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Protein–nanoparticle interaction in bioconjugated silver nanoparticles: A transmission electron microscopy and surface enhanced Raman spectroscopy study

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ABSTRACT

Understanding the mechanisms of interaction between proteins and noble metal nanoparticles (NPs) is crucial to extend the use of NPs in biological applications and nanomedicine. We report the synthesis of Ag-NPs:protein bioconjugates synthesized in total absence of citrates or other stabilizing agents in order to study the NP-protein interaction. Four common proteins (lysozyme, bovine serum albumin, cytochrome-C and hemoglobin) were used in this work. Transmission electron microscopy (TEM) and surface enhanced Raman spectroscopy (SERS) were mainly used to study these bioconjugated NPs. TEM images showed Ag NPs with sizes in the 5–40 nm range. The presence of a protein layer surrounding the Ag NPs was also observed by TEM. Moreover, the composition at different points of single bioconjugated NPs was probed by electron energy loss spectroscopy (EELS). The thickness of the protein layer varies in the 3–15 nm range and the Ag NPs are a few nanometers away. This allowed to obtain an enhancement of the Raman signal of the proteins in the analysis of water suspensions of bioconjugates. SERS results showed a broadening of the Raman bands of the proteins which we attribute to the contribution of different configurations of the proteins adsorbed on the Ag NPs surface. Moreover, the assignment of an intense and sharp peak in the low-frequency range to Ag–N vibrations points to the chemisorption of the proteins on the Ag-NPs surface.

1. Introduction

There is currently a strong interest in using noble metal nanoparticles (NPs) in nanomedicine, mainly for sensing applications in order to probe biological processes [1,2]. Understanding the effect of NPs on health is also a major issue [3,4] in which the interaction between nanoparticles and proteins is a key factor [5,6]. Recent works have shown that when NPs come into contact with a biological fluid their surface is covered by a layer of biological macromolecules [7,8]. Moreover, Ag-NPs are also investigated for their antimicrobial properties and potential applications in oncology [9]. Other works have shown the relevance of these adsorbed protein layers in understanding the interaction of NPs with living systems [10,11]. Nonetheless, little is known about the conformational changes of the proteins that are associated with these NP-biomolecule systems [12,13]. The changes in the structure of the protein affect its reactivity as well as the mobility of the NPs in the organism [14]. To elucidate these interactions, a wide range of physico-chemical techniques, such as UV–vis-IR absorption, fluorescence [15], surface plasmon resonance (SPR) [16], dynamic light scattering and circular dichroism spectroscopies [17], nuclear magnetic resonance and electron microscopy are used [2,13]. Surface enhanced Raman spectroscopy (SERS) can also help to identify these interactions thanks to the strong enhancement of the Raman signal coming from proteins adsorbed on noble metal NPs when these NPs are excited at or near their surface plasmon resonance [18–21]. After more than 30 years of development, SERS is increasingly used in the fields of Physics, Chemistry, Surface Science, Nanoscience, and Biomedical Science [22–24]. SERS has proved to be a very effective analytical tool because of its high sensitivity, high selectivity, and fluorescence-quenching properties. In the particular

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case of protein detection, different SERS strategies have been reported in the literature based on direct label-free detection or indirect detection of markers attached to the protein [20,25–27]. SERS is also very useful for detecting conformational and structural changes of molecules on a metal surface [9,15,28–32] which is of strong interest in the case of proteins adsorbed on metal nanoparticles.

The synthesis of NP:protein bioconjugates [32–37] enables the design of biocompatible metal NPs, which make them promising for many biomedical applications. Several works have reported the strong electron-donating nature of amino-acids, such as tryptophan, tyrosine and cysteine [33,38]. They can act as stable or transient intermediates in electron/hydrogen transport in biological systems through the corresponding radical intermediates. In this way, Si and Mandal [38] have proposed a mechanism where the tryptophan residue donates an electron to the metal ion and is itself converted to a transient tryptophyl radical, which eventually transform to the native tryptophan or ditryptophan/kynurenine form of the peptide.

In protein bioconjugated NPs suspensions, proteins around the NPs are charged, so there is repulsion between bioconjugated NPs [33,34,37] and proteins in the solution act as a buffer for pH, ionic strength and electrochemical activity. Moreover, there is also a steric effect due to the large size of the proteins. By coating an Ag NP, the proteins prevent other NPs or molecules from touching it like a polymer coating and they prevent the destabilization of the suspension.

Here, we report the synthesis of Ag-NP:protein bioconjugates using four common proteins: lysozyme, bovine serum albumin (BSA), cytochrome-C and hemoglobin. These proteins were chosen not only for their biological relevance and their common use in the laboratory [39–41], but for their different size and optical properties. Lysozyme and cytochrome-C are relatively small molecules (molecular weight: 14.3 and 12.3 kDa, respectively), whereas BSA and hemoglobin are big proteins (66 and 64.5 kDa, respectively). Moreover cytochrome-C and hemoglobin display strong absorption bands in the visible range, which is not the case of lysozyme and BSA. No citrates or other stabilizing agents were used in the synthesis process in order to investigate adsorption of proteins on the surface of Ag NPs. The size and morphology of the synthesized bioconjugates were mainly studied by transmission electron microscopy (TEM). Electron energy loss spectroscopy (EELS) and SERS were used to analyze the protein layer in order to investigate the protein–NP interaction.

2. Materials and methods

2.1. Synthesis of Ag:protein bioconjugates

All chemicals were purchased from Sigma–Aldrich and used without further purification. Briefly, 1 mg of protein was diluted in 10 mL of deionized water (18.2 mΩ). This solution was heated at 37 °C under strong stirring. 100 μL of 0.1 M $AgNO_3$ was then added. The solution was heated and stirred for 3 h. Ag NPs are formed in the solvent by the reduction of silver ions into metallic silver due to proteins acting as reducing agents. The use of UV irradiation (UVP inspection lamp, model UVGL-25 PL emitting at 365 nm) to increase the Ag NPs synthesis yield was also checked.

A different solvent (methanol) was used in order to improve the yield of the synthesis of Ag:lysozyme bioconjugates according to the method of Eby et al. [42] Indeed, methanol helps opening of the lysozyme structure. This process does not denature the protein and enhances its reactivity and capping properties. The yield of the synthesis process was too low in the absence of UV irradiation, thus all the Ag:lysozyme bioconjugates shown here were obtained under UV irradiation.

Prior to all the analyses, 1 mL of the synthesized NPs suspension was centrifuged (Eppendorf MiniSpin) at 13,400 rpm (G-force: 12,100×G) for 5 min. After removing a portion of the supernatant with the help of a pipette, 100 μL of deionized water was added and the suspension was sonicated.

It is worth noting that the obtained Ag:protein NPs suspensions are stable for more than three months.

2.2. Characterization methods

TEM and EELS analyses were carried out using a JEOL 2100 FEG with 200 kV acceleration voltage. The NPs morphology was studied in both TEM and STEM modes. Electron energy loss spectroscopy (EELS) analyses were performed in scanning transmission electron microscope (STEM) mode with a Gatan Tridiem system using a 1.5 nm probe size. The samples were prepared as follows: a drop of the concentrated NPs suspension was deposited on a holey carbon membrane supported by a copper grid. After evaporation of the solvent, only NPs placed at the border of the carbon membrane were observed by TEM in order to avoid interactions between the incoming electrons and the supporting film.

Optical absorption spectra of both diluted solutions of pure proteins and of the synthesized nanoparticles suspensions were obtained with a SHIMADZU UV-2550 spectrophotometer operating in the 200–800 nm range.

Raman spectra were obtained using a RENISHAW inVia spectrometer equipped with a 50 cm^{-1} cut-off edge filters and a 1800 groove/mm grating. The samples were prepared as follows for Raman analysis: after sonication of the concentrated NPs suspension, a volume of about 50 μL was deposited on an hydrophobic and reflective slide (Tienta SpectRIM) forming an almost hemispherical drop. SERS spectra were obtained under laser excitation with a frequency-doubled Nd:YAG laser at 532 nm using a macro objective (f = 30 mm) from RENISHAW. The laser power focused on the sample was typically about 5 mW and the acquisition time was about 10 × 10 s. Raman spectra of freeze-dried proteins were also measured for comparison. In the case of freeze-dried BSA, a 785 nm laser was used to reduce the protein fluorescence background.

3. Results and discussion

3.1. Structure and morphology of Ag:protein bioconjugates

TEM images (Figs. 1 and 2) were used to study the size and morphology of the different Ag:protein bioconjugates synthesized in this work. Fig. 1(a–d) displays images obtained for Ag:lysozyme bioconjugates. In dark field mode, brighter features in the images (Fig. 1a and b) correspond to Ag NPs, whereas lighter compounds appear in dark gray. In HRTEM mode (Fig. 1c and d), Ag NPs are dark, whereas organic compounds are brighter. The size distribution of NPs measured in TEM images is given in Fig. 3(a). The NP diameter varies in the 5–25 nm range. The images clearly show the presence of an amorphous layer coating the Ag-NPs. The thickness of this organic layer varies from about 3 to 15 nm. The distance between Ag-NPs is a few nanometers. This favors an enhancement of the protein Raman signal by the SERS effect.

In order to check the chemical composition of the Ag:lysozyme bioconjugates, localized EELS measurements have been performed in STEM mode (Fig. 4). They revealed the systematic presence of carbon, oxygen, nitrogen, silver and calcium (two peaks at 350 eV). Spectra obtained in the center of the NPs (spectrum (a)) show a