

# Control of magnesium corrosion and biocompatibility with biomimetic coatings

Sigrid Keim,<sup>1</sup> Johannes G. Brunner,<sup>1</sup> Ben Fabry,<sup>2</sup> Sannakaisa Virtanen<sup>1</sup>

<sup>1</sup>Institute of Surface Science and Corrosion, Department of Materials Science and Engineering (WW-4), University of Erlangen-Nuremberg, Erlangen, Germany

<sup>2</sup>Center for Medical Physics and Technology, Department of Physics, University of Erlangen-Nuremberg, Erlangen, Germany

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**Abstract:** The use of magnesium and its alloys as biodegradable metallic implant materials requires that their corrosion behavior can be controlled. We tailored the Mg release kinetics and cell adhesion properties of commercially pure Mg by chemical surface treatments in simulated body fluid, in Dulbecco's Modified Eagle's cell culture medium in the presence or absence of fetal bovine serum (FBS), or in 100% FBS. HeLa cells were cultured for 24 h on these Mg surfaces to characterize their biocompatibility. Cell density on all treated surfaces was significantly increased compared with a polished Mg surface, where almost no cells survived. This low biocompatibility of pure Mg was not caused by the high Mg ion release with concentrations of up to 300 mg/L

in the cell culture medium after 24 h, as cells grown on a glass substrate showed no adverse reactions to high Mg ion concentrations. Rather, the most critical factor for cell adhesion was a sufficiently reduced initial dissolution rate of the surface. A comparison among all surface treatments showed that an incubation of the Mg samples in cell culture medium gave the lowest dissolution rate and resulted in the best cell adhesion and spreading behavior. © 2010 Wiley Periodicals, Inc. *J Biomed Mater Res Part B: Appl Biomater* 96B: 84–90, 2011.

**Key Words:** magnesium, corrosion, cell culture, simulated body fluid, cytotoxicity

## INTRODUCTION

The use of magnesium alloys in biomedical applications as biodegradable metallic implant materials is of steadily growing interest, both for degradable bone implants<sup>1</sup> and for bioabsorbable cardiovascular stents.<sup>2</sup> For a safe application of these materials in the human body, their corrosion behavior needs to be evaluated, and the interactions between the dissolution process and the biological environment need to be explored. To date, still a limited number of studies on the use of Mg as a biodegradable material have been conducted (reviewed in [3]), with many open questions remaining.

To tailor the degradation rate of the implant for the specific biomedical application, novel biocompatible Mg alloys on the one hand and surface coating methods on the other hand are being developed. Coatings with calcium phosphate are of special interest. On titanium surfaces, such coatings have shown good biocompatibility and bioactivity (reviewed in [4]). Calcium phosphate coatings have been deposited on Mg surfaces, for instance, by ion-beam-assisted deposition<sup>5</sup> or various types of electrochemical and chemical treatments.<sup>6–13</sup> In each case, these coatings lead to a reduced corrosion rate but the crystal structure, chemical composition, and morphology of the coatings, and hence, also the measured degradation rates varied greatly. Of particular interest are Ca-P coatings that spontaneously form on Mg and Mg alloys on exposure to simulated biofluids.<sup>6,12–14</sup>

In addition to the corrosion behavior, another critical aspect in surface compatibility of biodegradable implant materials is the influence of the material on the biological environment. Cell survival on corroding Mg alloy surfaces is primarily limited by hydrogen liberation and pH increase in the medium. Data in the literature, however, show large differences because of different experimental approaches such as the use of different alloys and cell lines.<sup>15–23</sup> In a previous study, we showed that cell adhesion and survival on commercially pure Mg surface is drastically influenced by simple chemical surface pretreatments, such as passivation in NaOH or soaking in simulated body fluid (SBF).<sup>24</sup> The rationale behind these surface treatments was to reduce the initial corrosion rate of the surface by NaOH passivation and to biofunctionalize the surface with a carbonated (Mg, Ca)-phosphate layer for better cell adhesion. An interesting result was that even though the (Mg, Ca)-phosphate layer led to an initially good cell adhesion and spreading, the cells started to deadhere after longer times. We attributed this to the poor corrosion protection properties of the Ca-P surface layer formed in SBFs. In this study, we compare different sealing treatments of the SBF-formed layer on the corrosion behavior and biocompatibility. These data are then compared with biomimetic coatings formed in cell culture medium in the absence and presence of serum.

**Correspondence to:** S. Virtanen; e-mail: virtanen@ww.uni-erlangen.de  
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**TABLE I. Composition of the SBF 5 Solution<sup>25</sup>**

Ion	SBF
Na <sup>+</sup>	142.0
K <sup>+</sup>	5.0
Mg <sup>2+</sup>	1.0
Ca <sup>2+</sup>	2.5
Cl <sup>-</sup>	131.0
HCO <sub>3</sub> <sup>-</sup>	5.0
HPO <sub>4</sub> <sup>2-</sup>	1.0
SO <sub>4</sub> <sup>2-</sup>	1.0
TRIS buffer	50.0 mL/L

Concentrations are given in mmol/L.

## MATERIALS AND METHODS

For all experiments, samples were obtained by cutting a commercially pure Mg rod (9.5 mm diameter, 99.9% purity, ChemPur) into 5 mm slices. The samples were mechanically ground to a 1200 grit finish with SiC. The first set of samples was soaked in SBF (SBF 5)<sup>25</sup> at room temperature for 5 days, and each sample was exposed to 60 mL of SBF. Table I shows the composition of the SBF 5 solution used in this study. Different post-treatments of the SBF-soaked samples were then carried out: soaking in 1M NaOH at room temperature for 24 h (in a volume of 400 mL for six samples), heat-treatment at 150°C in air for 1 h, heat-treatment in steam for 1 h, and soaking in boiling water for 1 h. The second set of samples was incubated for 24 h in a cell culture incubator at 37°C, 5% CO<sub>2</sub>, in 100% fetal bovine serum (FBS), or in Dulbecco's Modified Eagle's cell culture medium (DMEM) with or without addition of 10% FBS and Penicillin/Streptomycin (the solution volume/sample was 60 mL). Samples soaked in SBF as described above, without sealing treatments, were used as a reference to these incubated samples in cell culture studies. After these surface treatments, the samples were rinsed with ethanol and dried in air. For the characterization of the sample morphology and composition, a field-emission scanning electron microscope (Hitachi FE-SEM S4800) equipped with an Energy dispersive X-ray (EDX) analyzer was used.

For cell culture experiments, the Mg samples were sterilized under UV irradiation with a wavelength of 260 nm. Cells from a human cervical cancer cell line (HeLa) were cultured in an incubator (5% CO<sub>2</sub>, 37°C, 95% relative humidity) for 24 h in 24-well plates containing the differently treated Mg samples. HeLa cells are a standard cell line used in many laboratories worldwide; they show robust growth under a range of conditions and form a dense monolayer when grown onto a flat substrate, which makes them suitable for cell attachment and spreading area quantification. DMEM with addition of 10% FBS and 100 U/mL Penicillin/Streptomycin was used as a cell culture medium. About 100,000 cells were seeded on the Mg samples, and 5 mL of cell culture medium was used. After 24 h in the incubator, the cells were fixed in 2% paraformaldehyde and stained with Alexa red phalloidin to visualize the actin cytoskeleton of the cells and with Hoechst 33342 to visualize the cell nucleus. Fluorescence microscopy of the stained cells was car-

ried out with a Leica DMI 6000B microscope. A minimum of three samples was measured for each condition. The cell densities reported show the mean of the different replicate experiments in each set of samples (set 1: SBF soaking with sealing treatments and set 2: DMEM incubation with SBF-soaked reference sample).

Corrosion behavior of the Mg samples was studied by electrochemical impedance spectroscopy with a Zahner IM6 set-up, in a frequency range of 10 MHz to 100 kHz with an amplitude of 10 mV. The experiments were carried out in the cell culture medium without cells, at 37°C. An Ag/AgCl (3M KCl) electrode was used as a reference electrode and Pt as the counter electrode. The impedance spectra were measured at the corrosion potential. The concentration of Mg ions in the medium after 24 h of culture was determined by atomic absorption spectroscopy according to the DIN 38406 standard.

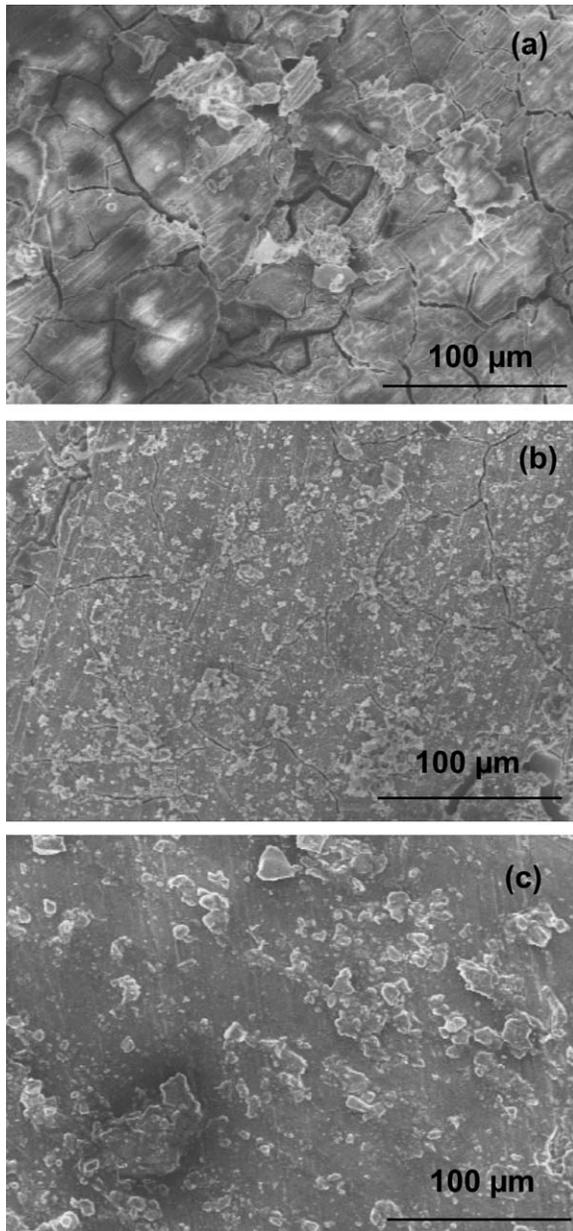
For cytotoxicity testing, HeLa cells were grown on glass and incubated in culture medium with increasing Mg<sup>2+</sup> concentrations ranging from 20 to 750 mg/L. Mg ion concentration in the cell culture medium was increased by adding MgSO<sub>4</sub>·7H<sub>2</sub>O, which had been previously sterilized by UV. The weighing and mixing of the sterilized salt with the cell culture medium was carried out under sterile conditions.

## RESULTS

### Sealing treatments of the coating formed in SBF

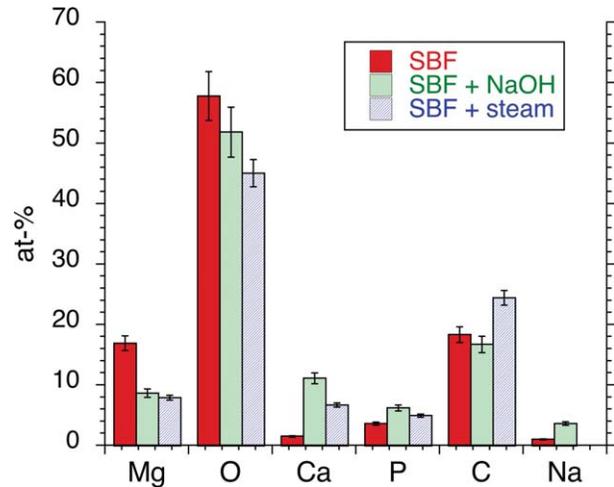
A previous study<sup>24</sup> showed a poor protective quality of the coating formed on Mg by soaking for 5 days in SBF due to cracks and pores in the layer. We therefore explored sealing strategies of the coating by soaking in NaOH or in hot water, as well as heat treatments in dry air or water vapor. Some of these treatments have been previously shown to significantly reduce the degradation rate of porous anodized Mg in simulated body solutions.<sup>26</sup> In our study, SEM characterization indicated that only soaking in 1M NaOH or steaming led to a noticeable sealing of large cracks and pores (Figure 1). However, many small particles appear on the surface after the sealing treatments that introduce an additional surface roughness. Heating the samples for 1 h at 150° in air led to widening of cracks in the layer formed in SBF, most likely due to a dehydration of the water-containing calcium-phosphate layer. Likewise, soaking in boiling water was not successful in sealing the pores or cracks. In the subsequent experiments, conditioning of the SBF-formed layer in hot air or in boiling water was therefore not further explored.

EDX analysis demonstrates that SBF-formed layers after all treatments contain Ca, P, and C in addition to Mg and O (Figure 2). Slight changes in the surface chemistry were noticeable after exposure to NaOH or to steam: the Ca- and P-signals increased, whereas the Mg and O signals decreased. This can be related to the sealing of the pores and cracks, which resulted in a lower signal from the Mg substrate. In addition, the increase of the C signal after steaming may indicate an increased carbonate content in the layer. All surface layers were also characterized by XRD; however, no crystalline phases of the surface layers were observed.



**FIGURE 1.** SEM images of the surface of Mg samples after different surface pretreatments: (a) 5 days soaking in SBF, 37°C, (b) SBF soaking as in (a) +1 h soaking in 1M NaOH, and (c) SBF soaking as in (a) +1 h in steam.

The influence of these surface treatments on the Mg ion release in the cell culture medium was studied by atomic absorption spectroscopy (Figure 3). Mg ion release from polished Mg samples in cell culture medium after 24 h was 299 mg/L. The polished Mg samples initially show a high corrosion rate in the cell culture medium as indicated by electrochemical measurements (the  $R_p$ -value determined from impedance spectra was ca.  $350 \Omega \text{ cm}^2$ ), as well as by strong hydrogen bubbling upon exposing the polished sample to cell culture medium. However, after 24 h, the surface is covered by a black layer, containing Ca, P, and C, and the corrosion rate fell noticeably. Surprisingly, Mg ion release

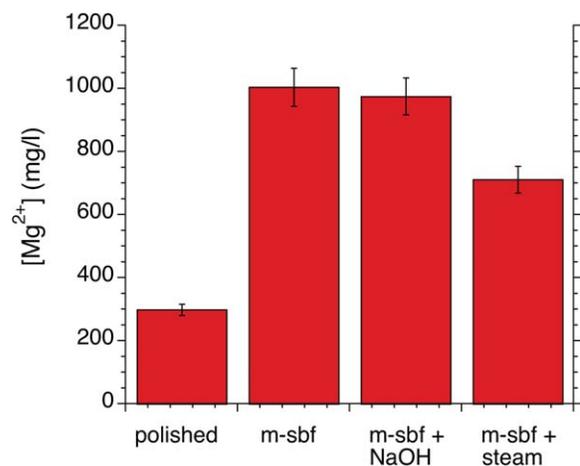


**FIGURE 2.** Composition of the surface layers formed on Mg during different surface pretreatments based on EDX measurements. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

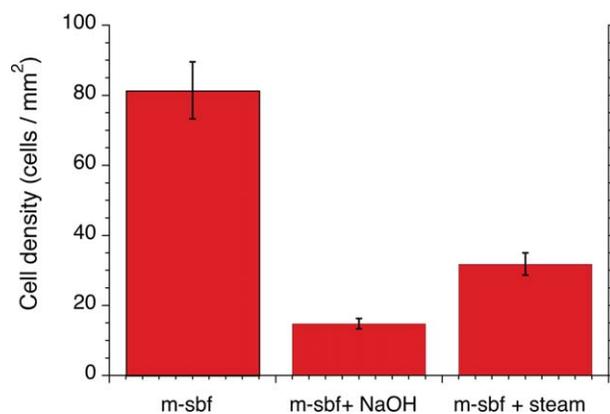
from all SBF-treated samples, regardless of sealing methods, was higher compared with polished Mg samples. Post-treatment of the SBF-formed layer in NaOH shows almost no effect on Mg ion release, whereas steaming decreased the Mg ion release by about 30%.

To address the biocompatibility of the SBF-formed layer, we cultured HeLa cells on Mg surfaces without pretreatment and with pretreatment in SBF both with and without a sealing step. No cells can be found on the surface of polished Mg without any pretreatment (Figure 4), which is in agreement with our previous study.<sup>24</sup> Soaking in SBF significantly improved Mg biocompatibility and allowed cells to adhere on the surface, but both sealing methods lead to reduced cell densities compared with the unsealed SBF-layer.

The efficiency of sealing treatments to improve the properties of the layer formed on Mg in SBF therefore was only



**FIGURE 3.** Mg<sup>2+</sup>-concentration in the medium after incubation for 24 h. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]



**FIGURE 4.** Cell densities after 24 h on Mg samples with different SBF surface treatments. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

of limited success: even though a small improvement in the corrosion behavior is observed (with steaming, reduction of Mg ion release of ca. 30% to nonsealed samples), the treatments lead to reduced cell densities.

#### Biomimetic coating formed on Mg in cell culture medium

The density of cells grown for 24 h on Mg samples pretreated in DMEM (with or without 10% serum and Pen/Strep) or pure serum was greatly increased compared with SBF-treated samples [Figure 5(a)]. No significant differences between serum-containing and serum-free media were observed. Also, the cells show good spreading on the surface of Mg pretreated in DMEM [Figure 5(b)].

No striking morphological features on the DMEM- or FBS-treated surfaces were observed using SEM. In fact, the surface morphologies were similar to the layer formed in SBF. EDX analysis indicated a high carbon content on the layer formed in culture medium when compared with SBF

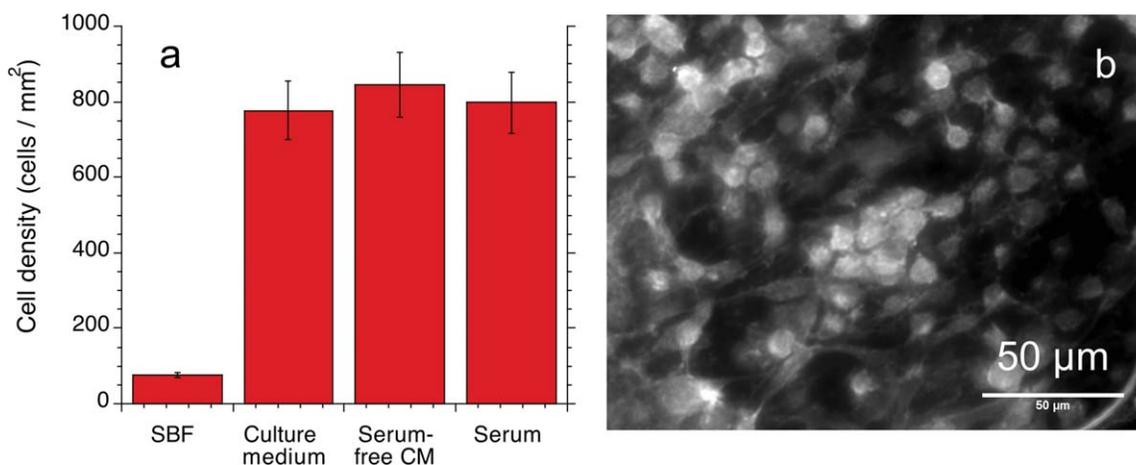
[Figure 6(a)]. When the samples with SBF-formed layers were further incubated for 24 h in DME, and the composition of the SBF-formed layers became more similar to the initially DMEM-formed layers [Figure 6(a)].

Unlike the SBF-formed layer, the coating formed in the cell culture medium was protective; Mg ion release in 24 h was only 112 mg/L, a decrease of about 60% compared with polished Mg [Figure 6(b)]. In contrast to the non-treated Mg samples, no vigorous H<sub>2</sub> gas evolution in the beginning of the experiment was observed for the samples treated in cell culture medium or FBS. Moreover, impedance measurements indicate a strongly increased corrosion resistance for these samples:  $R_p$ -values of about 5800  $\Omega$  cm<sup>2</sup> were measured for samples treated in cell culture medium, when compared with  $R_p \approx 350$   $\Omega$  cm<sup>2</sup> for polished Mg and  $R_p \approx 500$   $\Omega$  cm<sup>2</sup> for SBF-soaked samples. Our finding of a good corrosion protection of the surface layer formed in DMEM is in agreement with a recent study in which a significantly lower degradation rate of Mg in cell culture medium (E-MEM + FBS) compared with other types of SBFs was reported.<sup>27</sup>

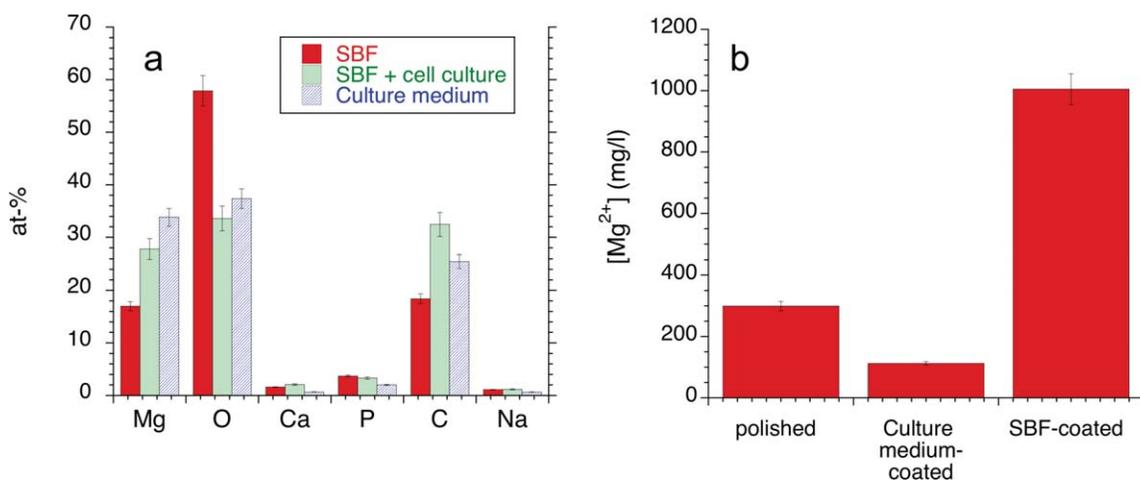
Taken together, sample pretreatment in cell culture medium was the most successful of all approaches studied here to reduce the dissolution rate of Mg and to simultaneously increase the number of cells that adhered to the surface.

#### Cytotoxicity

To assess the role of released Mg ions on the cell behavior, HeLa cells were grown on glass and incubated in culture medium with increasing Mg<sup>2+</sup> concentrations ranging from 20 to 750 mg/L, similar to the ion concentrations in the medium found after 24 h of Mg sample corrosion. Cell density, cell spreading, and actin stress fiber formation on glass was not affected even by the highest Mg ion concentration studied (Figure 7). A comparison of the cell counts for each Mg-concentration with the cell counts at the lowest Mg-



**FIGURE 5.** (a) Cell densities after 24 h on Mg samples pretreated in SBF, in cell culture medium, cell culture medium without serum (Serum-free CM), or in fetal bovine serum. (b) Fluorescence image (actin staining) of cells after 24 h on Mg surfaces pretreated in cell culture medium, showing a nearly confluent cell layer with good spreading and adhesion. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]



**FIGURE 6.** (a) EDX results on the composition of the surface layers formed by soaking in SBF or incubating in cell culture medium. In addition, the composition of the surface layer for the SBF-soaked sample after cell culturing is shown. (b) Mg<sup>2+</sup>-concentration in medium after 24 h for a polished Mg sample (no pretreatment), and samples coated by soaking in SBF or in cell culture medium. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

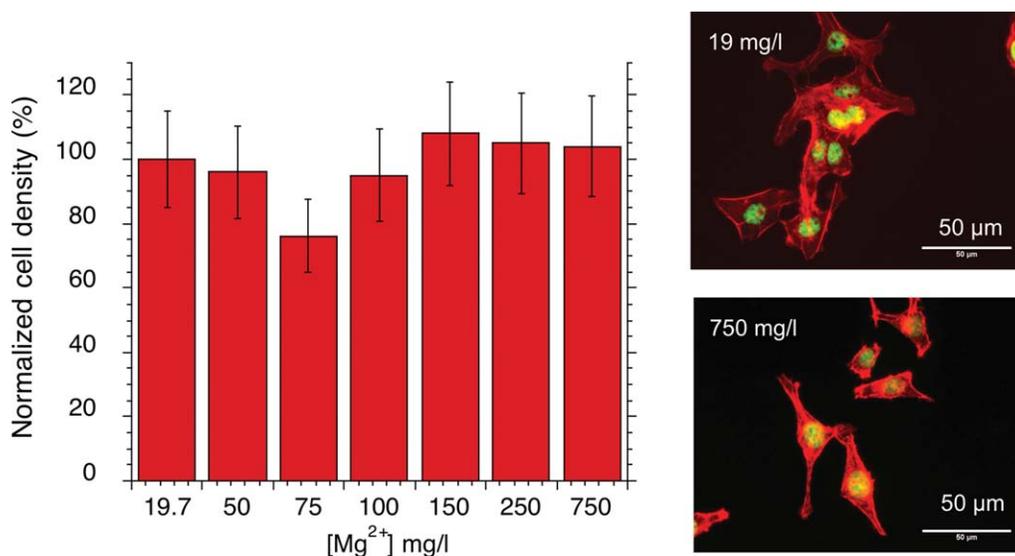
concentration using a two-tailed *t*-test shows no statistically significant differences ( $p > 0.3$ ), confirming that the Mg<sup>2+</sup>-concentration in the medium did not affect cell adhesion and growth.

#### DISCUSSION

The main finding of this study is that incubation of Mg samples for 24 h in cell culture medium (DMEM) resulted in the best corrosion protection and biocompatibility. The mechanism, however, is not fully understood. We could rule out that changes in the surface morphology were responsible for this effect, as the morphology of an SBF-formed layer was similar to the layer formed after incubation in DMEM.

We could also rule out that the excessive Mg ion concentrations in the medium due to Mg corrosion were responsible for the differences in biocompatibility, as no cytotoxic effects could be detected even at the highest Mg ion concentrations in the medium when the cells were grown on an inert surface such as glass.

The layer formed after DMEM incubation had a high carbon content; the precise chemical reaction between Mg and DMEM and chemical composition of the forming surface layer, however, is still a subject of further investigations. The presence or absence of proteins in DMEM had no impact on the surface layer. The electrochemical measurements indicate an initially high corrosion rate of the



**FIGURE 7.** Left: Influence of Mg<sup>2+</sup>-concentration in the medium on cell density for cells grown on glass. One hundred percent corresponds to the cell density in medium containing 19 mg/L Mg<sup>2+</sup>. Right: Fluorescence images (actin: red, nucleus: green) of cells grown at low- and high-Mg<sup>2+</sup> concentrations in the medium. Cells show no apparent differences in spreading area and cell morphology. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://wileyonlinelibrary.com).]

polished Mg sample but in a 24-h experiment a rather low Mg ion release. We therefore conclude that the protective C-containing layer forms rapidly upon contact of the bare Mg surface with the cell culture medium. This is also in agreement with visual observations of the fast decrease of the H<sub>2</sub> evolution rate on the sample surface.

The finding that the total Mg release during 24 h in cell culture medium was higher for samples pretreated in SBF than for polished samples—in spite of the somewhat higher polarization resistance values of the SBF-treated samples than of the polished samples—suggests that the SBF-formed layers prevent the formation of the protective carbon-rich layer in cell culture medium. It seems that free access of the components of culture medium to Mg surface is required to form a highly corrosion-blocking surface coating and hence lead to a low Mg release; this is provided for the polished sample but not for sample previously soaked in SBF.

From the different findings, we conclude that one decisive factor for the cell adhesion is the corrosion rate upon exposure of cells to the Mg surface. If the initial corrosion rate is high, the cells are exposed to H<sub>2</sub> bubbling and pH increase in medium. This can explain the low cell densities on the polished Mg, where vigorous H<sub>2</sub> evolution takes place upon introducing the cell culture medium on the surface. The SBF-formed surface layer, even though somewhat reducing the initial corrosion rate as compared with the polished surface (as demonstrated by the impedance measurements), is not sufficiently protective: a strong color change of the medium is observed when introducing the cell culture medium on the samples, indicating that pH increase in the vicinity of the surface takes place. This is in line with previous findings in which the pH in cell culture medium was measured.<sup>24</sup> This effect is not observed for the samples treated in culture medium. Moreover, the impedance measurements indicate a strongly increased corrosion resistance after 24 h soaking of Mg in cell culture medium.

Clearly, the corrosion rate, surface chemistry, surface morphology, and cell behavior on Mg surfaces is highly dynamic and can dramatically change during the course of an experiment. This in contrast to high corrosion resistant biomaterials such as Ti alloys, where the surface properties such as morphology and chemical composition change only slightly during cell culture experiments. For further studies of cell-interactions with corroding Mg surfaces, a continuous monitoring of the Mg ion release, cell density and spreading area, and Mg surface properties need to be carried out.

## CONCLUSIONS

The corrosion rate of pure Mg in cell culture medium can be tailored by simple chemical surface treatments. The most efficient treatment for reducing Mg ion release is a spontaneous formation of a biomimetic Ca, P, and C containing layer in cell culture medium. This reduces the Mg release by 60% compared with nontreated, polished Mg surfaces

and leads to strongly enhanced cell adhesion and spreading on Mg surfaces.

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