# SURFACE ENHANCED RAMAN SPECTRUM OF NANOMETRIC MOLECULAR SYSTEMS

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**ABSTRACT**

Surface-enhanced Raman spectroscopy (SERS) was demonstrated to be a useful and sensitive structural spectroscopic tool for the characterization of different types of supramolecular architectures, ideally suited for trace analysis. SERS spectra allowed to distinguish cell membrane components of the biomining bacterium Acidithiobacillus ferrooxidans and their physical and chemical variations caused by different growth media. Several SERS spectral modifications were arguments to propose different structures for two isolated model blue cooper proteins, i.e., hemocyanins belonging to mollusc Concholepas concholepas (CCH) and Megathura crenulata (KLH). The complex structure of a humic acid was demonstrated to be highly sensitive to the pH of the medium; a globular conformation predominated at lower pH, while a decurled structure was observed at higher pH. Finally, the SERS technique using different excitation laser lines allowed to characterizing a carbon nanotube as a single-walled species, with metallic electronic characteristics; these species coexisted with different diameters.

**Keywords**: SERS, Bacteria, Carbonnanotube, Blue copper proteins and Humic acid

## INTRODUCTION

Vibrational spectroscopy with its infrared (IR) and Raman techniques is a powerful molecular structural tool. The vibrational spectrum of giant molecular systems such as biological samples is normally studied as a contribution of their own different molecular components. For instance, Raman signals of bacteria are ascribed to membrane components rather than to the cellular machinery in the cytoplasm. These components are mainly protein subunits, phospholipids, nucleic acids and polysaccharides. Structure and conformation of isolated proteins are mainly analysed as a contribution of their different aminoacids on the basis of amide I, II or III and skeletal vibrations.

The IR or Raman spectra of high molecular weight systems such as humic acids started to be analysed as a contribution of each chemical function. The low weight carbon nanotubes display simple vibrational spectra; from the detailed band assignment it is

possible to infer about its physical parameters as diameter and length. Resonance Raman data allowed distinguishing about their metallic or semiconducting electronic characteristics.

Most of giant molecular systems display natural fluorescence; this is normally observed in biological samples, where the aqueous environment is inherent. The last situation is overcome by using Raman spectroscopy, and metal surfaces can be used to quench fluorescence. However, the usually weak intensity of the Raman process makes its application difficult. Thus, surface-enhanced Raman spectroscopy (SERS) combines both advantages and allows work even with analytical concentrations. Moreover, SERS can be used to study *in situ* biological samples, making possible to detect most of their components i.e. aminoacids, and determine their relative abundance.

The aim of the present contribution is twofold. First, to show a new methodology to prepare SERS samples of nanometric molecular systems like bacteria, isolated blue copper proteins, humic acids and carbon nanotubes, in silver colloidal solution. Secondly, assign their SERS spectra, to discuss about the biochemical, and chemical approaches, and the structural characteristics of these systems. In particular, to study spectral features of bacteria as a function of growth media conditions. Also, to discuss the SERS spectra of two different blue copper proteins coming from relative marine animal species, in order to establish eventual structure differences which were not possible to distinguish from X-ray measurements. In this second stage, we study at different pH and temperatures the possible conformations adopted by humic acids, and we determined structural and electronic characteristics of carbon nanotubes. Thus, the aim of the present contribution is to develop SERS as a rapid whole-organism or biomolecule fingerprinting method, without the need for any tagging or chemical modification, for the characterization of nanometric molecular systems.

## EXPERIMENTAL

*Sample preparation*

Origin, physical and chemical characteristics of some samples in the present work are confident and consequently they were used without further purification. Hemocyanin from C. concholepas (CCH) was provided by Biosonda Corp. (Chile) and hemocyanin from M. crenulata (KLH) was from Pierce-Endogen (USA). Stock solutions of 10 mg/ml were prepared in PBS (phosphate buffer saline, 0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2).

Colloidal silver nanoparticles were prepared by using hydroxylamine hydrochloride as reducing agent. These nanoparticles have the advantage of a more uniform distribution of size and shape together with the absence of citrate excess and its oxidation products. The adherence properties of these chloride-covered nanoparticles are better as concerning their immobilisation on glass giving rise to films by a simple deposition on the surface.

Metal films of Ag nanoparticles for micro-SERS measurements were prepared by immobilizing the colloidal nanoparticles. Previous to this immobilization, an aliquot of the sample in an adequate solvent was added to 500 mL of silver colloid up to the desired concentration. Afterwards, the colloid was activated by addition of 0.5 M aqueous potassium nitrate up to a final concentration in the range $10^{-2}$-$10^{-3}$ M. This activation is needed in order to increase the nanoparticles SERS activity by properly

modifying the morphology of the particles. Then, 20 mL of the final suspension was deposited onto a glass cover slide and dried at room temperature.

An aliquot of the original non-activated colloid was placed on a glass slide with a shallow groove, and then the cover glass slide containing the dried activated Ag nanoparticles was placed on the groove with the side containing the dried nanoparticles facing downwards so that the suspension is placed in the groove, as depicted in Fig. 1, scheme 1b. Only sample monolayers were assumed to contribute to the SERS spectra.

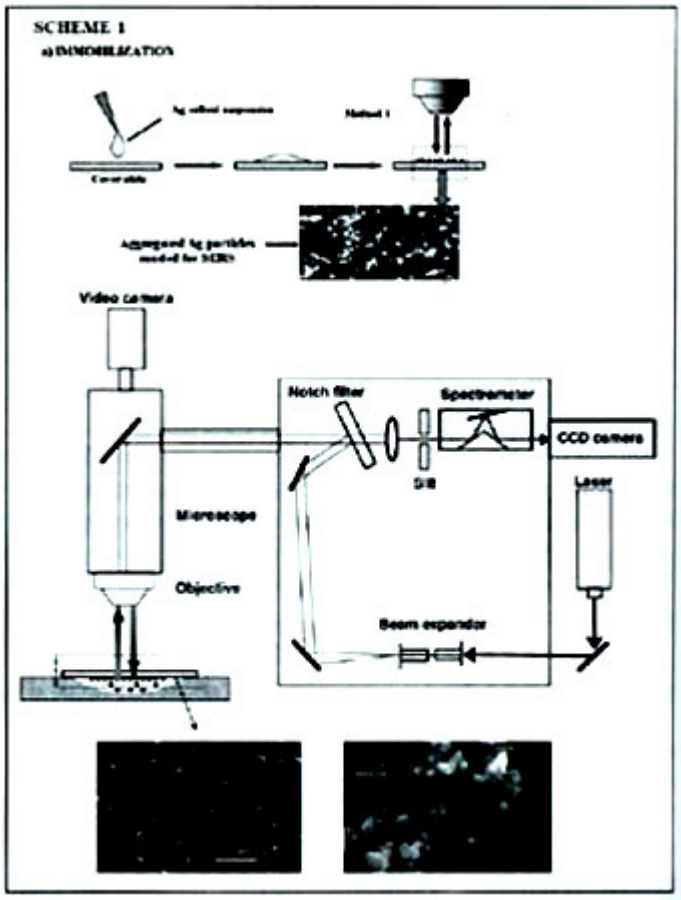


**Fig.1**. Raman instrument and sampling device employed for SERS measurements.

*Spectral reproducibility*.

The SERS spectra obtained from different aggregates were reproducible in what concerns the SERS profiles, although the intensities varied due to the dependence on the aggregate morphology. This reproducibility was maintained from batch to batch.

*Instrumentation*

The SERS spectra were recorded with a Renishaw Raman Microscope System RM1000 equipped with a diode laser (providing the 514 nm laser line) microscope and an electrically refrigerated CCD camera, and a notch filter to eliminate the elastic scattering. The spectra shown here were obtained by using a 50x objective. The output laser power was in the range 0.1-2.0 mW. Spectral resolution was 2 $cm^{-1}$. The spectral scanning conditions were chosen to avoid denaturation of the biological samples. A scheme of the Raman system employed is depicted in Fig. 1. Spectra reported are single scans.

## RESULTS AND DISCUSSION

Before any structural discussion a complete vibrational assignment must be carried out. For the present molecular systems bands were ascribed to the different groups and chemical functions following general published data[1-3] and recent publications[4-6].

*SERS of bacteria*

The chemolithoautotrophic bacterium Acidithiobacillus ferrooxidans is used in biomining due to capacity to solubilize minerals through oxidation reactions[7]. The surface components are essential for the bacterium-mineral interaction. SERS of the A. ferrooxidans grown in media containing thiosulphate, sulfur or iron (II) as oxidizable substrates are displayed in Fig. 2. Spectral signals mainly corresponded to different molecular components of the cell membrane. Main spectral differences arised in the region 1800-500 $cm^{-1}$. They concern some of the phospholipids bands at 1441 and 1270 $cm^{-1}$, which were not clearly observed in the spectrum of bacteria grown in thiosulphate. The phospholipid band at 1025 $cm^{-1}$ shifts in frequency by media effect. Three bands in the 1220-1110 $cm^{-1}$ region (amide III vibrations) and that observed at 1497 $cm^{-1}$ (amide II vibration) display frequency shifts; they are ascribed to protein bands. The phenylalanine (Phe) bands at 1649, 1002 and 614 $cm^{-1}$ were observed with relative medium intensity in the spectrum of Fe(II) and sulphur S media, but very weak in thiosulphate medium. The asparagine/glutamine (Asp/Glu) band at 1388 $cm^{-1}$ decreases in intensity in Fe(II) and S media. The band observed at 772 $cm^{-1}$, much less evident in SERS of bacteria grown in sulfur, could be attributed to polysaccharide structures present on the cell surface, most probably a glycoside ring mode. SERS of D-glucose exhibited this band at 730 $cm^{-1}$. This band has been also attributed in previous SERS studies of bacterial components to the lipid layer components of the cell walls and membranes[6].

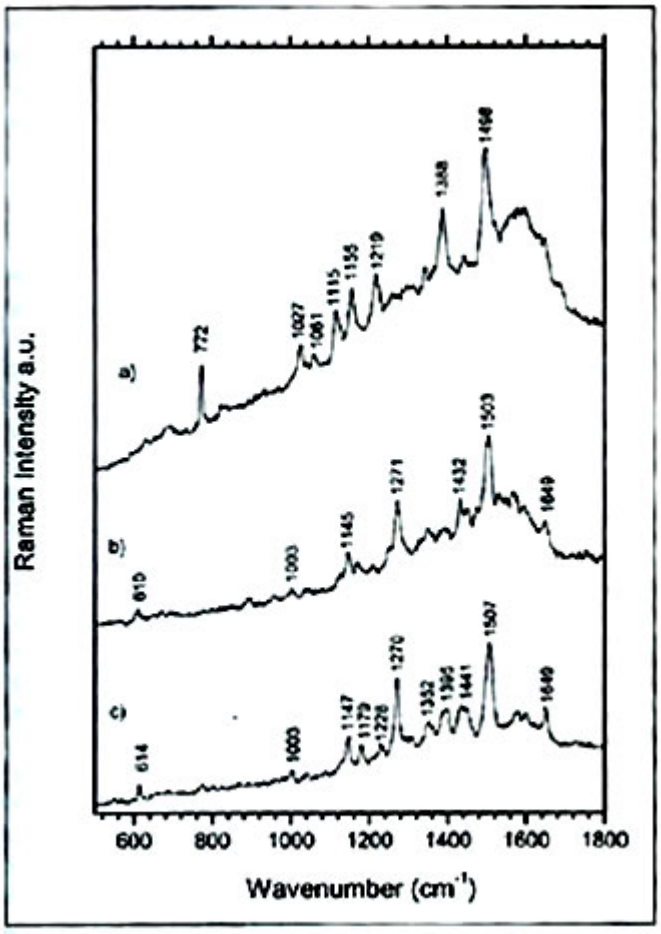


**Fig.2**. SERS spectra of bacteria in different growth media.
a) thiosulphate, b) sulphur and c) iron (II).

This set of results suggests that the membrane components, mainly phospholipids, are sensitive to the growth media of the bacteria. The aminoacids relative abundance also changes depending on the growth media. Different conformations of some aminoacids can be inferred from the observed frequency variations in the amide III spectral region by effect of the medium.

Changes in the surface components have been previously observed by polyacrylamide gel electrophoresis in A. *ferrooxidans* grown under different conditions[8,9]. Specifically, outer membrane proteins change their amounts when the microorganism grows in sulfur compared with ferrous iron10 or in the lack of phosphate[11]. Therefore, the changes seen here by using SERS may reflect some of these changes and more subtle changes not detected by biochemical methods.

*SERS of proteins*

SERS spectra of two purified blue copper proteins, molluscan hemocyanines[12] named CCH13 and KLH14 (Fig.3) displays clear and well isolated bands in the region 1800-400 $cm^{-1}$. Main differences arise from the relative abundance of aminoacids tryptophan

(Trp), bands at 1436, 1407, 1332 and 1029 $cm^{-1}$, and cystine (Cys), bands at 665 and 515 $cm^{-1}$ which is predominant in protein CCH. It should be emphasized that in usual methods for analysis of aminoacids content, hydrolysing the protein with 6N HCl, Trp and Cys are destroyed. On the other hand, these results support the greater stability of CCH as opposed to KLH in solutions without divalent ions, as consequence of a high content of extremely hydrophobic aminoacids as Trp and Cys. Histidine (Hys), band at 1524 $cm^{-1}$, is more abundant in protein KLH than in CCH; this peak correlates well with an imidazole ring vibrational mode. This result is in accordance with the aminoacid composition obtained for KLH and CCH by the analytical standard method15. Moreover, bands which belong to the same aminoacids show in both spectra mainly ascribed to tyrosine (Tyr) and Phe in the range 1300-1150 $cm^{-1}$, display different frequencies. This was also verified in the case of the Tyr band at 825 $cm^{-1}$ in CCH and at 878 $cm^{-1}$ in KLH. The Asp/Glu band at 1377 $cm^{-1}$ was observed with very low intensity in the CCH spectrum. These spectral differences suggest that both proteins are structurally different mainly from the conformational viewpoint. Thus, they differ in their primary and secondary structure. In fact, frequency changes of the amide III vibrations at 1280 and 1217 $cm^{-1}$ in CCH to 1291 and 1231 $cm^{-1}$ in KLH, are consistent with peptide bonds of different energy; this difference arises from a different electronic distribution around the peptide bond. The presence of such vibrations in both species along with the amide I vibration at 1651 $cm^{-1}$ and the skeletal mode at 970 $cm^{-1}$ clearly observed in the spectrum of KLH, is consistent with alpha helix conformations. The presence of the CCH band at 1217 $cm^{-1}$ and the KLH one at 1231 $cm^{-1}$ suggests the coexistence of an antiparallel beta sheet conformation in both species.

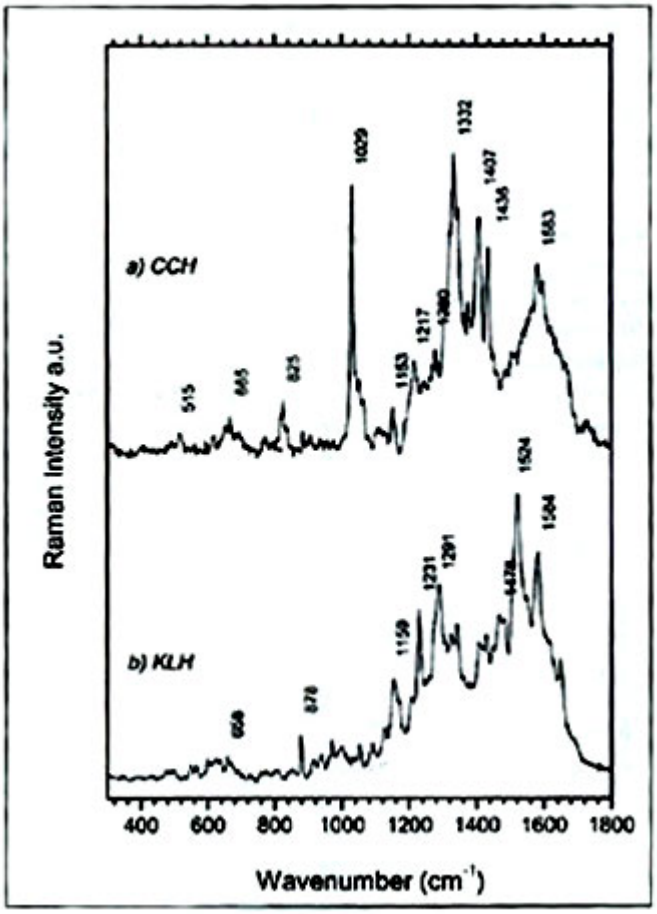


**Fig.3**. SERS spectra of molluscan hemocyanines. a) CCH and b) KLH.

In addition, the information available today supports that KLH and CCH have a similar tertiary structure (they have globular domains or functional units that include the active site, a copper atom pair that binds oxygen), in spite of the large differences in their quaternary structures. Indeed, KLH and CCH display a homodidecameric and heterodidecameric array of their subunits, respectively 12,13.

*SERS of humic acids*

In Fig. 4 we display the SERS spectrum of a 103Da humic acid. This spectrum registered at pH 4 shows two typical strong and large bands at about 1600 and 1400 $cm^{-1}$, which correspond to aromatic ring CC stretching modes. The SERS spectrum offers more information when the sample is dissolved at pH higher than 716. Some weak bands are now observed which are ascribed to different chemical functions of the humic acid: the nCOO- vibration at 1580 and 1380 $cm^{-1}$, $\nu$CC aromatic and dNH amide and amine modes at 1514 $cm^{-1}$, $\nu$CO and $\delta$OH modes at 1438 $cm^{-1}$, $\delta$CH aliphatic and $\nu$COC of ester of aromatic acids at 1304 and 1125 $cm^{-1}$, the last band is also ascribed to aliphatic ethers. Out of plane ring deformations and $\delta$NH vibrations of primary amides were observed at 925 and 705 $cm^{-1}$. This spectral behaviour by pH effect is interpreted in terms of conformational changes adopted by the molecules. Thus, at low

pH the molecule adopts a nearly globular structure; the increasing pH causes a decurling of the molecules. Most of bands enhanced by surface effect at the higher pH correspond to those modes ascribed to polar chemical groups; this is a consequence of the interaction of the corresponding ionic fragments with the metal surface.

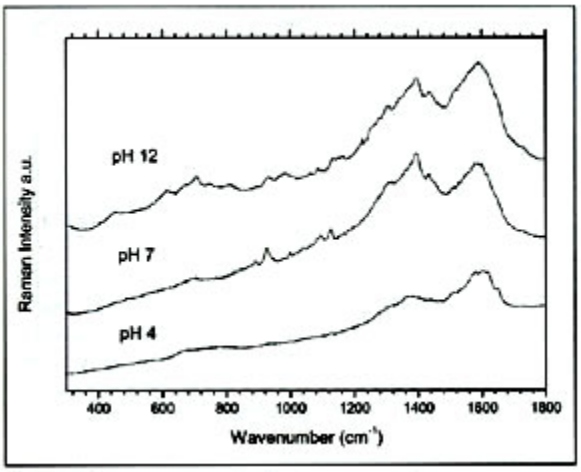


**Fig.4**. SERS spectra of humic acid at different pH.

The temperature effect in the range 30-150 °C on the humic acid conformation has been also studied but using the IR spectra. Results analysed with the same optic used in SERS suggest that molecules adopt a globular structure by increasing the temperature; as a consequence, most of bands decrease in intensity.

*SERS of carbon nanotubes*

The Raman resonance intensity depends on the density of the electronic states available for the optical transitions. In the case of a single-walled nanotube SWNT bundles, the Raman lines will be observed with the highest intensity[4] only in those nanotubes having the valence and conduction bands energy gap in resonance with the laser energy excitation. Figure 5A displays the resonance Raman spectra of the nanotubes obtained at two excitation wavelengths. The two most intense G peaks (G graphite) are labelled $G^+$ for atomic displacements along the tube axis, and $G^-$, for modes with atomic displacement along the circumferential direction. The asymmetric broadness of the line at 1540 $cm^{-1}$, obtained with 514 nm (2.41 ev) is characteristic of a $G^-$ band of a metallic nanotube. This band is drastically less intense when irradiating with the 785 nm laser line (1.58 ev) which indicates the presence of semiconducting species. The low relative intensity of the band at 1540 $cm^{-1}$ suggests a small amount of that species in the sample. Both spectral behaviours are consistent with single-walled nanotubes. The observed spectral frequency difference of the $G^-$ band by excitation energy effect (1556 $cm^{-1}$ at 785 nm and 1540 $cm^{-1}$ at 514 nm) suggests the coexistence of nanotubes with different diameters. The fact that the $G^+$ band frequency is practically invariant by the same effect corroborates this proposition. The $G^+$-$G^-$ observed difference indicates that the diameter of the metallic species is smaller than the diameter of the semiconducting species.

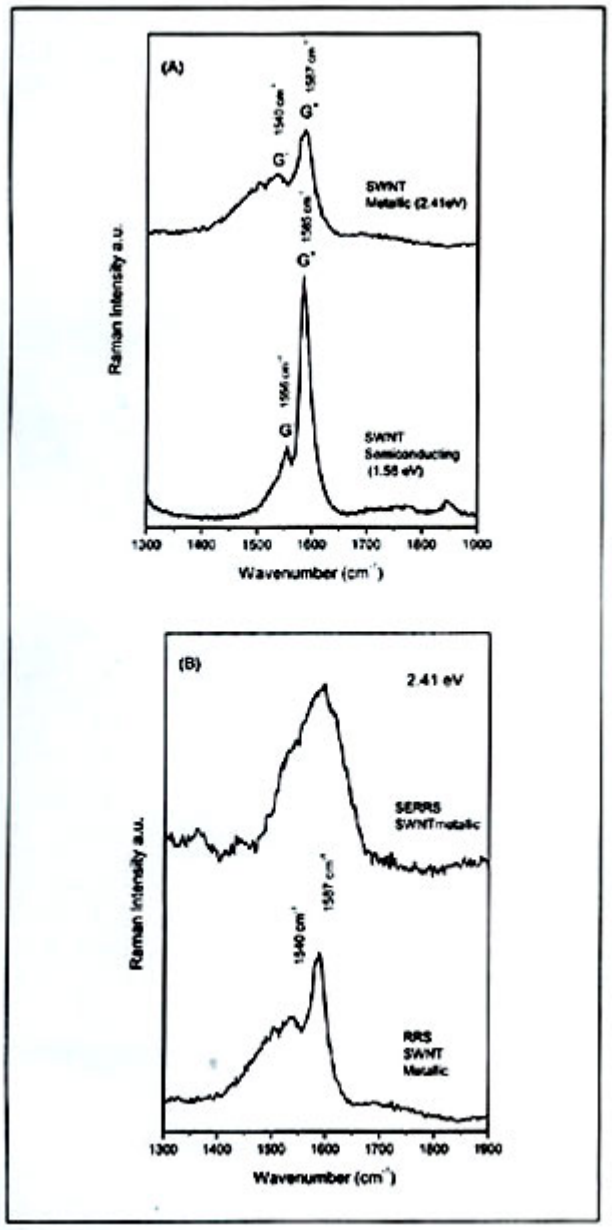


**Fig.5**. (A) Raman at different laser radiation wavelengths and (B) SERS spectra of nanotubes.

The Raman spectrum and the surface-enhanced resonance Raman SERRS spectrum of the nanotubes by exciting with the 514 nm laser line are displayed in Figure 5B. The main spectral feature concerns the $G^+$ band which broadens by surface effect. This behaviour is characteristic of metallic carbon nanotubes and it is the result of a significant perturbation of the electronic levels of the adsorbed nanotubes. These results are explained in terms of a charge transfer between the nanotube and the silver surface. In the case of semiconducting carbon nanotubes normal resonance Raman and SERRS spectra profiles are very similar.

## CONCLUSIONS

SERS technique is demonstrated to be a useful vibrational technique to study giant molecular systems. Results allowed determine physical and chemical changes in the bacteria cell membrane induced by different media growth. Both different conformational modifications of identical aminoacids and their relative abundance in two isolated blue copper proteins were arguments to propose different structures. The structure of a humic acid is demonstrated to be highly sensitive to the pH media; a globular conformation predominates at low pH, while a decurled structure is observed at pH higher than 7. Finally, the SERS technique using different excitation laser lines allowed characterize the present carbon nanotube as metallic-single walled species; it is also inferred the coexistence of nanotubes with different diameters.

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