Biomaterials 116 (2017) 174–185

Contents lists available at ScienceDirect

Biomaterials

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

Protein-crystal interface mediates cell adhesion and proangiogenic secretion

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ARTICLE INFO

Article history:
Received 5 August 2016
Received in revised form
30 October 2016
Accepted 24 November 2016
Available online 25 November 2016

Keywords:
Hydroxyapatite
Surface chemistry
Nano/microscale topography
Fibronectin
Breast cancer cells
Protein-crystal interactions

ABSTRACT

The nanoscale materials properties of bone apatite crystals have been implicated in breast cancer bone metastasis and their interactions with extracellular matrix proteins are likely involved. In this study, we used geologic hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$), closely related to bone apatite, to investigate how HAP surface chemistry and nano/microscale topography individually influence the crystal-protein interface, and how the altered protein deposition impacts subsequent breast cancer cell activities. We first utilized Förster resonance energy transfer (FRET) to assess the molecular conformation of fibronectin (Fn), a major extracellular matrix protein upregulated in cancer, when it adsorbed onto HAP facets. Our analysis reveals that both low surface charge density and nanoscale roughness of HAP facets individually contributed to molecular unfolding of Fn. We next quantified cell adhesion and secretion on Fn-coated HAP facets using MDA-MB-231 breast cancer cells. Our data show elevated proangiogenic and proinflammatory secretions associated with more unfolded Fn adsorbed onto nano-rough HAP facets with low surface charge density. These findings not only deconvolute the roles of crystal surface chemistry and topography in interfacial protein deposition but also enhance our knowledge of protein-mediated breast cancer cell interactions with apatite, which may be implicated in tumor growth and bone metastasis.

1. Introduction

Protein-crystal interactions play a fundamental role in many biological processes such as biomineralization [1–4]. For example, bone is a natural protein-crystal composite containing nanoscopic bone apatite crystals highly organized within a fibrillar protein extracellular matrix (ECM) that consists predominantly of collagen I [5]. Apatite surface chemistry and topography (ranging from the nano-to the microscopic level) are important mediators of protein-crystal interactions. Biological bone apatite has a platelet-like shape with (100) being the primary face, which interacts strongly with water [6]. Synthetic and geologic hydroxyapatites (HAP, $Ca_{10}(PO_4)_6(OH)_2$) are closely related to bone apatite but also develop other faces such as (001) and (101), although (100) is usually the prevalent face due to its highest hydrophilicity and stability in aqueous environment [7,8]. Biological and synthetic nanocrystalline apatites have high surface reactivity, such as ion exchange and protein adsorption, due to the presence of a metastable hydration layer [9]. The interactions between this sub-nanometer-thick hydration layer and crystal surfaces depend on face-specific HAP surface chemistry, and can significantly affect the adsorption of biomolecules [10–12]. A recent study reports that apatite-collagen interaction energies strongly influence the molecular scale organization of collagen on apatite surfaces [13]. Furthermore, interactions between mineral-modulating proteins and biologically relevant crystals are found to be primarily electrostatic in nature [14]. For example, fibronectin (Fn), another key skeletal ECM protein, adsorbs preferentially onto purely ionic crystal surfaces without structural water molecules incorporated in the crystal lattice [15]. On the other hand, both nanoscale and microscale surface topography have been shown to influence Fn adsorption (including total amount, spatial distribution, and molecular rigidity of Fn) as well as subsequent cell adhesion and

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http://dx.doi.org/10.1016/j.biomaterials.2016.11.043

signaling, although whether Fn conformational changes play a role in this process remains unclear [16–21].

Although the skeletal ECM is primarily composed of collagen I, Fn is the first bone matrix protein synthesized by osteoblasts and is required for subsequent deposition of collagen I [22–24]. In fact, the continuous presence of Fn is essential for maintaining the integrity of the mature bone collagen matrix [25]. Moreover, bone metabolism, including osteogenesis, induction of osteoblast differentiation, and survival of osteoblasts, all depend on interactions between osteoblasts and Fn [26,27]. In addition to cellular Fn originating from osteoblasts, circulating plasma Fn produced by the liver is also incorporated in the bone matrix. Interestingly, circulating plasma Fn represents the predominant source of Fn affecting bone mineralization and matrix properties [28]. Fn is a critical mechanotransducer whose conformational changes (*e.g.*, induced by interactions with crystal surfaces) result in either exposure or disruption of most of its binding sites. Among these sites are binding sequences for integrins, which are cell surface receptors regulating cellular responses to chemical, physical, and mechanical signals from the microenvironment [29–34]. Previous work using self-assembled monolayer substrates has reported that surface charge controls cell adhesion via Fn-mediated integrin engagement [35].

There has been increasing evidence that the nanoscale materials properties of bone apatite crystals likely modulate the pathogenesis of breast cancer bone metastasis [36–38]. Bone metastasis frequently occurs in patients at advanced stage of breast cancer and remains a major source of mortality in these patients [39]. Synthetic HAP has been widely used in previous studies to mimic bone apatite, although bone apatite has lower crystallinity and higher solubility; moreover, the crystal size, chemical composition, and distribution of bone apatite vary as a function of age and disease progression [40–44]. The presence of HAP in mineralized tumor models increases tumor cell adhesion, proliferation, and secretion of both proangiogenic and proinflammatory factors [36]. Furthermore, HAP materials properties including particle size, crystallinity, carbonate incorporation, and morphology, all have combined and/or individual effects on protein adsorption and breast cancer cell behaviors [37,38,45]. However, synthetic HAP nanoparticles usually present multiple faces and tend to form agglomerates, which makes it difficult to deconvolute effects of face-specific surface chemistry and surface topography during interactions with ECM proteins and tumor cells.

In this study, we investigated the individual effects of HAP surface chemistry and nano/microscale topography on both Fn molecular adsorption and subsequent cell behaviors. To obtain surfaces with homogeneous structural and chemical properties, Geiger el al. synthesized calcium-(R,S)-tartrate single crystals substrates that were tens of microns large for the study of epithelial cells adhesion [46,47]. Later, geologic calcite single crystals with identical surface chemistry but different surface roughness were used as model substrates for studying nano-topography sensing by osteoclasts [48]. Herein, we adopted a similar strategy by using geologic apatite single crystals to ensure uniform and well-defined surface chemistry (by controlling crystallographic orientation) and tune precisely nano/microscale surface topography (by controlling surface roughness). This strategy allowed us to (i) independently control HAP surface chemistry and topography, and (ii) avoid cytotoxicity associated with cellular uptake of HAP nanoparticles. Both molecular conformation and overall quantity of Fn adsorbed onto HAP facets were determined by Förster resonance energy transfer (FRET). MDA-MB-231 cells were subsequently cultured on Fn-coated HAP facets to evaluate the role of Fn-HAP interactions on cell adhesion and proangiogenic secretion, with likely implications in breast cancer bone metastasis.

2. Materials and methods

2.1. Geologic HAP crystals

Geologic apatite crystals with natural crystal termination faces were from Madagascar (Etsy, Inc). To determine the purities and elemental compositions of apatite crystals, a small portion was cut out of each crystal and ground into powders for powder X-ray diffraction (pXRD) and inductively coupled plasma atomic emission spectrometry (ICP-AES) analysis, respectively. The major phase of each crystal was determined to be HAP by pXRD (Scintag Inc. PAD-X theta–theta X-ray diffractometer, Cu Kα 1.54 Å, accelerating voltage 40 kV, current 40 mA, continuous scan, 1.0 deg/min). For elemental composition analysis, 3 mg of powders were dissolved in 25 mL 5% HNO_3 at 80 °C overnight. The solutions were then diluted 1:5 and analyzed using an ICP-AES spectrometer (Spectro, Ametek Material Analysis Division).

2.2. HAP facets preparation

HAP crystals were cut along natural faces to generate facets with two types of surface chemistry, (100) and (001), using a wafer cutting diamond saw. Each type of surface chemistry refers to a specific geometrical arrangement of ions and ionic groups, defined by the unit cell and the crystallographic orientation, resulting in specific surface properties (including interfacial energy, surface charge density, etc.) of each crystallographic facet. The crystallographic orientations of the facets were determined via XRD. These facets were then mechanically polished with a precision polisher (Allied High Tech, MultiPrep™ System) to obtain two types of topography/roughness. Micro-rough HAP facets 100 M and 001 M were polished using 30 μm diamond lapping films, resulting in a uniform array of grooves on the crystal surface. Nano-rough HAP facets 100 N and 001 N were polished using 30 μm, 9 μm, 1 μm diamond lapping films in sequence, and finished with 20 nm colloidal silica nanoparticles to smoothen any groove left by the lapping films. Lastly the nano-rough facets were polished against a polishing cloth in water for 5 min to remove the remaining silica nanoparticles. The dimension of original apatite crystals was approximately 1 cm. The facets used in this study were typically 6–8 mm in width and length, 2 mm in thickness. All lapping films, polishing cloths, and colloidal silica nanoparticles were obtained from Allied High Tech, Inc.

2.3. HAP surface chemistry and roughness characterization

The surface chemistry of HAP facets was characterized by measuring their surface zeta potential using phase analysis light scattering with Zetasizer Nano-ZS (Malvern Instruments Ltd. ZEN3600) [49]. The facets were mounted on a dip cell, immersed in aqueous buffer containing polystyrene latex standard at pH 9 (Malvern, DTS1235 ZP Transfer Standard, −42 ± 4.2 mV), and placed between the electrodes of the cell. The mobility of tracer particles in the vicinity of the charged surface was measured at 5 displacements (located at 125 μm–625 μm from the HAP surface), with 3 measurements per position, and fitted with linear regression to extrapolate the surface zeta potential at the HAP surface. The roughness of micro-rough HAP facets 100 M and 001 M were measured with an optical profiler (ADE phase shift microXAM optical interferometric profiler), using a 50× objective. The roughness of nano-rough HAP facets 100 N and 001 N were measured via atomic force microscopy (AFM, Veeco Dimension 3100), using silicon cantilevers holding tetrahedral silicon tips of radius 7 nm (Olympus, spring constant 26 N/m), in tapping mode, with scanning areas of 10 μm × 10 μm (512 samples/line).