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Original article

Immobilization of hesperidin on stainless steel surfaces and its blood compatibility

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ABSTRACT

The coating of hesperidin, a traditional Chinese medicine ingredient, was immobilized to the surface of stainless steel (SS) which was often used in the cardiovascular implant materials and evaluated for its blood compatibility in vitro. The immobilized coating showed prolonged activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) values compared with stainless steel control. The platelet adhesion and activation test on the hesperidin coating also showed significantly less data compared with the control. The test on conformational change of fibrinogen (fgn) demonstrated the hesperidin coating could reduce this parameter compared with the bare SS. It can be summarized that the hesperidin coating can effectively improve the blood compatibility of the SS surface. We envisage that this coating will provide a potential and effective selection for blood contact devices.

1. Introduction

Cardiovascular disease is the number one threat to global human health and life in today's world with its higher morbidity and mortality than neoplastic diseases. According to the reports of World Health Organization (WHO), the deaths caused by cardiovascular and cerebrovascular disease possess 30.3% of the total number of deaths in the world [1]. Atherosclerosis is one of the most common cardiovascular diseases [2], and its causes are not entirely clear. Early thrombus formation is considered to be the most important reason of the pathogenesis of atherosclerosis [3]. Vascular stent therapy is an effective means for the treatment of this disease currently [4]. So far, lots of anticoagulant drugs have been used for the drug-eluting stents (DES) to inhibit the thrombosis further formation. Yet, most of the DESs have a high cost and complicated production process [5,6]. Thus, DESs with low cost, simple production process and significant therapeutic are called for by both research and clinical treatment currently.

Hesperidin is the main ingredient of several traditional Chinese medicines, and naturally exists in the citrus and other plants, and can be isolated in large amounts from the rinds of some citrus species [7–9]. This flavanone has been proved to possess a wide range of pharmacological actions, such as effects on the vascular system, anti-inflammatory actions, platelet aggregation inhibition [7,10]. However, up to now, little has been reported on this biomolecule applied in the cardiovascular implant materials surface modification. Therefore, it was chosen to fabricate a medical coating on the biomaterials surface here.

In the present work, hesperidin coating was prepared onto the 316L stainless steel (316L SS) surface by a method of self-assembly [11] for blood-contacting implant applications. 316L SS plates are chosen as the substrates because stainless steel is the most widely used biomaterials for cardiovascular implants [12,13]. The modification of 316L SS substrate was characterized, as well as the quantity of the immobilized hesperidin was investigated. To determine whether the immobilization of hesperidin improved the blood compatibility, activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) of the samples were tested, and contact activation and adhesion of the platelets as well as conformational change of the fibrinogen (fgn) were evaluated in vitro. We anticipate that this hesperidin coating will be helpful to improve the biocompatibility of the SS-based biomaterial devices.

2. Materials and method

2.1. Materials and reagents

The 316L SS plates (Xin Zhe Steel Co., Ltd., China) were cut into small squares (10 mm × 10 mm) and polished, then the 316L SS discs were sonicated successively in acetone, ethanol, deionized water (dH_2O) and finally dried at room temperature (RT). Hesperidin powder (610.56 Da, INCPBP China) was dissolved in the Tris-buffer (2 mg/mL, pH 8.5). APTT, PT and TT kits for anticoagulation properties test were purchased from Sunbio, China.

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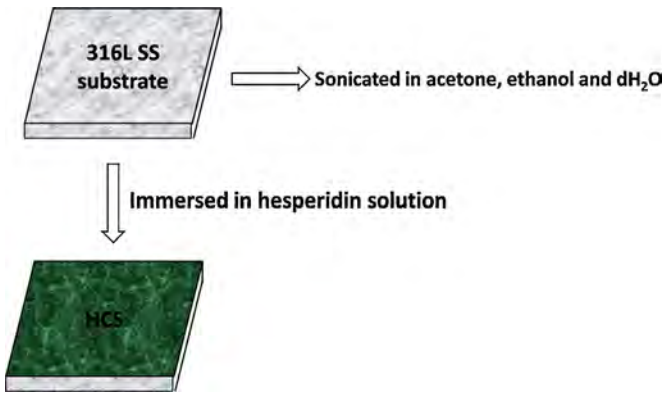

Fig. 1. The scheme of the immobilization of hesperidin onto the 316L SS surface.

Lactate dehydrogenase (LDH) and alpha-granular membrane protein (GMP140), conformational change of the fgn antibody and Rodamine123 were purchased from Boshide, China. All the other reagents used in the experiments were of the highest analytical purity (> 99.9%). Fresh anticoagulant (ACD) whole blood of a volunteer was centrifuged at 1500 rpm to obtain platelet-rich plasma (PRP) and 3000 rpm to obtain platelet-poor plasma (PPP), respectively.

2.2. Fabrication of hesperidin coating

Fig. 1 shows the scheme of the immobilization of hesperidin. The 316L SS samples were immersed in the hesperidin solution and incubated at 37 °C for 6 hours, and then rinsed with dH_2O for three times, finally dried in RT, and the sample was labeled as HCS.

2.3. Surface characterization of hesperidin coating

The surface morphology of the prepared hesperidin coatings on the 316L SS surface was depicted by a Nanowizard II AFM (JPK Instruments, Berlin, Germany) in tapping mode. Image was analyzed using the CSPM Imager software. The chemical composition of the coating was characterized by FTIR spectrometer (NICOLET 5700, USA) in diffuse reflectance mode. The wettability of the hesperidin coating was evaluated by static water contact angle measurement (DSA 100, Krüss, GmbH, Germany) [14]. The quantity of the immobilized hesperidin on the 316L SS was investigated using a weight loss method.

2.4. In vitro blood compatibility of the hesperidin coating

The anticoagulation property of immobilized sample and the controls was determined by means of an APTT, PT and TT assay. APTT was used to detect the intrinsic coagulation, PT was used to detect the extrinsic coagulation, and TT was used to detect the thrombin-mediated fibrin formation [14]. Lactate dehydrogenase (LDH) test and alpha-granular membrane protein (GMP140) test were used to determine the amount of adherent and activated platelets on samples, respectively [15]. The shape of the adherent platelets was observed under a fluorescence microscope after stained by Rodamine123 for 15 minutes. The conformational change of fgn on all samples was detected by immunochemistry [14]. In the blood compatibility examinations, 316L SS was used as positive controls, low-temperature isotropic carbon (LTIC) was used as negative controls.

2.5. Statistical analyses

The data were analyzed with SPSS 11.5 (Chicago, Illinois). Values were expressed as mean ± SD. $P < 0.05$ was the established level of significance.

3. Results and discussion

3.1. Surface characterization of hesperidin coating

Fig. 2 displays the AFM images of the HCS and 316L SS samples surface. The surface of the 316L SS was flat with an average roughness of 3.28 ± 4.16 nm, and became rougher with a roughness of 43.90 ± 56.40 nm after immobilized hesperidin. Obviously, the hesperidin coating caused a rougher surface.

The chemical structure of the 316L SS and HCS surface was analyzed by ATR-FTIR spectroscopy. Fig. 3A was the molecular structure of hesperidin which showed that hesperidin molecule has C=O bond, -OH group, and C-H bond. As shown in Fig. 3B, 316L SS and HCS both presented peaks at 1620 cm^{-1} (C=O) and the peak around 3500 cm^{-1} ascribed to -OH stretching vibrations. On the HCS sample surface, the new peaks of C-H at 2900 cm^{-1} and N-H at 1550 cm^{-1} confirmed that the 316L SS surface was successfully coated by hesperidin. The introduction of

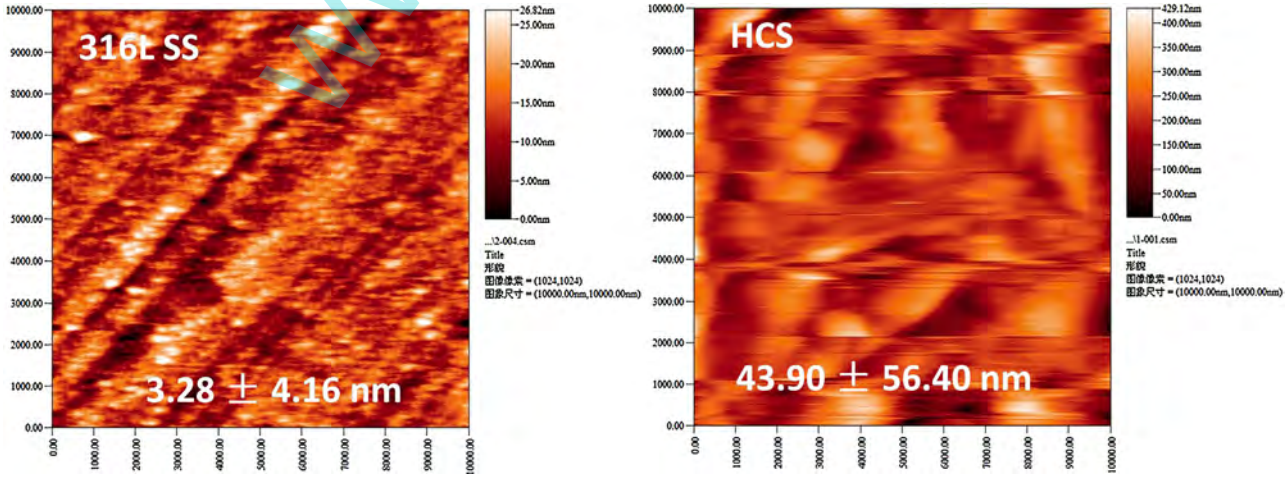

Fig. 2. AFM images of the HCS and 316L SS surface.

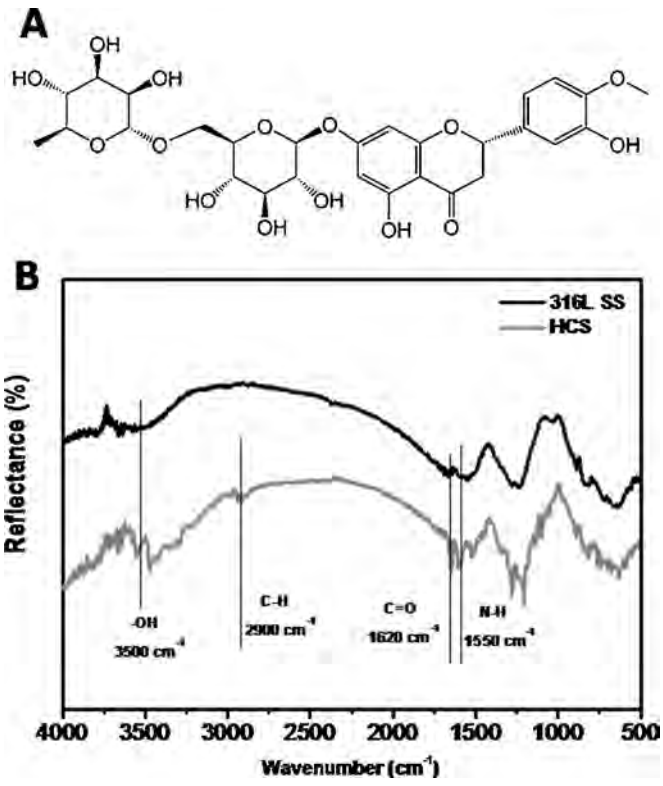

Fig. 3. (A) The molecular structure of hesperidin; (B) FTIR of the HCS and 316L SS surface.

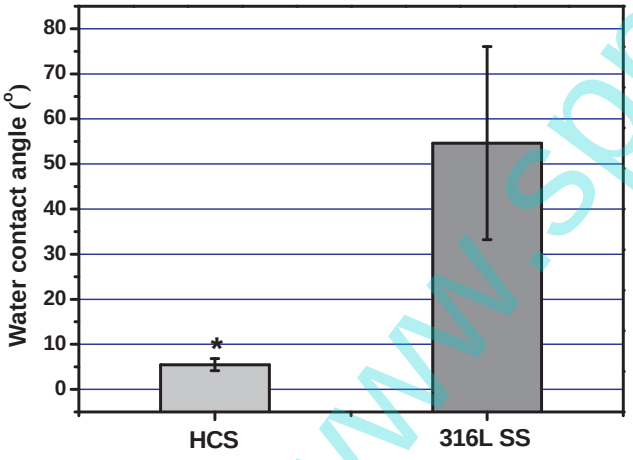

Fig. 4. Water contact angles of the HCS and 316L SS surface (*$P < 0.05$, mean ± SD, $n = 6$).

N-H may be attributed to the Tris-buffer that contained N element.

Fig. 4 shows the water contact angle measurement of the HCS and 316L SS surfaces. It could be seen that the contact angle dramatically decreased from 55.5 ± 17.5° to 6.9 ± 1.8° after coated by hesperidin, and this may be due to the large amount of hydroxyl group of the hesperidin molecules.

The quantity result of the immobilized hesperidin on the 316L SS by a weight loss method was about 299.6 ± 16.5 μg. This amount was still too little, and the reason may be part of the phenolic hydroxyl groups were oxidized at 37 °C in the air, thus could not be reacted with iron ions in weak alkaline condition. The prepared time of 6 hours may be also inadequate.

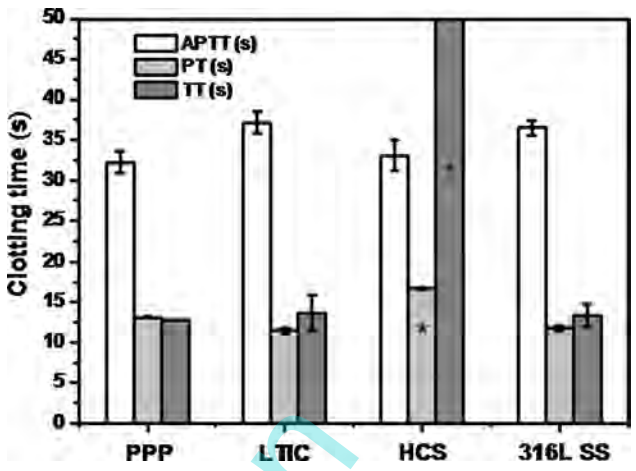

Fig. 5. APTT, PT and TT test of the HCS and PPP samples, 316L SS and LTIC were used as positive and negative controls, respectively (*$P < 0.05$, **$P > 0.05$, mean ± SD, $n \geq 3$).

3.2. In vitro blood compatibility of hesperidin coating

3.2.1. APTT, PT and TT

APTT, PT and TT tests were performed to evaluate the anticoagulant activities of the samples (Fig. 5). It could be seen that there were no significantly difference on the APTT value of each sample, indicating that hesperidin could not prolong the clotting time through inhibiting the intrinsic coagulation pathway. On PT values, the HCS samples displayed significant longer PT values than PPP and the controls, which indicated that hesperidin could prolong the clotting time through inhibiting the extrinsic coagulation pathway. Moreover, a not coagulation TT value appeared on the HCS sample, which indicated that the immobilized hesperidin had excellent effect on inhibiting fgn conformational change.

It has been reported that hesperidin can inhibit the collagen-, ADP-, AA-, and thrombin-induced aggregations in a dose-dependent manner and the anti-platelet effect of hesperidin is unlikely to be associated with cytotoxicity [10]. Our results also demonstrated that the dose of immobilized hesperidin on the 316L SS surface was enough to inhibit the thromin formation.

3.2.2. Platelet adhesion and activation

The LDH and GMP140 tests were performed to evaluate the amount and activation of the adherent platelets on hesperidin immobilized sample. 316L SS and LTIC were used as positive and

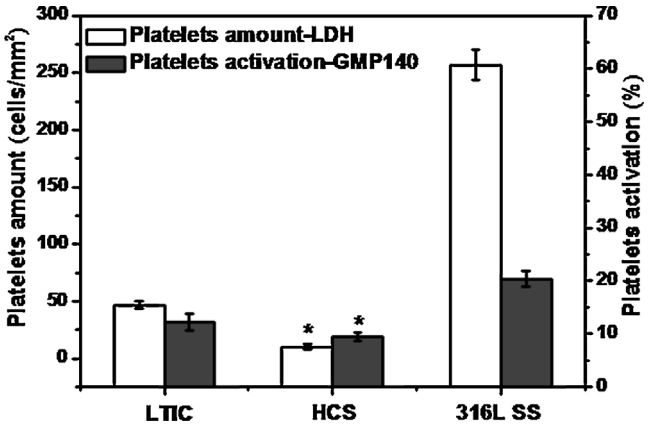

Fig. 6. Platelet adhesion (LDH) and activation (GMP140) on the HCS surface, 316L SS and LTIC were used as positive and negative controls, respectively (*$P < 0.05$, mean ± SD, $n \geq 3$).

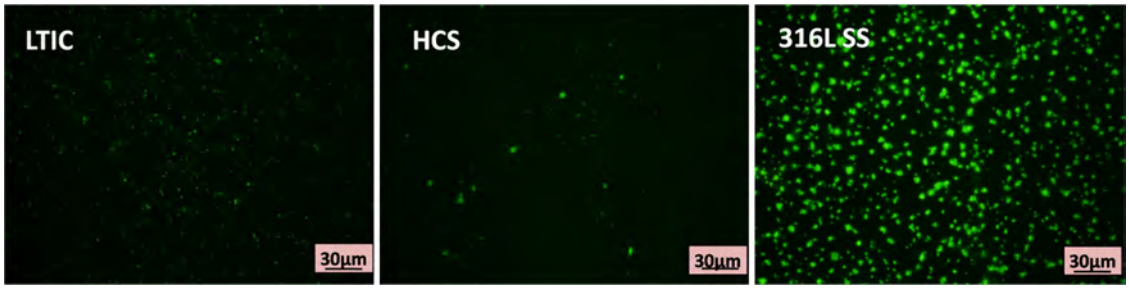

Fig. 7. Fluorescence images of the adherent platelets on the HCS, 316L SS and LTIC surfaces.

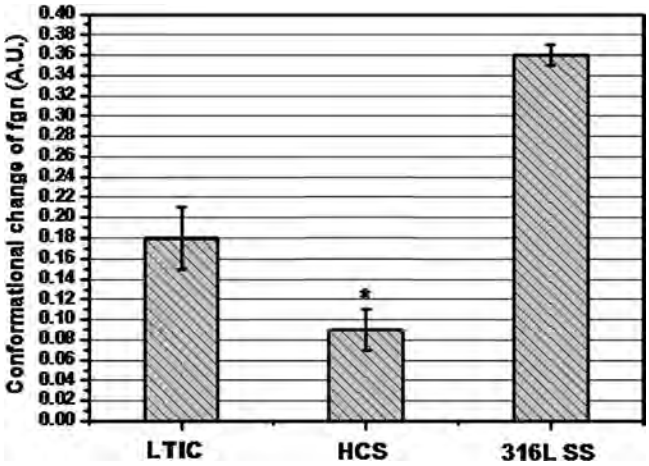

Fig. 8. Conformational change of fibrinogen (fgn) on the HCS surface, 316L SS and LTIC were used as positive and negative controls, respectively (*$P < 0.05$, mean ± SD, $n \geq 3$).

negative controls. The results of platelets adhesion and activation on each sample were shown in Fig. 6. It could be seen that after incubation in PRP for 1 hour, a remarkable platelet adhesion was observed on 316L SS surface, which also showed the highest platelets activation compared with LTIC and HCSF sample ($P < 0.05$). HCS showed the least platelets adhesion and activation compared with all the other samples. The results of platelets adhesion and activation suggested that the hesperidin immobilized surface possessed a better blood compatibility than 316L SS and even LTIC.

3.2.3. *Fluorescence staining of adherent platelets*

A fluorescence staining method was used to identify the adherent platelets on HCS sample and the results are shown in Fig. 7. It could be seen that the control samples showed a significantly larger amount of platelets adhesion than the HCS sample, and more platelets aggregated on 316L SS sample than that on HCS sample. The fluorescence staining of platelets also indicated that the HCS surface possessed better blood compatibility. This result was consistent with the results of clotting time, LDH and GMP140 tests.

3.2.4. *Conformational change of fgn*

The conformational change of fgn, i.e. exposure of γ chain (HHLGGAKQAGDV at γ 400-411) [14,16], plays an essential role in platelet activation and aggregation. Fgn conformational changes measured by the immunochemistry method could reveal the thrombosis tendency [14]. Fig. 8 displayed the conformational changes of fgn determined results from the control samples and the HCS sample. The HCS sample showed significantly lower absorbance compared with the controls ($P < 0.05$), suggesting a good blood compatibility of HCS sample and the results of fgn conformation change may be used to explain the results of PT, TT and platelets adhesion test.

4. Conclusion

In summary, the hesperidin coating was successfully prepared onto the stainless steel surface. The tests of APTT, PT, TT, platelet adhesion and activation, and conformational change of fgn demonstrated that the medical coating can significantly improve the blood compatibility of the stainless steel surface. Therefore, it may be helpful to thrombosis formation inhibition. This study may provide valuable and potentially application for cardiovascular implant materials such as anticoagulation DES.

Disclosure of interest

The author declares that he has no conflicts of interest concerning this article.

Acknowledgements

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