Magnesium Technology 2014
Edited by: Martyn Alderman, Michele V. Manuel, Norbert Hort, and Neale R. Neelameggham
TMS (The Minerals, Metals & Materials Society), 2014

IMPROVEMENT OF CYTOCOMPATIBILITY OF MAGNESIUM ALLOY ZM21 BY SURFACE MODIFICATION

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Keywords: biodegradable metal, ZM21, cytocompatibility, silane-coupling, PLLA

Abstract

Application of a biodegradable polymer coating is one of the methods to improve the initial corrosion resistance and cytocompatibility of magnesium (Mg) alloys. However, bulging of the coating film during long term immersion has been reported. Therefore, improvement of interface strength between the coating and the substrate surface is a key for the success of this method. Combination of surface modification [silanization with 3-(glycidyloxypropyl) triethoxysilane (GPTES)] and biodegradable polymer coating [poly-L-lactide (PLLA)] were applied to a Mg-2.0Zn-0.98Mn (ZM21) cast alloy.
Results of a cell proliferation assay show that PLLA and GPTES+PLLA coating successfully improved cell growth during 7 days of incubation and suppressed Mg^{2+} release after 4 days of incubation. The silanization process had no impact on suppression of corrosion. Calcification was observed on all samples after 1 week of incubation with calcification medium, but the calcified area was much larger on the GPTES+PLLA coated sample than on the uncoated sample.

Introduction

Recently, Mg and its alloys have been investigated as biodegradable metallic materials for biomedical applications. Because of their excellent mechanical properties close to that of human bone[1], biocompatibility[1, 2] and biodegradability[3, 4], Mg is recognized as an almost perfect candidate for temporary bone implants. However, Mg and its alloys have relatively high bio-corrosion rates, especially at the early stage of implantation in the human body. These high corrosion rates cause formation of gas bubbles in the tissue surrounding the implanted samples due to hydrogen generation. Additionally, corrosion reaction of the samples also generates OH^-, which increases the pH of the fluid around the Mg sample surface. This increase in pH influences surrounding cellular functions, decreases cytocompatibility, and even causes inflammation or necrosis[1, 5]. For biomedical applications of Mg and its alloys, their degradation rates should ideally match the rate of healing. For this purpose, their initial corrosion resistance and cytocompatibility must be improved. Song and colleagues explored corrosion rates of several Mg alloys in Hanks solution, finally concluding that elements such as Ca, Mn, and Zn could be suitable alloying elements for biomedical Mg alloys[5]. Zn and Mn are both essential elements for human body; Mg-Mn-Zn alloys, which gradually degrade in bone tissue, have good biocompatibility both in vitro and in vivo[6]. One method to supress biodegradation of Mg alloys at the early stage of implantation in human body is application of a biodegradable polymer coating. An additional advantage of this method is that, in the case of biodegradable polymers such as poly (L-lactic acid) [7, 8] and poly (ε-caprolactone)[7], the coating degrades in the human body over time.
In industrial applications, organic coatings for Mg alloys are used to improve their corrosion resistance or mechanical properties[9]. For better understanding of the protective behavior of a biodegradable polymer coating, it is important to achieve the preparation of a good quality, uniform and nonporous film. This can be done by spin coating, which involves depositing a polymer solution onto a solid substrate, and afterwards spinning the substrate at a high speed. Because of rotation, the solvent evaporates and the polymer forms a homogenous and non-porous coating on the substrate. However, with longer immersion time, bulging of the coating film is observed[10]. To improve the strength of the interface between the Mg substrate and the biodegradable polymer coating, silanization is employed before the polymer coating. Silanes are silicon-containing chemical compounds possessing a hydrolytically sensitive centre which is able to react with inorganic substrates, forming covalent bonds. They also possess an organic substitution, which can be used to easily change the surface physicochemical properties of the substrates, such as hydrophobicity[11].
In the present study, silanization was employed to improve the interface strength between the Mg substrate and the biodegradable polymer, and effects of silanization on cell proliferation and substrate degradation were investigated.

Materials and Methods

Sample Preparation

We used a cast Mg alloy with main alloying elements of 2.0 wt% Zn and 0.98 wt% Mn, abbreviated as ZM21 (ZFW gGmbH, Clausthal) as a substrate. Its slabs were cut into a dimension of 10 x 5 x 1.5 mm. Then, the samples were polished with SiC grinding paper (14 μm) damped with 99.9% ethanol and then separately, ultrasonically cleaned with acetone for 15 min. Prepared surfaces were modified with silane 3-(glycidyloxypropyl) triethoxysilane (GPTES; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and then coated with a biodegradable polymer, poly-L-lactide (PLLA, Mw 300 000, Polysciences, Inc. USA). GPTES was spin-coated on both sides of the sample (7000 rpm, 60s). Then, silane was reacted with substrate by incubation at 100 °C for 1 h in a Teflon vessel. Excess silane molecules were removed by ultrasonic treatment for 15 min in absolute ethanol. Then, 2% (w/v) PLLA chloroform solution was spin-coated on both sides of the samples (7500 rpm, 60s). This sample is referred to as GPTES+PLLA. For comparison, unmodified samples (castZM21), samples with silane coating alone (GPTES), and those with only polymer coating

(PLLA) were prepared. For cell culture tests, these samples were sterilized with ethylene oxide gas (EOG).

Polymer Film Characterization

PLLA film morphology was observed using an optical microscope (Axiotech 100, Carl Zeiss AG, Germany) and a low voltage scanning electron microscope (FE-SEM, Hitachi SU8000). Polymer film thickness was measured by a stylus surface profiler (Dektak 6M, ULVAC, Japan) for a sample whose polymer film is partially removed. At least 3 samples were tested for each kind of polymer film. For these purposes, mirror-like polished samples were prepared with a diamond past (1/4 μm).

Water Contact Angle

Wettability of the sample surface was measured by a contact angle meter (DM 700, Kyowa Interface Science Co. Ltd, Japan), observing the shape of a distilled water droplet in static condition. Measurements were repeated 3 times for each sample. For every measurement, 21 readings were taken with an interval of 1000 ms at room temperature.

Cell Proliferation

Human osteosarcoma cell line (SaOS-2) was used for the cell proliferation assay testing castZM21, GPTES+PLLA, GPTES and PLLA samples. Each sample was placed into a deep glass dish. SaOS-2 were inoculated at a density of 4 000 cells ml^{-1} in 27.5 ml of culture medium [Dulbecco's Modified Eagle Minimum Essential Medium supplemented with 10% (v/v) fetal bovine serum, abbreviated as D-MEM+10%FBS]. Then, cells were incubated under standard cell culture condition (37 °C, 5% CO_2) for 1, 4 and 7 days. After a certain incubation period, viable cell number was estimated by WST-1 assay. Briefly, the sample and 1 ml of the supernatant were transferred into a well of a 24-well microplate. Another 1 ml portion of the supernatant was transferred to another well as a blank. Aliquots of the supernatant were collected for quantification of released Mg^{2+}. Then, a 110 μl portion of the mixture of WST-1 and 1-methoxy PMS in Dulbecco's phosphate buffered saline [PBS (-)] was added. After 4h of incubation, the absorbance of the supernatant was measured at 450 nm by a Multiskan FC Microplate Photometer (Thermo Scientific, USA). The relative viability of cells (RVC) was calculated as following equation;

$$RVC = (A_s - A_b)/S_0$$

where A_s and A_b are the absorbance of the supernatant in the sample and blank wells, respectively, and S_0 is the top surface area of the samples. Next, the samples were fixed with 25% glutaraldehyde solution for 10 min and stained by 10% (v/v) Giemsa's staining solution for 15 min. Air-dried samples were observed using an optical microscope, and digital images were recorded by a CCD camera (DS-5M, Nikon Co. Ltd, Tokyo, Japan). Cell culture experiments were performed in triplicate.

Calcification

To investigate the effect of surface modification on cell functionalization, a calcification assay was carried out for all types of samples. On a sample in a glass deep dish, SaOS-2 were inoculated at a density of 10 000 cells ml^{-1} in 27.5 ml of D-MEM+10% FBS and incubated for 7 days. Then, the culture medium was exchanged into calcification medium, consisting of D-MEM+10%FBS with 0.5 mM β-glycerophosphate, 50μg/mL L-ascorbic acid, and 1% (v/v) antibiotic solution [penicillin (5000IU/mL) and streptomycin (5000 μg/mL)]. Cells were incubated for 7 and 14 days while the calcification medium was exchanged in every 7 days. At the designated time, cells were fixed with Mildform 10 NM (ca. 4% formaldehyde in phosphate buffer containing 10% of methanol) for 20 min and stained with 0.5% (w/v) Alizarin Red S staining solution for 3 min. Air-dried samples were observed using an optical microscope and digital images were recorded by a CCD camera.

pH Measurement

CastZM21, GPTES, PLLA and GPTES+PLLA samples were sterilized by EOG, separately placed in glass deep dishes, and immersed in 27.5 ml of D-MEM+10% FBS in a CO_2 incubator for 24h to monitor the pH of the medium. The distance between the pH electrode and the specimen surface was kept at 1 mm.

Quantification of Mg^{2+}

The degradation of the substrate during the cell proliferation assay was examined by the quantification of the released Mg^{2+} ions. Portions of the supernatant from each of the deep dishes were collected after 1, 4 and 7 days of incubation. The quantification of released Mg^{2+} ions was performed using the Magnesium B test Wako (Wako Pure Chemical Industries, Ltd, Osaka, Japan) by following the instruction supplied with the kit except the preparation of the calibration curve of Mg^{2+}, which was performed with D-MEM+10%FBS instead of water.

Results

Polymer Film Characterization

After the polymer spin-coating process, PLLA and GPTES+PLLA sample surfaces were investigated by optical microscopy. However, no crystalline structure was observed on the transparent polymer film. SEM observation showed a smooth and flat surface without any pores. The polymer film thickness is about 0.2 μm.

Water Contact Angle

The water contact angle was measured shortly after sample preparation. Obtained data shown in Figure 1 illustrates the influence of surface modification on wettability. The angles for castZM21, GPTES and PLLA samples are approximately 62°, 60° and 75°, respectively. The PLLA coating decreases hydrophilicity down to the level favourable for cell proliferation[12], but silanization increases hydrophilicity slightly. The values were stable with time, except at the beginning of the measurements for the castZM21 sample.

pH Changes

Table I lists the pH of D-MEM+10%FBS with castZM21, GPTES, PLLA or GPTES+PLLA sample in a CO_2 incubator. The pH values of the medium with and without a sample tended to decrease due to the dissolution of CO_2 into the medium and dissociation to be HCO_3^- for buffering, which occurred in the first

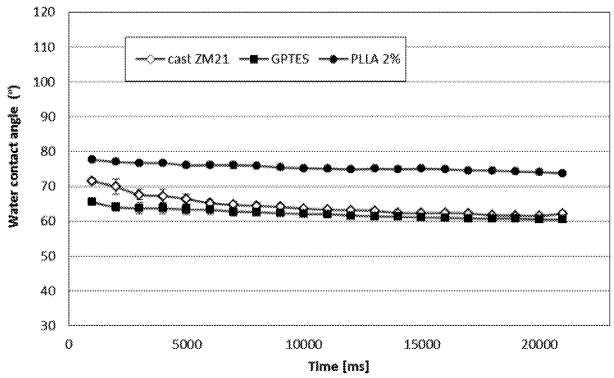

Figure 1. Time evolution of water contact angles of castZM21, GPTES, PLLA and GPTES+PLLA samples.

Table I. The pH of the D-MEM+10%FBS for castZM21, GPTES, PLLA.

	pH				
	Medium	**castZM21**	**GPTES**	**PLLA**	**GPTES+PLLA**
1h	8.49	8.60	8.25	8.54	8.48
4h	7.86	7.97	7.56	7.90	7.87
24h	7.02	7.76	7.43	7.60	7.15

hours of the measurements. After 24h, the pH value was the lowest in the medium without a sample. For medium with a castZM21 sample, decrease of pH was noticeably slower than in the medium with a PLLA, GPTES, or GPTES+PLLA sample.

Cell Viability

Results of the WST-1 assay for castZM21, GPTES, PLLA and GPTES+ PLLA samples are shown in Figure 2. There was no significant difference among samples after 1 day of incubation. On day 4, a slight increase in the number of cells was observed for PLLA and GPTES+PLLA samples. However, no statistically significant difference can be found ($p>0.05$). After 7 days of incubation, it becomes to be clear that the PLLA and GPTES+PLLA coating successfully improved cell growth comparing to castZM21 and GPTES; this effect is more visible for GPTES+PLLA. Moreover, on day 7, statistically significant increase in cytocompatibility can be found ($p\leq0.001$), both between castZM21 - GPTES+PLLA and GPTES - GPTES+PLLA samples.

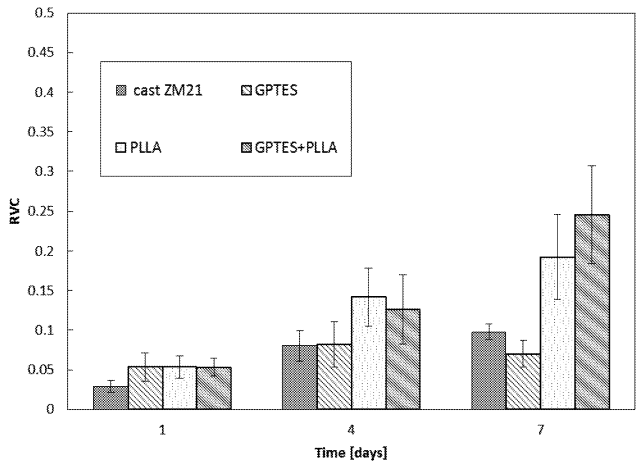

Figure 2. Proliferation of SaOS-2 cells on cast ZM21 substrate without/with silane, polymer, silane and polymer.

Cell Morphology

Optical microscopic images of castZM21, GPTES, PLLA and GPTES+PLLA samples after cell culture for 1 and 7 days are shown in Figure 3, indicating different responses of the cells to different samples. After 7 days, almost no cells were found on castZM21 and GPTES samples. For PLLA and GPTES+PLLA, elongated and spread cells were observed, but more cells are visible for GPTES+PLLA. The microstructure of the castZM21 substrate is observed after 1, 4 and 7 days of incubation (photos not shown). White corrosion products were observed on castZM21 and GPTES surfaces. In comparison with these samples, some part of the substrate underneath the polymer coating kept metallic gloss for PLLA and GPTES+PLLA samples. Other parts became black and were recognized as corroded area. White corrosion products were also observed underneath the polymer film. However, after 7 days of incubation, no polymer detachment was observed for PLLA and GPTES+PLLA samples.

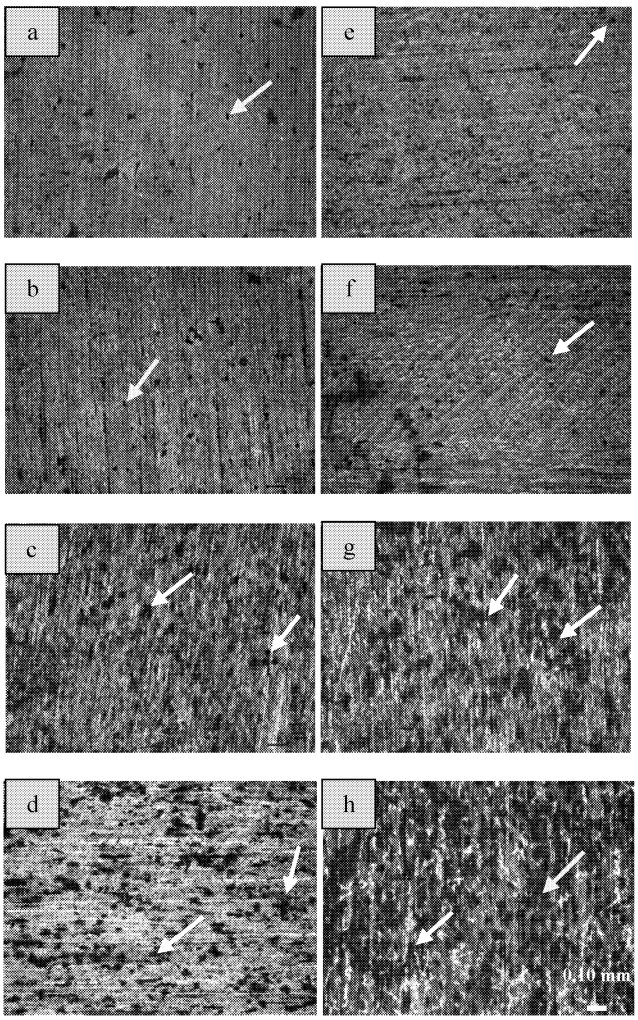

Figure 3. Typical cell morphology after incubation (indicated by white arrows) for 1 day on a) castZM21, b) GPTES, c) PLLA, d) GPTES+PLLA and 7 days on e) castZM21, f) GPTES, g) PLLA, h) GPTES+PLLA.

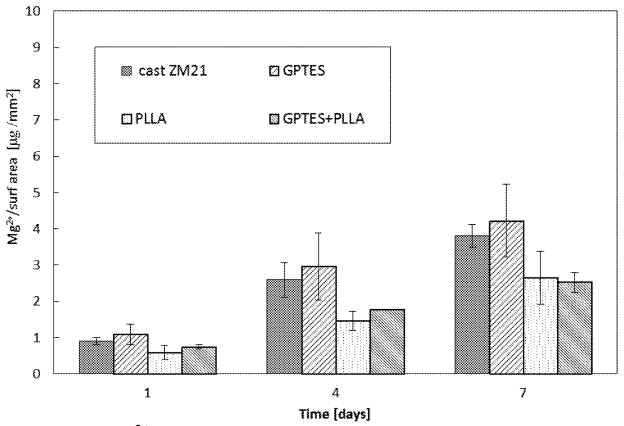

Figure 4. Mg^{2+} release for castZM21, GPTES, PLLA and GPTES+PLLA.

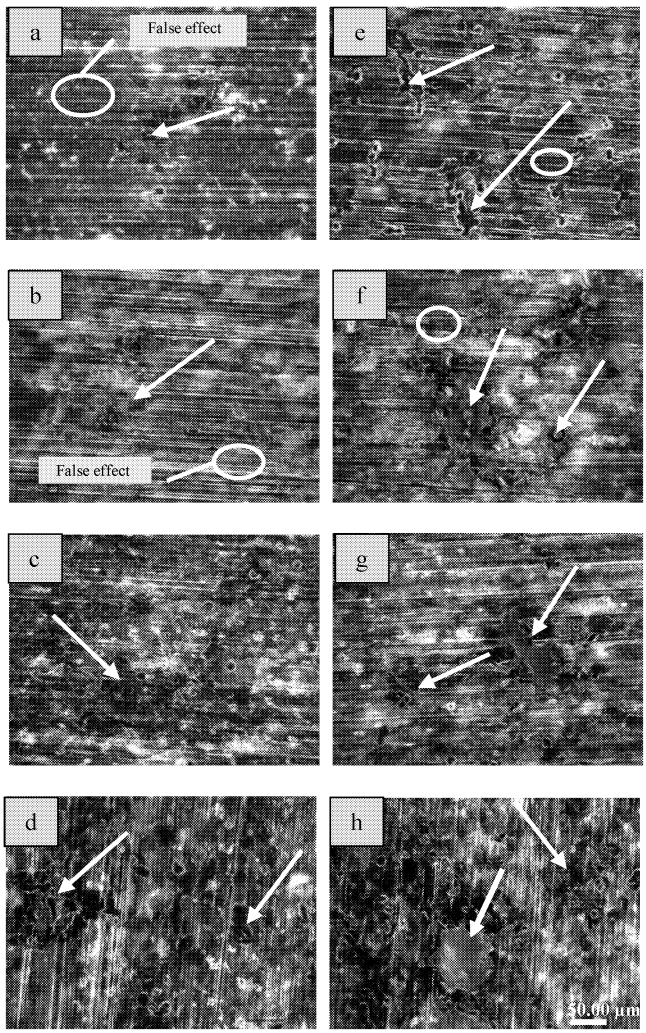

Figure 5. Typical morphology of calcified areas (indicated by white arrows) for 1 week on a) castZM21, b) GPTES, c) PLLA, d) GPTES+PLLA, and 2 weeks on e) castZM21, f) GPTES, g) PLLA, h) GPTES+PLLA.

Mg^{2+} Release

Mg^{2+} release from the samples during cell culture is presented in Figure 4. Ion release was observed even after 1 day of incubation for all samples, however, no statistically significant difference can be found ($p>0.05$). Along the incubation period, the amount of released Mg^{2+} increased for all samples. The lowest value was observed for PLLA, followed by GPTES+PLLA, castZM21, and GPTES. Statistically significant difference ($p<0.05$) was noticed between GPTES and PLLA on day 4, and between GPTES – PLLA and GPTES – GPTES+PLLA on day 7.

Cell Functionalization

The calcification process was examined on castZM21, GPTES, PLLA and GPTES+PLLA samples for 1 and 2 weeks of incubation with the calcification medium. It is difficult to see actual colours of the samples in a gray-scale, so they are marked in Figure 5. Pink and reddish color on castZM21 and GPTES samples indicates mostly false reaction of Alizarin Red S staining with corrosion products on the surface (circled in white). However, dark purple areas marked on Figure 5 for 1 week and brownish regions, mostly observed for 2 weeks, indicate calcified areas (white arrows). Notably, a much larger calcified area was observed on PLLA and GPTES+PLLA samples than on castZM21 and GPTES samples.

Discussion

Microscopic observation of the PLLA sample before cell culture clearly shows that spin-coated PLLA film is smooth, flat and without pores. Previous studies report poor adhesion, even film bulging, between polymer coatings and Mg substrate [10, 13], which may reduce device efficiency[13, 14]. Xu et al. mentioned the bulging of PCL film prepared on pure Mg substrate after 7 days' immersion in D-MEM+10%FBS[15]. However, this effect was not observed for low and high molecular weight PLLA (50 000 and 80 000-100 000, respectively) film prepared by spin-coating at 5 000 rpm resulting in the thickness of 0.34 and 0.97 µm, respectively[15]. In this study, PLLA coating film was prepared with higher molecular weight PLLA (300 000) by spin-coating at 7 000 rpm, generating a film of 0.2 µm thickness. No bulging was observed during 7 days of incubation in DMEM+10%FBS and an additional 7 or 14 days of incubation in the calcification medium. It is necessary to measure the adhesion strength between the polymer film and the Mg substrate for accurate determination of the strengthening effect of GPTES.

Mg^{2+} release during 1, 4 and 7 days of incubation clearly shows that surface modification influences the ZM21 substrate degradation under the cell culture condition. Silanization with GPTES increased Mg^{2+} release, but PLLA and GPTES+PLLA suppressed Mg^{2+} release from 1 day of incubation. However, PLLA has a slightly better protective effect than GPTES+PLLA in 1 and 4 days of incubation. This result suggests that PLLA film somewhat suppresses substrate corrosion, but GPTES may accelerate substrate corrosion. The PLLA film with 0.2 µm in thickness cannot stop the water molecules penetrating into and reacting with the ZM21 substrate. This reaction releases hydrogen, which diffuses through the polymer film. GPTES layer prepared on the ZM21 substrate surface causes a slight, but definite, reduction in the water contact angle at the beginning of the measurement, as shown in Figure 1. This result indicates a slightly increased affinity for water molecules, which might facilitate the

water-substrate reaction at the beginning of immersion into the culture medium and influence localized microcorrosion under the PLLA coating. When we compare Figure 3c and d, which show typical PLLA and GPTES+PLLA sample surfaces after 1 day of incubation in the culture medium, metallic gloss underneath the polymer film is more noticeable for GPTES+PLLA as well as black areas recognized as corroded area. For PLLA, substrate corrosion seems to be more uniform. This difference is also observed after 7 days of incubation in the culture medium. For further characterization of substrate corrosion, SEM observation of substrate surface following the removal of the polymer film is necessary and will be performed in the near future.

Attachment of anchorage dependant cells is a first step in the process of cell-material interactions. Surface physicochemical properties such as hydrophilicity and surface charge are well known to influence cell-material interaction. Hydrophilicity of samples can be easily changed by surface modification. Witecka and colleagues increased water contact angle of cast AZ91 alloy by silanization with 5 different silanes from approximately 30° to 54–100°[16]. In this study, applied surface modification also influences surface hydrophilicity. As already mentioned, GPTES slightly decreases water contact angle of castZM21 from approximately 62° to 60°. PLLA coating decreases hydrophilicity to 75°, which is favourable for cell proliferation[12]. Also pH values in Table I revealed that the medium above PLLA and GPTES+PLLA samples during the initial 24h of immersion has lower pH than that above castZM21, which is attributed to the protective effect of the polymer coating. Xu and colleagues reported that initial pH of the medium directly influences the cell survival, and the number of living cells in the initial stage is associated to the later cell growth[10]. Results of the cell proliferation assay for SaOS-2 after 1 day of incubation indicated slight differences in cell viability. During 7 days of incubation, PLLA and GPTES+PLLA coating successfully improved cell growth. However, the improvement of cytocompatibility is more visible for GPTES+PLLA samples. Surfaces of castZM21 and GPTES seem to suppress cell growth. Besides decreasing hydrophilicity, PLLA and GPTES+PLLA provide necessary conditions for cell attachment and growth. As other corrosion protective coatings, PLLA acts as a barrier and reduces water diffusion rate [17]. As already mentioned, it suppresses the corrosion of castZM21 substrate and may decrease the diffusion rate of OH^- from the substrate to the medium. However, GPTES+PLLA samples had slightly higher Mg^{2+} release than PLLA in the early stage of incubation, indicating more visible improvement of cytocompatibility. Probably, released Mg^{2+} can somehow encourage cell growth since Mg^{2+}, which is an essential element for human, has various physiological functions relating to the cellular energy metabolism as well as bone tissue metabolism.

The next stage in biocompatible studies is to investigate the effect of surface modification on cell functionalization. After 1 week of incubation in the calcification medium, SaOS-2 cells exhibit osteblastic differentiation. In Figure 5, calcified areas are observed after 1 week of calcification but become more viable after 2 weeks of calcification. This result can be correlated with that of cell proliferation assay. PLLA and GPTES+PLLA samples with higher cell growth in cell proliferation assay had larger calcified area than GPTES and castZM21. This suggests that better cytocompatibility in the initial stage of incubation supports better cell functionalization in the later stage of incubation. For quantification of relative osteogenic activity on the unmodified and modified samples, measurement of alkaline phosphatase (ALP) activity will be performed in near future.

Conclusions

Cast ZM21 magnesium alloy surface was modified by silane followed by the coating with a biodegradable polymer. Water contact angle measurements suggest that the silanization slightly changed the surface hydrophilicity, but the biodegradable polymer coating decreased hydrophilicity to a level known to be favourable for cell proliferation. Cell culture experiments show that, among the samples tested, GPTES+PLLA was the best at improving cell growth during 7 days of incubation, followed by PLLA. Surface modification reduced the Mg^{2+} release for PLLA and GPTES+PLLA samples. The silanization process has no impact on corrosion suppression, but may improve of cell viability for GPTES+PLLA.

Acknowledgements

This work was supported by the European Regional Development Fund within the Innovative Operational Programme in the frame of project BIO-IMPLANT (GRANT No. POIG.01.01.02-00-022/09).

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