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Fabrication of hydroxyapatite ultra-thin layer on gold surface and its application for quartz crystal microbalance technique

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Abstract

We present a method for coating gold quartz crystal microbalance with dissipation (QCM-D) sensor with ultra-thin layer of hydroxyapatite nanocrystals evenly covering and tightly bound to the surface. The hydroxyapatite layer shows a plate-like morphology and less than 20 nm in thickness. The hydroxyapatite sensor operated in liquid with high stability and sensitivity. The in-situ adsorption mechanism and conformational change of fibrinogen on gold, titanium and hydroxyapatite surfaces were investigated by QCM-D technique and Fourier-transform infrared spectroscopy. The change of secondary structures of fibrinogen adsorbed on the surfaces depended on the adsorbed amounts of protein. The secondary structure of fibrinogen adsorbed on the surfaces changes with increasing coverage. This is explained by repulsion among fibrinogens, affecting water structure and thus the strength of fibrinogen interactions on the surface. The study indicates that the hydroxyapatite sensor is applicable for qualitative and conformational analysis of protein adsorption.

Keywords: Hydroxyapatite; QCM-D; FT-IR; Fibrinogen; Adsorption

1. Introduction

Understanding and control of protein adsorption and conformational transformation on biomaterial surfaces is of great importance for their biocompatibility and motivates the development of reliable measurement techniques for these complex and dynamic processes. The quartz crystal microbalance with dissipation (QCM-D) technique is one of the high-sensitive and practical tools for in situ measurements of macromolecule adsorption and analysis of their conformational change [1–3]. The QCM-D measurements have been widely employed different substrates such as metals [4], polymers [5], and functionalized coatings [6].

Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (HAp) has been extensively applied in dental and orthopedic surgeries, due to its excellent biocompatibility and high osteoconductivity [7]. The porous HAp has been also investigated as a scaffold in bone tissue engineering for the rapid repair in larger defects [8]. The cytokines and its fragmented peptides, such as BMP, FGF, etc., can accelerate the new bone formation with simultaneous implantation of scaffolds [9]. The high biocompatibility of this material is partly explained by its ability for "mild" protein adsorption [10]. Therefore, a better control and understanding of the protein adsorption and detachment would be really useful for improvement of the biomaterial design.

QCM measurements have been applied before to study the crystal growth of HAp in simulated body fluid (SBF) [11,12] focusing on the inhibition role of functional groups, such as $-COO^-$, $-NH_2$ and $-SO_3^{2-}$ etc. The method has also been applied to monitor protein (albumin and histone) adsorption on 1-μm-thick HAp film deposited on gold by RF-magnetron sputtering method and subsequent hydrothermal treatment [13,14]. Stain adsorptions on HAp

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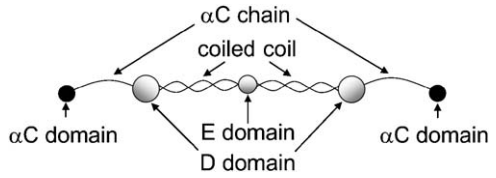

Fig. 1. Schematic picture of the secondary structure of fibrinogen. The detailed characteristics of the labeled domains are described in the text.

crystals were reported; with HAp deposited on the phosphate-terminated, polymer-modified gold surface by an electroassisted formation process in the accelerated calcification solution [14]. These studies described QCM technique as effective tool for the investigation of macromolecular adsorptions on HAp surface.

A number of coating techniques have been described for the fabrication of homogeneous HAp layer on metal surfaces: electrophoretic deposition (EPD) [15,16], plasma spraying [17], beam sputter deposition [18], laser ablation [19], and pulsed laser deposition [20]. The EPD method is a simple, rapid and low cost process; HAp nanocrystals with similar size and chemical component to the biological apatite, just precipitated and dispersed into a solvent, can be used in this process. However, the homogeneous HAp nano-layers tightly bonded on the metal surfaces by the EPD method have not been described to our knowledge.

Fibrinogen, a structural glycoprotein in blood plasma with isoelectric point 5.5 is often employed for QCM experiments of protein adsorption [4,21–23], due to its moderate molecular weight (340 kDa) and size (45 nm length [24]). The molecular structure is shown on Fig. 1: a central hydrophobic E domain is connected to two hydrophobic D domains by coiled-coil chain. These hydrophobic domains are charged negative under neutral pH condition. The αC domains, with Arg and Lys residues, are charged positive and are substantially more hydrophilic than the E and D domains.

Here we describe fabrication of ultra-thin layer of HAp nanocrystals on gold surface by EPD method and their use for protein adsorption analyzed with QCM-D technique. The HAp nanocrystals were prepared by a wet method with similar structure and composition to biological apatite. The desired properties of fabricated HAp layer are: (i) thickness less than 100 nm, to ensure clear detection of the dissipation (ΔD) changes [25] and (ii) tight accretion to substrate preventing detachment during protein adsorption. The structure and physical properties of HAp layers were elucidated by atomic force microscopy (AFM), contact angle analysis, Fourier-transform infrared reflection absorption spectroscopy (FTIR-RAS). The comparative adsorption behaviors of fibrinogen on gold, titanium and HAp were conducted by using the QCM-D analyses.

2. Materials and methods

HAp nanocrystals were precipitated at 80 °C by slowly dropping dilute H_3PO_4 solution into a $Ca(OH)_2$ suspension to a final pH value of 8.0. The resultant HAp suspension was centrifuged at 2000g for 15 min, washed three times with ethanol, and ultrasonically dispersed in ethanol at HAp concentration of 1 wt%. The crystalline phase obtained after drying the precipitation in air was identified by X-ray diffraction (XRD, 40 kV and 40 mA) analysis with a multiflex diffractometer (Ultima-III, Rigaku). The crystalline size was estimated from the half width of 100 and 002 diffractions according to Scherer's equation. The crystal morphology was observed by transmission electron microscopy (TEM, LEO 922). The TEM specimens were prepared by dropping the HAp suspension on microgrid and dried it in air for 30 min.

We used commercial (Q-Sence AB) gold and titanium plated QCM-D sensors. The sensors surface was cleaned before any measurements and treatments; the gold sensor was immersed in 5:1:1 mixture of Milli-Q quality distilled water, H_2O_2 (30%) and NH_3 (25%) for 10 min at 70 °C and the titanium surface was cleaned using 2% of sodium dodecyl sulfate (SDS) solution. The cleaning procedure was completed by 10 min UV/ozone irradiation.

The ultra-thin layer of HAp was deposited by EDP method using the gold-plated sensor as electrode and applying 10 or 100 V/cm DC voltage for 5 min. The electrolyte was 1 wt% of HAp suspension in ethanol, The ultrasonic treatment (28 kHz, 100 W) for 1 min in ethanol was conducted to remove the surplus HAp from the deposited layer on the cathode.

The surface morphologies, Z range images and root mean square (RMS) roughness for all sensors were analyzed in air by AFM (SPM-9500, Shimadzu Inc.). Silicon nitride probe mounted on cantilever was employed for the dynamic mode (tapping mode) to reduce surface damage. The surface wettability was analyzed at 22 °C in air by a sessile drop method of distilled water with automatic contact angle meter (CA-W200, Kyowa Interface Science). The thickness of the HAp layer deposited was examined with a spectral ellipsometer FE5000 (Ohtsuka electronics) at 300–800 nm wavelength, and polarizer and incidence angles of 60°, 65° and 70°, respectively.

We employed QCM-D apparatus (D300, Q-Sense AB) to study protein absorption on gold, titanium and HAp surface. The experimental arrangement is described in detail in Ref. [1]. For this model study, we used fibrinogen from bovine plasma (Wako Pure Chem. Ind., Ltd.) in solution of 1 mg/ml adjusted by using 10 mM phosphate buffer solution (NaH_2PO_4 and Na_2HPO_4, pH 7.0).

The resonance frequency (Δf) and dissipation (ΔD) were detected simultaneously driving the crystal at 15 MHz at 22 ± 0.05 °C.

FTIR-RAS spectra were recorded on a Spectrum GX-Raman (PerkinElmer Inc.) apparatus with accumulation times of 256 s and resolution of 1.0 cm^{-1}. The IR beam was introduced through a polarizer and a KBr window into the process chamber at an incident angle of 70°. The reflected beam from the substrate was detected by a mercury–cadmium–telluride (MCT) detector. The amide I band subtracted the baseline at the region of 1725–1575 cm^{-1} was fitted to β-turn, random, α-helix and β-sheet structures based on the Gaussian shape function. The peak positions were assigned following the literature [26] and analyzed without fixations in curve fitting. The secondary structure components were finally calculated with the refined peak positions to reduce residual errors.

3. Results and discussion

The HAp prepared at 80 °C has high crystallinity, according to measured XRD pattern and is a single phase without traces of other crystalline phases as tricalcium phosphate ($Ca_3(PO_4)_2$), calcium hydroxide and calcium carbonate. The crystalline sizes of d_{100} and d_{002}, calculated from Scherrer's equation ($K = 0.9$) were found to be 40–50 and 120–150 nm, respectively. The nanocrystals morphology (see Fig. 2) reveals a plate-like-faceted shape with approximately 10–20 nm in thickness. These sizes are consistent with the size estimated from the XRD analysis.