Applied Surface Science 459 (2018) 438–445

Contents lists available at ScienceDirect

Applied Surface Science

journal homepage: www.elsevier.com/locate/apsusc

Full Length Article

In-situ preparation of amino-terminated dendrimers on TiO_2 films by generational growth for potential and efficient surface functionalization

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ARTICLE INFO

Keywords:
Amino-terminated dendrimer
TiO_2 film
In situ generational growth
Platelet adhesion
Endothelial cell

ABSTRACT

Dendrimers, with their unique structure and polyfunctionality, have shown excellent performance in many biomedicinal applications, such as drug carriers and gene transfection. In this study, titanium dioxide (TiO_2) films with four generations (G1, G2, G3, G4) of amino-terminated dendrimers were prepared *in situ* using a simple iterative Michael addition and aminolysis reaction for generational growth. The TiO_2 films with different generations of dendrimers were characterized using X-ray diffraction, water contact angle measurements, and scanning electron microscopy. The chemical compositions of these films were confirmed by Fourier-transform infrared spectroscopy and X-ray photoelectron spectroscopy. Blood compatibility was evaluated using platelet adhesion and activation assays utilizing platelet-rich plasma. The results indicated that the dendrimers immobilized on the surface of the TiO_2 films effectively reduced platelet adhesion and aggregation. Endothelial cell culturing on the dendrimer-immobilized surfaces showed that the amino-terminated dendrimers exhibited a certain degree of cytotoxicity due to the positive charges of the amino groups. These results indicated that amino-terminated dendrimers immobilized on TiO_2 films provided numerous functional groups for the future immobilization of specific biological molecules and may be used for manufacturing future blood-contacting implants.

1. Introduction

Most existing biomaterials, particularly blood-contacting implants, must have multiple properties such as anticoagulant, anti-inflammatory, and antifouling properties. They should also be non-toxic and have good cell compatibility [1,2]. Therefore, various materials including metals, ceramics, polymers, and composites [3] have been used to manufacture medical implants. However, the biological functions of these bare materials have always been unsatisfactory. Consequently, to improve their biocompatibility and obtain relatively ideal biological materials, many researchers have performed various surface modification techniques and have concluded that these techniques are an important tool for promoting functionality, especially when designing biomaterials and biomedical devices [4,5]. The use of different organic functional groups, such as −NH2 and −OH, on the surfaces of biomedical metals helps achieve novel surfaces using chemical methods.

As special semiconductor materials, titanium dioxide (TiO_2) films have received considerable attention as promising candidates for developing implantable devices [6,7] due to their active hydroxylation [8], good biocompatibility [9] and corrosion resistance [10], and excellent mechanical properties [11]. Since TiO_2 is chemical inert, conventional methodologies to form surface organic coatings on TiO_2 films, such as siloxanation or carboxylation, are based on chemical reactions where the amino, hydroxyl, chloride, or alkane “head groups” attach to the surface of the film [4,12]. However, this attachment requires a low hydroxyl-group content on the surface [13]. Unlike siloxanation or carboxylation, phosphonate formation at the interface is not limited by the hydroxyl content on the surface [14,15]. This characteristic indicates that phosphonic acid forms strong bonds with TiO_2 [16]. Moreover, further functionalization on the surface using phosphonic acid can be achieved using chemical methods.

For the past two decades, studying the chemistry of self-assembled monolayers consisting of dendrimers has been one of the most intriguing and rapidly expanding areas in chemistry [17,18]. Dendrimers, a hyper-branched macromolecule with regular and well-defined three-dimensional structures, are synthesized by repeated growth and have numerous important chemical and physical properties [19,20].

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https://doi.org/10.1016/j.apsusc.2018.08.044
Received 20 April 2018; Received in revised form 29 July 2018; Accepted 4 August 2018
Available online 06 August 2018
0169-4332/

Although the utilization to dendrimers is restricted by their structural imperfections and polydispersity, large quantities can be manufactured using one-step procedures, thus making them attractive for various applications [20]. Moreover, their globular shapes have led researchers to believe that dendritic molecules can be prepared as ideal molecular building blocks on the surfaces and interfaces of biomaterials [20,21]. Using either divergent or covalent iterative methods [21], monodisperse macromolecules composed of primary structures having the same level of precision as biological molecules can be synthesized [22]. Moreover, due to the considerably large sizes and tunable peripheral functionality of dendrimers, they can be used as versatile building blocks for assembly on substrates [23]. Consequently, due to the unique structure and special physicochemical properties of dendrimers, the main goal behind pursuing their synthesis has become the strict control of their shapes and selective functionalization rather than numerous generations [24]. At present, conventional methods used for accurately controlling the structure of dendrimers include reactions in the core, at the periphery, or in the internal layers [25]. Therefore, precise tuning of structure, which is expected to eliminate the undesirable effects on the size and structural imperfections [26].

In this study, TiO_2 films were modified *in situ* using amino-terminated dendrimers via a simple iterative Michael addition and aminolysis reaction for generational growth. Thus, we could augment the number of functional groups and achieve efficient surface functionalization for potential applications in blood-contacting implants. The structure and properties of the surface of the amino-terminated dendrimers were evaluated, and the functions of the four generations of the dendrimers grown on the TiO_2 films were analyzed using platelet adhesion and endothelial cell proliferation tests.

2. Materials and methods

2.1. Materials

TiO_2 films were fabricated on a Si substrate using a magnetron sputtering deposition system. The 200-nm-thick deposited TiO_2 films were mainly of anatase phase. TiO_2 powder was purchased from Kelong Chemical Reagent Co., Ltd. (Chengdu, China) and subjected to heat treatment to obtain the anatase structure before use. Both 3-aminopropyl phosphonic acid (APPA) and tris-(2-aminoethyl)-amine (TAEA) were purchased from Acros Organics and used without further purification. Methyl acrylate (MA) and methanol were obtained from Fine Chemical Co., Ltd. (Shanghai, China). Prior to use, MA was purified by reduced pressure distillation and methanol was dried over metallic Na and distilled. All the other reagents were of analytical grade.

2.2. Preparation of samples

Silicon wafers coated with a TiO_2 film were neatly cut into 8 mm × 8 mm squares and used as substrates. Acetone, ethanol, and distilled water were successively used to wash the substrates. Briefly, the first layer of APPA was grafted onto the surface of TiO_2 by immersing the substrates in a 0.5 mL aqueous solution of 30 mmol/L APPA at 80 °C for 4 h [27]. The substrates were sonicated three times in water for 5 min to remove the weakly bonded APPA. The samples with APPA were obtained by repeating the incubation and sonication four times. Further, these samples were dried in vacuum and labeled as TiO_2-APPA.

Subsequently, the TiO_2-APPA samples were immersed in a graded MA solution (1/300; $V_{MA}/V_{methanol}$) for 24 h at 25 °C and cleaned with methanol, distilled water, and ethanol three times. Furthermore, the samples were transferred to a graded TAEA solution (1/60; $V_{TAEA}/V_{methanol}$) and magnetically stirred for 24 h in an argon atmosphere. The procedure is shown in Fig. 1(a) and the samples obtained were labeled as G1-NH_2. Furthermore, G2-NH_2, G3-NH_2, and G4-NH_2 samples, which immobilized different dendrimer generations, were obtained by repeating the above method two, three, and four times, respectively. Finally, four types of amino-terminated dendrimers were prepared and immobilized *in situ* on the TiO_2-APPA samples by generational growth according to a simple Michael addition and aminolysis reaction. Moreover, the structure of the amino-terminated G1-NH_2, G1.5-NH_2, and G2-NH_2 dendrimers is shown in Fig. 1(b), while the model diagram of the construction of the amino-terminated surface is shown in Fig. 1(c).

2.3. Surface characterization

The surface morphologies of the TiO_2 film, TiO_2-APPA, G1-NH_2, G2-NH_2, G3-NH_2, and G4-NH_2 were examined using field-emission scanning electron microscopy (SEM, Philips Quanta200). The surface chemical compositions of the specimens were measured using attenuated total reflectance Fourier transform-infrared (ATR-FTIR, NICOLET 5700) spectroscopy in the range of 400–4000 cm^{-1} and X-ray photoelectron spectroscopy (XPS, XSAM800, Kratos Ltd, UK) with a monochromatic Al Kα excitation radiation ($h\upsilon$ = 1253.6 eV). The C1s peak (binding energy 284.7 eV) was used as a reference for charge correction. A Shirley background was used, and the peaks were fitted using XPSPEAK 4.1 to obtain high-resolution information. The static water contact angles on the surfaces of the samples were measured using a goniometer with computer-assisted image analysis (DSA100, Krüss, Germany) at 25 °C. For each sample, the mean value of the water contact angle was calculated from at least three individual measurements taken at different regions on the examined samples.

2.4. Platelet adhesion

Fresh citrated human blood was taken in centrifuge tubes and centrifuged at 1500 rpm for 15 min to obtain the top platelet-rich plasma (PRP) layer for platelet adhesion experiments. Subsequently, 50 μL PRP was added onto the surface of each sample, and the samples were placed in a 24-well plate and incubated at 37 °C for 1 h. Furthermore, the samples were carefully rinsed with 0.9 wt.% NaCl solution to remove the platelets that did not firmly adhere. After fixation with 2.5 wt.% glutaraldehyde solution for 12 h, the samples were washed with 0.9 wt.% NaCl solution three times. The platelets adsorbed on the surface were dehydrated using ethanol solutions (50%, 75%, 90%, and 100%; $V_{ethanol}/V_{water}$) for 15 min for each ethanol concentration sequentially [28]. All the samples were critical-point dried (CPD030, BALZERS). Further, the resultant samples were sputtered with gold, and then examined using SEM (Quanta 200, FEI, Holland) to evaluate the morphology and quantity of adherent platelets.

2.5. Endothelial cell adhesion test

The cell compatibility of the samples was evaluated by adhesion and proliferation of human umbilical vein endothelial cells (HUVECs) *in vitro*. The HUVECs were derived from infant umbilical cords and cultured in humidified air containing 5% CO_2 at 37 °C [2]. The adhesion and proliferation behavior of the HUVECs after 1 and 3 days of culturing were investigated. The HUVECs were seeded onto the surfaces of the samples and incubated in 2 mL M199 medium (Gibco, USA) supplemented with 15% fetal bovine serum (Sigma, USA) and 20 μg/mL endothelial cell growth supplement (Millipore, Inc.) at a density of 1×10^4 cells/mL. The samples were removed after the predetermined time (1 and 3 days). Subsequently, the non-adhered cells were washed with phosphate-buffered saline three times and the adherent endothelial cells were fixed with 2.5% glutaraldehyde for 4 h. Further, the cells were stained with rhodamine-phalloidin for 15 min (50 μL per sample). Thereafter, all film samples were investigated by fluorescent microscopy (Zeiss Germany). Fluorescent images were acquired using a fluorescent microscope (Olympus IX51, Japan). For each sample, 15 images were captured and the results were averaged. The results were then converted to evaluate cell adhesion density.