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Effects of nanoparticle size and charge on interactions with self-assembled collagen

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ABSTRACT

Hypothesis: Insights into bone formation have suggested that the critical first step in the biomineralization process is the integration of small (nanometer dimension) mineral clusters into collagen fibers. Not only is such behavior of interest for understanding biomineralization but also should be important to nanotoxicology because collagen is a major component of structural tissues in the human body and accounts for more than 25% of the whole body protein content. Here, utilizing the current insights from biomineralization, we hypothesize that the binding affinity of nanoparticles to self-assembled collagen fibers is size and surface charge dependent.

Experiments: We developed a self-assembled collagen substrate compatible with Quartz Crystal Microbalance with Dissipation monitoring (QCM-D), which is very sensitive to mechanical changes of the substrate as a consequence of nanoparticle binding. QCM-D experiments were conducted with both positively and negatively charged gold nanoparticles between 2 and 10 nm in size. Complementary *ex situ* imaging Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) were used to confirm the QCM-D results.

Findings: We find that both positively and negatively charged nanoparticles of all sizes exhibited binding affinity for self-assembled collagen fibers. Furthermore, the smallest particles (2 nm) mechanically integrated with collagen fibers.

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1. Introduction

Collagen is the principal protein component of many tissues including bone, connective tissue and skin and provides critical support to their exceptional mechanical properties [1,2]. Collagen self-assembles to form fibers (hundreds of nanometers in diameter) with 67 nm repeating band structures consisting of less dense gap and more dense overlap regions [3]. Recent results suggest that during the biological mineralization process, the interior of collagen fibers is infiltrated by nanometer sized amorphous calcium phosphate clusters [4,5], which ultimately crystallize to form the mineral apatite. These calcium phosphate clusters are characterized by (1) their small size, generally less than 10 nm, (2) their negative surface charge, [4] and (3) their fluid like character commonly referred to as the Polymer Induced Liquid Precursor (PILP) [6]. Due to the abundance of collagen in the human body, the nature of nanoparticle interactions with collagen fibrils is of fundamental interest in at least two perspectives: (1) biomimetic materials synthesis: how do CaP nanoparticles bind to and integrate with collagen fibers? And (2) toxicology: how do nanoparticles interact with matrix biomolecules?

Here, we hypothesize that in the first step to biomineralization, the interaction between nanoparticles (NP) with self-assembled collagen fibers is driven by the charge of the NP. We further test NP size dependent association with collagen. We developed a model system with gold nanoparticles (AuNP), which are chemically and colloidally stable to study the nature of collagen–AuNP interaction (amount, spatial distribution and reversibility) as a function of particle size and surface charge polarity. Here, we use AuNP ranging from 2 nm to 10 nm in size and possessing positive or negative charges. To study the nanoparticle/collagen binding process, we used Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) along with Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). QCM-D has been used extensively to quantify binding and subsequent conformation changes of proteins [7,8], other macromolecules [9], nanoparticles

Abbreviations: QCM-D, Quartz Crystal Microbalance with Dissipation monitoring; AFM, Atomic Force Microscopy; SEM, Scanning Electron Microscopy; AuNP, gold nanoparticle; Δf, frequency change; ΔD, dissipation change; SD, Standard Deviation.

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[10] binding to surfaces, as well as mineralization processes at surfaces [11].

The findings of this study have potential applications in nanoparticle toxicology, where understanding the fate of nanoparticles in the body after exposure is important [12]. Because of the prevalence of collagen as a structural protein throughout the body, understanding the role of nanoparticle size and charge governed interaction with collagen is essential.

2. Experimental

2.1. Preparation of QCM-D sensors

To clean the gold-coated QCM-D sensors (QSX-301, Biolin Scientific) they were exposed to Ultraviolet Ozone (UVO, Jelight) for 10 min; soaked in a solution composed of Deionized Water (DI) water (Milli-Q), hydrogen peroxide (30%, Fisher Scientific) and ammonium hydroxide (Sigma–Aldrich), at a proportion of 5:1:1, respectively, at 70 °C for 5 min, N_2 dried and finally exposed to UVO for 10 min. Afterward, the QSX-301 sensors were made hydrophobic by exposure to 1 mmol/L dodecane thiol (Sigma–Aldrich) dissolved in anhydrous alcohol (Warner-Grantham), rinsed with DI water then anhydrous ethanol and stored in anhydrous ethanol.

2.2. Preparation of collagen films

Dilute neutralized collagen solutions were prepared by first, mixing acidified type I collagen solution (pureCol, Advanced Biomedicals, 3 mg/mL) with 10X phosphate buffered saline (PBS) (Gibco) and 0.1 mol/L NaOH, at proportions of 8:1:1, respectively, with the final pH of approximately 7.4. Then the solution was diluted to a ratio of 1:7, neutralized collagen solution to 1X PBS [13]. Hydrophobically-modified QSX-301 sensors were immersed in diluted neutralized collagen solutions for 12–16 h at 37 °C. The collagen functionalized QSX-301 sensors were rinsed in 1X PBS and DI water and stored in 1X PBS at 4 °C until used.

2.3. Nanoparticle solutions

All nanoparticles solutions were diluted, if necessary, with DI water to 0.001% by mass $AuCl_4$ and then adjusted to pH 7.4 with 0.01 mol/L NaOH. Where Au concentration of the as received nanoparticle solutions are reported by the manufactures as mass% of the $AuCl_4$ reactant prior to reduction. Citrate stabilized gold nanoparticles (2 nm, 0.001% by mass $AuCl_4$; 5 nm, 0.01% by mass $AuCl_4$; and 10 nm, 0.01% by mass $AuCl_4$; all from British Biosciences) and an amine terminated polyethylene glycol stabilized gold nanoparticle (3 nm, 0.05% by mass $AuCl_4$, nanoOCS) were used.

2.4. QCM-D

Measurements were performed using a single flow module on the Q-Sense E4 experimental platform, operating at 25 °C with a peristaltic pump at 20 μL/min under continuous flow. Prior to nanoparticle introduction, collagen films were equilibrated in flowing DI water for 2 h. To assure the collagen film successfully formed on the surface the stitched QCM-D data from the baseline before collagen attachment (sensor plus DDT) and after collagen attachment showed a significant change (≈900 Hz in frequency (Δf) and ≈200 in Dissipation factor (ΔD)). A baseline in flowing DI water was collected for ≈10 min prior to introduction of nanoparticle solutions (0.001% by mass $AuCl_4$). Collagen matrices were exposed to AuNP for 200 min and rinsed with DI water for 60 min. Changes in frequency (Δf) and Dissipation (ΔD) were recorded for the fundamental frequency (f = 5 mHz) and five overtones (3, 5, 7, 9 and 11 at 15 mHz, 25 mHz, 35 mHz, 45 mHz and 55 mHz). Each condition was repeated at least three times. Analysis of QCM-D data was performed with the QTools data analysis software. Data was fit using a one-layer Voigt model, with the following parameters: film density (1.36 g/cm^3 [14]), water density (1 g/cm^3) and fluid viscosity (1 mPa s) and using four overtones (3, 5, 7, 9). The following parameters were fit: film thickness, shear modulus and viscosity.

2.5. Preparation of N_2 dried samples

QCM-D sensors after exposure to AuNP were dried under a nitrogen stream for 1 min and then stored in a dry environment until imaging.

2.6. Preparation of critical point dried samples

QCM-D sensors after exposure to AuNP were removed from the flow module in a hydrated state, placed in DI water, incubated in ethanol/DI water mixtures of 10%, 25%, 50%, 75% then 100% ethanol for 10 min each and then placed into a critical point dryer (Tousimis Autosamdri – 814). Samples were stored in a dry environment until imaging.

2.7. Atomic Force Microscopy (AFM)

AFM images were collected on a Bruker Dimension Icon in Quantitative Nanomechanical Mapping (QNM) mode. All images were collected with a Tap150 probe (Bruker Inc.) (k = 4 N/m, 8 nm nominal tip radius). At least 10 images were collected per condition and images presented are characteristic of each sample.

2.8. Scanning Electron Microscopy (SEM)

FEI Helios 650 Focused Ion Beam (FIB) SEM was used to collect SEM images. All images were collected at 1 kV, 50 pA electron beam condition with 1 mm working distance using a through-the-lens detector in backscatter mode. The images presented are characteristic of each sample, at least 10 images were collected per condition.

3. Results and discussion

AFM was used to characterize self-assembled collagen fibers on functionalized gold QCM-D sensors, which exhibited a bimodal distribution of fiber diameters of approximately 15 nm and approximately 100 nm (Fig. S1). The smaller fibers formed a dense mat on the surface of the QCM-D sensor, with larger fibers overlying these structures at a lower areal density, consistent with previous findings [13,15]. The larger fibers are more characteristic of hierarchically assembled type I collagen fibrils *in vitro* [4,5,16,17] and of collagen fibers found in tissue [18–21].

QCM-D was used to monitor changes of mechanical properties for self-assembled collagen as a result of interaction with AuNP. Generally, changes in frequency (Δf) are qualitatively related to mass change in the system, either adsorption (negative shift) or desorption (positive shift) [7]. Conversely, changes in Dissipation factor (ΔD) are qualitatively related to mechanical properties of the system becoming more stiff (decrease) or more viscous (increase). Three replicate experiments were conducted for each condition. Between the replicate experiments the trends in changes of Δf and ΔD were reproducible, although some variation in the maximum values Δf and ΔD was observed (Table 1). Negatively charged (2 nm, 5 nm and 10 nm) and positively charged

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