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Considerations about sterilization of samples of pure magnesium modified by plasma electrolytic oxidation

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ABSTRACT

The use of Magnesium as biomedical implant has increased in recent years due to its ability to reabsorb in the body without causing adverse effects. For this kind of application, sterilization method should be considered to ensure the appropriate performance of the implant. The present study focused on the influence of different sterilization techniques such as steam autoclaving, dry-heating (170 °C), UV irradiation and steam formaldehyde on surface characteristics, composition and wettability of commercially pure Mg anodized in a silicate solution. Characterization of the samples was done by SEM, EDS, and XRD. Surface free energy was calculated by contact angle measurement. Changes in biological activity were assessed *in vitro* as a hemolysis assay. Our results showed that UV treatment generated only minor surface changes, but that irregularly shaped surface pores might prevent full penetrance and sterilization of the assessed materials. Dry-heat induced cracks on the surfaces and formaldehyde affected the surface morphology. Despite autoclave was the treatment that showed the highest changes in the surface energy, it did not induce structural surface changes and therefore it was considered as the choice option for the sterilization of Mg samples.

1. Introduction

During the past years, the use of magnesium (Mg) in the biomedical field has increased significantly [1–5]. Its high biocompatibility and the fact that it is degradable, rendered magnesium a promising and versatile option for transient implants [6–8]. In contrast, the chemical element Mg is highly reactive and upon its oxidation causes an increase of pH and releases hydrogen, which are cytotoxic. After implantation, increased pH and hydrogen may affect the stability of the implant and also of the surrounding tissue [9–11]. This problem can be managed by generation of a protective coating that decreases the rate of degradation of the base material. Plasma electrolytic oxidation (PEO) is a technique in which the material is oxidized in a controlled way [12–15]. The PEO generates an oxide/hydroxide Mg layer which by itself degrades slowly and also reduces the degradation of the underlying pure Mg. Previous research suggests that this surface modification as a versatile way to improve the corrosion properties of the Mg and currently is used in different medical devices [16–18]. Since Mg is highly reactive in aqueous solutions, it is important to assess the influence of the sterilization process on the material, in particular those that employ steam (autoclaving). The aim of this work was to show the influence of the most commonly used sterilization processes on the integrity of the PEO coatings formed on commercial pure magnesium (c.p Mg), looking to understand how this can lead changes at the biological level.

2. Material and methods

2.1. Anodization of samples

Square samples of c.p. Mg (99.9%) of 1 cm × 1 cm and a thickness of approximately 1 mm were mechanically polished with silicon carbide paper 1000 grade. All specimens were cleaned in water for a few seconds with the purpose of removing particulate material from the polishing process and then degreased with acetone in an ultrasonic bath for 30 min. After that, samples were anodized in a two-electrochemical cell where the c.p Mg was set as the anode and a stainless steel beaker was

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used as cathode. The experiment was carried out under potentiostatic mode at 320 V during 600 s by using a DC power supply (Kepco BHK 500–0.4 MG). As electrolyte solution, 0.0352 M $NaSiO_3 \cdot 9H_2O$/0.07 M KOH was used. The voltage data were recorded electronically by Labview 8.1 software (National Instruments) interfaced with a personal computer. After anodizing, the specimens were removed immediately from the electrolyte; subsequently, they were rinsed with water and dried at room temperature. AFM analysis was performed in order to verify the morphology and roughness of the obtained coating.

2.2. Sterilization protocols

Samples were sterilized by using four techniques: Autoclaving (A), dry-heat (DH), UV-irradiation (UV) and formaldehyde (F) under the following conditions:

- Steam Autoclaving (A): 121 °C during 45 min at 1 bar (CISA Autoclave Model: 4270).
- Dry-heat (DH): 170 °C for 1 h after the desired temperature is reached
- UV-irradiation (UV): Samples were exposed to UV–light (40 W·cm^{-2}) in a biosafety cabinet during 1 h per side and in total 2 h per sample.
- Steam Formaldehyde (F): 55 °C, 0.35 bar during 6–7 h. (CISA Autoclave Model: 4270).
- A set of samples without sterilization were used as a control.

2.3. Characterization of the samples

After the respective sterilization, each group of samples was observed by SEM to identify changes in the surface morphology and in the cross-sections. Changes in the composition of the coating were initially studied by EDS in which the samples are stimulated with electrons and the outgoing spectrum gives the composition and the percentage of each element. As an additional technique, XRD was performed for analysis of the crystal structure of the material, which was compared against a database of known structures.

2.4. Wettability

The contact angle of the samples before and after sterilization was measured. For this procedure, a drop of deionized water was dispensed on each sample then the contact angle was measured with the help of a Goniometer/Tensiometer Ramé-hart Model 250 Standard. The measurement was performed in triplicate, in three different samples and the average of the all measurements was calculated. Those values were used to calculate the surface energy (γ_S) by the numerical iterations and using the Neuman method described by the (Eq. (1)), where $\beta = 0.0001247$ (m^2/mJ)^2, $\gamma_L = 72.8$ mJ·m^{-2} and θ (contact angle)

$$\cos\theta = 2\left(\frac{\gamma_S}{\gamma_L}\right)^{0.5} \exp[-\beta(\gamma_L-\gamma_S)^2] - 1 \quad (1)$$

2.5. Hemolysis test

Healthy human blood from volunteers was collected in a flask containing sodium citrate (0.109 M, 3.2%) in a ratio 9:1. Then, the blood was diluted with a saline solution in a ratio of 4:5. After that, samples were dipped in tubes containing 10 ml of saline solution and incubated at 37 °C for 30 min. Then, 0.2 ml of diluted blood were added to each tube, mixed carefully and incubated at 37 °C for 60 min. After that, tubes were centrifuged at 3000 rpm for 5 min. Supernatants were removed and transferred to a plate in order to do spectroscopic analysis at 545 nm. Normal saline solution was used as a negative control and deionized water as positive control. The hemolysis was calculated based on the Eq. (2):

$$Hemolysis = \frac{OD(test) - OD(negative\ control)}{OD(positive\ control) - OD(negative\ control)} \times 100 \quad (2)$$

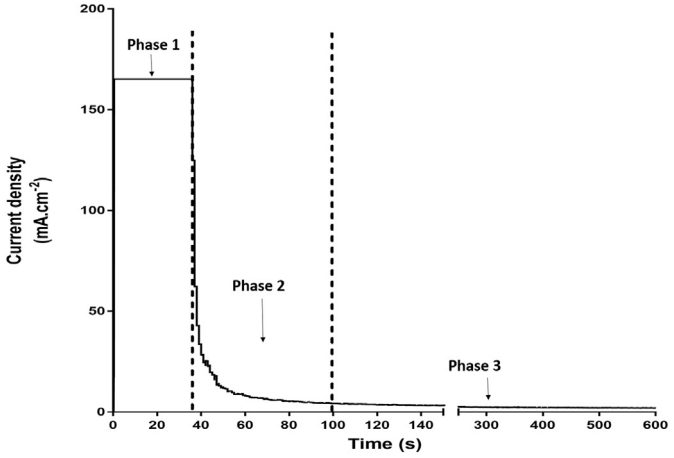

Fig. 1. Anodizing curves of the anodizing process of the c.p. Mg in silicate solution. Voltage-time curve (left) and current density-time curve (right).

3. Results

3.1. Characterization of the sample

The samples were treated under potentiostatic mode in a solution base of silicate. According to the current density-time curve we observed that at the beginning of the process, the system reached the maximal value of 166 mA·cm^{-2} due to the low resistance of the system (given for the substrate and electrolytic solution) and after 36 s, in response to the growing of the barrier layer, the current dropped down drastically until 60 mA·cm^{-2}. Then, the current decreased slowly during 60 s until it reached a value of around 2 mA·cm^{-2} and finally the current remained constant (Fig. 1).

The anodized samples were analyzed by AFM to assess the morphology of the coating and its roughness. The Rq was 166.0 ± 10.4 nm Fig. 2.

Once the materials were anodized, these were sterilized and the composition of the coatings was analyzed by XRD and EDS.

Additionally, the EDS analysis indicated as expected that in addition to magnesium and oxygen, Si was present also in a lower fraction (12–15%). The silicate ions (SiO_3^{2-}) get incorporated from the electrolyte into the film during anodization, under the effect of the electric field and facilitated with the formation of channels in the anodic film, as a product of the occurrence of sparks. The only variation observed in the chemical composition of the treated surfaces was an increment of the oxygen content for the sample exposed to UV radiation. EDS chemical composition of the coatings is showed in Table 1.

Results from XRD (Fig. 3) showed the crystalline composition of all samples. All diffractograms showed peaks corresponding to MgO besides to signals from the Mg substrate. The diffuse background observed at approximately 45° in the untreated sample (Fig. 3A), revealed the presence of amorphous compounds, which was clearly reduced for the sterilized surfaces. As the temperatures employed in all the sterilizing treatments are relatively low, there is no possibility of occurrence of phase transformation. Therefore, the reduction in the presence of amorphous material on the treated surfaces indicates the cleaning effect of the various procedures, which resulted in decrease of the amount of anodization by-products. There was not a clear indication of the existence of crystalline phases containing Si such as Mg_2SiO_4, which could be related with the amorphous nature of these species, the low content of those compounds in the oxide film and/or the concealing effect of the

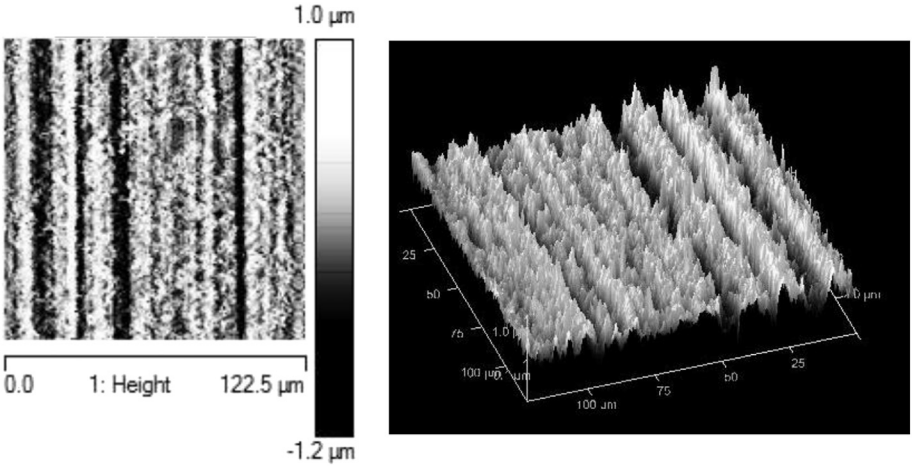

Fig. 2. AFM pictures (122.5 × 122.5 µm scans) of the Mg surfaces anodized.

Table 1
Composition of the anodized coatings sterilized by different methods by using EDS analysis (% weight).

Sample	Mg	O	Si
Untreated	36.3	50.2	13.5
Steam autoclaving	37.7	50.3	12.0
Dry-heat	37.5	47.1	15.4
Uv-irradiation	29.8	58.3	11.9
Formaldehyde	37.7	48.8	13.5

high Mg peaks; both structures diffract at similar angles.

On the other hand, analysis of the surfaces and the cross-section was carried out to determine the integrity of the coatings after the different sterilization processes. In Fig. 4 it is possible to observe at different magnification (1000× and 3000×) the general appearance of the surfaces. A homogeneous distribution of interconnected pores was observed with an average size of the pores of 1.02 ± 0.1 µm. The thickness of the anodic film was about 2 µm. The images of the surface of the anodic film samples treated with steam autoclaving and UV-irradiation did not show any changes on morphology. On the other hand, samples sterilized by dry-heat showed random cracks on the surfaces that can be a product of internal stresses induced in the coating by the heating of the samples. Additionally, for samples treated by steam formaldehyde sterilization, changes in the morphology of the coating surface was evident, both porosity and topography appears to have been modified by the sterilization treatment. However, cross-section images for all the treatments showed no major effects on the internal structure and thickness of the anodic films. In a similar manner, it was not observed any effect on the substrate material, in all cases, the interface coating/

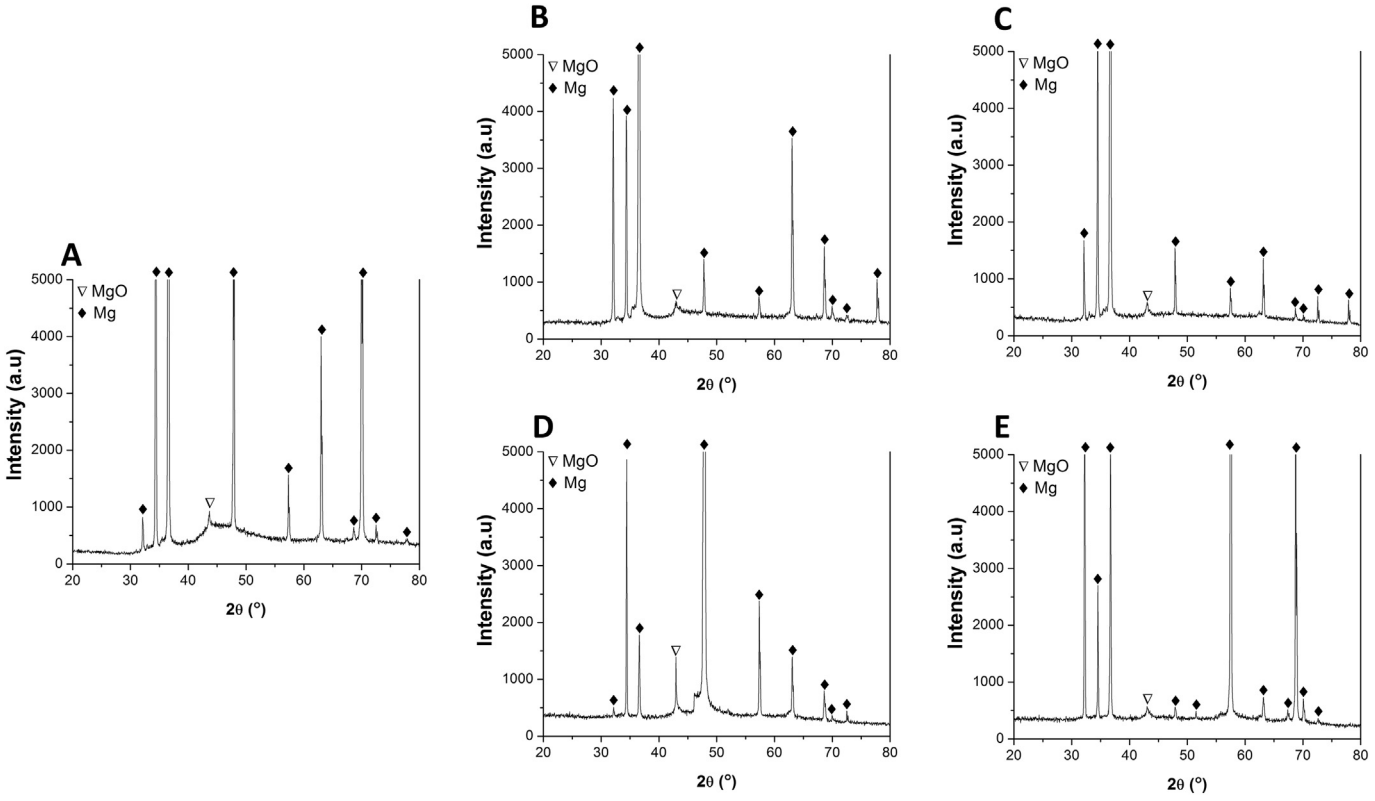

Fig. 3. XRD patterns of samples sterilized by different methods. Untreated sample (A), steam autoclaved (B), dry-heat (C), UV-radiation (D) and formaldehyde (E).

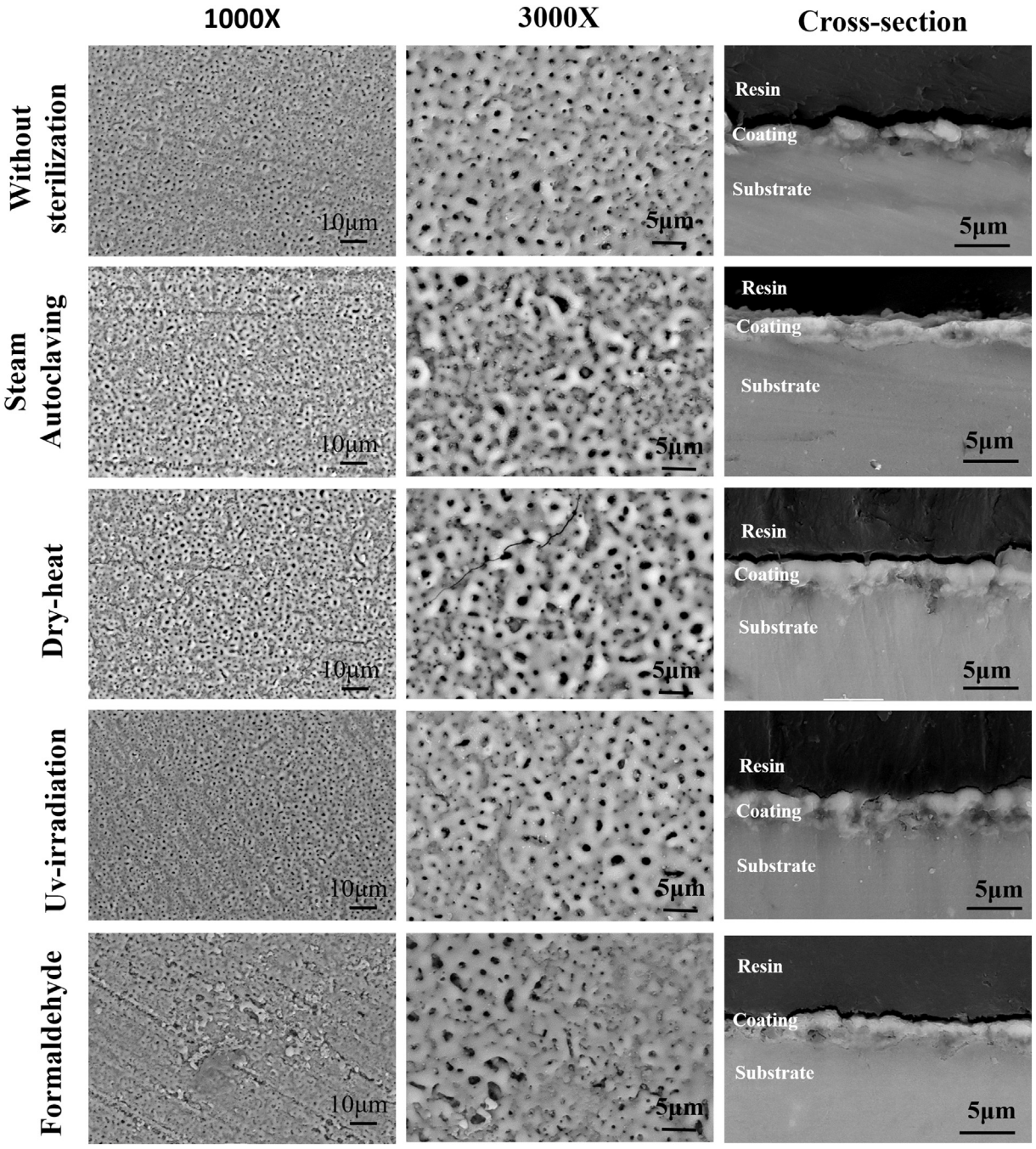

Fig. 4. Top-view SEM image of the surface and cross-section for anodized samples of Mg before and after different sterilization procedures.

substrate appears to be unaltered.

As an additional evaluation, the contact angle was measured and the corresponding surface energy was calculated. In Table 2, these results are summarized showing that samples treated by autoclaving and in a lesser amount by steam formaldehyde sterilization, showed an increment for the contact angle values and consequently these samples also presented a decrease in surface energy compared to the other treatments and the untreated sample.

Finally, as an approach to the biological performance of the samples, evaluation of the hemocompatibility was carried out. Results showed that none of the evaluated samples presented hemolysis after contact with human blood (Fig. 5).

Table 2
Contact angle and surface energy of sterilized samples.

	Untreated	A	DH	UV	F
Contact angle (°)	27,8 ± 3,4	84,3 ± 10,0	26,7 ± 3,0	34,8 ± 11,6	42,3 ± 10,4
Surface energy (mJ/m^2)	65.5	32.8	66.0	62.1	58.1

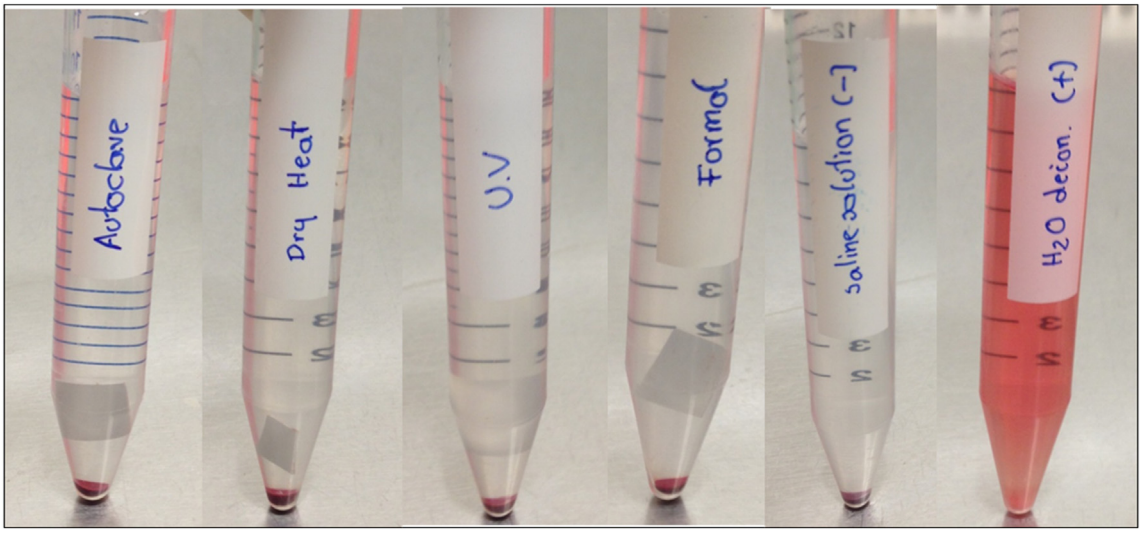

Fig. 5. Hemocompatibility of Mg samples sterilized by different methods. Samples showed no hemolysis after being in contact with human blood.

4. Discussion

The high reactivity of Mg makes this material complicated in its manipulation as an implant. Some considerations must be taken into account to be sure that the product obtained after anodization is the same to be evaluated *in vitro* or to be implanted in a living organism. Within the proposed sterilization techniques, irradiation by UV light is the method that could produce the least modification on the material at structural level, however, a rise in the oxygen content at this sample surface was observed by EDS analysis. This result is consistent with the reports of Ito et al. [19], who stated that the process of adsorption of oxygen is promoted by the exposition of MgO to UV irradiation where oxygen species (ozonide and superoxide) are formed. Additionally and according to studies from Att et al. [20,21] on zirconia surfaces, the effect of the changes in the surface of the material by UV exposition will be highly dependent on the time and intensity of exposure. They reported that the wettability of the material can be increased, therefore improving its biological performance. However, considering the porosity of the samples studied in the present case, the UV treatment may be not enough to ensure a proper sterilization process, as it is very superficial and therefore it does not guarantee that within the porous structure, microbial agents (bacteria or fungi) have been completely eliminated.

On the other hand, dry-heat sterilization is generally used to avoid deterioration and/or corrosion of the material, which could be particularly important for magnesium substrates due to its high reactivity. In addition, this is an effective and low-cost technique. The results obtained with this technique for anodized Mg were similar to those found by other authors in other biomaterials such as titanium and zirconia [22,23]. However, as relatively high temperatures are used in this technique and although Mg does not present structural or compositional modifications at these temperatures, shielding of tensions, recrystallization processes and release of tensions can be generated at the substrate-coating interface [24]. As a consequence of this, cracks can appear generating points of failure or defects in the coating that can affect its corrosion and mechanical resistance.

In steam autoclaving water vapor, temperature and pressure are combined to kill microorganisms, also microbial spores are neutralized [25]. Additionally, during steam autoclaving and formaldehyde treatments, in contrast to UV, vapor will penetrate all cavities of the sample, efficiently sterilizing all the surfaces. Passivation of material is initiated by its oxidation and the release of corrosion products decreasing the reactivity of the material by this protective layer. For the case of Mg, and according with previous studies performed for the authors [26], during the first hours in which the material is exposed to an aqueous solution, the highest levels of corrosion rate are presented, which subsequently decrease due to the passivation of the material. The use of water vapor could produce such passivating effect on the implant material, which would be advantageous as this high corrosion stage will not affect the evaluation with cellular components due to otherwise, augmented hydrogen production and pH changes.

Sterilization with steam formaldehyde is often used when materials are thermally sensitive, as it is the case of some polymers with low melting points such as PLGA, nylon, polystyrene, poly(N-isopropyl acrylamide) hydroxylpropylcellulose, poly(vinylcaprolactame), polyvinyl methyl ether or heat-sensitive surgery instruments *e.g.* cryo-instruments, probes or catheters. The low temperatures (around 55 to 75 °C) and mainly the chemical action (chemical crosslinking) of formaldehyde generate an atmosphere with high penetration that allows killing bacteria and spores. This process is carried out under reduced pressure. One of the disadvantages of this method is that formaldehyde is a toxic, hazardous chemical considered carcinogenic, and poses a potential health risk for personnel that operates steam formaldehyde equipment [27–29]. For samples of anodized Mg, the use of an agent such as formaldehyde shows that the surface undergoes changes at a morphological level which means that in terms of surface configuration, the evaluated material is different from that obtained once it was anodized. Formaldehyde is an aldehyde *i.e.* reducing agent, thus could potentially chemically interact with the surface layer or the underlying magnesium. That would adversely alter the chemical-physical properties of the PEO-treated Mg.

According to the cross-sections, none of the evaluated treatments showed evidence of degradation of the substrates. Therefore, it is considered that the coating was sufficiently protective to avoid the reaction of the Mg substrate in any of the environments employed for sterilization.

Wettability is a parameter directly related to the contact angle and the surface energy of the surfaces. According to the results of this study, the porosity of all the coatings increased the contact surface area between the drop of water and the material during the contact angle test. As the area is larger the water can spread more on the surface. In the sample of formaldehyde, the surface porosity of the sample was modified, these change on the surface configuration lead to the increase of the contact angle of the surface and consequently a decrement on the surface energy. Autoclaving increased the contact angle, which might be due to the altered surface topography *i.e.* the sealing of pores in the anodic film or formation of $Mg(OH)_2$ on the coating surface, both phenomena addressed by the contact of the sample with the water

during the autoclave process. Hiromoto et al. [30] suggested that due to autoclaving the anodized samples could increase the oxygen concentrations and improve the resistance corrosion of the material. Our results corroborate findings of Park et al. [31], that showed that surface-anodized Ti cleaned and sterilized by autoclave, gamma irradiation, oxygen plasma, and ultraviolet light presented changes in the hydrophobicity and roughness which at the same time affected the biological performance of the surface. Samples sterilized by autoclaving showed an increment in the contact angle, which was explained by the authors as a consequence of the temperature and pressure during the process, which affected the oxidation states and the oxide layer thickness on the treated surface. Similar results were found by Pegueroles et al. [32] when grit-blasted titanium samples with different roughness, which were sterilized with steam autoclaving, presented a hydrophobization of the surface after this procedure. Also, other authors explain the increment in the contact angle due to the contamination of the sample with organic impurities present in the environment during the process [22]. Finally, although further biological assays will be required, such as cell viability and proliferation, the results from hemolysis presented here could be an indication that none of the methods evaluated here induced changes in the biological behavior of the surfaces.

5. Conclusions

Different options of magnesium sterilization were studied to know the impact of this process in the conservation of the anodic films previous to *in vitro* and *in vivo* evaluation. In general, all sterilization methods did not affect the main composition of the material or cause degradation of the substrate. However, dry-heat and formaldehyde techniques showed modification on the surface morphology. In terms of surface energy, samples treated by autoclave and formaldehyde showed a decrement in wettability. Although the results of the present work did not provide any evidence of sample passivation during the autoclave process, if this took place in some extension, it will be an advantage of this sterilization procedure, as it will reduce its occurrence during *in vitro* assays.

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