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# Vascular endothelial cell compatibility of superhard ternary Ti–Si–N coatings with different Si contents

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ABSTRACT

Superhard ternary Ti–Si–N coatings with different Si atomic concentrations are deposited on titanium alloy substrates by arc-enhanced magnetron sputtering (AEMS) and the vascular endothelial cell compatibility is studied. *In vitro* studies show that the surface hydrophilicity and hemolysis are about the same as those of the titanium alloy control and platelet adhesion and protein adsorption tests suggest a close relationship between the surface energy and blood compatibility. Endothelial cells cultures reveal better proliferation and anti-platelet adhesion on the coatings compared to the titanium alloy control and the Ti–Si–N coating with 20 at% Si exhibits excellent endothelialization.



## 1. Introduction

In spite of continuous improvements, the blood compatibility of biomedical implants such as cardiovascular stents and artificial heart valves remains one of the critical problems that trigger failure [1,2]. The blood compatibility of biomaterials is closely related to surface properties such as roughness, composition, and elemental chemical states [3], and surface modification is an effective way to enhance the surface blood compatibility without affecting the favorable bulk properties such as materials strength and sturdiness. Coatings such as gold, titanium nitride, silicon nitride, silicon carbide, iridium oxide, and diamond-like carbon (DLC) have been prepared on cardiovascular stents in order to prevent or alleviate formation of wear debris and inhibit platelet adhesion and activation [4–6].

Endothelium can maintain vascular homeostasis, regulate vascular tone and thrombosis, and foster muscle cell proliferation and migration. Damaged vascular endothelial cells and sub-endothelial matrix exposure may cause thrombosis and neointimal hyperplasia [7,8]. This is very important to re-endothelialization of injured parts to repair vascular functions. Studies have shown that cultivation of endothelial cells on the material surface in contact with blood is a good choice and clinical trials have been conducted. In arteries and heart valves, there are high impact and force imposed by the blood flow and hence, it is important to improve the adsorption capacity of endothelial cells on biomaterials in order to maintain endothelial cell functions.

Recently, ternary Ti–Si–N coatings have drawn much attention because of their excellent mechanical performance such as superior hardness attributable to its phase and microstructure of the nanocrystalline TiN and amorphous $Si_3N_4$ matrix [9]. They are thus suitable for bio-tribological applications such as artificial heart valves and hip joints. Although much is known about their attractive mechanical properties, the biological properties of Ti–Si–N coatings, especially the blood compatibility [10], are relatively not well known. Ternary Ti–Si–N coatings can be prepared by chemical vapor deposition (CVD) and physical vapor deposition (PVD) [11–13] and their mechanical properties depend on the microstructure and processing parameters. In biological applications, the blood compatibility is related to the surface properties of the biomaterials. In particular, surface endothelialization can produce anti-thrombogenic coatings that mimic natural blood vessels [14] and so it is of both scientific and technical significance to investigate the interactions between Ti–Si–N coatings and vascular endothelial cells. In this work, Ti–Si–N coatings with different Si atomic concentrations are deposited on medical titanium alloy by arc-enhanced magnetron sputtering (AEMS) and the relationship between the surface properties and blood compatibility as well as vascular endothelial cell compatibility is systematically investigated.

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## 2. Experimental details

### 2.1. Deposition of Ti–Si–N coatings

Arc-enhanced magnetron sputtering (AEMS, Beijing Powertech Co. Ltd) was used to deposit the Ti–Si–N coatings on polished titanium alloy ($Ti_6Al_4V$) samples with a diameter of 20 mm and thickness of 2 mm. A columnar titanium target with a diameter of 60 mm and length of 450 mm produced the arc discharge in the presence of a hollow and permanent magnet placed in the center of the hole. The two ultra-pure Ti (99.99%) and two ultra-pure Si (99.99%) targets with dimensions of 435 mm × 94 mm × 8 mm were used to prepare the Ti–Si–N coatings. A Ti interlayer was first deposited using the columnar Ti target (operated at 20 V and 60 A) at 0.3 Pa in the arc discharge mode for 10 min to improve adhesion between the substrate and coating. The important deposition parameters are listed in Table 1.

### 2.2. Microstructure and surface characterization

The structure of the coatings was characterized by X-ray diffraction (XRD, XRD-7000, SHIMADZU LIMITED) using Cu $K_\alpha$ radiation. The surface morphology was examined by atomic force microscopy (AFM, SPI3800-SPA-400, Seiko Instruments Inc.) and the chemical states were determined by a X-ray photoelectron spectroscopy (XPS, AXIS ULTRA, KRATOS ANALYTICAL Ltd.) using Mg (1253.6 eV) radiation.

### 2.3. Contact angle measurements

The static (sessile drop) water contact angles were determined on the Rame-Hart imaging system (USA) [15]. The mean contact angle was calculated from at least three individual measurements taken at different locations on each sample.

### 2.4. Hemolysis ratios

In order to obtain the hemolysis ratios of the samples, blood samples were obtained from healthy human donors. 4 ml of blood was diluted with 5 ml of 0.9% (w/v) sodium chloride solution and then 10 ml of 0.9% (w/v) sodium chloride was added. Additionally, 10 ml of the 0.9% (w/v) sodium chloride solution (negative control, $n = 4$) and 10 ml of double distilled water (positive control, $n = 4$) were prepared for antitheses. All the samples were kept at 37 °C for 30 min and immediately incubated in 0.2 ml of the whole blood at the same temperature. After 60 min, the samples were centrifuged for 5 min and the supernatant was analyzed at 540 nm to determine the absorbance of cells undergoing hemolysis using a microplate reader (Powerwave XS MQX200R). The hemolysis ratios were calculated by the following relationship [16]: $R = (A - C1)/(C2 - C1) \times 100\%$ with $R$ being the hemolysis ratio (%), $A$ the absorbance (%), $C1$ the absorbance of the negative controls (%), and $C2$ the absorbance of the positive control (%).

**Table 1**
Typical instrumental parameters adopted in the fabrication of the Ti–Si–N coatings arc-enhanced magnetron sputtering.

| Parameters | Values |
|---|---|
| Working pressure (Pa) | 0.4 |
| $N_2$ flow rate (sccm) | 12 |
| Ar flow rate (sccm) | 4 |
| Ti targets current (A) | 10 |
| Si targets current (A) | 1, 3, 5, 7 |
| Substrate temperature (°C) | 120 |
| Negative bias voltage (V) | 100 |
| Deposition time (min) | 120 |

### 2.5. Adsorbed human plasma proteins and cell culture medium proteins

Fresh whole blood from a volunteer was centrifuged at 3000 rpm to obtain the platelet-poor plasma (PPP). The samples without PBS immersion were put on a 12-well culture plate and 2 ml of PPP was added and incubated for 2 h at 37 °C. Afterward, each sample was rinsed ten times with PBS to detach the loose proteins. The plates were soaked in 1 wt% aqueous sodium dodecyl sulfonate (SDS) solution for 30 min to desorb the protein and a protein analysis kit (Micro BCA, Pierce, Rockford) was utilized to determine the concentration of the PPP in the SDS solution based on the BCA method [17]. The amount of PPP adsorbed on the polymer surface was calculated from the concentration in the SDS solution. We also do a protein adsorbed assay of cell culture medium, method as described above. The data were expressed as means ± standard deviation ($n = 4$) based on the analysis of variance and Student $t$-test.

### 2.6. Platelet adhesion

Platelet adhesion on different samples was examined by fluorescence staining. The membrane probe DiI was purchased from Beyotime (China) and the fresh human platelet-rich plasma (PRP) incorporated with the DiI solution was incubated at 37 °C for 10 min. The platelets were separated from the red dye by high speed centrifuging and then the samples were immersed in PRP (1 ml per sample), incubated at 37 °C for 1 h, and rinsed with PBS three times. The stained samples were observed under an inverted fluorescence microscope.

### 2.7. Cell culture

The endothelial cell line (EAhy926) was provided by Shanghai cell bank (catalog number GNHu39) of The Chinese Academy of Sciences. They served differentiated endothelial cell functions, namely angiogenesis, homeostasis, thrombosis, blood pressure, and inflammation. Furthermore, they could be cultured to high passages without appreciable changes in the growth rate and phenotype, thus avoiding the diversity of primary isolated endothelial cells from different individuals, limitation of replication potential, and senescent tendency in cultures. The cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin in culture dish and incubated in a humidified atmosphere of 5% $CO_2$ at 37 °C [18].

### 2.8. Cell proliferation

After sterilization with 75% alcohol, the samples were placed on a 12-well culture plate and 1 ml of the DMEM medium was added. The concentration of endothelial cells was $5 \times 10^4$ cells/ml. Endothelial cell proliferation was investigated by the CCK-8 kit (Biotime, China) after incubation for 1 and 5 days. The medium was removed and the samples were washed twice with PBS. The fresh medium containing CCK-8 reagent was added to each sample and incubated at 37 °C for 3 h under standard culturing conditions. Afterward, 100 μl of the blue solution were transferred to a 96-well plate. The absorbance was measured at 570 nm on a microplate reader and all the proliferation experiments were performed in triplicates [19].