Cytocompatibility of porous P-containing coating prepared by plasma electrolytic oxidation of Mg alloy

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Keywords: phosphate, biological activity, magnesium

Abstract

Magnesium alloys are potential biodegradable hard-tissue implant materials because of their excellent biomechanical compatibility, biosafety, and absorbability. In order to further improve the biocompatibility of these alloys, a ceramic coating was prepared on the surface of a magnesium alloy specimen by plasma electrolytic oxidation (PEO) in a phosphate solution. The ceramic coating has a rough surface, porous structure, and no harmful elements in the ceramic membrane. Further, experiments with MG63 cells show that the PEO ceramic coating can promote cell adhesion and proliferation, and it causes no obvious cytotoxicity. In conclusion, PEO can be used to prepare a suitable ceramic coating on the surface of the magnesium alloy, thereby increasing its potential for clinical applications.

1. Introduction

Magnesium-based metals have a high specific strength and stiffness, and their elastic modulus is 41–45 GPa. Compared with metallic biomaterials such as stainless steel and titanium alloy, their elastic modulus is the closest to human natural bone (3–20 GPa), which can reduce the stress shielding effect to a certain extent [1]. More importantly, magnesium is one of the essential nutrients and an indispensable mineral element involved in the metabolism in the human body. Magnesium can catalyze or activate many enzymes in vivo, participate in many metabolic processes in vivo, and stabilize the DNA and RNA structures. It can regulate and stabilize the synthesis of proteins and nucleic acids, and also affect the transport of calcium and potassium ions. In addition, it has a signal-transmission-regulating effect, and it is essential for maintaining the cell membrane structure and regulating cell growth. Magnesium also regulates the cell cycle and maintains the integrity of the mitochondrial structure.

The life process of cells mainly manifests as proliferation and differentiation (that is, the proliferative state and functional state). The early adhesion and growth of cells on the surface of implant materials is necessary for the cells to further secrete the extracellular matrix and synthesize related proteins. The ideal implant material requires the ability to actively induce a large number of cells to attach, spread, and proliferate on the surface quickly. The proliferation of cells on the surface of the material also reflects the interaction between the cell and the material. For the adhesion of cells to the surface of the material, the proliferation reaction is the result of a longer-term interaction. Traditionally, biomaterials have been expected to exhibit good biocompatibility, that is, they cannot have toxic effects on surrounding cells, tissues, and systems. In recent years, in addition to good biocompatibility, high biological activity has also been desired for biomaterials. Specifically, in addition to close contact between the implant surface and bone tissue after bone formation, chemical bonding between the implant surface and bone tissue is necessary. The magnesium ion promotes the differentiation and proliferation of osteoblasts. Magnesium-based metal implants show good in vivo biocompatibility and osteoinduction performance, and have the substantial effect of stimulating the formation of new bone [2, 3].
However, the electrochemical activity of magnesium-based metallic materials is high, which tends to result in stress corrosion, galvanic corrosion, and fatigue corrosion, as these materials are unable to generate a protective oxide coating [6]. In particular, there are a large number of chloride ions in the body that react with magnesium, thereby accelerating its degradation. Therefore, it rapidly undergoes corrosion after implantation into the body and is seriously damaged before the body recovers, greatly deteriorating the mechanical properties of the implant. Stable, released hydrogen in the body too late to spread and accumulate around the implant, forming bubbles, will affect the physiological function of the tissue around the implant and the treatment of the implant site to a certain extent [5]. Thus, the rapid degradation rate of magnesium alloys and a series of concurrent problems restrict the clinical applications of these alloys.

Many studies have shown that factors such as the microscale morphology, surface energy, roughness, chemical composition, and structure of the material surface may affect osteoblasts, and the mechanism of these effects is very complex and interrelated. Therefore, many studies have focused on the surface modification of magnesium alloys. In summary, surface modification mainly includes surface morphology modification and surface chemical composition modification. The commonly used surface treatment methods for magnesium alloys are electrochemical plating, chemical conversion, anodizing, vapor deposition, laser surface modification, and the formation of an organic coating [6–9].

Plasma electrolytic oxidation (PEO) is a new technology based on anodic oxidation for the in situ growth of homogeneous and dense ceramic coatings with high hardness on the surface of titanium, magnesium, aluminum, and their alloys. Electrochemical methods are used to generate spark discharge spots in the micropores on the surface of metal substrates. Because of the combined effects of electrochemistry, thermochemistry, and plasma chemistry, a ceramic layer is formed with a high bonding strength between the PEO coating and the substrate. This technique is the result of the diversified development and application of anodizing technologies such as anodizing and pulse anodizing at the current stage. This technique offers many advantages over other techniques, including excellent wear resistance, high hardness, corrosion resistance, strong adhesion of the resultant ceramic coating to the substrate, and no toxic substances [10, 11]. In addition, the coating has a rough surface that is conducive to osteoblast attachment. Therefore, this technique is more suitable than others for the surface treatment of magnesium alloys for biomedical applications. Researchers worldwide have conducted in vitro studies on PEO-treated magnesium alloys and reported that the surface shows no cytotoxicity. Ma et al [12] prepared the PEO coatings on the surface of Mg alloy, and further study revealed that the coatings could improve the corrosion potential of magnesium and reduce its degradation rate. What’s more, the PEO-coated Mg alloy showed no cytotoxicity and more new bone was formed around it during in vivo degradation. Zhang et al [13] observed the blood compatibility of PEO-treated Mg alloy, and found PEO-treated Mg alloy showed favorable blood compatibility characteristics, and PEO might be an attractive surface modification technology on Mg implant materials.

Phosphorus (P) is an important bioactive element in human body. The introduction of P element through PEO method on the surface of magnesium alloy is rarely studied. In this study, the surface of a magnesium alloy specimen was modified by PEO in a sodium phosphate solution, and the microstructure, phase composition, element distribution, and biocompatibility were studied. In addition, the biological activity of osteoblasts on the specimen after PEO was studied to evaluate its potential for clinical applications.

2. Materials and methods

2.1. Sample preparation and surface characterization

An AZ91D magnesium alloy specimen was cut into a round piece with a diameter of about 20 mm and a thickness of 2 mm. After sequential grinding with 300 ×, 600 ×, 800 ×, 1000 ×, 1500 ×, and 2000 × SiC sandpaper until almost no obvious scratch was found on the surface, the specimen was polished. Finally, the specimens were sonicated in acetone, anhydrous ethanol, and deionized water for 20 min to remove the scraps on the surface of the magnesium sheet. This was followed by vacuum drying. This PEO electrolyte consists of sodium phosphate, sodium hydroxide and deionized water.

A 20 kW pulse PEO device developed by Chang’an University was used to treat the alloy specimen. The frequency was 1000 Hz, the duty cycle was 30%, and the reaction time was 5 min. In this study, the specimen subjected to PEO in the phosphate solution was denoted PEO-AZ91D, while that not subjected to PEO was referred to as the AZ91D specimen. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were used to observe the surface morphology of the PEO coating. The elemental composition of the coating was analyzed by energy-dispersive x-ray spectroscopy (EDS), the chemical structure of the coating surface was analyzed by x-ray photoelectron spectroscopy (XPS), and the phase composition of the oxide coating was tested by x-ray diffraction analysis (XRD).
2.2. Osteoblast culture
MG63 cells were purchased from the cell bank of the Shanghai Chinese Academy of Sciences. The cell culture medium was Dulbecco’s Modified Eagle Medium (DMEM), a high-glucose complete medium containing 10% fetal bovine serum and 1% penicillin-streptomycin mixture. The cells were cultured in a cell incubator at 37 °C containing 5% CO₂. The medium was changed every two days. When 80%–90% confluence was achieved, 0.25% trypsin-EDTA was used for digestion and subculture.

2.3. Live/dead staining
The osteoblasts were inoculated to the surface of the two specimens at a density of 2 × 10⁴ cells/well. After inoculation, the culture plates were placed in the cell incubator and cultured for 3 days separately. Then, the old medium in the wells was discarded, and washed with phosphate-buffered saline (PBS) thrice. The inoculated cell samples were transferred to new 24-well plates. Next, 50 μl of the live/dead staining agent was added to the surface of each specimen, which were then placed in a 37 °C cell incubator for 1 h to avoid light staining.

2.4. EDU staining
An EDU solution diluted with the cell culture medium at the ratio of 1000:1 was added to each well and the cells were cultured with materials for 3 days. Then, the cell fixing solution was added at room temperature for incubation, followed by glycine addition for incubation and then washing. Next, the Hoechst 3342 reaction solution was added, followed by incubation in the dark at room temperature on a decolorizing shaking table for 30 min. The final step was washing with PBS thrice, before observation with a laser confocal microscope.

2.5. Statistical analysis
The data were expressed as mean ± standard deviation and analyzed using the SPSS 16.0 software. p < 0.05 means that there is a statistical difference.

3. Results and discussion
The structure and phase composition of the PEO coating are affected by the composition of the matrix alloy, the composition of the electrolyte, and the process parameters.

Figure 1 presents a SEM image of the magnesium alloy after PEO in a phosphate solution. The PEO coating has a gray-white color. Its surface is dense, granular, and porous, but the pores are small. In addition, there are obvious cracks on the surface, which may be due to the contact between the solution and the coating surface during PEO. Caused by excessive thermal stress during rapid solidification of the melt.

Figure 2 shows the AFM image of the alloy after PEO in the phosphate solution. As shown in the figure, the surface after PEO is rough, porous, and uneven, and there is a fusion phenomenon between pores.

Figure 3 shows the EDS mapping images for the alloy after PEO in the phosphate solution. The image shows that the coating layer formed after PEO in the phosphate solution contains Mg (36.94, at.%), P(5.39, at.%), O(53.20, at.%) and small amounts of other elements (Al, Na and C etc.). This shows that all these elements are involved in the response. Further, it can be seen that Mg, P and O are uniformly distributed in the PEO coating.

Figure 4 shows the XRD patterns of the alloy after PEO. From the figure, it can be seen that the presence of Mg, Mg₁₇Al₁₂ and MgO peaks is detected on the surface of the magnesium alloy after PEO. Combined with EDS mapping, we speculate that P may exist in the coating in amorphous form.

The chemical composition and valence of the surface of the magnesium alloy after PEO were measured by XPS. The results are shown in figure 5. In the full spectrum (a), Mg, O, and P peaks are clearly visible. Figure 5(b)
shows the high-resolution spectrum of Mg elements. The binding energy corresponding to the Mg 1s peak position is 1034.4 eV, and the binding energy corresponding to the O 1s peak position is 531.8 eV, indicating that the Mg and O elements in the coating exist as MgO [14]. The binding energy corresponding to the P 2p peak position is 133.3 eV, and we speculate that phosphorus may exist in the form of P2O5.

The results of MG63 osteoblast toxicity on the different specimen surfaces are shown in figure 6. All sample surfaces show almost green, i.e., living, cells with the exception of individual dead cells (red), indicating that the coating formed was not significantly toxic to MG63 osteoblasts. Studies have shown that factors such as the microscale morphology, surface energy, roughness, chemical composition, and structure of the material surface may affect osteoblasts, and the good properties of implant surface can promote the proliferation and differentiation of osteoblasts [15, 16]. Our results are consistent with those of other researchers worldwide who have conducted studies on PEO-treated magnesium alloys [17, 18].

Figure 7 shows the EDU staining of MG63 osteoblasts on the surface of different samples. The results clearly show that PEO increased the proliferation of osteoblast cells significantly (three regions, p < 0.05).
Designing magnesium-based metal materials with controlled degradation and suitable mechanical properties remains a challenge in the field of magnesium metal research, although researchers have attempted it. In this study, the PEO technique was used to prepare a biological coating on the surface of AZ91D magnesium alloy. This ceramic coating is composed of magnesium, phosphorus, and oxygen elements, and does not contain harmful components. This treatment can reduce the rate of degradation of the metal, improve its corrosion resistance, and provide stable support for fracture healing for a sufficient time; moreover, because the coating has good biocompatibility, it also improves the overall biological properties of the AZ91D implant material. In addition, the ceramic coating has a porous morphology, which is conducive to the adhesion and proliferation of osteoblasts. Therefore, the resultant implant material is expected to be suitable for potential clinical applications.

Acknowledgments

This work was supported by the Open Project of State Key Laboratory of Military Stomatology (No. 2018KA02).
Conflicts of interest

The authors declare no competing financial interests.

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Figure 6. Fluorescence images of live/dead staining of MG63 cells on different surfaces (green represents living cells and red represents dead cells).

Figure 7. EDU staining of MG63 osteoblasts on the surface of different samples.
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