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ARTICLE

Hemocompatibility of Fluoride Treated AZ31B Magnesium Alloys Used for Intravascular Stents

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Abstract: Due to the excellent biodegradability, biocompatibility and mechanical properties of magnesium alloy, the possibility of magnesium alloy as intravascular stent was studied. Firstly, based on our previous work, a compact fluoride conversion coating was prepared on the surface of AZ31B magnesium alloy. The hemolytic tests, blood coagulation tests and platelet adhesion tests were carried out to evaluate the hemocompatibility of the fluoride treated AZ31B magnesium alloy. The surface energy was measured to analyze the mechanism of the hemocompatibility of the samples. Results show that the fluoride treated AZ31B magnesium alloy has potential application value in intravascular stenting.

Key words: magnesium; fluoride; hemocompatibility; intravascular stents; coronary interventions

The advent of intravascular stent is regarded as a revolutionary and remarkable achievement in coronary interventions. Currently, the permanent metallic stents, such as 316L stainless steel[1], Zr-Al-Fe-Cu[2], Co-based alloy[3,4], are most clinically used. However, the disadvantages of the permanent metallic stenting, including early late restenosis caused by neointimal hyperplasia and late complications, have attracted more and more clinical doubts[5]. Thus, the polymer-based stents, which are used as biodegradable intravascular stents, present the highlighted research direction due to technology accumulation in polymers[6-12]. As ideal biodegradable stents, they should initially provide sufficient mechanical support, and disappear after the service period. Unfortunately, the poor mechanical performance of the biodegradable polymer-based stents restricts their clinical implication.

As the requests for an ideal biodegradable intravascular stent continue to increase, the biodegradable metallic stent presents more and more advantages, which seems to be a better solution[13-16]. Specially, magnesium and its alloys have attracted much attention as potential biodegradable metallic stents owing to their biocompatibility and mechanical properties. As early as 2005, Zartner[17] et al have reported a biodegradable magnesium intravascular stent, made by BIOTRONIC, implanted into the left pulmonary artery of a preterm baby, and concluded that bioabsorbable stents with different diameters may help develop new strategies in the therapy of vessel stenosis in pediatric patients. However, it is noticed that problems such as alkalization, hydrogen release and high concentration of magnesium ions[18,19], caused by high corrosion rate in body fluid, will be the main clinical application barriers of magnesium. The key issue to address in development of the biodegradable magnesium intravascular stents is to reduce the corrosion attack to a reasonably low level.

In our previous work, we have reported a fluoride treatment used for biodegradable AZ31B magnesium alloy[20-22]. The fluoride treatment can significantly improve the corrosion resistance of the magnesium alloy in the simulated body fluid. We are also interested in the possibility of using the fluoride treated AZ31B magnesium alloy as intravascular stent. In light of the above, a fluoride conversion coating was prepared on the surface of AZ31B magnesium alloy in the present investigation, blood compatibility and surface energy tests

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were carried out to examine the hemocompatibility, and the possibility of using the fluoride treated AZ31B as intravascular stent was discussed.

1 Experiment

1.1 Fluoride conversion treatment

The AZ31B intravascular stent, with a composition (mass fraction) of 1.2% Al, 0.74% Zn, 0.35% Mn, 0.026% Si, 0.003% Fe, 0.0028% Cu, 0.0003% Ni and balance Mg, was used as the treating substrate. For conversion treatment, the specimens were immersed in a plastic bottle containing 50wt% HF at 30 °C for 48 h according to our previous work[21]. The treated specimens were rinsed with distilled water and dried in an oven at 40 °C.

1.2 Characterization

The morphology of the fluoride conversion coating on the surface of the AZ31B intravascular stent was examined by scanning electron microscope (SEM, JEOL JSM-6301F). The composition of the layer was analyzed by thin-film X-ray diffraction (TF-XRD, BRUKER AXS D8 ADVANCE).

1.3 Hemocompatibility test

Hemocompatibility tests including coagulation, hemolysis and platelet adhesion tests, were conducted in a 10 000 class clean room. Tests were performed according to ISO-10993-4[23]. The human whole blood containing 10% ACD was used for the tests. Platelet-rich plasma (PRP) was prepared by centrifugation at 1500 r/min for 10 min and platelet-poor plasma (PPP) was prepared by centrifugation at 3000 r/min for 15 min.

1.3.1 Hemolytic test

The samples for hemolytic test were 316L stainless steel, untreated and fluoride treated AZ31B magnesium. Six parallel samples were laid in each group. Blood testing solution was prepared by 4 mL fresh human whole blood with an ACD medium and then diluted with 5 mL simulated blood plasma. The test was performed as follows. First, the samples were soaked in normal saline according to the ratio of 3 cm^2/mL and incubated at 37 °C for 30 min. Deionized water and simulated blood plasma were used as the positive and negative control, respectively. Then, 50 μL diluted blood was added into each tube. All the tubes were incubated at 37 °C for 60 min, followed by centrifugation at 3500 r/min for 5 min, and finally the optical density (OD) of supernatant was evaluated by a microplate reader (GF M-2000) at 545 nm wave length. The hemolytic rate expressed as percentage was calculated according to the following equation:

$$\text{Hemolytic rate}=[(D_t-D_{nc})/(D_{pc}-D_{nc})]\times 100\% \quad (1)$$

where D_t is the OD value of the test group, D_{pc} and D_{nc} are the OD values of the positive and negative group, respectively.

1.3.2 Blood coagulation test

The samples for blood coagulation test were 316L stainless steel, pure Mg, untreated and fluoride treated AZ31B magnesium. The in vitro blood coagulation time, including plasma recalcification time (PRT) and prothrombin time (PT) was determined using an automated blood coagulation analyzer (GF-2000, Caihong, China). The PRT was measured to compare substrate-induced delay in clotting of platelet-poor plasma following activation of prothrombin (Factor II) in the presence of Ca^{2+}. In brief, the PRT was measured as follows. 150 μL PRP was placed on the sample at 37 °C for 2 min, and then 100 μL incubated PRP was transferred to testing tube. Simultaneously, with the addition of 100 μL 0.025 mol/L $CaCl_2$ solution, an autotimer ran and the equipment recorded the clotting time automatically. PT was measured to assess the interdiction of the extrinsic coagulation pathway. The measurement of PT was similar to that of PRT except that the PPP and the thromboplastin solution replaced PRP and $CaCl_2$ solution, respectively.

1.3.3 Platelet adhesion test

The samples for platelet adhesion test were 316L stainless steel, fluoride treated and untreated AZ31B magnesium. PRP was used for the test. After rinsing and sterilizing, samples were distributed evenly in two culture plates. A certain amount of PRP was poured into each well of the culture plates. After incubating at 37 °C for 30 min and 3 h, the PRP was taken out of the wells. The platelets that were non-specifically adsorbed on the surface were removed by washing with phosphate buffer solution (PBS) for 3 times. Then 2.5vol% glutaraldehyde was added into the plates. The platelets adhered on the surface were fixed at 4 °C for 4 h. After rinsing with distilled water, the samples were subsequently dehydrated through 50%, 75%, 95% and 100% ethanol water solutions for 10 min and dried at 4 °C overnight. The surface of the samples was observed by SEM and the photographs of platelets were randomly taken from the observation.

1.4 Contact angle and surface energy measurement

The contact angle was measured by the sessile drop method, using a contact angle meter (JC2000A, Powereach, China). The applied liquids were water and naphthalene bromide, drop volume 1~2 mL; each measurement was performed in triplicate. The surface energy was calculated according to Owens and Wendt[24].

1.5 Statistical analysis

All statistics were performed using SPSS 16.0. The hemolytic and blood coagulation analysis were all expressed as the mean ±SD. Differences between two groups were analyzed by the paired-samples t-test. Statistical significance was defined as $p<0.05$.

2 Results

2.1 Characteristics of the coating

Fig. 1 shows the surface morphology of fluoride treated AZ31B intravascular stent after balloon inflation. It can be observed that a dense and smooth coating with some scattered small irregular pores forms on the surface of intravascular stent sample. The irregular pores in the coating may be generated by the evolution of hydrogen and reduced or filled by precipitation of MgF_2 and MgO particles[22] during fluoride conversion treatment. Meanwhile, according to our previous

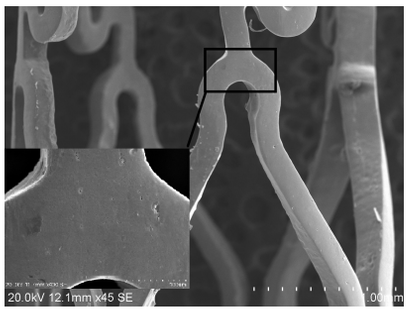

Fig.1 Surface morphology of fluoride treated AZ31B intravascular stent

work[22], the thickness of the fluoride coating is about 1.9 μm.

The result of TF-XRD, as shown in Fig.2, which is similar to our previous work[21], clearly indicates that the conversion coating is composed of MgF_2 and MgO. The magnesium alloy substrate is also detected and shows high resolution in the spectrum because of the small thickness of the coating.

2.2 Hemolytic test

Table 1 shows the results of the hemolytic tests performed on 316L, treated and untreated AZ31B samples. Untreated AZ31B samples induce hemolysis of (94.45±2.8)%. The hemolytic rate of 316L and fluoride treated AZ31B is 0. The pH values of the saline were also measured after the hemolytic test. The results show that pH value of simulated blood plasma incubated untreated AZ31B sample reaches about 12.20, whereas that of saline incubated 316L and treated AZ31B samples remains at about 7.4.

2.3 Blood coagulation test

Fig. 3 shows the result of blood coagulation test. It can be seen that the prothrombin time (PT) of pure Mg, treated and untreated AZ31B is longer than that of 316L, whereas the difference is insignificant. The plasma recalcification time (PRT) of pure Mg, untreated and fluoride treated AZ31B is 1.4, 1.38 and 1.5 times longer than that of 316L, respectively. It is evident that fluoride treated sample has better anticoagulation characteristics than 316L, pure Mg and untreated AZ31B.

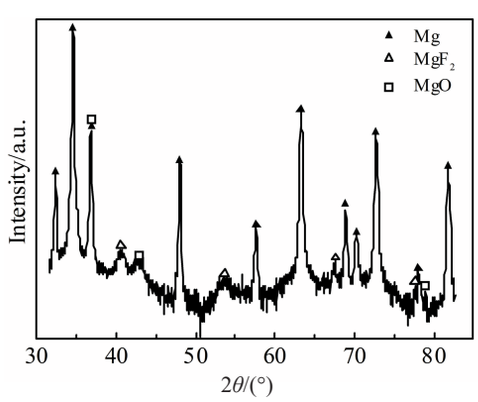

Fig.2 TF-XRD spectrum of fluoride treated AZ31B intravascular stent

Table 1 Results of hemolytic test

Materials	Hemolytic rate/%	pH value of solution after test
316L stainless steel	0	7.38
AZ31B	94.5±2.8	12.20
Treated AZ31B	0	7.42

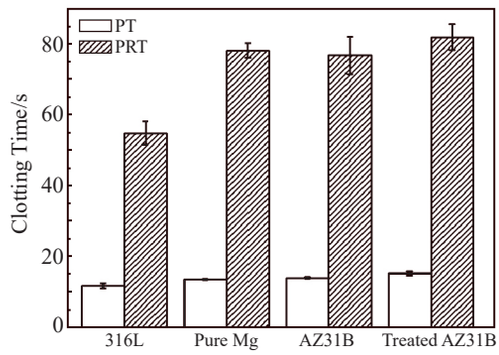

Fig.3 Comparison of anti-coagulation property of samples in terms of PRT and PT (n=5, mean±SD)

2.4 Platelet adhesion test

Fig. 4 presents the numbers and the morphologies of platelets attached on the surface of fluoride treated AZ31B, untreated AZ31B and 316L stainless steel incubated in PRP for 30 min and 3 h. It can be observed that: (1) the number of platelets on treated and untreated AZ31B is both less than that on 316L, (2) the number of platelets on 316L after incubating for 3 h is more than that for 30 min, while no significant difference is found on treated AZ31B, and (3) platelets on 316L and untreated AZ31B have some agglomeration and distortion, while no agglomeration or distortion is found on treated AZ31B.

3 Discussion

Hemolysis is the destruction or dissolution of red blood cells, with subsequent release of hemoglobin. Besides endogenic hemolytic factors such as autoimmune hemolytic anemia, there are some kinds of extrinsic factors such as physic agent on material's surface which can lead to cytotoxicity or may result in machinery damage to red blood cells (RBC). RBCs have a limited lifetime before they are recycled by the body. However, foreign materials and excessive stress can cause RBCs to rupture and to release excessive hemoglobin (the oxygen carrier in RBCs) in the blood stream. The hemolytic test shows the sensitivity of cells by measuring the amount of hemoglobin released during interaction with a foreign material. According to the ISO 7405[25], a hemolytic ratio below or equal to 5% indicates that the concerned material fits well according to the international requirements. On the contrary, a hemolytic ratio higher than 5% is a sign of hemolyzation. It can be seen from the results of hemolytic tests that treated AZ31B and 316L have no hemolysis, while

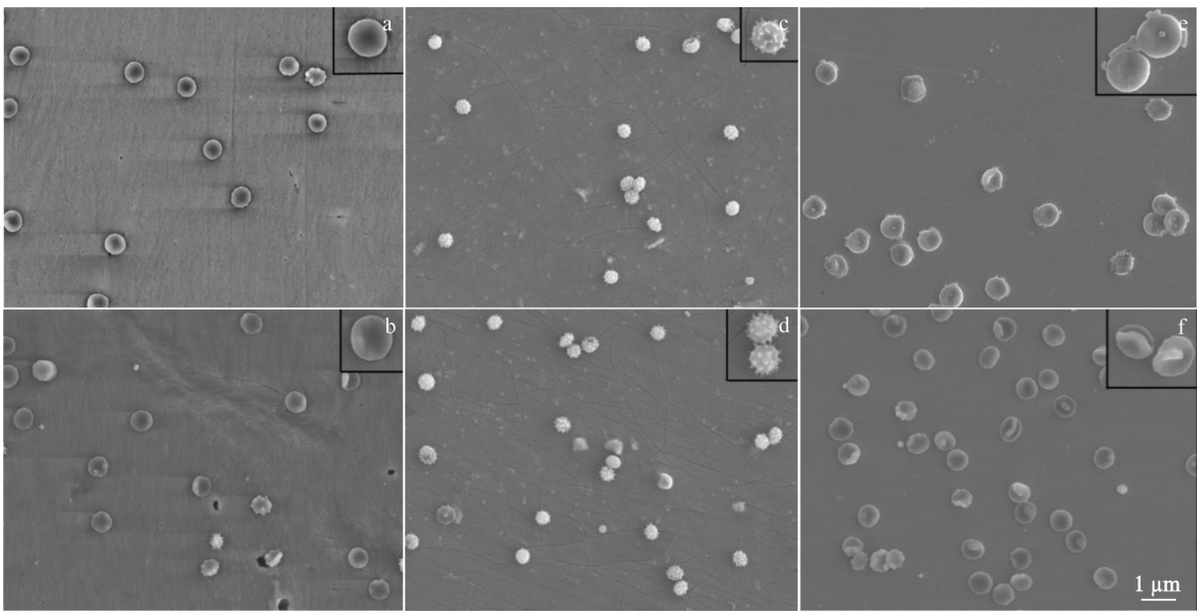

Fig.4 Platelet adhesion on samples incubated in platelet-rich plasma for 30 min (a, c, e) and 3 h (b, d, f): (a, b) fluoride treated AZ31B, (c, d) untreated AZ31B, and (e, f) 316L stainless steel

acute hemolysis appears on untreated AZ31B samples. It is found that human erythrocytes can be fused with Ca^{2+} at pH= 10.5[26] and a sharp increase in hemolysis occurs at a high pH value[27]. The pH value of solution incubating untreated AZ31B samples reaches about 12.0[21], whereas that of solution incubated 316L and treated AZ31B samples keep about 7.4[20,21]. The low value of pH is considered to be the main reason for the decrease of hemolysis rate in this study.

The blood coagulation cascade includes the intrinsic pathway, the extrinsic pathway, and the common pathway. The plasma recalcification time (PRT) and prothrombin time (PT) are used to examine the intrinsic and extrinsic pathways, respectively. Results of blood coagulation tests reveal that the PT of pure Mg, treated and untreated AZ31B is longer than that of 316L. The PRT of pure Mg, treated and untreated AZ31B is 1.4, 1.38 and 1.5 times, respectively, which is longer than that of 316L. The best blood compatibility can be achieved by the longest clotting time.

When blood comes in contact with a foreign material, plasma proteins are always adsorbed onto the material surfaces, and provoke the adhesion of platelets to the plasma protein layer. Adherent and aggregated platelets release substances such as ADP and ATP, thereby inducing more platelet aggregation on the surface. In the final phase, the system will lead to the formation of non-soluble fibrin network or thrombus. Therefore, platelet adhesion is an important means to evaluate blood compatibility of biomaterial surfaces[28]. The less the distortion and the adhesion of platelets to the material surface, the less the probability of blood coagulation. Thus, the results of the platelet adhesion test obtained in the present study illustrate that the fluoride treated AZ31B samples have better blood compatibility.

For biomaterials, surface energy and wetting properties are the key factors that affect the protein's adhesion and proliferation. Along with contacting between body fluid and implant material, proteins adhere to the surface of material[29]. When samples are immersed in the blood plasma, platelets and other organic agents in the plasma adhere to the surface until the thickness of adhered protein film reaches a critical value. It is found that the coating with good wetting ability does not possess good protein adhesion[30]. Therefore, the small contact angle and high surface energy of material do not promote adhesion of proteins, thereby preventing the adhesion of platelets and other organic agents in the plasma to induce the prothrombin. The results of contact angle measurements and surface energy given in Table 2 indicate that the fluoride treated AZ31B has the smallest contact angle (26.3°±1.5°) and the highest surface energy (71.6 mJ/m^2) as compared to AZ31B (76.1° ±1.7° , 40.7 mJ/m^2) and 316L (67.5° ±2.1° , 44 mJ/m^2). The results suggest that the good wetting ability of fluoride treated AZ31B with smaller contact angle and higher surface energy is one of the main reasons that are attributable to its better blood-compatibility in the platelets adhesion and anti-coagulation.

Table 2 Results of contact angles of water and surface energy of samples

Sample	Contact angle/(°)	Surface energy/ $mJ·m^{-2}$
Fluoride treated AZ31B	26.3±1.5	71.6
AZ31B	76.1±1.7	40.7
316L	67.5±2.1	44

4 Conclusions

1) In order to promote the possibility of clinical application

of magnesium alloy as intravascular stent, a compact fluoride conversion coating can be prepared on AZ31B magnesium alloy based on our previous work.

2) The fluoride conversion coating on the AZ31B alloy results in a better biocompatibility compared to untreated AZ31B and 316L.

3) The enhanced corrosion resistance and high surface energy lead to better hemocompatibility of the fluoride treated AZ31B magnesium alloy. The fluoride treated AZ31B magnesium alloy has potential application value in intravascular stenting.

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氟转化处理AZ31B镁合金冠脉支架的血液相容性

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摘　要：基于镁及镁合金优异的生物降解性、生物相容性和综合力学性能，对镁合金在血管支架领域的应用进行了探索。采用转化处理在AZ31B镁合金表面成功制备出氟转化涂层；随后分别采用溶血实验、凝血试验和血小板黏附试验研究了氟转化处理后的AZ31B镁合金的血液相容性能；为了分析其血液相容性能的机制，同时开展了材料表面能的测定。结果表明，氟转化处理AZ31B镁合金在冠脉介入治疗领域极具应用潜力。

关键词：镁；氟转化；血液相容性；血管支架；冠脉介入治疗

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