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Effect of surface pre-treatments on biocompatibility of magnesium

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Abstract

This study reports the influence of Mg surface passivation on the survival rate of human HeLa cells and mouse fibroblasts in cell culture experiments. Polished samples of commercially pure Mg show high reactivity in the cell culture medium, leading to a pH shift in the alkaline direction, and therefore cell adhesion and survival is strongly impaired. Passivation of the Mg surface in 1 M NaOH can strongly enhance cell survival. The best initial cell adhesion is observed for Mg samples incubated in simulated body fluid (M SBF), which leads to the formation of a biomimetic, amorphous Ca/Mg phosphate layer with high surface roughness. This surface layer, how ever, passivates and seals the Mg surface only partially. Subsequent Mg dissolution leads to a significantly stronger pH increase com pared to NaOH passivated samples, which prevents long term cell survival. These results demonstrate that surface passivation with NaOH and M SBF together with the associated changes of surface reactivity, chemistry and roughness provide a viable strategy to facil itate cell survival on otherwise non biocompatible Mg surfaces.

Keywords: Magnesium; Corrosion; Cell culture; Passivation

1. Introduction

Magnesium and its alloys are of growing interest for biomedical applications, in particular as a biodegradable material for cardiac or orthopedic implants [1 16]. Bioabsorbable Mg implants might provide a solution for a number of problems associated with permanent metallic implants such as permanent physical/mechanical irritation, and inability to adapt to growth and other ongoing shape changes in the human body. Bioabsorbable Mg implants might also be able to prevent problems associated with long-term release of metallic ions and/or particles through corrosion or wear processes.

To reliably predict the behavior of Mg implants in the human body, the in vivo corrosion behavior as well as the biological interactions with the Mg surface need to be characterized. Corrosion behavior of commercial Mg alloys has been widely studied in typical environments the alloys may encounter in different applications, e.g. in NaCl solutions. General information on the electrochemical corrosion behavior of Mg and its alloys have been reviewed in Ref. [17,18]. Mg corrosion takes place under H_2 gas formation, and leads to alkalization of the surroundings. On the one hand, both factors could strongly impair the biocompatibility of Mg, e.g. by preventing cell adhesion on the implant surface. On the other hand, release of Mg ions by implant dissolution is not expected to lead to toxic reactions, as the concentration of Mg in the body is controlled by homeostatic mechanisms [6].

Even though the interest in Mg in medicine is increasing, only a few studies on cell behavior on Mg alloy surfaces can be found in the literature. It has been reported that osteoblasts and human bone derived cells adhere, prolifer-

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ate and survive on the corroding surface of the Mg alloy AZ91D [19]. However, in comparison to tissue-culture-treated plastic surfaces, a reduction in cell viability was observed which was attributed either to an increase in pH (+0.39) or to the release of ions into the medium. Another study reported that the presence of commercially pure Mg samples in the cell culture medium had no inhibitory effect on cell growth in murine bone marrow cells [20]. A very recent paper reported that cells do not survive on untreated Mg alloy AZ91 for longer than 1 day, whereas samples coated with hydrogenated amorphous silicon show good biocompatibility [21]. An evaluation of binary Mg Ca alloys using L-929 cells showed no cytotoxic effects; instead the viability of cells cultured in the alloy extraction medium was even better than under control conditions [22]. A similar study was carried out on fibroblasts and osteoblasts grown on a wide range of binary Mg alloys [23]. In that study, however, the cell viability was shown to depend on the type of corrosion products produced by the dissolution of the different binary alloys.

In conclusion, the effect of Mg corrosion on cell behavior is ambivalent, as reports indicating either survival or death of cells on corroding Mg surfaces can be found in the literature. The type of alloys and cell lines used for these experiments vary widely, and therefore it is not possible to draw any generalizing conclusions on the critical factors influencing cell behavior on Mg alloy surfaces.

In the present work, we study the behavior of human HeLa cells and mouse fibroblasts in cell cultures in the presence of commercially pure Mg, either without or with specific surface modifications, which were used to control the reactivity, chemical composition and/or roughness of the surface. The results demonstrate the importance of surface treatment for initial cell adhesion and for cell survival on Mg surfaces.

2. Materials and methods

For all experiments, samples were obtained by cutting a commercially pure Mg rod (25.4 mm diameter, 99.9% purity, ChemPur) into 2 mm slices. The samples were mechanically ground on a microcut paper disc (600 grit soft), then polished with diamond paste (6 μm) using an ethanol/glycerol (3:1) mixture as lubricant. Subsequently the samples were cleaned by sonicating in pure ethanol for 3 min, and air dried.

To modify the corrosion resistance, chemical composition and morphology of the Mg surface, the samples were either passivated by soaking in 1 M NaOH for 24 h, or by soaking in simulated body fluid (M-SBF) [24] at 37 °C for 5 days. Table 1 shows the composition of the M-SBF solution used in this study. Soaking in 1 M NaOH reduces the surface reactivity due to the formation of a thin $Mg(OH)_2$ passive layer. Soaking in M-SBF leads to the formation of mixed Ca/Mg-phosphate layers on the surface (e.g. [25–27]).

After these surface pre-treatments, the samples were rinsed with ethanol and dried in air. The morphology and composition of the sample were characterized by field-emission scanning electron microscopy (FE-SEM, Hitachi S4800) and energy-dispersive X-ray (EDX) analysis. The wettability of the surface was determined by optically measuring the water droplet contact angle. A UBM Microsoft Expert laser profilometer with a wavelength of 780 nm was used to determine the roughness of the differently treated surfaces.

Table 1
Composition of the simulated body fluid (M SBF) [24] used in this study. All concentrations are given in mmol l^{-1}.

Ion	M SBF
Na^+	142.0
K^+	5.0
Mg^{2+}	1.5
Ca^{2+}	2.5
Cl^-	103.0
HCO_3^-	10.0
HPO_4^{2-}	1.0
SO_4^{2-}	0.5
HEPES	75.0

pH changes that were induced by Mg dissolution were optically monitored in situ in 2 ml of Eagle's minimum essential cell culture medium incubated at 37 °C, 5% CO_2 and 95% humidity in order to ensure environmental conditions identical to those used for the cell culture tests. The cell culture medium contains phenol red as a pH indicator; the color changes from red at pH 7.4 to pink at pH 9.0 were monitored with an RGB CCD camera (Canon PowerShot G5). A calibration curve was determined in the pH range 7.4–9.0. The pH values in the presence of the Mg samples were then calculated colorimetrically from the ratio of the red-to-blue channel intensities.

For cell culture testing the Mg samples were sterilized under UV irradiation with a wavelength of 260 nm. Human HeLa cells were cultured in the dish with the differently treated Mg specimens under 5% CO_2 in air atmosphere at 37 °C, 95% relative humidity for 24 h in an incubator. Eagle's minimum essential medium with addition of 10 vol.% fetal bovine serum (MEM + FBS) was used as a cell culture medium. About 1,000,000 cells were seeded on the dish. Three dishes were measured for each condition. Under selected conditions, additional experiments were carried out with GSP-C12 mouse fibroblasts stably transfected with EGFP-actin to study the time-series of their attachment and spreading. Both cell lines are standard cell lines and are used in many laboratories worldwide; they show robust growth under a range of conditions, and they form a dense monolayer when grown onto a flat substrate which makes them suitable for cell attachment and spreading area quantification.

After 24 h in the incubator, the cells were fixed with a 3% paraformaldehyde/0.3% Triton-X100 solution in PBS and stained with Alexa red phalloidin to visualize the actin cytoskeleton of the cells, or with Hoechst 33342 to visualize the cell nucleus. Fluorescence microscopy of stained cells was carried out with a Leica DMI 6000B microscope.

For SEM observation, cells were fixed with 2.5% glutaraldehyde solution (Merck) for 15 min at room temperature. Samples were rinsed in PBS solution, dehydrated in a graded series of ethanol (33%, 66%, 95%, 98%, 100%), hexamethyldisilazan (5 min in 33%, 5 min in 66%, 15 min in 100%), and then air dried.

3. Results and discussion

3.1. Surface characterization after pre-treatments

The surface morphology was studied by SEM and by laser profilometry. Fig. 1 shows the SEM top view of the samples soaked in NaOH and in M-SBF solution. After passivation in 1 M NaOH, the original grinding marks on the surface are still clearly visible, indicating that the passive film formed is very thin – the thickness of passivation layers formed in alkaline solutions is of the order of nanometers. A much thicker, rough surface layer is formed by soaking in M-SBF for 5 days – the thickness of this layer is of the order of tens of micrometers. The roughness of the differently treated surface was determined by laser profilometry as shown in Fig. 2. The average R_a roughness is strongly increased for the sample soaked in M-SBF (R_a values given in Fig. 2). The surface morphology of the sample soaked in M-SBF also leads to a strongly hydrophilic behavior of the surface as indicated by the measured water droplet contact angles (Table 2).

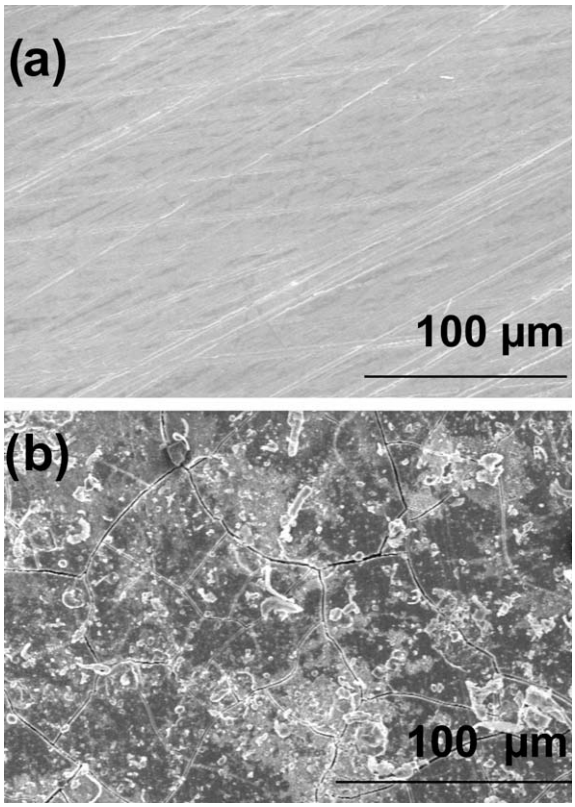

Fig. 1. SEM images of the surface of Mg samples soaked in: (a) 1 M NaOH for 24 h and (b) M SBF at 37 °C for 5 days.

The chemical composition of the surface layers was determined by EDX analysis (Table 3). For the sample passivated in NaOH, only signals from Mg and O are detected. Due to the low thickness of the surface layer formed in NaOH (nanometer scale) as compared to the sampling depth of EDX (micrometer scale), the composition determined by EDX does not correspond to the $MgO/Mg(OH)_2$ passive layer, as the signal mostly stems from the Mg substrate. For the sample soaked in M-SBF, strong signals from Ca, P and C in addition to Mg and O peaks were observed. This composition is very similar to that reported previously on a rare-earth-containing Mg alloy soaked in M-SBF under identical conditions as in the present study (5 days, 37 °C, M-SBF) [27]. Also, similarly to the layer formed on the rare-earth-containing Mg alloy in the previous study, no apatite peaks could be detected by X-ray diffraction (XRD) for the Mg samples of the present study, indicating that the carbonated Ca/Mg-phosphate surface layer formed on Mg in M-SBF is amorphous.

In summary, soaking in NaOH leads to passivation of the surface by a thin (nanometer scale thickness) $MgO/Mg(OH)_2$ layer, whereas a significantly thicker (tens of micrometer scale thickness) amorphous, carbonated Ca, Mg-phosphate layer with a high surface roughness is formed on Mg by soaking in M-SBF.

3.2. pH measurements in cell culture medium

The pH values determined optically in the cell culture medium show for all samples an increase in the pH from the reference value 7.68 during 2 h contact with Mg samples. The pH increase is the strongest for the sample soaked in M-SBF (pH 8.96), followed by the polished sample (pH 8.01), and is the lowest for the NaOH-passivated sample (pH 7.88). These values represent average values of the solution; as the solution was not stirred during the experiments, the local pH in the vicinity of the Mg sample surface could be significantly higher. The finding of a strong pH increase on the sample soaked in M-SBF indicates that the surface layer formed on Mg during soaking is not highly protective; this is due to the porosity of this layer. This is in line with investigations on the time-dependent corrosion behavior of a Mg alloy in M-SBF [28]. A comparison of the electrochemical behavior of the differently treated Mg surfaces in the cell culture medium indicates that the initial dissolution rate of Mg in the cell culture medium is decreased by approximately a factor of five by soaking in M-SBF, and by a factor of ~20 by soaking in 1 M NaOH. The dissolution rate (electrochemical behavior) is strongly time-dependent, as the passive film formed in the alkaline solution shows a limited resistance in the neutral cell culture medium containing chloride ions.

3.3. Cell culture experiments

For each experiment, the number of cells on the Mg substrate and on the glass reference was determined. Fig. 3a

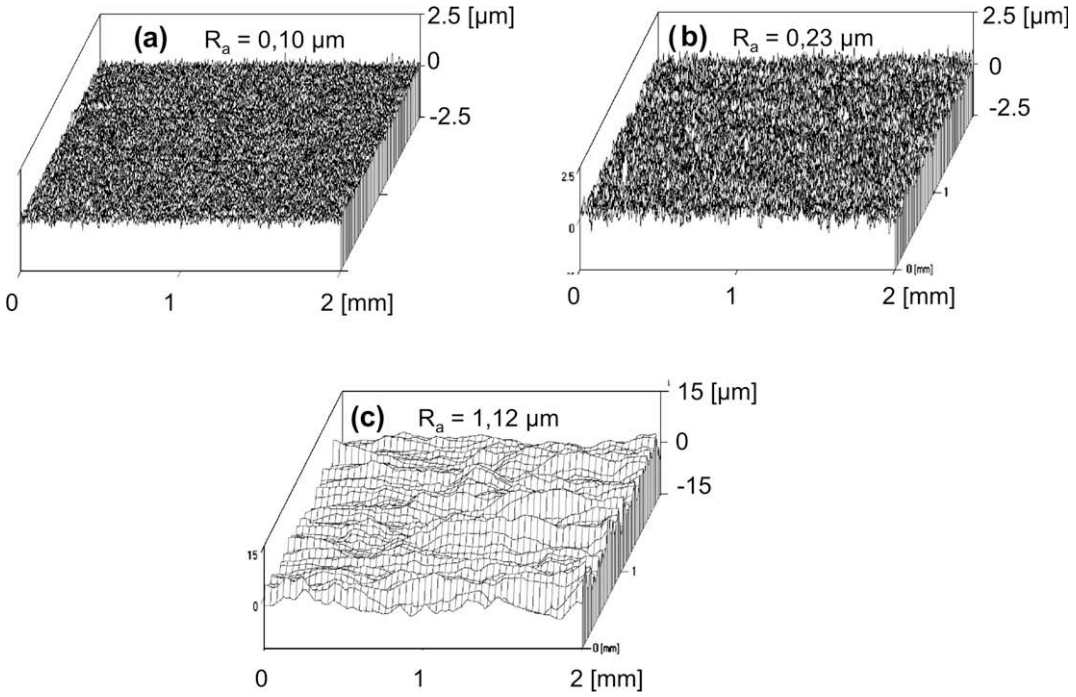

Fig. 2. Laser profilometry of the Mg surface: (a) polished; (b) soaked in 1 M NaOH for 24 h; and (c) soaked in M SBF at 37 °C for 5 days.

Table 2
Water drop contact angles on differently treated Mg surfaces.

Surface treatment	Water contact angle (°)
Polished	25
NaOH passivation	15
M SBF soaking	0

Table 3
EDX analysis of the surface layer formed on Mg surface by different surface pre treatment: soaking in 1 M NaOH for 24 h, or soaking in M SBF at 37 °C for 5 days.

Element	at.%	
	NaOH	M SBF
Mg	91,47	10,50
O	8,53	61,97
C		10,90
Ca		6,45
P		8,60
Na		1,58

shows the average cell density from three independent experiments. The reference sample (glass without Mg sample in the petri dish) shows the highest cell density on the surface. A large range of factors including H_2 gas evolution, pH increase due to Mg dissolution in the cell culture medium, as well as the chemical and physical properties of the native or corroded Mg surface may all have contributed to a decrease in cell adhesion and growth on the Mg substrate in comparison to glass. Dissecting those factors is possible but requires extensive experiments that go beyond the scope of this study. The following data only address the overall effect of Mg surface treatment on cell density and spreading but do not attempt to separately measure cell adhesion, growth and survival as a function of physical and chemical changes in the cells' environment.

Strong differences in cell density after 24 h of culture can be found between the differently treated Mg samples. Without pre-treatment (polished sample), no or a very low number of cells are found on the Mg surface. Visible strong gas evolution is observed upon introducing the cell culture medium onto the sample. There is little doubt that the high reactivity of the non-treated Mg sample largely prevents cell adhesion and survival on the surface, although it is not clear whether the H_2 gas formation or the pH increase is the more dominating factor.

Both surface-modified samples show a significantly higher number of cells on the surface than the polished surface, and soaking in M-SBF seems to be even more beneficial for cell adhesion than passivation in NaOH. The differences in cell behavior are likely be attributable either to the different surface chemistry (presence of Ca and P on the surface of the M-SBF-soaked sample, see Table 3), or to a strongly increased surface roughness (see Fig. 2).

We further investigated cell spreading and actin cytoskeleton formation by fluorescence microscopy imaging as shown in Fig. 3b e. For the polished samples without pre-treatment, only single isolated cells can be seen, and cell spreading was poor. For the NaOH-passivated sample, however, strong formation of the actin cytoskeleton can be observed, and the cells are well spread, covering most of the surface. Good cell spreading and filopodia formation on the NaOH-passivated surface can also be seen with a higher resolution using SEM as shown in Fig. 3f. For other type of surfaces, no filopodia formation could be detected (data not shown).

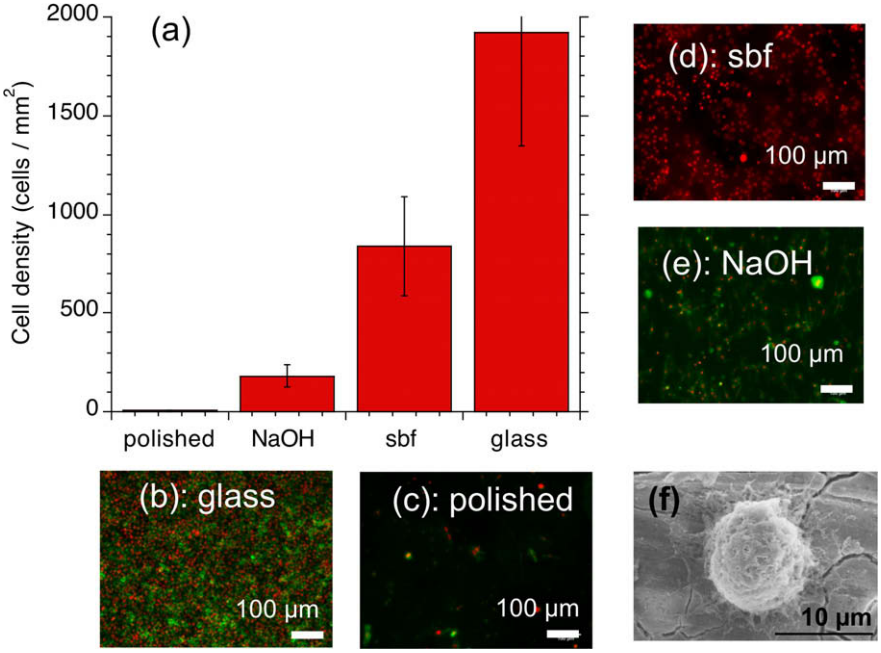

Fig. 3. Behavior of HeLa cells on differently pre treated Mg surfaces as well as on glass: (a) cell density after 24 h in cell culture; (b–e) fluorescence imaging of stained cells; (f) SEM image of a cell attached to the surface of Mg soaked in M SBF.

For the sample soaked in M-SBF, a large number of cell nuclei can be seen, but cell spreading was poor and the actin cytoskeleton not well formed. This finding suggests that the surface of the SBF-soaked sample is favorable for the initial cell adhesion, but cell spreading after 24 h of culture is poor, with cell death possibly occurring in the 24 h timeframe of our experiments. This could result from the strong pH changes taking place in the cell culture medium upon exposure of the SBF-soaked Mg sample in the medium as explained above.

In order to study the time dependence of the cell adhesion and spreading on the surface soaked in M-SBF, preliminary experiments were carried out with mouse GSP-C12 fibroblast cells stably transfected with EGFP-labeled actin. The same samples could be microscopically studied after different cell culture times. Fig. 4 shows microscopy images of cells after different times in culture, as well as the cell spreading area as a function of culture time. Up to 6 h of culture, the cell spreading area is increasing, and cells form filopodia, indicating that the surface layer formed in M-SBF indeed facilitates cell adhesion. However, after 6 h, the decrease in cell spreading area and the cell shape indicate strongly decreased viability of the cells. After 24 h, most cells have detached from the surface, and the remain-

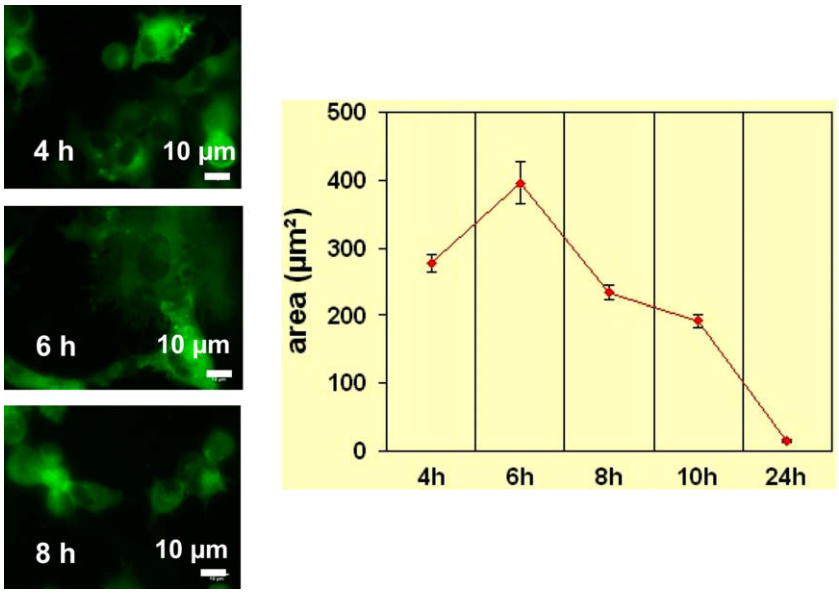

Fig. 4. Mouse fibroblasts on Mg surface which had been pre treated by soaking in M SBF at 37 °C for for 5 days. Fluorescence imaging of cells and cell spreading area as a function of culture time.

ing cells have rounded up with a spreading area close to zero. These results indicate that the surface chemistry and/or roughness of the layer formed in M-SBF leads to good cell adhesion, but the limited protection afforded by the layer and the subsequent alkalization of the cell culture medium prevents long-term survival. As a conclusion, to ensure a strong adhesion of cells and long-term cell survival on the surface, the surface chemistry and roughness must be optimized, and at the same time the reactivity of the Mg surface must be strongly reduced. A possible route to this may be a combined passivation/biomineralization treatment of Mg surfaces.

Regarding biomedical applications of Mg and Mg alloys in the human body, it should be considered, however, that pH changes could be largely prevented by mass transport processes and the continuous exchange with a large volume of buffered body fluids. To assess the influence of Mg corrosion on the behavior of cells under more physiological conditions, we suggest that in vitro cell culture experiments should be carried out in flow cells or with medium exchange. On the other hand, the pH in the immediate vicinity of a Mg implant might be even higher than in the in vitro experiments because of limited fluid exchange through the extracellular matrix surrounding the implant.

Nevertheless, the results clearly indicate that by optimizing the surface pre-treatment of Mg, the interaction of Mg surface and the biological environment could be tailored to the desired performance. Future work will be carried out to systematically study the critical surface parameters for adhesion and survival of different cell types on Mg surfaces. Moreover, it will be of interest to compare the behavior on pure Mg to different Mg alloys, since alloying can decrease the corrosion rate but also changes the surface chemistry.

4. Conclusions

Without pre-treatments the initially high reactivity of Mg surface does not enable adhesion and survival of HeLa cells on the surface. Passivation of the Mg surface in 1 M NaOH can significantly increase the cell survival rate. Soaking in M-SBF leads to a formation of a rough, Ca- and P-containing surface layer, which initially enhances cell adhesion on the surface. However, due to the poor protective properties of this layer, long-term cell survival is prevented.

As cell culture testing is typically carried out with a relatively low medium volume/surface area ratio, and without solution exchange, deleterious effects of Mg corrosion, such as pH increase, most probably are over-critically assessed in comparison with the in vivo case. However, H_2 gas formation on the surface and rapid dissolution leading to a very dynamic interface may directly hinder the formation of the cell surface bond, and these processes are independent of the solution volume. Hence, a control of the reactivity of the Mg surface for the initial cell reactions in the body remains an important issue for further research.

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References

[1] Heublein B, Rohde R, Kaese V, Niemeyer M, Hartung W, Haverich A. Biocorrosion of magnesium alloys: a new principle in cardiovascular implant technology? Heart 2003;89:651–6.
[2] Witte F, Kaese V, Haferkamp H, Switzer E, Meyer Lindenberg A, Wirth CJ, et al. In vivo corrosion of four magnesium alloys and the associated bone response. Biomaterials 2005;26:3557–63.
[3] Waksman R. Metallic bioabsorbable stents: concepts, experimental findings, early clinical results, and the future. ACC Rev 2005;14:36–43.
[4] Staiger MP, Pietak AM, Huadmai J, Dias G. Magnesium and its alloys as orthopedic biomaterials: a review. Biomaterials 2006;27:1728–34.
[5] Witte F, Reifenrath J, Muller PP, Crostack H A, Nellesen J, Bach FW, et al. Cartilage repair on magnesium scaffolds used as a subchondral bone replacement. Mat wiss u Werkstofftech 2006;37:504–8.
[6] Williams D. New interests in magnesium. Med Device Technol 2006;17:9–10.
[7] Erne P, Schier M, Resink TJ. The road to bioabsorbable stents: reaching clinical reality? Cardiovasc Intervent Radiol 2006;29:11–6.
[8] Witte F, Fischer J, Nellesen J, Crostack H A, Kaese V, Pisch A, et al. In vitro and in vivo corrosion measurements of magnesium alloys. Biomaterials 2006;27:1013–8.
[9] Witte F, Ulrich H, Rudert M, Willbold E. Biodegradable magnesium scaffolds. Part I: appropriate inflammatory response. J Biomed Mater Res 2007;81A:748–56.
[10] Witte F, Ulrich H, Palm C, Willbold E. Biodegradable magnesium scaffolds. Part II: peri implant bone remodeling. J Biomed Mater Res 2007;81A:757–65.
[11] Xu L, Yu G, Zhang E, Pan F, Yang K. In vivo corrosion behavior of Mg Mn Zn alloy for bone implant application. J Biomedical Mater Res 2007;83A:703–11.
[12] Zartner P, Buettner M, Singer H, Sigler M. First biodegradable metal stent in a child with congenital heart disease: evaluation of macro and histopathology. Catheter Cardiovasc Interv 2007;69:443–6.
[13] Erbel R, di Mario C, Bartunek J, Bonnier JJ, de Bruyne B, Eberli FR, et al. Temporary scaffolding of coronary arteries with bioabsorbable magnesium stents: a prospective, non randomised multicentre trial. Lancet 2007;369:1869–75.
[14] Muller WD, Nascimento ML, Zeddies M, Corsico M, Gassa LM, de Mele MAFL. Magnesium and its alloys as degradable biomaterials. Corrosion studies using potentiodynamic and EIS electrochemical techniques. Mater Res 2007;10:5–10.
[15] Zheng R, Dietzel W, Witte F, Hart N, Blawert C. Progress and challenge for magnesium alloys as biomaterials. Adv Eng Mater 2008;10:B3–B14.
[16] Sikora E, Shaw B, Virtanen S. Fix, heal and repair – a new approach to using metals in the human body. Interface 2008;17:45–9.
[17] Song GL, Atrens A. Corrosion mechanisms of magnesium alloys. Adv Eng Mater 1999;1:11–33.
[18] Song GL, Atrens A. Understanding magnesium corrosion – a framework for improved alloy performance. Adv Eng Mater 2003;5:837–58.

[19] Witte F, Feyerabend F, Maier P, Fischer J, Stormer M, Blawert C, et al. Biodegradable magnesium hydroxyapatite metal matrix com posites. Biomaterials 2007;28:2163 74.
[20] Li L, Gao J, Wang Y. Evaluation of cyto toxicity and corrosion behavior of alkali heat treated magnesium in simulated body fluid. Surf Coating Technol 2004;185:92 8.
[21] Xin Y, Jiang J, Huo K, Tang G, Tian X, Chu PK. Corrosion resistance and cytocompatibility of biodegradable surgical magne sium alloy coated with hydrogenated amorphous silicon. J Biomed Mat Res A, in press.
[22] Li Z, Gu X, Lou S, Zheng Y. The development of binary Mg Ca alloys for use as biodegradable materials within bone. Biomaterials 2008;29:1329 44.
[23] Gu X, Zheng Y, Cheng Y, Zhong S, Xi T. In vitro corrosion and biocompatibility of binary magnesium alloys. Biomaterials 2009;30:484 98.
[24] Oyane A, Kim HM, Furuya T, Kokubo T, Miyazaki T, Nakamura T. Preparation and assessment of revised simulated body fluids. J Biomed Mater Res 2003;65:188 95.
[25] Cortes DA, Lopez HY, Mantovani D. Spontaneous and biomimetic apatite formation on pure magnesium. Mater Sci Forum 2007;539 543:589 94.
[26] Hiromoto S, Shishido T, Yamamoto A, Maruyama N, Somekawa H, Mukai T. Precipitation control of calcium phosphate on pure magnesium by anodization. Corrosion Sci 2008;50:2906 13.
[27] Rettig R, Virtanen S. Composition of corrosion layers on the magnesium rare earth alloy in simulated body fluids. J. Biomed Mater Res 2009;88A:359 69.
[28] Rettig R, Virtanen S. Time dependent electrochemical characteriza tion of the corrosion of the magnesium rare earth alloy in simulated body fluids. J. Biomed Mater Res 2008;85A:167 75.