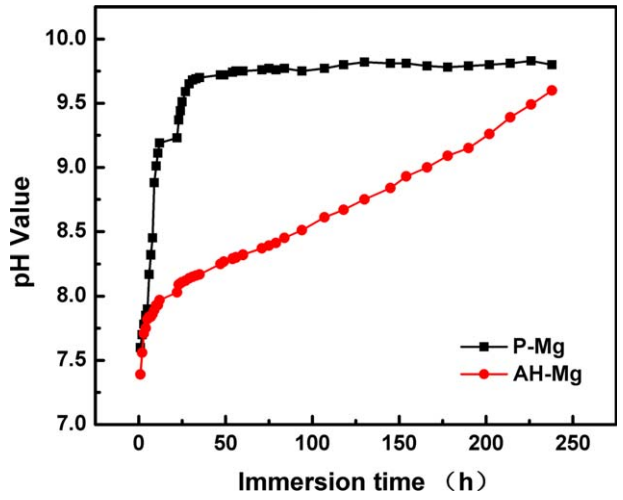


FIGURE 5. The pH changes of P-Mg and AH-Mg samples as a function of the immersion time in SBF. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

higher absorbance than that of AH-Mg at the first few hours, and the cells cultured on the surfaces of AH-Mg for 8 h proliferated and extended well. The SEM photographs (Fig. 9) showed that MG63 on all the samples exhibited spreading with characteristic spindle-like morphology. The results indicated that the MG63 proliferated well on the samples that were pretreated in serum cell culture medium for 24 h.

Free radicals scavenging ability of alkaline-heated and untreated Mg metals in MG63

Figure 10 showed the average OD values of free radicals concentration in single MG63 cell cultured on P-Mg and AH-Mg samples for 4, 6, and 8 h. After the MG63 cells were cultured for 4 and 8 h, the average OD values of cells on P-Mg and AH-Mg groups were lower than the control group ($p < 0.05$), and the average OD values of cells on AH-Mg group were higher than the P-Mg ($p < 0.05$).The average OD values of cells on P-Mg and AH-Mg groups showed no statistically significant difference ($p < 0.05$) at 6 h culture and were higher than those groups at 4 and 8 h.

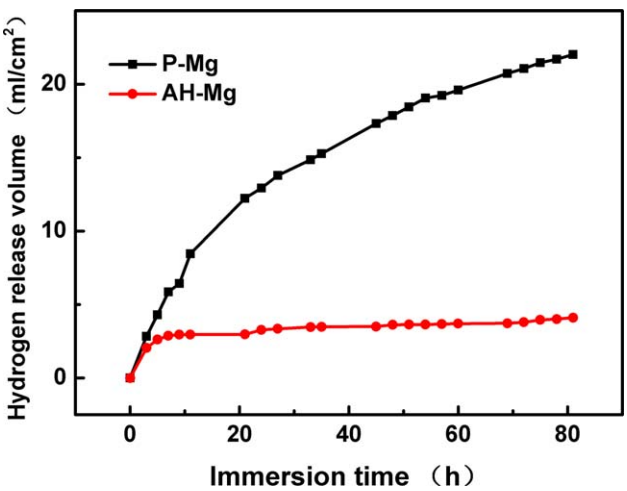


FIGURE 6. The hydrogen release volumes of P-Mg and AH-Mg samples as a function of the immersion time in SBF. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

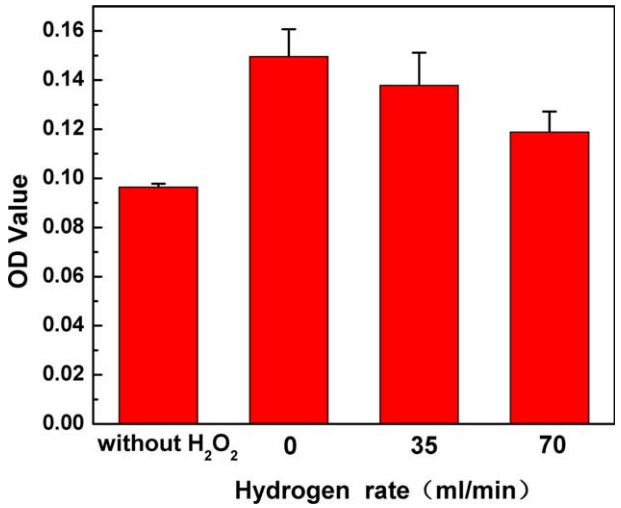


FIGURE 7. The OD values of hydroxyl radical concentration in different systems. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

Controlled Mg degradation and controlled H_2 release

Our results showed that the corrosion resistance of Mg plates subjected to alkali heat treatment in 1*M* $NaHCO_3$ solution followed by 10 h heat treatment at 773 K in air had been raised. Alkaline heat treatment produced a surface protective layer of MgO on Mg metal with characteristic morphology, structure, and chemical composition. In our preliminary study, which we did not report in this paper, we had got the samples by only heat treatment. The amount and crystallinity of MgO formed by only heat treatment were both lower than that subjected to $NaHCO_3$-heat treatment. The amount and crystallinity of MgO might be the decisive factors for controlling corrosion resistance of sample. Therefore, the protective layer (MgO) produced by $NaHCO_3$-heat treatment could effectively improve the corrosion resistance of Mg metal in simulated body fluid. During alkaline treatment, the following reactions Eqs. (5–8) would happen.[24] After heat treatment, the hydrated $MgCO_3$ decomposed into MgO and CO_2 [Eq. (9)]. All the following reactions explained the results in Figure 1.

$$Mg \rightarrow Mg^{2+} + 2e \tag{5}$$

$$2H_2O + 2e \rightarrow 2OH^- + H_2 \tag{6}$$

$$Mg^{2+} + CO_3^{2-} \rightarrow MgCO_3 \tag{7}$$

$$MgCO_3 + 3H_2O \rightarrow MgCO_3 \cdot 3H_2O \tag{8}$$

$$MgCO_3 \rightarrow MgO + CO_2 \tag{9}$$

The pH change (Fig. 5) and hydrogen release (Fig. 6) indicated the MgO layer had a protective effect on corrosion

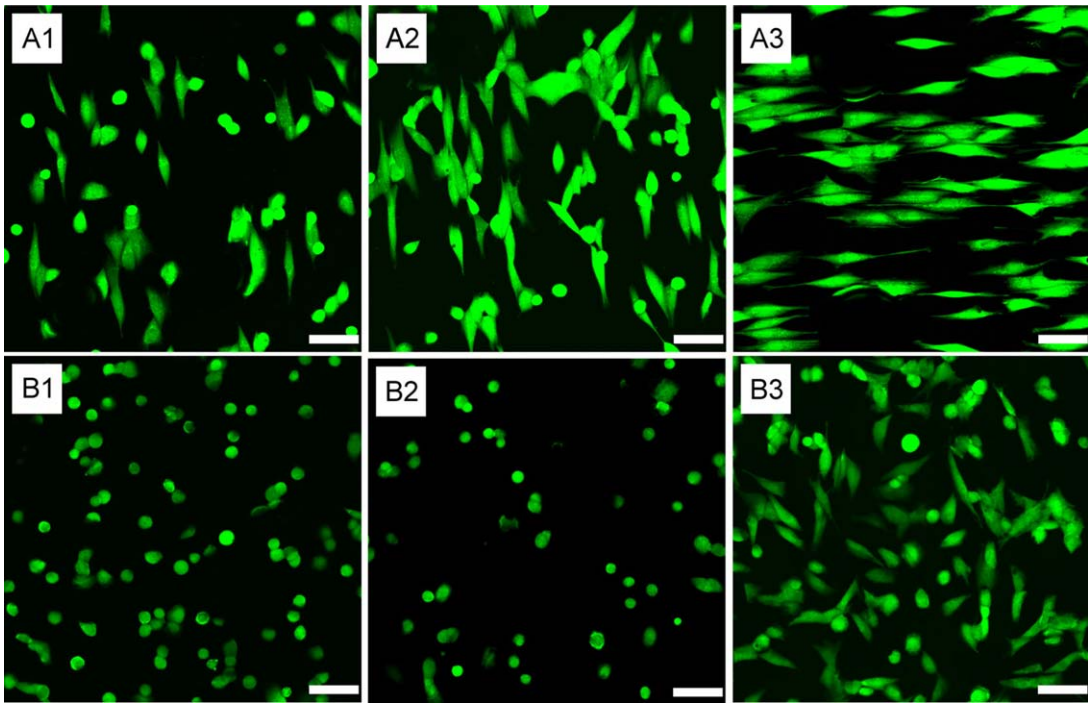


FIGURE 8. Fluorescence coloration results of MG63 cultured on P-Mg (A) and AH-Mg (B) samples for 4 (A1,B1), 6 (A2,B2), and 8 h (A3,B3). The bar is 75 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

resistant in SBF. Therefore, Mg degradation could be controlled by alkaline heat treatment. Equation (1) as previously mentioned showed that Mg degradation process was the process of releasing H_2. Thus, the controlled H_2 release was achieved by controlling the degradation rate of Mg metal.

Cell culture and controlled free radicals scavenging

In our study, cell density on the surfaces of AH-Mg pretreated in serum cell culture medium was significantly increased compared with the surfaces without pretreatment. Almost no cells survived on the surfaces without pretreatment. Sigrid found that incubation of Mg samples for 24 h in cell culture medium (DMEM) resulted in good corrosion protection and biocompatibility. Biomimetic coating was formed on the samples that were pretreated in cell culture medium. The formed coating played as a protective role for the metal. The mechanism, however, was not fully understood.[25] The average OD values of single cell on P-Mg and AH-Mg groups were lower than the control group ($p <$

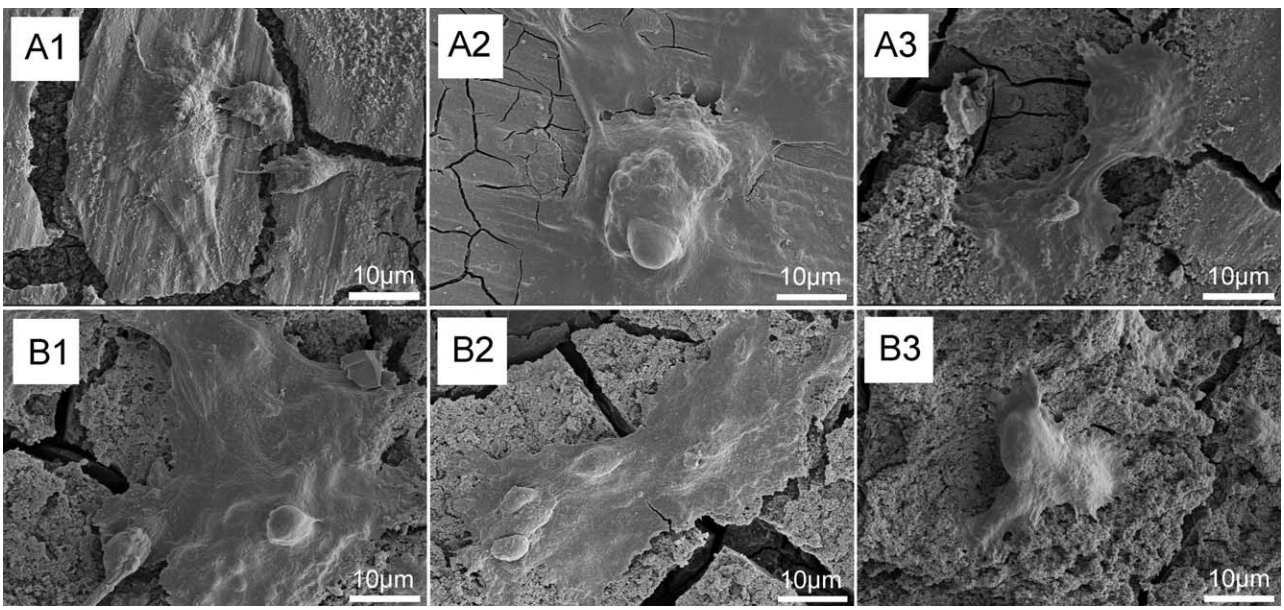


FIGURE 9. SEM photographs of MG63 cultured on the P-Mg (A) and AH-Mg (B) samples for 4 (A1,B1), 6 (A2,B2), and 8 h (A3,B3). The bar is 10 μm.

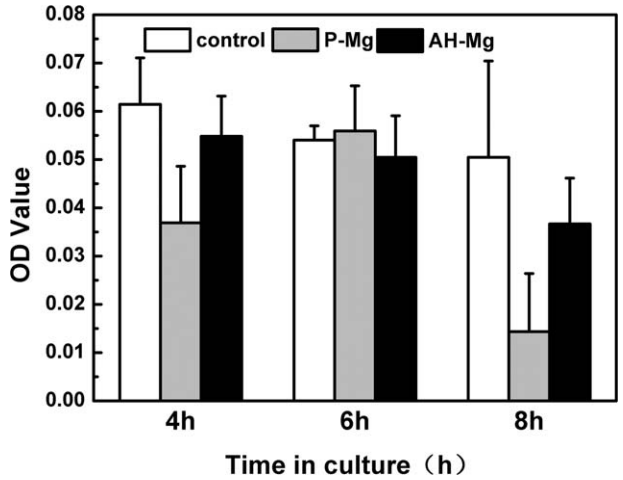


FIGURE 10. The relative OD values of free radicals concentration in MG63 cultured on P-Mg and AH-Mg samples for 4, 6, and 8 h.

0.05) after 4 h culture. This phenomenon indicated that H_2 from P-Mg and AH-Mg degradation could effectively scavenge free radicals in MG63. In addition, the average OD values of cells on AH-Mg were higher than that on the P-Mg ($p < 0.05$), which was because of the faster hydrogen release rate of P-Mg than AH-Mg (Fig. 6).

The average OD values of cells on P-Mg and AH-Mg showed no statistically significant difference ($p < 0.05$) after 6 h culture and were even higher than those groups after 4 and 8 h. This phenomenon suggested that H_2 was not the only influence factor on the level of free radicals in MG63 cells. According to the report,[26] the pH value had a great impact on the enzymatic activities of superoxide dismutase, peroxidase, and catalase in cells. The high pH value might lead to reduction on activity of these enzymes in cells.[27] Therefore, the ability of cell itself on scavenging free radicals decreased, and excess free radicals accumulated in cells. In our study, the pH value of DMEM rising after 6 h culture should result in the higher level of free radicals in MG63 cells. We concluded that the pH value had a negative effect and H_2 had a positive effect on scavenging free radicals. The result at 6 h (Fig. 10) may be because of that the negative effect of pH value exceeded over the positive effect of H_2 on scavenging free radicals.

Compared to the levels of free radicals at 6 h, the levels had decreased at 8 h, which was because of the positive effect of H_2. The result that the relative OD values of AH-Mg group were higher than the P-Mg ($p < 0.05$) for 8 h culture was also attributed to that the hydrogen release rate of P-Mg was faster than AH-Mg (Fig. 6).

We could conclude that H_2 releasing from P-Mg and AH-Mg degradation in cell culture medium could effectively scavenge free radicals in MG63 cells. In our study, by controlling H_2 release, scavenging free radicals in tumor cells under control had been achieved.

CONCLUSION

In summary, H_2 can effectively scavenge free radicals. By regulating the surface structure of Mg metal, H_2 release was controlled by alkali heat treatment and scavenging free radicals in bony cancer cells under control was achieved. It is expected that the Mg metal is a potential biomaterial with anti-bony cancer properties.

ACKNOWLEDGMENT

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