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Single-molecule detection of yeast cytochrome *c* by Surface-Enhanced Raman Spectroscopy

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Abstract

The giant enhancement of Raman signal near silver colloidal nanoparticles is exploited to study the Raman spectrum of Cytochrome *c* from *Saccharomyces cerevisiae* (Yeast Cytochrome *c*—YCc) in the limit of single-molecule. The investigation is performed on proteins both in solution and immobilised onto a glass slide using a quasi resonant laser line as exciting source with low excitation intensity. In both cases, spectra acquired at different times exhibit dramatic temporal fluctuations in both the total spectrum and in the specific line intensity, even though averaging of several individual spectra reproduces the main Raman features of bulk YCc. Analysis of the spectral intensity fluctuations from solutions reveals a multimodal distribution of some specific Raman lines, consistent with the approaching of single molecule regime. Among other results, the statistical analysis of the spectra from immobilised samples seems to indicate dynamical processes involving the reorientational of the heme with respect to the metal surface.

Keywords: Single-molecule; Cytochrome *c*; SERS

1. Introduction

In recent years, the study of proteins in single molecule (SM) regime is attracting increasing interest. The extraordinary development of some optical techniques permitting the detection of specific signal in very low molecule concentration limit [1–6] has opened new opportunities in many disciplines such as biophysics and biomedicine [6]. Measurements on individual molecules have the main advantage of enabling the investigation of phenomena that are usually hidden to ensemble average [7].

Different spectroscopic approaches have so far been applied and developed up to reach single molecule detection, and particular attention has been focused on vibrational spectroscopies [1,2] because they are of fundamental importance for the understanding of internal configurations, structure and dynamics of proteins. In particular, Raman spectroscopy provides significantly a wealth of structural information and can be used to specifically identify molecules with no need of estrinsic labelling of the samples. On the other hand, it generally presents a very low cross-section. The use of a radiation resonant with an electronic transition leads to the Resonance Raman (RR) effect that is characterized by a higher cross section. The finding that Raman signals are enhanced in presence of nanostructured metal surfaces [8] has dramatically boosted Raman spectroscopy as a powerful tool for the vibrational studies in life science in the low concentration limit, because the related cross section can be increased up to a factor 10^{14} [9–11]. The use of silver or gold colloidal nanoparticles to enhance Raman effect on one hand, and the rapidly growing capability (based on advances in both lasers and detectors) to detect very low signals using high-resolution microscopy on the other, has allowed a very wide use of Surface-Enhanced Raman Scattering (SERS) and Surface-Enhanced Resonance Raman Scattering (SERRS) to study the vibrational features of very low concentrated samples, even down

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to single molecule regime [1,2,6,9–13]. This possibility could be particularly appealing in the study of biomolecules, because SM-SERS can be an extraordinary device to go deep inside the functionality and structure of biological proteins also by following an individual molecule throughout the course of events or analyzing the conformational states as function of time.

Recently, a number of works using SERS for the investigation of biomolecules (studies on horseradish peroxidase [15], tyrosine [16], myoglobin [17], haemoglobin [18]) has confirmed such abilities, providing the tool for obtaining very insightful information on a class of biomolecules, the Electron Transfer (ET) proteins, that is attracting much interest from both scientific and bionanotechnological standpoints [19,20]. Their natural redox properties and low dimensions make them good candidates for an integration in hybrid submicrometer-sized electronic components, as well as in novel biosensor configurations [20]. Furthermore, SERS study on ET proteins could give some insights in the explanation of Raman enhancement effect. As a matter of fact, it is acknowledged that two main processes are responsible for Raman enhancement: an electromagnetic effect (EM) and a chemical one that probably involves a charge transfer (CT) between molecule and enhancing surface.

In the main stream of research about ET proteins, we have recently investigated the topological, spectroscopic and electron transfer properties of cytochrome *c* from *Saccharomyces cerevisiae* (Yeast Cytochrome *c*-YCc), directly self-chemisorbed on bare gold electrodes through the free sulfur-containing group [19,21]. YCc is an ET protein having the peculiarity of bearing an additional free sulphur-containing group (Cys102). Thus it results to be highly suitable for specifically oriented interactions with metals with an expected minor perturbation for the heme group and a good electrical contact of YCc with a metal surface. Our results, from Scanning Tunneling Microscopy (STM), Atomic Force Microscopy (AFM) and cyclic voltammetry studies, clearly indicate that this variant of cytochrome *c* is adsorbed on electrodes with preservation of morphological properties and redox functionality showing good coupling with the electrode. To get a deeper insight into charge transfer dynamics and orientation of YCc near a metallic surface, an SM-SERS investigation could be appropriate. Here, we present a preliminary SM-SERS study of YCc, adsorbed on colloidal silver nanoparticles, on both solutions and immobilised samples in order to compare single molecule behavior to the well known average ensemble Raman behavior.

2. Materials and methods

All chemicals ($AgNO_3$, YCc, Trizma, APES) have been purchased from Sigma. Solutions of colloidal silver have been prepared by Lee-Meisel standard citrate reduction method [22]. The concentration of silver particles, estimated by optical absorption, is about 10^{-11} M. AFM studies have revealed that the colloids consist of an heterogeneous size and shape (spheres and rods) particle distribution characterized by an average size of about 70 nm [17].

YCc from *S. cerevisiae* (M.W. 12588 Da) is a small single-domain heme-containing protein, which represents an essential component of the mitochondrial respiratory chain, playing a major role in the ET between two membrane-bound enzyme complexes, cytochrome *c* reductase and cytochrome *c* oxidase. As in many others heme cytochromes, in YCc the heme group is covalently bound to the protein matrix through thioether linkages involving two cysteine residues (Cys14 and Cys17). In addition, YCc bears a free sulphur-containing group (Cys102). YCc solutions, used without further purification, have been prepared dissolving the powder in 1 mM TRIS buffer (pH 8.0) at a concentration of 2.6 mM. Successive dilutions have allowed us to obtain the desired concentrations. For Raman measurements, samples have been prepared at a concentration of 1.6×10^{-4} M YCc, while for SERS measurements an aliquot of a solution has been incubated with colloidal particles for 1 h at room temperature in order to obtain a ratio of 6:1 between the number of colloidal particles and the number of cytochrome molecules at 1.7×10^{-12} M concentration of YCc. SERS measurements on samples in solution have been carried on immediatly after the deposition of a droplet (20 μl) onto a glass slide, previously coated with polymerized 3-aminopropyltriethoxysilane (APES) [23]. Dry samples have been prepared leaving in a dryer for 1 h at room temperature the droplet previously deposited on glass slide.

Raman spectra have been recorded using a Jobin-Yvon Super Labram confocal system equipped with a liquid nitrogen-cooled CCD (EEV CCD10-11 back illuminated; pixel format: 1024×128) detector and a spectrograph with a 1800 g/mm grating allowing a resolution of 5 cm^{-1}. A 100× objective with a numerical aperture N.A.=0.9 has been implemented. The laser source has been an Argon ion laser (MellesGriot) providing a 514.5 nm radiation that is preresonant with the $Q(0,1)$ (520 nm) band, associated with the $Q(0,0)$ band electronic transitions. The provided laser power has been kept below 4 mW (corresponding to about 20 kW/cm^2); 20% of such power really impinging on the sample.

When liquid samples are under investigation, the scattering volume (V_S) can be modelled as a double right cone. From geometrical considerations we have obtained: $V_S=(2\pi/3)\Delta z\cdot(r^2+\omega_0^2+\omega_0 r)$, where Δz=10.7 μm (from technical sheets of the system) is the depth of field, ω_0 is the beam waist of laser ($\omega_0=(4/3)(\lambda/\text{N.A.})$=0.791 μm), r, the spread due to the objective, is $r=\Delta z\cdot\tan(\arcsin(\text{N.A.}/n))$=10 μm, n is the refraction index of the sample, λ is the laser wavelength. In such a way, we have estimated $V_S\approx 2.5$