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Selective characterization of proteins on nanoscale concave surfaces

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ABSTRACT

Nanoscale curvature plays a critical role in nanostructure-biomolecule interactions, yet the understanding of such effects in concave nanostructures is still very limited. Because concave nanostructures usually possess convex surface curvatures as well, it is challenging to selectively study the proteins on concave surfaces alone. In this work, we have developed a novel and facile method to address this issue by desorbing proteins on the external surfaces of hollow gold nanocages (AuNG), allowing the selective characterization of retained proteins immobilized on their internal concave surfaces. The selective desorption of proteins was achieved via varying the solution ionic strength, and was demonstrated by both calculation and experimental comparison with non-hollow nanoparticles. This method has created a new platform for the discrete observation of proteins adsorbed inside AuNG hollow cores, and this work suggests an expanded biomedical application space for hollow nanomaterials.

1. Introduction

Nanostructured materials have exhibited significant potential for biomedical applications, rendering the investigation of nanostructure-biomolecule (nano-bio) interactions critical regarding both the efficacy and safety of nano-biomaterials [1–4]. In particular, nanoscale morphology has been shown to elicit significant biomolecular responses, as proteins appear to "sense" variations in the topography of their nanoscale environments, and alter their conformation and hence their function [5,6], accordingly. During the last decade, studies on these "morphology effects" have demonstrated the critical nature of surface curvature in affecting biomolecule adsorption, conformation, and activity [7–11]. While the vast majority of these studies have focused on surfaces with positive (convex) curvature, there remains a dearth of systematic studies of the structure and function of biomolecules adsorbed on negatively curved (concave) surfaces. To help address this lack of understanding, galvanically-synthesized gold nanocages (AuNGs) were previously employed by our group to study the effect of concave surfaces [12]. AuNG have hollow cores with concave surfaces [13] and are distinct from other concave nanostructures due to their excellent morphological controllability, stability and protein compatibility [14]. Moreover, due to the surface plasmon coupling effect, additional hotspots can exist inside the hollow core of AuNGs as they aggregate, further facilitating the selective spectroscopic characterization of internally adsorbed macromolecules [15]. In our earlier work, we demonstrated that AuNG provide an excellent platform to study this type of nano-bio interaction. However, it remained a challenge to clearly distinguish the effects on internally and externally adsorbed proteins on AuNG.

While some experimental methods can, to a limited extent, serve this purpose of distinguishing between internally and externally adsorbed proteins (e.g., additional modification of nanoparticle external surfaces [16] or proteolysis of external proteins [17,18]), a simple procedure to minimize damage to the adsorbed proteins is still desired. In the present study, we employed a simple ionic strength-based method to selectively remove the externally bound proteins on AuNG, taking advantage of hindered through-pore diffusion in AuNG to regulate protein leaching from the internal concave cavities into the highly ionic buffer. Non-hollow gold nanocubes (AuNCs) were used as a reference to verify external protein removal, and surface-enhanced Raman spectroscopy (SERS)

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was employed to characterize ligand and protein conformation. Lysozyme was chosen in this study to probe the nano-bio interactions, as the enzyme's structure is well characterized and it is known to undergo structural changes upon adsorption to nanoparticle surfaces [19,20]. This combination of methods enabled the specific observation of proteins on nanoscale concave surfaces selectively, without interference from otherwise adsorbed proteins.

2. Materials and methods

2.1. Materials and instrumentation

Gold (III) chloride trihydrate (99.9%), silver nitrate ACS reagent (99+%), 11-mercaptoundecanoic acid (11-MUA) (90%), sodium sulfide nonahydrous, 1,5-pentanediol (96%) (PD), and polyvinylpyrrolidone (M_w ~ 55000) (PVP) were purchased from Sigma–Aldrich (St. Louis, MO). Ethylene glycol was purchased from J. T. Baker (Center Valley, PA). Lysozyme (Lyz) from hen egg white, and 4-methylumbelliferyl β-D-N, N′, N″-triacetylchitotrioside (4-MU-β(GluNAc)$_3$), and *Micrococcus lysodeikticus* were purchased from Sigma–Aldrich as dry powders and used without further purification. Bicinchoinic acid (BCA) and micro-bicinchoninic acid (μBCA) assay reagents were purchased from Pierce Biotechnology, Inc (Rockford, IL). Scanning electron microscopy (SEM) was performed via a Carl Zeiss Supra-55 VP field-emission SEM (Jena, Germany); samples were prepared by drop-drying aqueous nanoparticle suspensions on silicon wafers. Inductively coupled plasma mass spectroscopy (ICP-MS) was performed using a Bruker Varian 820-MS ICP-MS (Billerica, MA), and the samples were prepared by dissolving nanoparticle suspensions into *aqua regia* and then diluted in 5% nitric acid. Raman and surface-enhanced Raman spectroscopies (SERS) were performed on a Renishaw Ramascope (Gloucestershire, UK) attached with a Carl-Zeiss optical microscope. The 785 nm excitation laser was from a semiconductor cw diode and used with a holographic notch filter and a transmission grating of 1200 lines per millimeter. Drop-dried samples were used to measure nanoparticle ligand conformation, and liquid samples were used to measure protein conformation. In both cases, Raman spectra were collected in backscattered configuration and processed in Wire 3.3 software, also provided by Renishaw. UV–vis spectroscopy measurements were made on a Hitachi U-2910 spectrophotometer.

2.2. Preparation of nanobioconjugates and removal of externally adsorbed proteins

AuNG and AuNC nanoparticles were synthesized and surface-modified according to previously described protocols [21,22] with minor modifications [12] and Lyz–nanoparticle conjugates were also prepared as previously described [12]. Briefly, ~100 μg/ml Lyz was incubated with ~2 nM AuNG or AuNC in the 2 mM, pH 7.4 PBS for 24 h to ensure thorough protein internalization. To remove externally bound proteins in the present work, both AuNG-Lyz and AuNC-Lyz nanobioconjugates were subjected to a high-salt (1 M NaCl) buffer to screen the electrostatic attractions between nanoparticles and proteins. A solution comprised of 800 μl of nanobioconjugates in PBS mixed with 200 μl, 5 M NaCl aqueous solution was shaken for 5 min, making sure the nanobioconjugates were well dispersed from macro-aggregation. After shaking, nanobioconjugates were gently centrifuged at 5000 rpm for 3 min twice to remove any unbound protein, and finally re-dispersed in 500 μl of the 2 mM, pH 7.4 PBS. The centrifuged supernatants were subjected to a μBCA assay to measure the protein concentrations, and the protein amount remaining on AuNG or AuNC was calculated by subtracting the protein amount in the supernatant from the total added protein amount. A control group treated with DI water instead of NaCl solution was used for each nanobioconjugate titration to determine the amount of adsorbed proteins before NaCl wash. Moreover, according to our experiments, the presence of 1 M NaCl in μBCA had no observable effect on protein concentration measurements.

2.3. Characterization of internally adsorbed proteins

The enzymatic activity of Lyz-NP conjugates (either Lyz-AuNG or Lyz-AuNC) was determined spectrofluorometrically for the Lyz-catalyzed hydrolysis of 4-MU-β-(GluNAc)$_3$ to yield 7-hydroxy-4-methylcoumarin (4-MU), and were also measured by a turbidity assay (*M. lysodeikticu*). The measurements followed existing protocols as described in detail by Qian et al. [12]. Protein conformation was analyzed via SERS. To prepare the Raman spectroscopy sample, AuNC-Lyz and NaCl-washed AuNG-Lyz nanobioconjugates were dropped onto a glass slide and covered by a microscope cover slip. The 785 nm wavelength laser was carefully focused between the slides via a 40X objective lens. To minimize the photon-induced alteration of the samples, the laser power was set at its lowest value (2 mW before the lens), the scanning time was 30 s and the final spectrum was accumulated in 5 scans. Scans were performed in both extended mode at 400–1800 cm^{-1}, to obtain a general spectrum of the protein's Raman shift, and in static mode at 1100–1500 cm^{-1}, to obtain the specific information of the Amide III band (1200–1350 cm^{-1}). The baseline of the colloidal AuNG or AuNC solutions at the same measuring condition was subtracted from the spectra of the respective nano-bio samples. Lyz secondary structure was characterized using the analytical method reported by Cai and Singh [23]. Briefly, the spectra were deconvoluted and the areas under each of the deconvoluted peaks were calculated. The peaks around 1300 cm^{-1} were designated as α-helix peaks, and their areas were compared with the summed areas of all the deconvoluted peaks, which gave the percentage of α-helix in the Lyz secondary structure. The final secondary structural content was averaged from triple measurements. A 1 mM free Lyz solution in the 2 mM, pH 7.4 PBS, with 20X stronger excitation intensity due to the absence of any Raman enhancement effect, was used as a control to examine the free protein conformation.

3. Results and discussion

3.1. Nanoparticles and surface ligands

Typical SEM micrographs of AuNG and AuNC are shown in Fig. 1A–D, exhibiting excellent nanoparticle uniformity in both size and morphology. The average size of the AuNG is 54 ± 10 nm, and AuNC is 74 ± 14 nm. The pores on the AuNG corners are 5–10 nm in diameter (Fig. 1B), enabling access and protein internalization in the AuNG. The AuNG wall thickness varies from 5 to 10 nm, due to the curvature of the internal hollow. The 3-D structure of the AuNG's hollow core has been characterized by multiple advanced microscopic methods [13,24] and has been shown to be an irregular sphere or rounded cube. For example, Goris et al. characterized the 3-D morphology of such AuNGs via high-angle annular dark field-scanning transmission electron microscopy (HAADF-STEM) [13], as shown in Fig. 1C. Quantitatively, our previous studies on protein–nanoparticle conjugates indicated that proteins (2–4 nm in size) are able to respond to the variation of surface curvature with radii over the range from 4 to 100 nm [7–9,17]. The sizes of the internal hollow cores of the AuNGs are also within this scale range (15–20 nm in radius), rendering them to be suitable platforms in this regard. The quantification of AuNG geometry followed the method developed by Qian et al. [12]. By combining the measured