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ARTICLE

Evaluation of Haemocompatibility of TLM Titanium Alloy with Surface Heparinization

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Abstract: A layer of TiO_2 film was prepared by sol-gel method on the surface of TLM(Ti-3Zr-2Sn-3Mo-25Nb) alloy. Then the coated samples were treated by hydroxyl solution and amination solution in order to introduce the OH^- and NH_2^- active groups, and the heparin was linked on the surface of TiO_2 film through these active groups. The phase identification and the surface characteristics of the samples were successively undertaken using XRD, SEM and EDS. The in vitro blood compatibility of the TLM alloy specimens with and without heparinization treatments was evaluated by the contact angle test, the hemolysis test and investigation of their platelet adhesion behavior. The results showed that the haemocompatibility of the TLM alloy could be significantly improved by surface modification via heparinization.

Key words: haemocompatibility; heparinization; TLM alloy; film; surface modification

In recent years, the β titanium alloys have been increasingly used in the research and development of medical devices designed for applications in direct contact with human blood, for example, haemodialysis systems, extracorporeal circulation circuits, heart valves, blood by-pass tubes, prosthetic devices, catheters, etc. A major effort in this field of biomaterials technology has been directed towards developing biomaterials with improved haemocompatibility. It is known that when blood contacts an artificial surface a complex series of interacting events occur: protein adsorption, cellular (mostly platelet) adhesion, activation and aggregation, activation of blood coagulation systems, contact and complement activation and finally fibrin and thrombus formation[1].

However, the "perfect haemocompatible" material is far from a reality in a field rather too complicated to provide simple and easy solutions. A significant part of current research on haemocompatible materials is focused on the surface modification of already available materials with satisfactory mechanical properties rather than the development of new materials. Surface modification methods that seek to improve the haemocompatibility of these materials include chemical treatments (fluorination, introduction of reactive groups, surface grafting, etc.)[2]. More recently new surface modification techniques have been developed which include immobilization of specific biological molecules on the surface of materials, mostly heparin and albumin[3-5].

The novel near β titanium TLM alloy (Ti-3Zr-2Sn-3Mo-25Nb) exhibits a set of satisfactory mechanical properties such as high strength, good ductility and a high fatigue limit, which make it suitable for the use in many applications requiring contact with blood[6-8]. In order to improve the haemocompatibility (especial anticoagulation) of the TLM alloy, the investigations of the in vitro blood haemocompatibility of TLM titanium with and without surface heparinization treatments were made.

1 Experimental

All the chemical reagents used in this investigation such as ethylene glycol monomethyl ether, ethyl acetoacetate, for-

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mamide, persulfate, acrylamide and ceric ion were all of an analytically pure grade; the TLM(Ti-3Zr-2Sn-3Mo-25Nb) alloy was provided by Northwest Institute for Nonferrous Metal Research.

TiO_2 films were prepared by a Pechini sol-gel process and dip-coating method. Ti(OC_4H_9) was added into ethylene glycol monomethyl ether solvent as precursors of TiO_2 sol, and then small amounts of hydrolysis inhibitor of ethyl acetoacetate and complexing agent of formamide were added into the sol. After magnetic stirring for 30 min, a pH regulating solution was trickled into the mixture. The uniform transparent pale yellow sol was prepared after magnetic stirring for 2 h. Finally, the sol was aged for 24 h.

The TLM samples were dipped in the sol. After coating using the Czochralski Technique, the samples were moved to a resistance furnace for heat treatment. In order to obtain the films of different structures and composition, the heat treatment temperatures were varied(500, 700, 1000 ℃), and then kept at that temperature for 1 h. After cooling-off, three kinds of TiO_2 thin films with different phase compositions were prepared[8,9].

The films were treated with an aqueous solution of potassium peroxydisulfate(100 g/L) for 6 h at 75 ℃ dynamically, and finally the samples were washed with hot water and dried in vacuum. Then the films were submitted to grafting reactions in an aqueous solution with the aid of the ceric ion technique using acrylamide(120 g/L) in nitric acid(0.04 mol/L) and ceric ammonium(0.04 mol/L) solution at 65 ℃ for 24 h under a stream of nitrogen. Films were washed extensively with NaOH solution followed by hot water.

Heparin immobilization was carried out on the treated film surfaces using 1 g/L heparin solution (heparin sodium, Serva, 174 000 IU(International Unit)/g) at a pH of 5.5 containing 2 g/L carcodiimide hydrochloride (EDC-Sigma) as the coupling agent. The reaction lasted for 48 h at ambient temperature under static conditions.

The analysis of the phase identification of the TiO_2 films has been performed using X-ray diffractometry, X radiographic source(CuKα, Scanning rate was 0.03°/s, Scanning angle: 20°～80°, deflection angle: 1°). Surface characteristics of the films were observed by using a scanning electron microscope (JSM-6460, JEOL). Contact angle measurements by the sessile droptechnique using a contact angle geniometer (Phoenix-300) were conducted at 25℃ with double distilled water. The results presented in this paper represent the statistical averages of three measurements on different regions of the sample surface.

The heparinization samples and 10 mL saline were immersed into the silylation tube. After reacting in a water bath at 37℃ for 30 min, the saline was added to ACD rabbit blood. The supernatants were obtained using centrifuge process. The degree of hemolysis was determined spectrophotometrically by the concentration of hemoglobin in the supernatant at 540 nm.

The formula for calculating the haemolysis rate is as follows:

$$D_\eta = \frac{D_e - D_n}{D_p - D_n} \times 100\%$$

Where, D_η is the haemolysis rate (%), D_e is the absorbance in the experimental group, D_n is the absorbance in the negative control group, and D_p is the absorbance in the positive control group. The value of $D_\eta \leqslant 5\%$ is taken as normal.

In vitro platelet adhesion testing was performed to investigate the quantity, morphology, aggregation and pseudopodium of the adherent platelets. Blood was obtained from a healthy adult volunteer free of aspirin or other drugs that could bias the results. The whole blood was treated with the anticoagulant (sodium citrate). After centrifuging, the red blood cells and platelets were separated, and a platelet-rich plasma(PRP) was obtained. The samples were immersed in PRP and incubated at 37 ℃ for 2 h. The samples were subsequently rinsed with a 0.09 g/L NaCl solution to remove weakly adherent platelets[4,13].

The adhered platelets were fixed in 2.5% glutaraldehyde solutions at room temperature for 12 h, then dehydrated in a graded ethanol series, and critical point dried, and covered using a sputter coater with a layer of gold. Platelet morphology in the case of heparinized and reference materials was examined by scanning electron microscopy using a scanning electron microscope at 20 kV (JSM 6700, JEOL, USA).

2 Results and Discussion

2.1 XRD analysis

Fig.1 shows the X-ray diffraction patterns of the TiO_2 film specimens with different heat treatments. The anatase and rutile phases can easily be identified with the diffraction peaks being sharp but not splitting. At 500 ℃, the film is mainly composed of anatase, and there is only a small amount of rutile. TiO_2 can transform into rutile from anatase at 700 ℃. When the temperature is 1000 ℃, the film exists mainly in the form of rutile. The phase composition of the TiO_2 films can be controlled by adjusting the temperature of the heating treatment and aging time. The 1000 ℃ is the final temperature selected at last because the blood compatibility of rutile is better[10].

It is clear from Fig.2a and 2b that the TiO_2 films cover the whole surface of the TLM samples, without cracking and spalling behavior. The surface of the films is smooth, uniform and dense. The results of the Scan Energy Spectrum Analyses of Fig.2c and 2d indicate that the oxide film on the TLM samples is TiO_2 film.

2.3 Hydrophilicity test

The lower contact angles of the biomaterials are of benefit to the antithrombotic performance of the biomaterials.

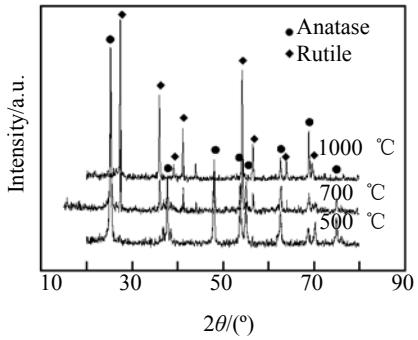

Fig.1 XRD patterns of the films under different heat treatment

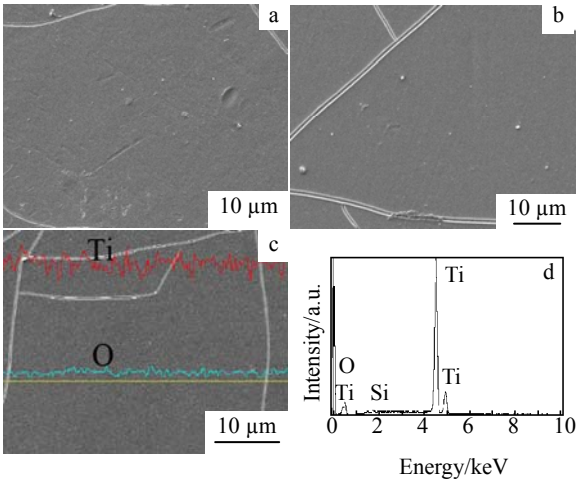

Fig.2 Micrographs and EDS spectra analysis of the TiO_2 films: (a, b) SEM micrographs, (c) line scan energy spectrum analysis, and (d) plane scan energy spectrum analysis

Table 1 shows that the heparinization treatment can reduce the contact angles made with the water droplets. The contact angles of the samples without heparinization treatment are higher than that of those subjected to heparinization treatment. This is because the natural TiO_2 film on the TLM sample is very thin(10-90 nm), and it is amorphous. But the thickness of sol-gel film is over hundreds of nanometers, and with high crystallinity characteristics. More importantly, it is believed that the sol–gel TiO_2 film rich in Ti–OH groups can reduce the contact angles. In addition, the active functional group of OH^- and NH_2^- which are introduced on the surface of the films are all hydrophilic active groups, and thus the hydrophilicity of heparinization samples are better than that of the samples without heparinization.

Table 1 The contact angle of the TLM alloy before and after heparinization

Samples	1#	2#	3#	Average value/(°)
TLM alloy	33.9	34.8	34.7	34.5
Heparinization TLM alloy	17.1	16.8	15.9	16.6

2.4 Hemolysis test

The hemolysis rate of the TLM alloy before and after heparinization treatment is shown in Table 2. It can be seen that the average hemolysis rate of the TLM alloy before heparinization treatment is 0.70%, and the value is 0.08% after heparinization treatment, which is obviously lower than the national standard (5%) and could not cause the hemolytic reaction of the body.

Hemolysis can result from the comprehensive factor, while hemolysis is promoted by the aid of chromatic dispersion action stemming from the surface and fully humidifying of water on the surface. The OH^- and NH_2^- are hydrophilic groups along with hydrogen bonding existing easily on the surface through physical absorption of water. Hydrophilicity of the activated films thus can be enhanced, which is the main reason leading to a decrease in the hemolysis ratio of the heparinization films[11,12]. In addition, the theory of charge transport that the electron transfers to the surface of materials is related to the adsorption and conformational changes of the plasma protein, fibrin on the surface. TiO_2 is an N-type semiconductor whose lower Energy Gap favor electron transition between valence and conduction bands which is helpful in increasing the Fermi Level and decreasing the Work Function of the films. So the heparinization films have good haemocompatibility[13,14].

2.5 Platelet adhesion test

Fig.3 shows the adherence and morphology of platelets on the TLM alloy specimens with and without heparinization treatment. It is clear from Fig.3a and 3b that the TLM alloy samples with heparinization treatment show a fairly clean surface with no red blood cell adhesion and no fibrin formation. The number of adhered platelets on the surface of the TLM alloy samples with heparinization treatment is much less than that on the untreated sample with almost all platelets adherent to the preadsorbed surface in a less activated pattern (round and individual), and also there is no deformation, agglomeration and pseudopod formation of the platelet on the heparinization surface. But as shown in Fig.3c, 80% of the platelets on the surface of the TLM alloy specimens without heparinization treatment are in aggregation and in the pseudopodium state. Thus, platelets are strongly surface-activated by the TLM alloy, and this process is promoted by preadsorption of fibrinogen. It is consistent with the result of fibrinogen adsorption, and much more fibrinogens are adsorbed on the TLM alloy surface.

Table 2 Hemolysis rate of TLM alloy before and after heparinization

Samples	1#	2#	3#	Average value/%
TLM alloy	0.65	0.69	0.76	0.70
Heparinization TLM alloy	0.07	0.09	0.09	0.08

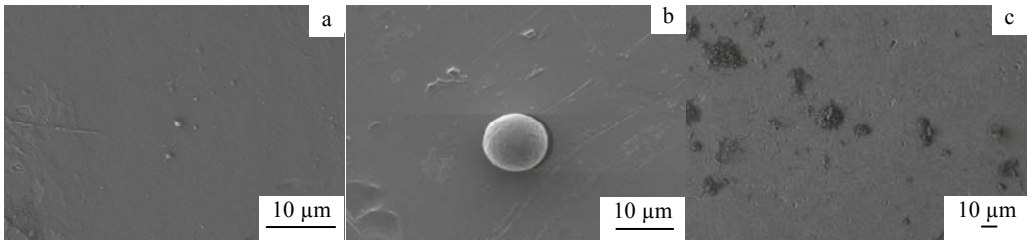

Fig.3 SEM micrographs of platelet adhesion for TLM samples with: (a,b) with heparinization treatment and (c) without heparinization treatment

As is apparent from the results, there is an improved blood-compatibility response of the heparinized TLM alloy compared to the untreated samples concerning both platelet retention and activation of the coagulation system. This, in our opinion, means that heparin molecules remain biologically functional in the heparinization procedure, because the active groups(OH^- and NH_2^-) used to connect heparin to a substrate ensure that the active site of the heparin is held outward away from the support so as to contact the body fluid efficiently.

At least two different hypotheses have been proposed to theoretically explain this effect. The older one is the AT-III theory, and it assumes that the reduced thrombogenicity of the heparin-coated surfaces is attributable to the catalytic effects of heparin on AT-III and the accompanying forces of thrombin complexes[15]. More recent studies assume that the advantage of heparin coatings lies much more in the reduction or selective adsorption of plasma proteins as well as in the maintenance of the native conformation of these proteins once adsorbed on the heparinized surface[16]. Furthermore, some researchers note that the inhibitory effect of heparin on coagulation and complement is independent and shows different structure requirements on the heparin molecule[17]. They also mention that the better response of the heparinized materials in terms of platelet adhesion and activation could be attributed to the improved response of the complement and contact activation system.

3 Conclusions

1) An heparinization surface can be obtained on the TLM alloy through preparing a layer of TiO_2 film by sol-gel and covalent immobilization of heparin onto the surface.

2) The contact angle and hemolytic rate of the heparinization surface of the TLM alloy are both smaller than those of the one without treatment. The number of adherent platelets on the surface associated with the heparinization treatment is much smaller than that on TLM alloy without heparinization treatment, and the aggregation and pseudopodium is much less than that of the untreated one. Therefore, the TLM alloy with heparinization shows improved haemocompatibility.

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表面肝素改性TLM钛合金的血液相容性评价

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摘　要：在新型近β钛合金 TLM(Ti-3Zr-2Sn-3Mo-25Nb)表面用溶胶-凝胶法镀上一层 TiO_2 薄膜，再将薄膜依次用羟基化溶液和胺基化溶液处理以在薄膜表面引入活性 OH^-和 NH_2^-，然后通过该活性官能团将肝素共价键接在薄膜表面。利用 XRD、SEM 和 EDS 研究了 TiO_2 薄膜的相结构和表面特性；通过测定材料表面的接触角、溶血率和血小板黏附行为对肝素化 TLM 合金的血液相容性进行分析、评价。结果表明，表面肝素化处理后 TLM 合金的血液相容性得到了明显的改善。

关键词：血液相容性；肝素化；TLM 合金；薄膜；表面改性

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