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ARTICLE

Effects of Drug-contained Poly-β-hydroxybutyrate Coating on Degradation Behavior of Mg-4.1Y-2.8Nd-0.2Zn-0.4Zr Alloy in Simulated Body Fluid and Cell Function

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Abstract: Magnesium and its alloys have great potential as absorbable biomaterials in clinical medicine, but their rapid corrosion might lead to implant failure. Poly-β-hydroxybutyrate (PHB) coating with drug was prepared on the surface of WE magnesium alloys using layer-by-layer self-assembly technique. The corrosion behavior of magnesium alloys with drug coating in simulated body fluid (SBF) was studied. The solution concentration and microstructure change during degradation process were observed. The effects of drug coating on tissue cells were investigated by cell migration, cytotoxicity and apoptosis. The results show that the bioactive drug coating effectively reduces the corrosion rate of the samples in SBF, cytotoxicity, apoptosis, and promotes the migration of the cells.

Key words: magnesium alloy; biomaterials; PHB; cell; degradation

The clinical use of magnesium and its alloys as biodegradable materials is widely discussed[1-3]. It is well known that magnesium alloys have good biodegradability, biocompatibility and good mechanical properties[4-6]. However, the main problem of magnesium alloys as vascular stent materials is that their degradation rate is too fast in the biological environment, and the degradation rate is not matched with the body healing rate, which easily causes inflammation[7,8]. Therefore, it is necessary to study how to reduce degradation of magnesium alloys to meet clinical needs.

PHB as a natural polymer exists within the cell, has good biodegradability and biocompatibility, and facilitates cell adhesion and differentiation[9-11]. PHB is used as a base material of coating which can control the degradation rate of the magnesium alloy[12,13]. Based on the above-mentioned studies, PHB was used as coating material in this study. Layer by layer (LbL) assembly technique, based on electrostatic attraction between positively and negatively charged molecules, is a convenient and substrate-independent method, and has become one of the most promising surface modification strategies to prepare multifunctional blood and tissue contacting materials[14-16]. After aminated PHB, heparin with anticoagulant effects and vascular endothelial growth factor (VEGF) promoting angiogenesis were assembled on the surface of the PHB coating by LbL assembly method. The cytotoxicity and cell apoptosis were used to evaluate the biocompatibility of magnesium alloy with bioactive drug coating.

1 Experiment

1.1 Materials

Cell Titer-Glo®3D Cell Viability Assay kits were purchased from Promega Corporation, Beijing, China. Hoechst 33258 was purchased from Beyotime Biotechnology, Shanghai,

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China. Magnesium alloys used in this study were Mg-4.1Y-2.8Nd-0.2Zn-0.4Zr alloy (4.1% Y, 2.8% Nd, 0.2% Zn, 0.4% Zr and Mg balance). PHB was purchased from Sigma-Aldrich. (RPMI) 1640 medium and fetal bovine serum (FBS) were obtained from Hyclone Company, China. VEGF and heparin were purchased from Jiancheng Bioengineering, Nanjing, China.

1.2 Preparation of PHB coating

Magnesium alloys were cut into pieces of 8 mm×2 mm. They were polished until the surface was bright with the metallographic sandpaper of 400#，800#，1000#，2000# and 4000#. Each sample was then ultrasonically cleaned in acetone, absolute ethanol and ultrapure water for 10 min, and dried at room temperature. After the pre-treatment, Mg alloys were placed in 0.5 g/L PHB solution, and the PHB film formed on the surface of the sample. The coating thickness and the contact angle were used to investigate the optimal soaking time.

Later, the samples containing PHB coating were placed in the 1,6-hexanediamine/n-propanol solution, heated in water bath at 40 °C for 30 min, and rinsed with ultrapure water to remove residual hexane diamine. The samples containing ammoniated PHB coating were dried in vacuum oven at 30 °C until a constant mass, which was positively charged on the surface.

The concentration of heparin was 3 mg/mL in ultrapure water. And the pH value was 4.2. The concentration of VEGF was 100 mg/mL in PBS. Heparin was negatively charged by adjusting the pH value of the solution. In the same way, VEGF was positively charged. Repeat three times to obtain six layers of drug coating on the surface. The LbL assembly method was used to prepare PHB coating containing drug on the surface of Mg alloys.

1.3 Degradation test

The degradation was investigated by immersion test and electrochemical test. The immersion test was performed according to ASTM G31-2012a Standard 9.8. The main ion concentration of simulated body fluid (SBF) is shown in Table 1. The pH of solution was adjusted to 7.4 with HCl and NaOH solution, and it was filtered through a 0.2 μm filter to remove bacteria. The ambient temperature during the degradation process was maintained at 37 °C, and the duration was 7 d. The pH value was recorded after different immersion durations. The change of Mg^{2+} was detected by continuous titration. The PHB concentration was detected with UV spectrophotometer.

Table 1 Main inorganic ion concentrates in simulated fluids

Ion	Concentration/mg·L^{-1}
Na^+	3265.8
K^+	195.1
Ca^{2+}	100.2
Mg^{2+}	36.02
Cl^-	5240.91
HCO^{3-}	254.18
HPO_4^{2-}	96.13

After spraying gold, the samples were examined by scanning electron microscope (SEM), and the surface topography changes in different periods were recorded.

The electrochemical test of modified materials was evaluated by potentiodynamic polarization curve in SBF. Pt was used as counter electrode, saturated calomel electrode (SCE) was used as a reference and the modified material with exposed area of 0.05 cm^2 was used as the working electrode. The potentiodynamic polarization data were analyzed by CorrView software and painted by Origin Lab 8.5 software.

1.4 Cell culture

HUVECs were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum, in an incubator containing 5% CO_2 at 37 °C.

1.5 Cellular activity test

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) is reduced by cells into a formamidine compound which is soluble in the medium. The amount of formazan product is directly proportional to the number of active cells in the culture. 100 μL of the cell suspension (1×10^5 cells/mL) was inoculated into a 96-well plate and placed in a CO_2 incubator for 24 h. Normal cultured cells were used as negative controls. After the cells formed a semi-confluent monolayer, the medium was removed and 100 μL of the extract was added. Then 20 μL of MTS was added into each well, and the absorbance of the sample was measured at 490 nm at 37 °C. The calculation formula of cell viability is follow: Cell viability=Average absorbance value of the test group/Average absorbance value of the negative control group ×100%

1.6 Apoptosis test

1 mL cell suspension (1×10^5 cells/mL) was inoculated into a 6-well plate. The original culture solution was removed after 24 h of culture. 1 mL material extract with different concentrations was added and incubated for 24 h. The morphology of the cells was examined by staining analysis. After aspirating the culture solution, 0.5 mL fixing solution was added, and then fixed at 4 °C for at least 10 min. The fixative was then removed and the cells were washed with PBS for 3 min, repeated twice. Drain the liquid, add 0.5 mL Hoechst 33258 staining solution, and stain for 5 min. The staining solution was removed, washed with PBS for 3 min, repeated twice. The control was performed by neglecting nuclear staining. The anti-fluorescence quenching of the sealing liquid was observed by a fluorescence microscope.

1.7 Cell migration test

HUVECs were placed into 24-well plates (1×10^6 cells/well) for 24 h. Then the original medium was removed. 0.5 mL extracts with different concentrations were added into the 24-well culture plate and incubated for 24 h. When the cell fusion degree reached 90%, the HUVECs would synchronize for 24 h with RPMI1640 medium lacking serum. When the

cells grew into monolayer, several vertical and parallel lines of about 0.7 mm were drawn with the sterile pipette tip in the same position, then washed two times using sterile PBS, and incubated normally with RPMI1640 medium containing 10% fetal bovine serum. The cell migration was observed after 0 and 12 h. The samples were compared by the migration area ratio. The migration area ratio of the samples is calculated using the following formula[17]:

Migration area ratio=$(1-A_t/A_0)\times 100\%$

where A_0 is the starting scratch area and A_t is the migration of the scratch area.

1.8 Statistical analysis

Statistical Program for Social Sciences (SPSS, version 22.0) was performed for statistical analysis. Comparisons among group means were determined by one-way analysis of variance (ANOVA). Multiple comparisons between two means were conducted by Duncan's multiple range method. $p<0.05$ was considered statistically significant.

2 Results and Discussion

2.1 Preparation of PHB coating

The thickness and hydrophilicity of the coating do not change significantly after 24 h (Fig.1, Fig.2). The hydrophobicity of the PHB film has a contact angle of about 100°[18-21]. After 24 h of coating, the average thickness and contact angle of the coating on the surface of the material no longer change significantly. The surface of the material is completely covered with a layer of PHB film. It can be considered that 24 h is the optimal coating time.

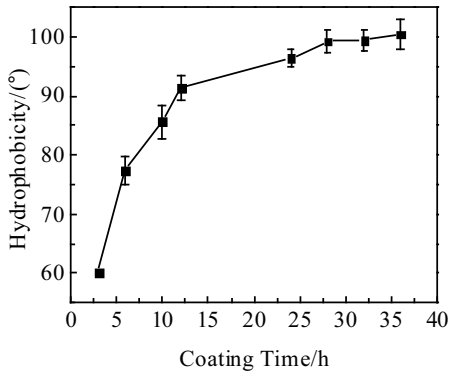

Fig.1 Change of hydrophobicity with coating time

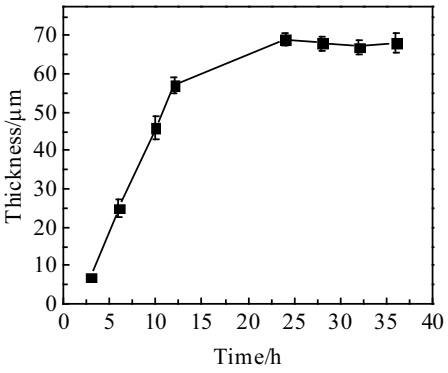

Fig.2 Change of thickness with time

2.2 Degradation test

Fig.3 shows the pH value change of the SBF during the degradation process. It can be seen that pH value of naked samples increases from 7.4 at 0 d to 10.2 at 7 d. The growth trend is similar to Watseka's research[22]. But the pH value of the coated sample fluctuates gradually, and increases less than 7.9 after 7 d. In addition, Mg^{2+} concentration variation is shown in Fig.4. The concentration increase of naked samples is much higher than that of the coated sample. Due to increase of carboxyl and hydroxyl in polymer structure, the pH value of solution is gradually reduced[23].

The degradation of naked magnesium causes more hydroxyl in solution, which increases the pH value gradually. The increase rates of pH and Mg^{2+} concentration for naked samples ae much faster than those of the coated samples. The difference in rate increase is caused by the protection of the coating against the magnesium alloy. It has been demonstrated that LbL is mild technique and does not change the original properties of materials[24-26]. Contrast to other samples with coating, the solution change of bioactive drug sample is similar with that of other samples. It can be considered that the coating improves corrosion resistance of Mg alloys and the process of bioactive drug assemble has no effect on the degradation resistance of PHB coating.

Research shows that PHB degradation is slow in SBF[27-29]. The carbon-carbon bonds in the polymer backbone are not susceptible to biodegradation, because high molar mass polymers are not easily degraded in a short term[30]. During the degradation process (Fig.5), the PHB concentration slowly increases.

Fig.6 shows the sample surface microstructure during the degradation process (from 1 d to 7 d). In the early stage of the degradation process (0~3 d), there is no significant change on the surface of the coating. After 4 d, some cracks and layered structures are observed on the surface of the samples. These

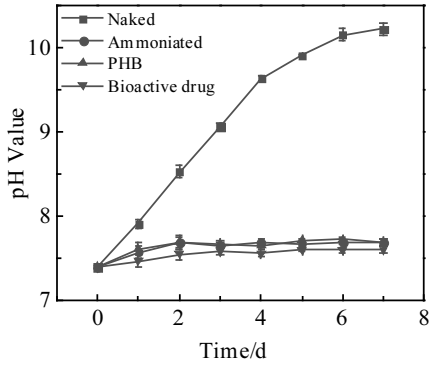

Fig.3 pH value change of SBF during the degradation process

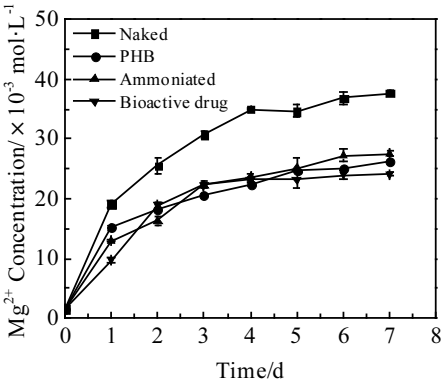

Fig.4 Mg^{2+} concentration change of SBF during the degradation process

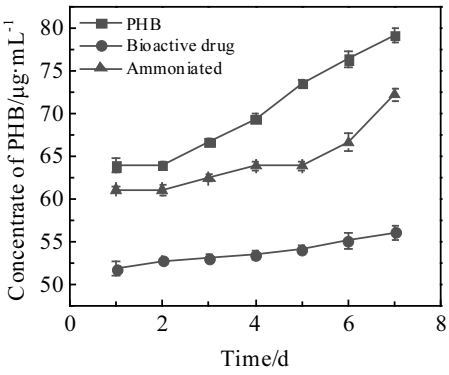

Fig.5 PHB concentration change of SBF during the degradation process

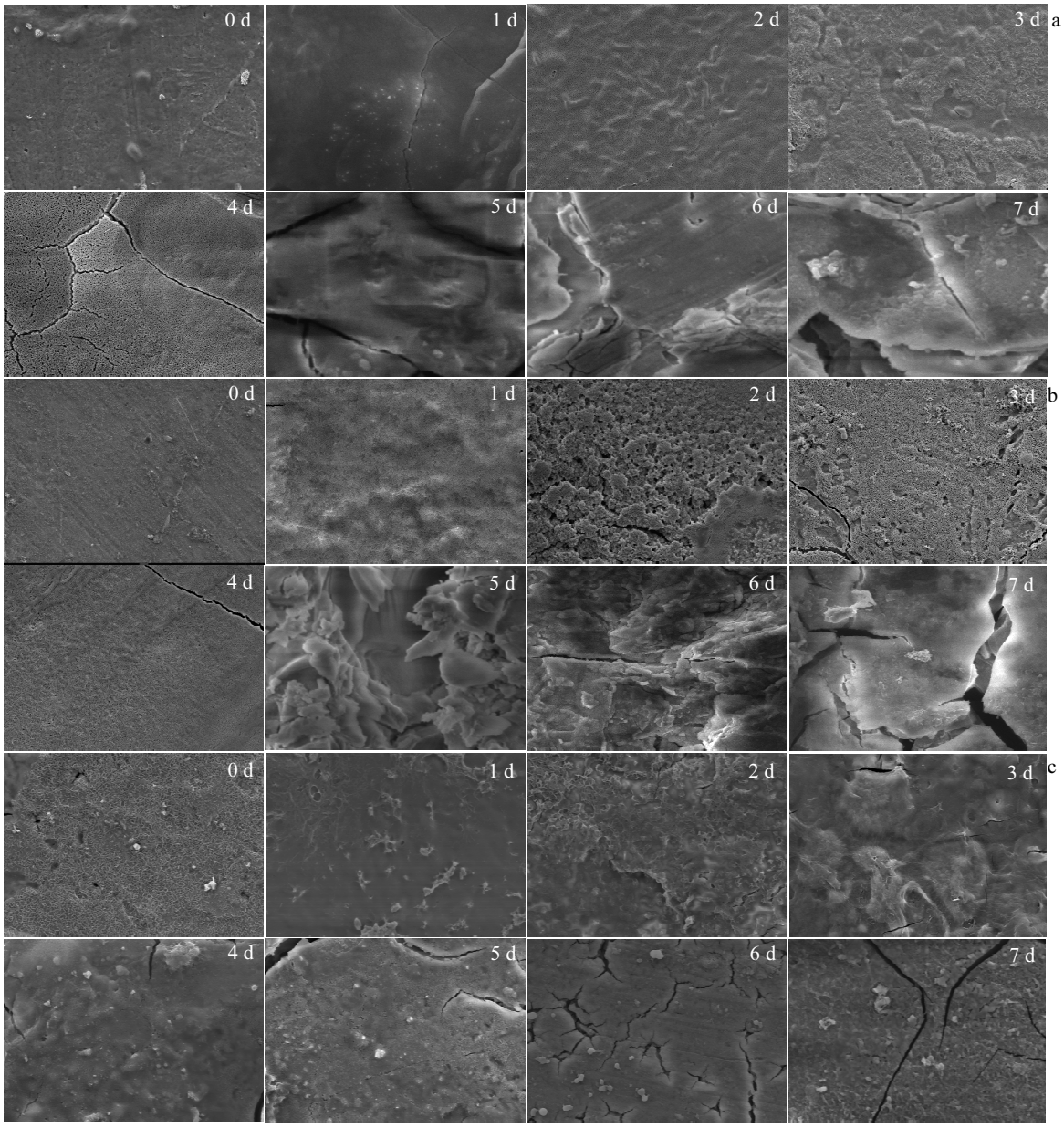

Fig.6 SEM images of surface microstructure of samples: (a) PHB coating samples, (b) ammoniated PHB coating samples, and (c) PHB coating samples with bioactive drug

layered structures of images are similar to other studies[31-33].

It is considered that PHB coating on the surfaces of biomedical magnesium alloy materials makes it possible to control the degrading rates of the materials[34]. It indicates that the degradation of PHB coating remains intact during degradation and protects magnesium alloys effectively[1,10].

Fig.7 shows potentiodynamic polarization curves of the Mg alloy substrate and the coated samples in SBF. Generally, it is assumed that the cathodic polarization curves represent the cathodic hydrogen evolution through water reduction, while the anodic polarization curves represent the dissolution of magnesium[35]. It can be seen that the cathodic polarization current of hydrogen evolution reaction in sample with coating is much lower than that of the naked sample. As a result, the coating of samples protects the samples effectively. The current plateaus with different breakdown potentials can be observed in the anodic parts of the curves, which indicate the existence of some protective film on the surface of the samples. Higher breakdown potentials of samples with PHB coating are observed. The bioactive drug sample is not obviously different from other samples with PHB coating, which indicates that the surface films are protective for alloy samples and the bioactive drug on the coating does not have a negative effect on degradation resistance of the PHB coating.

2.3 Cellular activity test

As shown in Fig.8, similar to the results of Mao et al[36-38], PHB is believed to be safe for cells. When PHB is assembled with VEGF and heparin sodium, it is also believed to be non-cytotoxic. However, the process of producing the blends as well as elaborating the films includes not only the base materials but also the solvents such as hexanediamide and chloroform, which may contribute to cytotoxicity. The magnesium alloy materials and PHB are not cytotoxic, and do not affect cell growth. The cellular activity of 100% extract for each sample is more than 70%, indicating that the samples are not potentially toxic. The cellular viability of the aminated samples is inversely proportional to the concentration of the extract. The cellular activity of 25% extract is 95%, but the

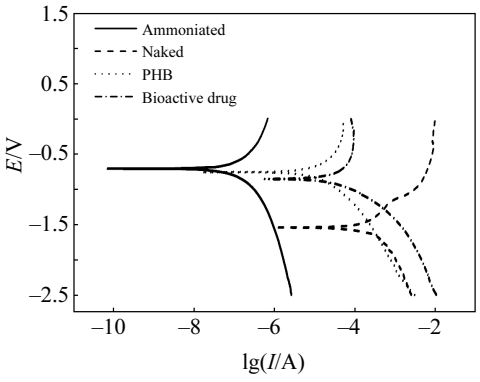

Fig.7 Potentiodynamic polarization curves of the samples

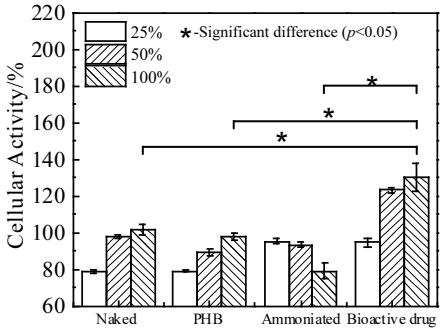

Fig.8 Cellular activity of different samples

cellular activity of 100% extract is 79%. VEGF can increase VEGFR2 signaling to promote the expression of angiogenic molecules, such as CD31 and VEGFR2[39-41].

The results show that chloroform does not affect samples' biocompatibility during the process of PHB coating preparation. The cellular activity of samples with the bioactive drug coating is significantly higher than that of other samples. The cellular activity increases with increasing the extract concentration. It indicates that the process of LbL effectively assembles the bioactive drug on the PHB basement and the drugs can release and increase the cellular activity of the materials. The cellular activity of bioactive drug samples is significantly higher than that of other samples.

2.4 Cell apoptosis

The cell apoptosis is determined by monitoring the changes of cell staining and count[42]. As shown in Fig.9, when the concentration of the extract increases, the number of cells of naked magnesium alloys decreases, and the proportion of apoptotic cells increases after 24 h. For the samples with coating, compared with the control group, there is no significant change in the number of cells with increasing the extract concentration. The proportion of apoptotic cells for the samples containing ammoniated PHB coating increases. However, for samples with the bioactive drug coating, the apoptotic cells are reduced. Especially for the 100% extract, no obvious apoptotic cells are observed. It indicates that the magnesium alloy samples are obviously detrimental to the cells, while the coated samples have less toxicity to the cells. In summary, the bioactive drug coating can reduce the apoptotic destruction, which is also supported by Ref.[43]. VEGF levels are negatively correlated to apoptotic endothelial cell counts.

2.5 Cell migration

The cell migration process is observed by an inverted microscope (Fig.10). The mobility is calculated by IPP software (Fig.11). Compared with the control group, the cell migration rate on the surface of the bioactive drug coating sample during the period is slightly higher than that of the control group. The cells cover the scratch surface of the control

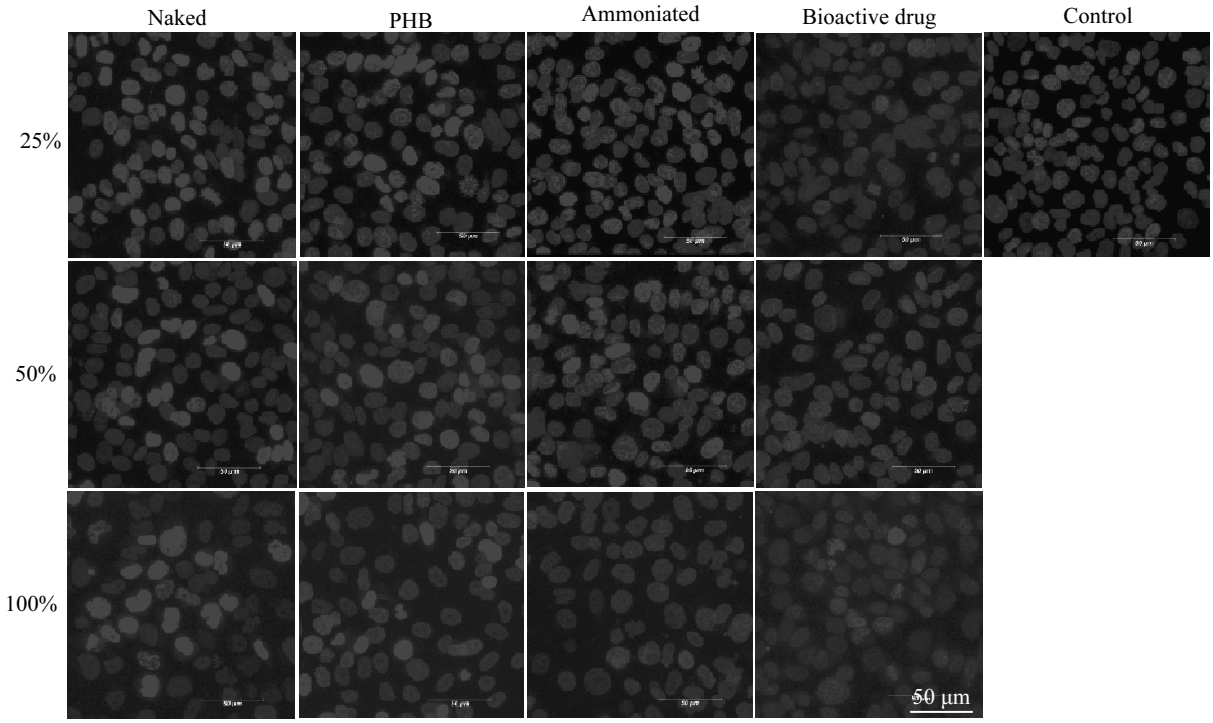

Fig.9 Effect of extracts with different concentrations on cell apoptosis

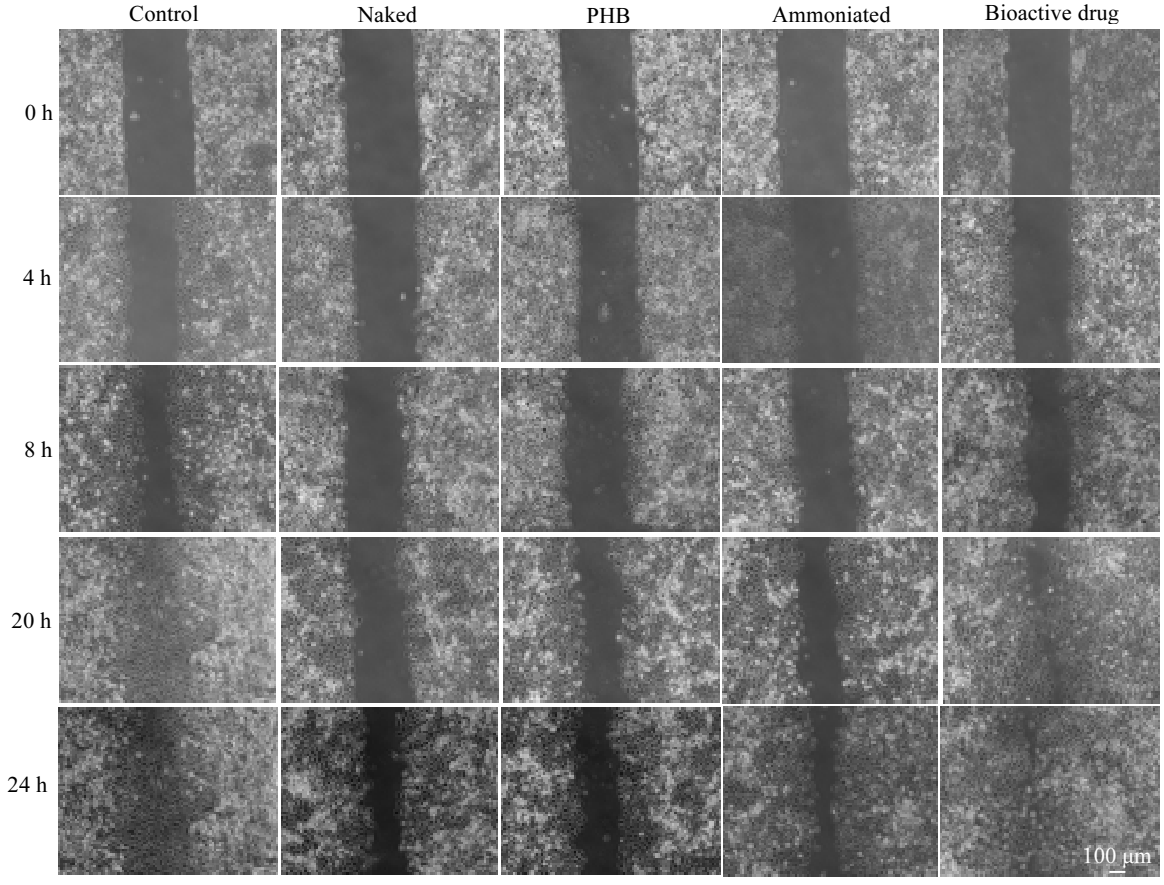

Fig.10 Micrographs of cell transmigration process

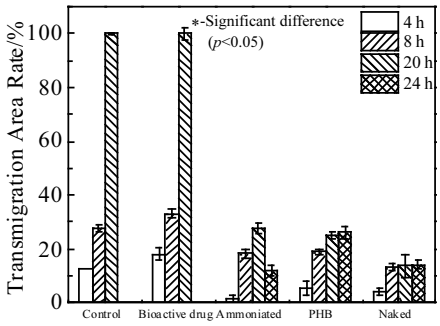

Fig.11 Transmigration area proportion of cells

group and samples with bioactive drug coating within 20 h. After 24 h, other sample scratches are still not completely covered. The maximum mobility of PHB coating samples during the experiment is 26.15%. This indicates that the samples containing bioactive drug coating have a slight boost for cell growth and migration, while other samples are not conducive to cell migration.

3 Conclusions

1) The Poly-β-hydroxybutyrate (PHB) coating containing heparin and vascular endothelial growth factor (VEGF) on the magnesium alloy surface is achieved by layer-by-layer self-assembly. PHB coating can increase the corrosion resistance of Mg alloys in simulated body fluid (SBF). The PHB coating provides strong protection for magnesium alloys. The corrosion resistance of bioactive drug sample is not affected by the layer by layer (LbL) assemble process.

2) The comparison of cell migration assay, cellular activity and apoptosis results indicates that heparin and VEGF can assemble on the PHB coating and be released in solution. While the bioactive drug coating can greatly reduce the cytotoxicity of the material and have a certain promoting effect on cell survival and cell migration.

3) The biological functions of coating include the degradation control of magnesium alloys and endothelialization promotion. However, for the PHB coating with drug, the coating uniformity, effects on tissues and cells during the dynamic degradation process, and in vivo experiments are not settled yet. The release behavior of heparin and VEGF assembled by LbL also needs further research.

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负载活性药物的聚 *β*-羟基丁酸酯涂层对 Mg-4.1Y-2.8Nd-0.2Zn-0.4Zr 合金在模拟体液中降解行为和对细胞功能的影响

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摘　要：镁及其合金作为可吸收生物材料在临床医学中具有巨大潜力，但其过快的腐蚀速率可能会导致植入失效。通过使用层-层自组装技术在 WE 镁合金表面制备一种带有生物活性药物的聚 *β*-羟基丁酸酯（PHB）复合涂层对镁合金的耐降解性能进行改善，并对具有该药物涂层的镁合金在模拟体液（SBF）中的降解行为以及在降解期间溶液中的离子浓度和微观结构的变化进行观察。通过细胞迁移，细胞毒性和细胞凋亡来研究药物涂层对细胞以及组织的影响。结果表明，该活性药物涂层有效降低了样品在 SBF 中的腐蚀速率，细胞毒性以及细胞凋亡率，并促进了细胞在材料表面的迁移。

关键词：镁合金；生物材料；PHB；细胞；降解

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