On the biodegradability, mechanical behavior, and cytocompatibility of amorphous Mg$_{72}$Zn$_{23}$Ca$_{5}$ and crystalline Mg$_{70}$Zn$_{23}$Ca$_{5}$Pd$_{2}$ alloys as temporary implant materials

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Abstract: The evolution of microstructure and mechanical properties of almost fully amorphous Mg$_{72}$Zn$_{23}$Ca$_{5}$ and crystalline Mg$_{70}$Zn$_{23}$Ca$_{5}$Pd$_{2}$ alloys during immersion in Hank’s balanced salt solution (HBSS), as well as their cytocompatibility, are investigated in order to assess the feasibility of both materials as biodegradable implants. Though the crystalline Mg$_{70}$Zn$_{23}$Ca$_{5}$Pd$_{2}$ sample shows lower wettability and more positive corrosion potential, this sample degrades much faster upon incubation in HBSS as a consequence of the formation of micro-galvanic couples between the nobler Pd-rich dendrites and the surrounding phases. After 22-h immersion, the concentration of Mg ions in the HBSS medium containing the Mg$_{70}$Zn$_{23}$Ca$_{5}$Pd$_{2}$ sample is six times larger than for Mg$_{72}$Zn$_{23}$Ca$_{5}$. Due to the Zn enrichment and the incipient porosity, the mechanical properties of the Mg$_{72}$Zn$_{23}$Ca$_{5}$ sample improve within the first stages of biodegradation (i.e., hardness increases while the Young’s modulus decreases, thus rendering an enhanced wear resistance). Cytocompatibility studies reveal that neither Mg$_{72}$Zn$_{23}$Ca$_{5}$ nor Mg$_{70}$Zn$_{23}$Ca$_{5}$Pd$_{2}$ are cytotoxic, although preosteoblast cell adhesion is to some extent precluded, particularly onto the surface of Mg$_{70}$Zn$_{23}$Ca$_{5}$Pd$_{2}$, because of the relatively high hydrophobicity. Because of their outstanding properties and their time-evolution, the use of the Pd-free alloy in temporary implants such as screws, stents, and sutures is envisioned. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 2013:101A:502–517, 2013.

Key Words: magnesium, biodegradation, mechanical properties, metal ion release, cytotoxicity

INTRODUCTION

The need for temporary implants such as plates, screws, pins, stents, and sutures has recently prompted a renewed interest and, in turn, stimulated a lively research activity in the field of biodegradable materials. Polymers and metals have been advocated as potential bioabsorbable implants. Polymers and metals have been advocated as potential bioabsorbable implants. 1, 2 Compared with polymers (the poly-l-lactic acid (PLLA) is by far the most studied), metals show superior mechanical properties (e.g., higher strength to bulk ratio) which make them the material of choice in many applications, for example for replicating the properties of SS316L—the reference material for coronary stents 3—or as internal bone fixation screws/pins. 4 Among the metallic candidates, Mg-based alloys have captured a great deal of attention because of their outstanding properties such as appropriate corrosion rates in physiological media and Young’s modulus close to that of bone tissue ($E_{\text{bone}} = 3-20$ GPa). Pure Mg implants had been tested in the 1940s as bone plates and screws but were ultimately abandoned mostly due to the generation of hydrogen gas during in vivo implantation. 5 Alloymi Mg with suitable elements has been demonstrated as an effective means to minimize or even block hydrogen gas release while improving the mechanical properties of the base metal. Furthermore, the corrosion rates of pure Mg, which are exceedingly large in physiological conditions, are reduced. In this line, Mg alloys including Mg-Al-Zn (e.g. AZ31), Mg-Al-Zn-Mn (e.g. AZ91D), Mg-Zn-Mn, and rare-earth (RE) containing alloys (e.g. WE43 and LAE442) have been

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investigated as re-absorbable bone implants in rats and guinea pigs with rather great success. Yet, some of them contain aluminum and/or RE elements that preclude their medical use in clinical patients. Al is known to have adverse effects on neurons and osteoblasts and has indeed been linked to dementia and Alzheimer’s disease, while RE might lead to hepatotoxicity. Hence, there is currently a need for Mg alloys free from Al, RE or other heavy metals that show good biocompatibility and acceptable degradability rates in physiological conditions. Some investigators have shown that binary Mg-Zn and ternary Mg-Zn-Ca alloys are two of the most promising candidates. Compared with other alloying elements, which could cause serious adverse effects when released into the blood serum, zinc and calcium are rather harmless. Zinc is an essential trace element to the body and is vital to keep a healthy immune system, whereas calcium is present in large amounts in bone tissues.

Examples of either fully crystalline or amorphous (i.e., metallic glass) alloy compositions have been reported for Mg-Zn and Mg-Zn-Ca families. Because of the lack of dislocations, metallic glasses can exhibit mechanical properties that are often better than those encountered in their crystalline counterparts. In addition, they typically show improved corrosion performance due to both their chemical homogeneity and the absence of grain boundaries. In a previous work we studied the mechanical properties of Mg72Zn23Ca5 (2 ≤ x ≤ 6) alloys for use in biomedical applications. It was found that alloying Pd with Mg-Zn-Ca glassy alloy gave rise to changes in the mechanical performance of the material in the as-cast state. The addition of 2 at% Pd was particularly favorable in terms of improving hardness and wear resistance. However, the biodegradability of the newly developed alloys was not explored in detail, neither the evolution of the mechanical properties of these materials upon immersion in bodily solutions. Furthermore, the cytotoxicity remained mostly overlooked. This is a serious concern when it comes to Pd since, compared with other metals, the available information on the release and accumulation of Pd into the body is rather scarce. Pd biocompatibility has been analyzed mostly in relation to dentistry application where it is used for dental inlays, crowns and bridges, usually mixed with other materials (gold, silver or copper and zinc). Several reports have described oral symptoms (inflammation of gingival and periodontal tissues) in individuals exposed to dental material containing Pd. Similarly, skin allergy has been reported in persons after ear-piercing with jewels containing Pd. Other detrimental effects in Pd-sensitized patients include: a specific Interferon (IFN)-γ production in peripheral blood mononuclear cells (PBMC) challenged with a Pd compound, an increase of Pd concentration in biological fluids in patients with dental prosthesis, and skin sensitization to Pd. Scarce articles have tested the toxicity of Pd in vitro. One of them demonstrated that pure Pd metal did not decrease cell viability after 24 h in contact with human fibroblasts-keratinocytes cocultures, while another reported that Pd produced a 50% of cell mortality in mouse fibroblast cultures at a concentration of 0.3 mM. Hence, the eventual release of Pd into the body from biodegradable Pd-containing alloys poses some risk. The goal of the present work is to assess the feasibility of Mg72Zn23Ca5 and Mg72Zn23Ca5Pd2 alloys as temporary implants by focusing on two aspects: (i) biodegradability in a simulated body fluid, including electrochemical characterization, solution pH evolution with time, concomitant monitoring of release of metal ions to the adjoining medium and evolution of the mechanical properties, and (ii) mouse preosteoblast cells adhesion onto alloy surfaces and cytocompatibility. The present study provides baseline information to fathom out the mechanisms responsible for in vitro biocorrosion of Mg72Zn23Ca5 and Mg72Zn23Ca5Pd2 alloys and gives some insights regarding their cytotoxicity in a physiological environment.

MATERIALS AND METHODS

Materials

Ingot alloys were obtained by melting a mixture of pure elements (≥99.9 at. %) of Mg, Zn, Ca and Pd in an induction furnace. Rod samples with diameter of 2 mm and up to 20 mm length were obtained by remelting the master alloy in a quartz tube and subsequent injection into copper mould in an inert gas atmosphere. For most experiments (corrosion, mechanical testing, cytotoxicity) the disks were about 1 mm thick. They were ground with SiC paper up to 4000 grit and then thoroughly rinsed in ethanol and distilled water prior to the experiment.

Structural characterization

The structure of the samples was studied by X-ray diffraction (XRD) (Philips X’Pert) with monochromated Cu Kα radiation (30–90° 20 range, counting time: 10 s, step size: 0.030°). The microstructure was observed with a scanning electron microscope (SEM) (Zeiss Merlin) equipped with energy dispersive X-ray (EDX) analysis. The surfaces of the samples after being immersed in Hank’s balanced salt solution (HBSS, purchased from Aldrich) were observed both before and after removal of the corrosion products. For removal of the corrosion products, the specimens were soaked in a CrO3 solution (180 g/L) for a few minutes, and afterwards thoroughly rinsed with ultrapure water followed by ethanol and finally dried with nitrogen gas.

Contact angle measurements

To assess the wettability of the alloy surfaces, the contact angle of aqueous drops (<5 μL) deposited onto the surface of the alloys was measured using a contact Angle Measuring System DSA 100 from KRÜSS at room temperature (sessile drop technique). Both HBSS and ultrapure (MilliPore MilliQ) water were tested.

Electrochemical measurements

Electrochemical experiments were performed in a thermostatted, one-compartment three-electrode cell filled with 50 mL HBSS at 37°C. A double junction Ag|AgCl reference electrode was used with 3M KCl inner solution and 1M NaCl outer solution. A Pt sheet acted as a counter electrode. Initially, the specimens (3.14 mm2 exposed area) were immersed in the HBSS to determine the open circuit potential (OCP). The potential became usually stable after 3 h of...
immersion. Immediately afterwards, the potential was swept at a rate of 0.1 mV s\(^{-1}\) from 300 mV below the OCP towards 300 mV above the OCP. The corrosion current density \(i_{\text{corr}}\) values were determined by extrapolation of the anodic and cathodic Tafel slopes to the corrosion potential \(E_{\text{corr}}\). Four replicas per sample were tested.

**Metal cations release and pH evolution**

In order to evaluate the time-dependent release of metal cations from the alloy samples and pH evolution, disk samples were introduced in sterilized plastic containers filled with 45 mL of HBSS (free from CaCl\(_2\) and MgCl\(_2\)) from Invitrogen. The containers were then sealed and placed in a thermostatized bath at 37°C. The pH was monitored with a pH-meter from Metrohm. At certain time intervals aliquots of 5 mL HBSS were pipetted off and placed in tube tests for inductively coupled plasma-optical emission spectroscopy (ICP-OES) measurements. Both tests (metal cations release and pH evolution) were extended over 22 h.

**Mechanical characterization**

The dependence of mechanical properties of the two alloy samples with the immersion time in HBSS was evaluated by nanoindentation at room temperature in the load control mode. Prior to the initial test (i.e., before immersion of the samples in HBSS), the specimens were polished until the two flat surfaces of the disks exhibited a mirror-like appearance. Then, the disks were immersed in HBSS for different times. After removing the samples from the HBSS bath the disks were not polished again (i.e., polishing was only carried out once, before the immersion experiments), but the corrosion products were chemically etched away using the CrO\(_3\) solution.\(^{15,22}\) Nanoindentation tests of the as-cast and immersed specimens were performed on one of the two faces of the disks using a diamond Berkovich-type tip. The indentation function consisted of a loading segment of 150 s, to a maximum load of 500 mN, followed by a load holding segment of 50 s and an unloading segment of 150 s. The thermal drift was kept below 0.05 nm s\(^{-1}\).

The results shown in this work correspond to the average of a total of 30 indentations per sample. The hardness \((H)\) and reduced elastic modulus \((E_r)\) values were derived from these load-displacement curves using the method of Oliver and Pharr,\(^{23}\) while the wear resistance was estimated as the ratio between \(H\) and \(E_r\), \(H/E_r\). Proper calibrations for the instrument compliance, tip blunting, thermal drift and initial penetration depth were applied. It should be noted that \(E_r\) takes into account the elastic contributions from both the sample and the indenter tip. However, due to the high Young’s modulus of the indenter tip, \(E_r\) is usually very close to the Young’s modulus of the investigated sample.\(^{24}\)

**Cytotoxicity studies**

Mouse preosteoblasts (MC3T3-E1, ATCC) were maintained in α-minimum essential medium (Invitrogen) supplemented with 10% foetal bovine serum in standard conditions (37°C, humidified atmosphere and 5% CO\(_2\)). Sample disks were first glued individually onto glass coverslips using silicone, and the coverslips introduced into a 4-multiwell culture plate. Samples were sterilized by ultraviolet light for at least 2 h. Preosteoblasts were then seeded into each well (75,000 cells/well) and cultured for 4 and 8 h. Control cells were seeded directly on the glass coverslip in the absence of the alloy and cultured also for 4 and 8 h. Alloy cytotoxicity was evaluated by detecting the activity of intracellular esterases, using the live/dead Viability/Cytotoxicity Kit for mammalian cells (Invitrogen) according to the manufacturer’s protocol. This kit allows distinguishing live cells (showing green color because of their esterase activity) from dead ones (presenting red color because of permeability of their damaged plasma membrane to propidium iodide). Samples were analyzed under an Olympus IX71 inverted microscope equipped with epifluorescence. Several images from different regions of each sample were captured and live/dead cells were counted (minimum 400 cells per experiment). Differences in the number of live/dead cells between the experimental groups were analyzed using the Fisher test. Statistically significance was considered when \(p < 0.05\).

**Cell adhesion analysis**

After the cell viability assay, control cells and cells grown onto disk alloys were processed for SEM analysis. Cells were rinsed twice in 0.1M sodium cacodylate buffer (SCB), fixed in 2.5% glutaraldehyde in 0.1M SCB for 45 min at room temperature and rinsed twice in SCB. Cell dehydration was performed in a series of ethanol washes (50, 70, 90, and two 100%) for 8 min each. Dehydrated samples were then dried using a substitute for critical point drying: 50% of hexamethyl disilazane (HMDS) in absolute ethanol followed by 100% of HMDS. Finally samples were mounted on stubs and examined using the SEM (Zeiss Merlin) equipped with EDX analysis.

**RESULTS**

**Microstructural characterization**

Figure 1(a,c) shows the electron back-scattered (EBS) SEM images of Mg\(_{72}\)Zn\(_{23}\)Ca\(_5\) and Mg\(_{70}\)Zn\(_{23}\)Ca\(_5\)Pd\(_2\) alloy samples, respectively. While the former is mostly featureless and just a few scratches from the polishing procedure are seen, a clear microstructure consisting of graded-contrast features is observed in the latter. Such a drastic change in the microstructure is ascribed to the addition of Pd to the alloy, which is known to decrease the glass-forming ability. The corresponding XRD patterns [see Fig. 1(b,d)] are in agreement with the EBS images. Namely, a broad halo centered at around 2θ = 39° is observed for the Mg\(_{72}\)Zn\(_{23}\)Ca\(_5\) sample. This confirms the mainly amorphous nature of the Pd-free alloy. Conversely, several reflections appear in the XRD pattern of the Mg\(_{70}\)Zn\(_{23}\)Ca\(_5\)Pd\(_2\) sample. These peaks match the angular positions expected for hexagonal CaZn\(_6\), hexagonal MgZn, cubic MgPd, and trigonal Mg\(_6\)Zn\(_2\)Ca\(_5\) phases.\(^{25}\) The local compositional variations within each region of the Mg\(_{70}\)Zn\(_{23}\)Ca\(_5\)Pd\(_2\) sample were evidenced by EBS observations. Figure 2 shows a representative EDX mapping corresponding to a central area of the Mg\(_{70}\)Zn\(_{23}\)Ca\(_5\)Pd\(_2\) disk.
Apparently, Pd is concentrated within the bright dendrites, which are thus attributed to Mg₆Pd. Instead, Ca and Zn elements mainly contribute to the grey and black zones surrounding the bright dendrites, together with Mg element. Hence, the hexagonal CaZn₅ and MgZn phases and the trigonal Mg₂Zn₃Ca₂ phase are presumably located in the regions surrounding the dendrites.

**Electrochemical and contact-angle measurements**

Figure 3(a) shows representative potentiodynamic polarization curves for Mg₇₂Zn₂₃Ca₅ and Mg₇₀Zn₂₃Ca₅Pd₂ alloys. Though the $j_{corr}$ values are comparable (1.7 mA cm⁻² and 2.1 mA cm⁻², respectively), $E_{corr}$ for the Mg₇₀Zn₂₃Ca₅Pd₂ alloy is shifted 110 mV towards more anodic values, thus suggesting a delayed onset of material degradation in physiological conditions. Indeed, it has been demonstrated that the formation of superhydrophobic surfaces onto magnesium alloys improves their corrosion resistance.²⁹ Several treatments have been pursued to this end, such as anodic oxidation²⁷ or chemical vapor deposition.²⁸ As a result of such treatments, an $E_{corr}$ shift towards more positive values is typically observed.²⁹ Figure 3(b,c) shows photographs of droplets of HBSS deposited onto the surfaces of Mg₇₂Zn₂₃Ca₅ and Mg₇₀Zn₂₃Ca₅Pd₂ alloys. The measured contact angles are 86.7° and 102.7°, respectively. A similar trend was found when depositing ultrapure water droplets, that is, a higher contact angle is measured for Mg₇₀Zn₂₃Ca₅Pd₂ (100.4°) compared with the Pd-free sample (82.7°). Although Mg-based and Mg-containing crystalline materials and, in general, multiphase crystalline alloys typically exhibit poorer corrosion resistance compared with their homologous amorphous counterparts, there are some examples in the literature where the opposite trend is observed.³⁰,³¹ In this study, not only the microstructure changes from amorphous to crystalline but also the chemical composition varies. Hence, both the evolution from a single amorphous structure to a multiphase crystalline alloy and the change in the nominal chemical composition need to be taken into account.

**pH dependence and metal cations release on immersion time**

In order to get a better understanding on the degradation performance of the alloy samples, as-polished disk specimens were immersed into HBSS at 37°C under nonpolarized conditions. Figure 4 shows the time evolution of HBSS pH for the two samples. An increase in the bulk pH of the solution was observed in both cases, though higher values were measured for the Pd-containing alloy from the very beginning. The alkalinization effect stems from the release of OH⁻ ions due to the oxidation of Mg metal to Mg²⁺ according to the following reaction:

\[
\text{Mg} + 2\text{H}_2\text{O} \rightarrow \text{Mg}^{2+} + 2\text{OH}^- + \text{H}_2
\]  

After 21.5 h, the pH readings were 7.93 and 8.58 for the Mg₇₂Zn₂₃Ca₅ and Mg₇₀Zn₂₃Ca₅Pd₂ incubated samples, respectively. Though it has been reported that local pH at sample surface may differ in several pH units compared with bulk pH,³² these results point to a higher corrosion rate of the Pd-containing alloy from the very beginning. The alkalinization effect stems from the release of OH⁻ ions due to the oxidation of Mg metal to Mg²⁺ according to the following reaction:

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such apparent contradiction between the results from immersion experiments (non-polarized conditions) and the electrochemical data (polarized conditions) can be understood on the basis of micro-galvanic couples originating between the nobler Mg-Pd phase and the surrounding Mg-Zn matrix that leads to selective dissolution of phase boundaries in the Mg70Zn23Ca5Pd2 alloy. Such a selective etching spreads all over the sample ultimately leading to its rapid disintegration. Figure 5(a,b) shows the SEM morphologies of the corroded surfaces after incubation of the alloys in HBSS for 2 h. While the Mg70Zn23Ca5Pd2 sample displays a rougher surface and, apparently, larger fraction of corrosion products, the Mg72Zn23Ca5 alloy shows more uniform corrosion morphology, similar to that observed in other Mg-Zn-Ca alloy compositions. Differences in the surface morphology for the two investigated samples are also observed after selective etching of the corrosion products. As shown in Figure 5(c), immersion of the Mg72Zn23Ca5 alloy in HBSS for 2 h already causes certain surface corrugation as a result of the dealloying process. The effects are much more pronounced in the Mg70Zn23Ca5Pd2 sample [Fig. 5(d)], where deep pores with sizes in the micrometer range are seen. This large porosity is closely related to the occurrence of micro-galvanic couples, which causes severe dissolution of Mg metal.

The effects of dealloying have been carefully investigated by assessing the levels of metal cations released from the alloy disk samples. Figure 6 shows the concentration of Mg, Zn, Ca (and Pd) ions in the HBSS extraction medium as a function of the immersion time. First of all, the concentration of Mg cations released from the Pd-containing sample notoriously increases from 4 h onwards and at 21.5 h of immersion is six times higher than for the Mg72Zn23Ca5 sample, which confirms its faster degradation. The release of Zn ions from Mg70Zn23Ca5Pd2 also surpasses the levels of the Mg72Zn23Ca5 sample. Remarkably, no Pd was detected in the aliquots pipetted off from the Mg70Zn23Ca5Pd2-containing HBSS medium. This means that Pd is not released into the solution.

![Figure 2](image-url)  
**FIGURE 2.** EDX mapping of a central region of Mg70Zn23Ca5Pd2 disk sample. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

![Figure 3](image-url)  
**FIGURE 3.** (a) Potentiodynamic polarization curves of Mg72Zn23Ca5 and Mg70Zn23Ca5Pd2 alloys in HBSS at 37°C. Photographs of as-deposited HBSS droplets onto the surface of (b) Mg72Zn23Ca5 and (c) Mg70Zn23Ca5Pd2 alloys. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
but rather remains in the metallic form. These results reinforce the hypothesis that micro-galvanic couples exist in the Mg$_{70}$Zn$_{23}$Ca$_5$Pd$_2$ sample. The compositional analyses, performed by EDX on the alloy samples, as a function of HBSS immersion time (see Table I) are in agreement with the faster dissolution rate of Mg compared with the other constituent elements. The disintegration of the Mg$_{70}$Zn$_{23}$Ca$_5$Pd$_2$ alloy in a short period of incubation (around 4 h) seriously precludes its use in hard-tissue repair. This means that mechanical integrity of the implant is spoiled before the tissue has sufficient time to heal the bone.

Mechanical properties

Shown in Figure 7(a,b) are the dependences of the hardness, $H$, and the reduced Young’s modulus, $E_r$, on the immersion time in HBSS for the Mg$_{72}$Zn$_{23}$Ca$_5$ and Mg$_{70}$Zn$_{23}$Ca$_5$Pd$_2$ alloys. Results from macroscopic compression experiments (not shown) revealed that the two investigated alloys are rather brittle but confirmed that the Young’s modulus of Mg$_{72}$Zn$_{23}$Ca$_5$ is lower than for Mg$_{70}$Zn$_{23}$Ca$_5$Pd$_2$. Interestingly, while $E_r$ tends to simply decrease with the immersion time in both cases, the dependence of $H$ is more complex, particularly in the case of Mg$_{72}$Zn$_{23}$Ca$_5$. For this alloy, $H$ steeply increases after short-term immersion, decreases for longer times and increases again, but only slightly, for immersion times longer than 16 h.

From a tribological point of view, it is known that the wear resistance of a given material not only depends on its hardness (harder materials typically exhibit a higher wear resistance) but also on the reciprocal of the Young’s modulus. Actually, the elastic strain to failure, which is related to the $H/E_r$ ratio measured by nanoindentation, has been shown to be a suitable parameter to estimate the wear resistance, even more than the hardness itself.$^{34,35}$ Figure 7(c,d) reveals that larger $H/E_r$ are observed for the amorphous Mg$_{72}$Zn$_{23}$Ca$_5$ alloy than for the crystalline Mg$_{70}$Zn$_{23}$Ca$_5$Pd$_2$ sample.
Remarkably, a peak in $H/E_r$ is observed after 2h of immersion (due to the increase of hardness) and this ratio again increases for immersion times longer than 16h, indicating that the wear resistance shows a nonmonotonic behavior during corrosion of the Mg72Zn23Ca5 alloy. Conversely, due to its high corrosion rate and concomitant large porosity, the crystalline Mg70Zn23Ca5Pd2 alloy practically only exhibits a decrease in $H/E_r$ with immersion time.

Cytotoxicity analysis

The potential toxic effect of Mg72Zn23Ca5 or Mg70Zn23Ca5Pd2 alloys on preosteoblasts cultures was analyzed through the live/dead Viability/Cytotoxicity Kit. For each sample, fluorescence images of cells growing on top of the disk and of cells growing on top of the coverslip were taken (Fig. 8). No significant differences were found in the number of live cells between cultures in the presence of Mg72Zn23Ca5Pd2 alloy and control cultures after 4 or 8 h of incubation, neither when comparing control cultures with cultures in presence of Mg72Zn23Ca5 after 8 h of incubation (Fig. 8). In all cases more than 93% of cells were alive regardless of the presence or absence of the alloy. However, for both alloys and time-points tested, fewer cells were found growing on top of the disks than on the coverslips. Whereas after 8 h of culture many cells were still attached to the Mg72Zn23Ca5 disk, and 59.9% were still alive, very few cells were attached to the Mg70Zn23Ca5Pd2 disk and most of them were dead. In fact, very few live cells were seen on the top of Mg70Zn23Ca5Pd2 after 4 h of culture.

Cell adhesion studies

Adhesion studies of cells growing on top of the coverslips and of cells growing on top of the disks were also carried out. Figure 9 shows the morphology and adhesion degree of cells growing on top of the coverslip at 4 and 8 h. After 4 h in culture, some control cells were completely attached to the coverslip and thus presented a flat morphology, whereas others appeared to be in the process of attachment. Cell confluence was about 60–80% depending on the area. Completely and partially attached cells were also observed on the coverslip of culture containing the Mg70Zn23Ca5Pd2 disk; preosteoblasts appeared healthy with the only difference that cells were covered by debris of soft appearance. After 8 h of culture, almost all control cells were completely attached to the coverslip, and in a large number of cells nucleoli could be observed in their nuclei (Fig. 9). The morphology of cells attached to the coverslip of cultures in presence of Mg72Zn23Ca5 or Mg70Zn23Ca5Pd2 at 8 h was similar to that of cells from the control culture, also presenting nuclei with several nucleoli. Soft debris was also observed in cultures in presence of Mg72Zn23Ca5 and in lower quantity in the presence of the Mg70Zn23Ca5Pd2 alloy. The cell density was similar in all cultures analyzed after 8 h and was equivalent to that seen after 4 h of culture.

The morphology and adhesion degree of cells growing on top of the alloy disks at 4 and 8 h can be seen in Figure 10. Cells adhered to the Mg70Zn23Ca5Pd2 disk after 4 h

![Figure 6. Mg, Ca, Zn (and Pd) elements concentration in 5-mL aliquots withdrawn from the HBSS extraction medium at different periods of immersion for (a) Mg72Zn23Ca5 and (b) Mg70Zn23Ca5Pd2 disk samples at 37°C. The inset in (a) shows a zoomed detail of the concentration profiles. Volume corrections have been applied to account for the successive withdrawn of aliquots from the test solution. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE I. Summary of Compositional Results, Obtained Using EDX, on the Mg72Zn23Ca5 and Mg70Zn23Ca5Pd2 Samples in Their As-Cast States and after Being Immersed in HBSS for 2 and 50 h (the Latter Only for Mg72Zn23Ca5)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mg</th>
<th>Zn</th>
<th>Ca</th>
<th>Pd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg72Zn23Ca5 as-cast</td>
<td>72.0</td>
<td>23.0</td>
<td>5.0</td>
<td>–</td>
</tr>
<tr>
<td>Mg70Zn23Ca5Pd2 as-cast</td>
<td>70.0</td>
<td>23.0</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Mg70Zn23Ca5 2 h HBSS</td>
<td>63.5</td>
<td>32.0</td>
<td>4.5</td>
<td>0</td>
</tr>
<tr>
<td>Mg70Zn23Ca5Pd2 2 h HBSS</td>
<td>62.0</td>
<td>31.5</td>
<td>4.5</td>
<td>2.0</td>
</tr>
<tr>
<td>Mg70Zn23Ca5Pd2 50 h HBSS</td>
<td>50.5</td>
<td>46.0</td>
<td>3.5</td>
<td>–</td>
</tr>
</tbody>
</table>

For such analyses the corrosion products were previously removed using a CrO3 solution. Data reported has been normalized to 100% after neglecting the O content (around 2%).

AMORPHOUS Mg72Zn23Ca5 AND CRYSTALLINE Mg70Zn23Ca5Pd2 ALLOYS

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culture [Fig. 10(a,b)] were difficult to distinguish because of the large amount of debris. The disk had visible cracks and cells were completely covered by debris, which looked more compact than that covering the cells grown onto the coverslips. Protrusions that seemed to correspond to a cell were analyzed using EDX analysis. Such protrusions displayed larger amounts of carbon than the surroundings, hence confirming the presence of cells [Fig. 10(e,f)]. After 8 h of culture, many healthy-looking cells were found attached to the disk of Mg72Zn23Ca5 alloy, but most of them were not firmly attached. Cells were also covered with soft debris [Fig. 10(c,d)]. After 8 h of culture, the Mg70Zn23Ca5Pd2 alloy disk was completely fractured, and only small fragments could be seen scattered on top of the coverslip with no cells adhered to them.

DISCUSSION

Wettability and corrosion

A correlation is usually observed between the wettability of a given material and its anticorrosion performance. Materials with more hydrophobic character are typically more resistant against corrosion in aqueous environments. This accounts for crystalline as well as for amorphous solids.36 The wettability can be determined by means of the sessile drop technique from the measurement of the contact angle between a liquid drop and the polished surface of the studied material. In a first approximation, if the wetting is non-reactive, the Young's equation correlates the contact angle with the existing interfacial tensions:

$$\cos \theta = \frac{\sigma_{SV} - \sigma_{SL}}{\sigma_{LV}}$$

where $\sigma_{SV}$, $\sigma_{SL}$ and $\sigma_{LV}$ are the interfacial tensions between solid-gas, solid-liquid and liquid-gas phases, respectively. Our results indicate that the crystalline Mg70Zn23Ca5Pd2 alloy exhibits markedly larger contact angles than the amorphous Mg72Zn23Ca5, in spite of their close composition. Although the wettability of metallic glasses can vary depending on the existing amount of free volume frozen in the glassy structure during the casting,37 it has been shown that, quite often, metallic glasses are more hydrophilic (lower contact angles) than crystalline materials with the same composition.38,39 Since $\sigma_{LV}$ depends mainly on the liquid used for the sessile drop experiments, its value can be regarded as constant. Hence, the change in contact angle between amorphous and crystalline structures of the same composition has to be attributed to variations in $\sigma_{SV}$ and/or $\sigma_{SL}$. It has been argued that since the structure of metallic glasses is metastable, their atomic activity is rather high and this would tend to favor the spreading of the liquid drop on the solid surface, hence decreasing $\sigma_{SL}$ with respect to the value in the crystalline alloy.38,39 In turn, due to the
high atomic activity and the presence of not-fully bonded states at the surface of metallic glasses, the value of $\sigma_{SV}$ could be higher than for the crystalline alloy. Consequently, the value of $\cos \theta$ would turn out to be larger for the amorphous structure [see Eq. (2)], resulting in a lower contact angle. It should be pointed out that in some highly corrosive Ca-based metallic glasses, suitable surface treatments, such as coating with appropriate materials (e.g., fluoroalkylsilane or pure Fe films) has been reported to result in a significant enhancement of hydrophobicity and
improvement of the corrosion resistance. In this sense, the nobler character of Pd, as compared with Mg or Ca, could assist to provide an effective anti-corrosion protection of the Mg\textsubscript{72}Zn\textsubscript{23}Ca\textsubscript{5}.

Aside from the wettability, one would expect instead the crystalline Mg\textsubscript{70}Zn\textsubscript{23}Ca\textsubscript{5}Pd\textsubscript{2} alloy to show more cathodic $E_{\text{corr}}$ and larger $j_{\text{corr}}$ values than the amorphous Mg\textsubscript{72}Zn\textsubscript{23}Ca\textsubscript{5} if their microstructure is considered. Actually, when dissimilar metallic phases are in intimate electrical contact in a corrosive environment, the less noble metal is preferentially corroded at the expense of the more noble metal. This phenomenon is termed galvanic corrosion and could in principle develop in the Mg\textsubscript{70}Zn\textsubscript{23}Ca\textsubscript{5}Pd\textsubscript{2} alloy. However, the occurrence of galvanic corrosion can be retarded if the material gets covered by a protective passive layer, which acts as efficient barrier to corrosion. In such a case, the resulting $E_{\text{corr}}$ is typically near to that of the more noble metal (or phase). Hence, the shift in $E_{\text{corr}}$ with the addition of Pd to the alloy is, to some extent, to be expected and is in agreement with the sessile drop technique measurements. However, if just the $E_{\text{corr}}$ values are taken into account, one can underestimate the hazards of galvanic attack. Indeed, the $j_{\text{corr}}$ values retrieved by Tafel extrapolation of the potentiodynamic polarization curves can be

**FIGURE 10.** SEM images showing the morphology and adherence of cells growing on top of alloy disks (direct analyses). (a) Cells attached to the Mg\textsubscript{70}Zn\textsubscript{23}Ca\textsubscript{5}Pd\textsubscript{2} disk after 4 h in culture, detail in (b). (c) Cells attached to the Mg\textsubscript{72}Zn\textsubscript{23}Ca\textsubscript{5} disk after 8 h in culture, detail in (d). The EDX analyses of the zones indicated with “1” and “2” in (b) are displayed in panels (e) and (f), respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
regarded as "instant" values; that is, they do not reflect the changing nature of corrosion rate with time. Results from immersion experiments in the HBSS indicate a higher corrosion rate of the Pd-containing sample that seems inconsistent with the previous electrochemical polarization measurements and contact angle tests. To understand this apparent contradiction, one should bear in mind that in a potentiodynamic polarization test, both cathodic and anodic reactions are artificially accelerated, which induces a significant disturbance of material surface. This has been demonstrated to be particularly tricky in Mg-based alloys due to the so-called "abnormal polarization behavior". This implies that the polarization curve might not necessarily follow the Tafel equation; henceforth, using the Tafel extrapolation method or the Stern-Geary equation to calculate the \( j_{\text{corr}} \) values can provide biased data. There is another feature in the polarization curves shown in Figure 3(a) that further proves the vulnerability of Mg\(_{70}\)Zn\(_{23}\)Ca\(_{5}\)Pd\(_2\) sample. The anodic branch corresponding to this sample is especially noisy, or at least is noisier than the curve corresponding to Mg\(_{72}\)Zn\(_{23}\)Ca\(_{5}\). This can be attributed to the slow scan rate used (0.1 mV/s), which makes the potential to be forced away from the steady value for extended periods, thereby allowing significant corrosion to occur. A proposed scheme of the natural biodegradation process (i.e., under non-polarized conditions) for the Mg\(_{70}\)Zn\(_{23}\)Ca\(_{5}\)Pd\(_2\) alloy is depicted in Figure 11. When the sample is immersed in the HBSS, preferential anodic dissolution of the Mg-Zn matrix occurs at the expense of the Pd-containing dendrites, so that the release of Mg\(^{2+}\) ions (and of Zn\(^{2+}\) to a less extent) into the medium is accompanied by intense hydrogen gas evolution [Fig. 11(a,b)]. This brings about the formation and progress of cracks across the disk sample. The divalent ions precipitate onto the sample surface forming a Mg(OH)\(_2\) (and Zn(OH)\(_2\)) passive layer, and their formation is promoted as the pH of the HBSS increases [Fig. 11(c)]. However, the chloride anions of the HBSS make the passive layer extremely vulnerable and cause its rapid solubilization [Fig. 11(d)]. The protective layer is thus progressively thinned and the release of the ions coming from the less noble regions of the sample (i.e., Mg\(^{2+}\), Zn\(^{2+}\) and Ca\(^{2+}\)) is increased [Fig. 11(e)]. At some point, the disk completely breaks due to mechanical disintegration rendering nondissolved Pd-containing fragments as final products [Fig. 11(f)].

Mechanical properties

Although the evolution of the mechanical properties with time during in vitro or in vivo experiments using biodegradable implants is of upmost importance for the applicability of such materials, this issue has been only investigated in some detail in bioabsorbable polymers but has been largely overlooked in biodegradable metallic alloys. The results presented in Figure 7 show that the hardness in Mg\(_{72}\)Zn\(_{23}\)Ca\(_{5}\) increases by roughly 50% during the first 2 h of immersion, whereas that of Mg\(_{70}\)Zn\(_{23}\)Ca\(_{5}\)Pd\(_2\) mainly decreases with time, although a modest increase is also observed after immersing the alloy for a few minutes in the HBSS. The observed increase of \( H \) after short-term immersion could be mainly attributed to the fast dissolution of Mg into the HBSS. Indeed, the dissolution rate of Mg is faster than that of Zn and Ca (see Fig. 6), therefore an enrichment in Zn occurs at the surface of both materials as they get progressively corroded [see Fig. 12(a) and Table I]. Since the hardness of Zn is almost
twice that of Mg, this could therefore induce a mechanical hardening effect. However, for longer immersion times the dissolution process is such that both alloys not only undergo surface chemical change but also a physical modification with the formation of corrugations and pores (Fig. 5). When these physical flaws become large enough, their softening effect can compensate the hardening caused by Mg dissolution and the hardness decreases.

The lowering of reduced Young’s modulus is probably related also to the increase of porosity as the samples get progressively dealloyed, as evidenced in Figure 12(b,c) for Mg72Zn23Ca5 and in Figure 5(d) for Mg70Zn23Ca5Pd2. Indeed, it is well known that the porosity level has a strong influence on the measured values of Young’s modulus. In a first approximation, if the porous material is treated as an open-cell metallic foam, the Young’s modulus, \( E_{\text{porous}} \), can be described as follows:

\[
E_{\text{porous}} = C_1 E_{\text{bulk}} \left( \frac{\rho_{\text{porous}}}{\rho_{\text{bulk}}} \right)^2
\]

where \( E_{\text{bulk}} \) is the Young’s modulus of the bulk, nonporous metal, \( C_1 \) is a geometry constant close to 1 and \( \rho_{\text{porous}}/\rho_{\text{bulk}} \) is the relative density of the foam.

Remarkably, while both \( H \) and \( E_r \) decrease rather fast in the Mg70Zn23Ca5Pd2 alloy (after 2 h both parameters deteriorate considerably), in the Mg72Zn23Ca5 alloy relatively large values of \( H \) and \( E_r \) are retained even after 30 h of immersion. This can be understood bearing in mind the faster degradation rate (and larger degree of porosity) in Mg70Zn23Ca5Pd2 as compared with the Mg72Zn23Ca5 alloy [cf. Figure 5(d) and 12(b)].

At this point it should be noted that enrichment in Zn may also bring about an increase of \( E_r \) since the Young’s modulus of Zn is higher than that of Mg. This is not observed experimentally, probably because the effects of porosity overcome any eventual increase of \( E_r \). It is worth mentioning that while the \( H/E \) ratio tends to slightly decrease with the porosity level in ceramic materials, the opposite trend is often encountered in metals. In our case, \( H/E_r \) increases from around 0.053 (in as-cast Mg72Zn23Ca5) to ~0.1 (in Mg72Zn23Ca5 immersed in HBSS for 2 h), thus indicating that the effect of porosity on the Young’s modulus after short-term immersion is more pronounced than on hardness, which is in agreement with what is observed in many other metallic alloys.

Despite the large porosity level, the Young’s modulus of the Mg72Zn23Ca5 alloy does not decrease drastically, even after long-term immersion. Actually, the value at 50 h immersion time (\( E_r \approx 37 \text{ GPa} \)) is very close to that of human bone. In turn, \( H \) tends to slightly increase again after long-term immersion in HBSS. Such trends can be ascribed to the densification effect that occurs in this alloy during nanoindentation, particularly when the porosity level is sufficiently high [see Fig. 12(c)]. Densification is known to cause an increase of the compressive yield stress and could also slow down the decrease in \( E_r \). The dissolution rate of Mg, which is particularly high for long immersion times [see Figs. 6(a) and 12(a)], could also contribute to the observed increase in \( H \).

Figure 13 compares the mechanical properties (compressive yield stress, \( \sigma_{\text{yc}} \), Young’s modulus, \( E \), and resilience, which is proportional to \( \sigma_{\text{yc}}^2/E \)) of different families of materials suitable for bioabsorbable implants, including biodegradable polymers, metallic alloys and ceramics. Mechanical hardness is directly proportional to \( \sigma_{\text{yc}} \), that is,
H ≈ C σ_{y,C} (where C is the so-called constraint factor and is usually around 3 for crystalline metallic alloys and a bit larger for metallic glasses\(^{53}\)). Figure 13(a) reveals that Mg-Zn-Ca bulk metallic glasses exhibit rather large values of yield stress, thus suggesting that these alloys are amongst the hardest biodegradable materials so far reported in the literature. In turn, Mg-Zn-Ca glassy alloys exhibit Young’s modulus values closer to that of cortical bone (\(E_{\text{bone}} \approx 3-20\) GPa) than most synthetic hydroxyapatites or crystalline Mg-based alloys (which are also mechanically softer). Taking into account that Mg\(_{70}\)Zn\(_{23}\)Ca\(_{5}\)Pd\(_{2}\) is fully crystalline, its hardness is also much larger than that of most Mg-Zn-Ca crystalline alloys. Mg-based bulk metallic glasses are also harder than most Fe-based biodegradable alloys, which have the additional drawback that they are often ferromagnetic at room temperature (except FeMn, which is antiferromagnetic), hence precluding the use of nuclear resonance imaging techniques for diagnostics purposes. After long-term immersion in HBSS, the Young’s modulus of Mg\(_{72}\)Zn\(_{23}\)Ca\(_{5}\) approaches that of Ca-based or Sr-based biodegradable metallic glasses, as well as that of polymeric materials reinforced with glassy fibers. At the same time, the Mg\(_{72}\)Zn\(_{23}\)Ca\(_{5}\) alloy remains mechanically harder than all these materials.

Resilience is another important mechanical parameter in materials intended to be used as biological implants. This property refers to the ability of a given material to absorb energy when it is deformed elastically and then release it upon unloading (i.e., the maximum energy per unit volume that can be elastically stored in the material, without causing any plastic deformation) and it is determined as the ratio \(\sigma_{y,C}^2/2E\). Biomaterials such as articular cartilage or intervertebral disks require high mechanical resilience. Figure 13(c) reveals that Mg-Zn-Ca bulk metallic glasses show rather high values of resilience, similar to Ca-based metallic glasses or some biodegradable polymers, particularly those reinforced with glassy fibers. The high resilience of Mg-Zn-Ca glasses, as compared with their homologous crystalline alloys, stems from their rather high yield strain.

**Cytotoxicity and cell adhesion**

Cytotoxicity can be measured indirectly, that is, culturing cells in the presence of a solution that has been previously in contact with a specific material, or directly, growing the cells on top of the specific material.\(^{56}\) In this work, cells were seeded in wells of 2 cm\(^2\) where the alloy (3.14 mm\(^2\)) was glued on top of a coverslip with nearly the same area of the well. Cells were distributed randomly onto the alloy disk and the coverslip, allowing to carry out indirect and direct analyses at the same time. Cells growing on the coverslip were exposed to metal cations released from the corroded alloy but were not directly in contact with it (indirect studies), whereas cells growing on the alloy surface were exposed to the corroded material and in close contact with the material (direct studies).

Since the corrosion rate of the alloys tested was very different, cytotoxicity analyses were carried out at different time-points depending on the rate. For the Pd-free alloy, with a slow corrosion rate, cell adhesion and viability was...
of surface wettability. For the Mg72Zn23Ca5 alloy the contact angle of the Pd-containing alloy was even more hydrophobic than the Mg72Zn23 alloy, in agreement with those reported by Schmalz et al.20, who did not observe any negative effect in the viability of fibroblast-keratinocyte cells cultured onto pure Pd during 24 h.

When direct cultures (cells grown onto the alloy) were analyzed, significant differences were found in viability and cell adhesion between both alloys and between these and control cultures. The total number of cells observed (8 h culture) on top of the Mg72Zn23Ca5Pd2 alloy was lower than over the coverslip in the presence of the same alloy. Moreover, the number of dead cells was higher on the alloy than on the coverslip. This decrease in the number of cells was observed both in fluorescence and SEM images. The Pd-free alloy also presented fewer cells than the corresponding coverslip, and the cells were not completely adhered to the surface, as both round cells and fusiform cells could be observed. It has been reported that one of the most important items to be considered when culturing cells is the hydrophobicity of the surface on which the cells are grown.58,59 Osteoblast adhesion increases with an increase of surface wettability. For the Mg72Zn23Ca5 alloy the contact angle is around 87° and it has been shown that it is insufficient to allow a good cell spreading.60 Thus, some cells remained attached, but not spread, after 8 h of culture and some probably died because they could not be attached.

These differences are much more dramatic when cells grown over Mg72Zn23Ca5Pd2 alloy are analyzed. The number of cells observed on top of Pd-containing alloy was already scarce at 4 h of culture and most of them were dead. At 8 h the alloy had disintegrated into pieces and it was very difficult to find a piece with cells faced up. The small fragments that were analyzed presented a high number of dead cells. No fragments with attached cells were found when analyzing the alloy by SEM, but that could be due to the protocol used to fix and dehydrate cells, which involves several solution changes. If the contact angle of the Mg72Zn23Ca5 alloy was too hydrophobic for the growth of cells, the contact angle of the Pd-containing alloy was even more hydrophobic, around 103°, and according to Wei and collaborators60 the adherence is very poor in these surfaces. In fact, the Pd-containing alloy prevents osteoblast adhesion, as few cells found on the alloy surface (4 h culture) maintained a round morphology and no signs of spreading were observed. Another issue that hinders the adhesion of cells to the substrate is the roughness of the sample.58,61 As mentioned before, the Pd-containing alloy is more prone to corrosion than the Pd-free alloy, i.e., after incubating both alloys in HBSS for 2 h the former displays a rough surface with roughness of the order of micrometers (see Fig. 5) whereas the latter shows a more uniform corrosion. In addition, the Pd-containing alloy disintegrates into pieces after 4 h of culture. It has been described that osteoblast adhesion and proliferation is progressively inhibited when roughness (micropore size from 200 nm to 8 μm) is increased.81 According to the same authors, surface discontinuities produced in this case by the track-etched pores could prevent cell adhesion. In round-shaped cells stretching is inhibited because no focal adhesions can be formed.

Cell adhesion to the extracellular matrix or to a surface is a complex process mediated by transmembrane proteins called integrins, which cluster to form focal adhesions. Several authors have described the induction of anoikis (a form of apoptosis) when cells are prevented to adhere. For instance, when human intestinal epithelial cells are kept in suspension, 90% of the cells activate anoikis after 3 h.62 On the other hand, it has been shown that hydrophobic and rough materials, such as the Mg72Zn23Ca5Pd2 alloy, prevent integrin clustering and the formation of focal adhesion complexes due to the spatial conformation of the adsorbed molecules in the material.59 Thus, we believe that the increase in dead cells found after 8 h in culture in the presence of the Mg72Zn23Ca5Pd2 alloy is not due to alloy toxicity but to the hindrance of osteoblasts adhesion onto the alloy surface, which may cause osteoblasts to undergo apoptosis after 8 h in culture.

CONCLUSIONS

In this work, a rational framework to understand the mechanisms responsible for the biodegradation of amorphous Mg72Zn23Ca5 and crystalline Mg72Zn23Ca5Pd2 alloys in a physiological environment is provided. Moreover, apparent inconsistencies between different characterization tests that might lead to dissimilar conclusions are outlined and thoroughly discussed. It is shown that the microstructural characteristics of the materials (i.e., amorphous vs. crystalline) bring about differences at various levels (wettability, degradation rate, mechanical properties, preosteoblast cell adhesion, etc.). Due to the occurrence of micro-galvanic couples between the crystalline phases, the Mg72Zn23Ca5Pd2 alloy degrades too fast in physiological conditions. Conversely, Mg72Zn23Ca5 shows a slower degradation rate and an improvement of the mechanical properties within the first hours of immersion in the bodily fluid, which makes this particular alloy certainly appealing for use as temporary implants such as screws, stents and sutures. In any case, none of these two alloys are cytotoxic when preosteoblast cells are cultured in their presence, though cell adhesion

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onto alloy surfaces is significantly better in the amorphous Pd-free alloy due to its lower hydrophobicity. Appropriate surface plasma treatments could be performed to increase the hydrophilic character of the Pd-containing alloy. The present article demonstrates that the crystallographic nature of two alloys with a very similar composition clearly determines their biodegradation and cell compatibility, a result that can be used as a valuable roadmap in future works.

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