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Synthesis and characterization of collagen–hydroxyapatite immobilized on polydopamine grafted stainless steel

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ABSTRACT

Hydroxyapatite (HA) and collagen have been coated on metallic implants to accelerate osseointegration. Most methods to coat HA require high sintering temperature, high cost and high energy power while the methods to coat collagen commonly produce unstable coating. Therefore, in this study, a polydopamine film was used as an intermediate layer to immobilize HA and collagen type I on a medical grade stainless steel (SS316L) implant to overcome those disadvantages. The SS316L disks were pre-treated and grafted with a polydopamine film. It was then covalently immobilized with collagen fibers at different immersion times (6, 12 and 24 h). The disks were further biomineralized with HA in simulated body fluid (SBF) for 7 days. The film surfaces were characterized by FTIR, FESEM-EDX, XRD and contact angle analyses to investigate the chemical composition, morphology, crystallinity and wettability properties. The collagen and carbonated HA (lath-like surface) were successfully immobilized on the polydopamine film with less agglomeration as the immersion time in the collagen solution increased. Increasing the immersion time accelerated the activation of carboxylic groups in the collagen to form an amide cross-linkage for heterogenous nucleation of HA. Furthermore, the crystallinity and wettability properties were also enhanced with the closest theoretical Ca/P ratio. As a conclusion, the immobilization of collagen at 24 h has produced better HA formation and wettability property that might be beneficial for the attachment and proliferation of osteoblast cells on biomedical implants.

1. Introduction

Hydroxyapatite (HA) is a bioactive ceramic that is composed of two main components of mineral bone (calcium and phosphorus) [1]. It has been coated on metallic implants to promote osseointegration [1]. Most techniques and technologies to deposit and coat HA on an implant such as plasma spray, electrophoretic deposition and hot isostatic pressing, require high sintering temperature which leads to crack formation due to mismatch of thermal expansion [2]. The crack formation further contributes to coating instability and practically interrupts the bone–implant fixation [3]. Furthermore, these techniques are expensive and consume high energy power to operate those instruments [4].

The biomimetic technique is one of the methods to coat HA on a metallic implant [5]. It does not require high energy power and high processing temperature, preventing the formation of crack and coating instability [3]. Recently, a polydopamine film was utilized to form the biomimetic HA on medical grade stainless steel (SS316L) through a functionalization process [6]. The application of polydopamine as an intermediate layer to functionalize biomolecules is adopted from the work of Lee et al. [7]. The functionalization mechanism is based on the existence of amine and thiol/catechol functional groups which will participate in the binding process [8]. This mechanism produces strong and stable anchorage properties [9]. Besides, the biomimetic HA grafted on the polydopamine film mimics the natural properties of bone. Those properties cause the polydopamine film to become a favorable way to optimize the surface of metallic implants [10].

Collagen fibers are another type of component, existing as the main organic composition of bone extracellular matrix [11]. It is commonly used to improve the biocompatibility of implant surfaces [12,13]. These fibers act as a building template for bone formation and provide mechanical strength to bone [14]. The immobilization of collagen on material surfaces through a physical absorption technique shows simplicity and flexibility, but generally this method produces instability of the coating film [15]. Meanwhile, a covalent immobilization technique compromises better control of coating parameters such as coating thickness, ligand density and molecular orientation [16]. There are various strategies to covalently immobilize the collagen onto metallic surfaces that usually involve complex chemistry and regularly induce additional toxic factors [17,18]. Therefore, the exploration of a simple and versatile covalent immobilization technique is crucial to immobilize the collagen fibers to promote osseointegration without producing toxic residues.

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The aim of this study was to immobilize collagen type I and biomimetic HA on a polydopamine grafted SS316L. Fig. 1 shows a schematic diagram of the sample preparation process. The chemical composition of the grafted film was characterized using Fourier transform infrared spectroscopy (FTIR) and energy dispersive X-ray spectroscopy (EDX). The crystallinity and morphology of the grafted film were investigated by X-ray diffractometer (XRD) and field emission scanning electron microscope (FESEM), respectively. Finally, the contact angle (CA) analyses were performed to determine the wettability property of the grafted film.

2. Materials and methods

2.1. Sample preparation

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, Missouri, US). Stainless steel sheet (Goodfellow Cambridge Limited, Huntingdon, England) was wire cut into a disk shape with 12.5 mm diameter and 0.5 mm thickness. The disks were cleaned by ultrasonic cleaning in acetone, deionized (DI) water and methanol for 10 min, accordingly. The cleaned disks were then electropolished in an electrolyte solution composed of glycerol, phosphoric acid (H_3PO_4) and DI water at a ratio of 10:7:3 for 3 min. The attached electrolyte solution was removed by dipping the disks in an acid mixture of nitric acid (HNO_3), hydrofluoric acid (HF) and DI water at a ratio of 5:1:44 [19]. Finally, the disks were rinsed with DI water, dried using an air compressor and hereafter referred to as pre-treated disk.

The pre-treated disks were grafted with a polydopamine film by ageing the disks in a dopamine solution for 24 h at ambient temperature. The dopamine solution was prepared by dissolving 20 mg dopamine chloride, $C_3H_{11}NO_2 \cdot HCL$ in 10 mM Tris $(CH_2OH)_3CNH_2$ buffer at pH 8.5 [20]. The grafted polydopamine disks, known as dopa, were then immersed in a collagen solution for 6, 12 and 24 h, separately (*x*Coll-dopa; $x = 6$, 12 or 24 h). The collagen solution was prepared by dissolving 1 mg collagen calf skin type I in 1 mL of 5 mM acetic acid, $C_2H_4O_2$ for 1 h. The pH was adjusted between 4 and 6 by sodium hydroxide, NaOH. After 1 h, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) with a concentration of 2.5 mg/mL and N-hydroxysuccinimide (NHS) with a concentration of 0.63 mg/mL were added to the collagen solution [17] to activate the functional groups of collagen. Finally, the *x*Coll-dopa disks were ultrasonically cleaned in DI water to remove excess non-attached collagen and dried under vacuum.

Biomimetic HA was formed on the *x*Coll-dopa disks through a biomineralization process in 1.5× simulated body fluid (SBF) solution. The solution was prepared based on a method described by Kokubo and Takadama [21] involving the dissolution of NaCl (12.053 g), $NaHCO_3$ (0.533 g), KCl (0.338 g), K_2HPO_4 $3H_2O$ (0.347 g), $MgCl_2 \cdot 6H_2O$ (0.467 g), $CaCl_2$ (0.438 g), Na_2SO_4 (0.108 g) and Tris (9.177 g) in 1 L of DI water at 36.5 °C. The solution was buffered at pH 7.40 using an appropriate amount of 1 M HCl. The *x*Coll-dopa disks were immersed in 30 mL of SBF solution and incubated at 37 °C for 7 days. The SBF solution was refreshed every 24 h to provide adequate ions for HA nucleation. After 7 days, the biomineralized disks (HA-*x*Coll-dopa) were removed from the solution, rinsed with DI water and dried under vacuum. The biomineralized disks without the immobilization of collagen were also prepared and mentioned as HA-dopa disk.

2.2. Surface characterization

The surface functionalities of the samples were investigated by FTIR (Nicolet IS-5-IR Spectrometer, Thermo Fisher Scientific, US) in an attenuated total reflectance (ATR) mode. The data were analyzed at a frequency interval of 600 to 4000 cm^{-1} using a crystal germanium. The number of scanning and resolution were set at 36 and 4, respectively. The crystallinity and crystal orientation of the biomineralized HA were then characterized by XRD (D5000, Siemens, Germany) in a thin film mode at 40 kV and 40 mA. The data were recorded in 2θ range using CuKα radiation of 1.5406 Å.

The surface morphologies of the samples were visualized under FESEM-EDX (SU8020, Hitachi, Japan). Each sample was coated with an ultrathin platinum film as a conductive layer to enhance image resolution. The images were taken at 5000×, 10,000× and 40,000× magnification. The elemental analyses were also performed at an operating voltage of 15 kV. Finally, a video contact angle instrument (VCA-Optima, AST Products Inc., US) was used to measure the surface wettability property. The measurements were recorded at five different spots by dropping 1 μL DI water on the disk surface at 6 μL/min. The water drop images were captured after 10 s to measure the angle of the water drop.

3. Results and discussion

3.1. Chemical functionalities

Fig. 2 shows FTIR spectra of pre-treated, dopa, pure collagen, *x*Coll-dopa, HA-dopa and HA-*x*Coll-dopa. In Fig. 2(a), both peaks at 2750 cm^{-1} and 2924 cm^{-1} were denoted by C–H vibrations in the polydopamine film as these peaks were not visible on the pre-treated disk. After the immobilization of collagen on the polydopamine film (Fig. 2(b)), several notable functional groups were noticed such as amide A (3350 cm^{-1}) and amide B (2985 cm^{-1}). Amide A was associated with N≡H stretching vibration that showed the existence of hydrogen bond while amide B indicated asymmetrical stretch of CH_2 stretching vibration.

Other functional groups were also observed on the 12Coll-dopa and 24Coll-dopa included amide I (1639 cm^{-1}), amide II (1550 cm^{-1}) and amide III (1280 cm^{-1}). Amide I was associated either with C═O stretching vibration or a hydrogen bond coupled with COO^-, amide II presented the bending vibration of N≡H coupled with stretching vibration and amide III showed the vibration absorption of C═N. However, amide I and III were not clearly seen in the 6Coll-dopa due to insufficient time for the activation of all carboxylic groups. According to Ao et al. [15], the activation of carboxylic groups (collagen) requires a minimum of 6 h that is crucial for the cross-linkage between the amine groups from the polydopamine and the carboxylic groups from the collagen, to form the amide bond.

In Fig. 2(c), the phosphate (PO_4^{3-}) groups were clearly observed in the HA and HA-*x*Coll-dopa consisted of anti-symmetric v_1 and v_3 vibration modes at 961 cm^{-1} and 1000 cm^{-1}, respectively. The additional

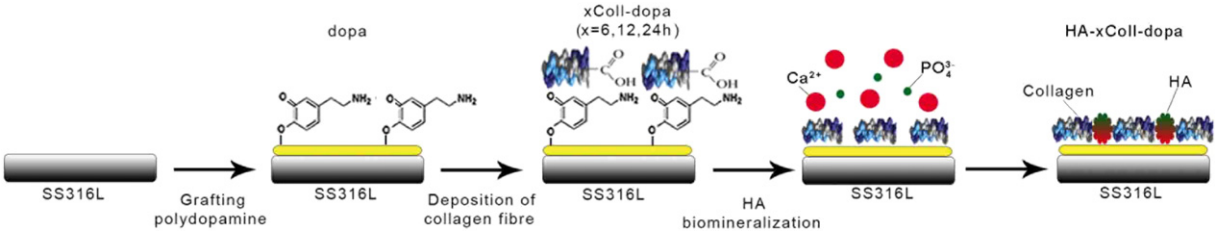

Fig. 1. Schematic diagram of sample preparation process.