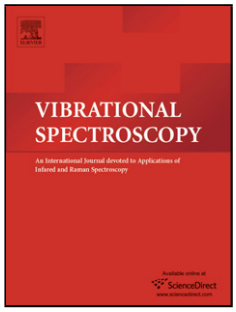

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Interaction of the C-terminal peptide from pigeon cytochrome C with silver nanoparticles. A Raman, SERS and theoretical study

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ABSTRACT

The Raman and surface-enhanced Raman scattering (SERS) spectra of the C-terminal peptide of pigeon cytochrome C (PCC_{87-104}), were recorded. This peptide is widely used to study the immune response in vivo. Hydrophobicity and net charge parameters of PCC_{87-104}, allowed prediction of the nature of its interaction with colloidal nanostructured silver surfaces. The SERS spectrum provided information about the organization and orientation of PCC_{87-104} on the surface of silver nanoparticles (AgNPs). The batch to batch reproducible SERS spectra were obtained by adding the colloidal AgNPs solution onto the dried analyte sample. On the basis of the SERS information and the analysis of the net charge of each amino acid residue in the peptide sequence, it is concluded that the interaction of the peptide and the AgNPs is mainly induced and oriented by the lysine residues. The spectroscopic results are supported by quantum chemical calculations, performed by using Extended Hückel theory for a model of PCC_{87-104} interacting with a silver surface.

Keywords. Raman, Surface-enhanced Raman scattering, PCC_{87-104} peptide, hydrophobicity, net charge, Extended Hückel theory.

1. Introduction

This contribution deals with the Raman and SERS study of PCC_{87-104}, a specific antigenic peptide found in the pigeon cytochrome C structure [1]. PCC_{87-104} binds to the I-E major histocompatibility complex molecule on antigen presenting cells which activates T helper lymphocytes of a transgenic mouse strain called AND. This system is widely used to study the immune response in vivo [2-4].

Raman spectroscopy allows structural information of biological systems such as amino acids and peptides to be obtained. Several papers on the vibrational characterization of amino acids and peptides have been published [5-17]. Bioanalytes commonly display high fluorescence in normal Raman spectra whereas the surface enhanced Raman scattering (SERS) technique suppresses fluorescence and so is advantageous. In fact, the metallic nanometer-sized structures used as substrates enhance the Raman signal and partially or totally quench fluorescence. SERS spectroscopy has other advantages when compared to conventional Raman spectroscopy, such as to obtain structural and conformational information in systems of analytes at very low concentrations, working in aqueous solution at controlled pH.

Results on the application of SERS in peptides have been published by Podstawka-Proniewicz et al. [18]; L-valine phosphonate dipeptides were studied by Fourier-transform infrared (FT-IR) spectroscopy, Fourier-transform Raman spectroscopy (FT-RS) and SERS. The band assignment was performed on the basis of B3LYP/6-311G**++ calculations. The orientation of these dipeptides as well as the specific-competitive interactions of their functionalities with the silver substrate was proposed. In another SERS study Podstawka et al. [19] compared the adsorption behavior of bombesin (BN) and five BN-related peptides in silver colloidal solution. The peptide-metal interaction occurs mainly through the pyrrole

ring of tryptophan and the aromatic ring of phenylalanine. They also inferred a weak interaction through particular skeletal fragments of the peptide chain. These results are slightly modified in the series of peptides. Other results by Di Foggia et al. [8] concern the peptide EAK 16 (AEAEAKAKAEAEAKAK). Five alternating polar/hydrophobic oligopeptides derived from EAK 16 were examined in comparison with EAK 16 both after solubilisation/lyophilisation and deposition on TiO_2 surfaces. Infrared (IR) and Raman spectroscopies were used to investigate the influence of the amino acid substitution on the self-assembling properties of the peptides under both experimental conditions. The β-sheet conformation prevails after deposition on TiO_2. The interaction with the surface was mainly due to carboxylate groups with a bidentate bridging coordination and C=O peptidic groups. Ryu et al. [20] demonstrated, using SERS and gold nanoparticles, that the HKHAHNYRLPASGGKK peptide selective to the well-known *Anthrax* biomarker, protective antigen (PA), can be used for the low concentration range detection (down to 6.1 fM) of target biomarkers.

Deckert-Gaudig et al. [21] performed measurements by Tip-enhanced Raman scattering (TERS) and theoretical calculations of the aromatic amino acids phenylalanine, tyrosine, and tryptophan in systems sandwiched between gold and silver layers. These results are also compared with SERS data in colloidal silver and solid state Raman. On the basis of the spectral data, they concluded that the carboxylate and amino groups are the preferential moieties attached to the surface's cavities. The experimental results were supported by theoretical calculations.

Seballos et al. [22] studied the SERS spectra of several peptides composed of different combinations of proline, tryptophan and tyrosine. They concluded that the binding with

a silver surface occurs through both the carboxyl terminus and the aromatic amino acid moieties.

Guiffo-Soh et al. [23] used Raman spectroscopy and circular dichroism (CD) to study aqueous solutions of peptides with the formula $(KL)_n K$, n=1,4,7. The results demonstrated the usefulness of Raman spectroscopy to eliminate ambiguity of the conformational assignments in peptides.

Our recent SERS results dealt with the oligopeptides MRKDV, ADEDRDA and LGRGISL [13]. These oligopeptides display different net charges and hydrophobic characteristics which were related to particular structural aspects of the adsorbate-substrate interaction. In all cases the SERS spectrum displays signals coming from the guanidinium moiety of the arginine (R) which induces the orientation of the peptides on the metal surface. These spectroscopic results were supported by quantum chemical calculations. In order to contribute to knowledge about the drug delivery research area and following the already commented protocol, we have recently published SERS data for the C-terminal peptide of the β-subunit human chorionic gonadotropin βHCG constituted by 37 amino acids and deposited on Ag nanoparticles [6]. SERS and theoretical data allowed us to propose that the peptide nanoparticle interaction is mainly electrostatic and governed by positively charged Thr, Arg and Lys amino acids.

The SERS protocol is dependent on the nature of the peptide. The length and the sequence are important factors related to the three-dimensional conformation of the peptide, which in turn determines the accessibility of the molecules and the adsorption on a metal surface. Moreover, the hydrophilicity, the residual electrostatic charge and the existence of side chains containing chemical groups displaying affinity for the metal surface are factors determining the interaction strength of the peptide with a metal surface.

On the basis of the SERS data of the present peptide along with its hydrophobicity and charge characteristics, and the interface's nature, we intend to determine the influence of the individual amino acids on the interaction of the peptide with Ag metal surfaces. The SERS spectrum is obtained by adding the colloidal AgNPs solution onto the dried analyte sample. This procedure already used in previous works [5,6,13] allows to obtain a batch to batch reproducible SERS spectrum. In order to complete the analysis of the SERS experiments, a theoretical study based on the Extended Hückel Theory (EHT) method for a molecular model of the peptide surface interaction was performed.

2. Experimental

2.1. Materials

Solid peptide $PCC_{87\text{-}104}$ from New England Peptides was kindly supplied by Professor M.R. Bono from the Laboratory of Immunology, Faculty of Sciences, University of Chile. The amino acid sequence of the peptide is KKAERADLIAYLKQATAK; the carboxyl terminal group was modified by the amide group ($CONH_2$). The molecular weight is 2017.4 g/mol. Stock solutions of the peptide in nanopure water were prepared to a final concentration of 10^{-4} M. pH was controlled to a constant value close to 7. This guarantees the coexistence of a small numbers of conformers. Nanopure water used in the experiments has a conductivity in the range 18.1-18.3 Mohm.

2.2. Preparation of silver nanoparticles

Silver nanoparticles were prepared by chemical reduction of silver nitrate with hydroxylamine hydrochloride [24]. The resulting colloids display a final pH near 7.

The aqueous solutions utilized for the AgNPs formation were prepared by using nanopure water. The colloid shows a milky grey colour.

2.3. Preparation of Raman and SERS samples

20 μL of 10^{-4} M aqueous solution of the peptide were deposited onto a quartz slide. Solution samples were dried at room temperature. The Raman spectrum of the solid was obtained after evaporation of the solution. The colloidal AgNPs solution was dropped onto the dried $PCC_{87\text{-}104}$; a room temperature dried sample was used for the SERS measurements. The SERS spectrum of lysine was obtained under the same experimental procedure and conditions used to obtain the SERS spectrum of the peptide.

2.4. Instrumentation

The Raman and SERS spectra of the peptide were measured with a Renishaw micro-Raman system (RM1000) using as excitation the 785 nm laser line. This instrument was equipped with a Leica microscope, and an electrically cooled CCD camera. The signal was calibrated by using the 520 cm^{-1} line of a Si wafer and a 50x objective. The laser power on the sample was 2 mW. The resolution was set to 4 cm^{-1} and 5 to 20 scans of 10 s each were averaged. Spectra were recorded in the 200-1800 cm^{-1} region. The spectral scanning conditions were chosen to avoid sample degradation.

2.5. Spectral reproducibility

No reproducible SERS spectra were obtained by using the traditional method, that is, by addition of the sample solution to the colloidal suspension or the vice versa [5,13]. The reproducible SERS spectra from batch to batch were obtained by adding the colloidal AgNPs solution onto the dried analyte sample.

3. Molecular models and calculations.

The silver surface was simulated as follows. A big Ag cube with a face centered-cubic structure (a = 0.408 nm) was built as in our previous studies [15-16, 25-26]. The resulting structure was trimmed to get a planar surface of Ag composed of 356 atoms. Molecular mechanics was employed to optimize the $PCC_{87\text{-}104}$-Ag surface geometry. The layer geometry was kept constant. The peptide was placed at different distances and orientations from the center of the Ag layer. EHT was used to calculate the wave function of $PCC_{87\text{-}104}$ as an isolated system and interacting with the metal surface. The Hyperchem program was used [27]. EHT calculations produce qualitative or semiquantitative descriptions of molecular orbital and electronic properties [26]. The combination of EHT with molecular mechanics was able to give, for example, a qualitative explanation of our previous SERS works in arginine [5], lysine [15], tryptophan [16], nanotubes [25] and humic acids [28] interacting with Ag surfaces. EHT was chosen because (apart from the system's size) it was shown that, within the Hartree-Fock-Rüdenberg picture (HFR), EHT is compatible with the nonempirical Hartree-Fock method in Roothaan's form. HFR thus explains why EHT proved to be qualitatively successful [29-30].

4. Results and discussion

4.1. Physicochemical properties of $PCC_{87\text{-}104}$

Colloidal AgNPs display a negative charge imposed by chloride ions resulting from the reduction agent hydroxylamine hydrochloride used in the synthesis. The $PCC_{87\text{-}104}$ peptide has a +4 net charge and the hydrophilic index is 0.6 [31] at pH 7. The hydrophilic index and net charges of $PCC_{87\text{-}104}$ are shown in Table 1. From these data one could expect that the interaction occurs through the positively charged amino acids, lysine and arginine, or

fragments containing them in the sequence **KK**AE**R**ADLIAYL**K**QATA**K**. Arginine is surrounded by two negatively charged amino acids, glutamic and aspartic acid, thus hindering its interaction with the negatively charged surface. This is not the case for lysine in positions 87, 88, 99 and 104. Thus, one can expect that the peptide-metal interaction be mainly governed by lysine.

Table 1. Structural amino acid components, hydrophilic index [31,37] and net charges of $PCC_{87\text{-}104}$.

4.2. Raman and SERS spectra of $PCC_{87\text{-}104}$

The Raman and SERS spectra of the peptide are displayed in Fig.1. No fluorescence was observed. The band assignment was performed on the basis of previous and related work [5,6,13,15,16] and our own data [10,14,32-38]. Table 2 displays the proposed assignments. Two intense bands observed in the SERS spectrum of the peptide at 1054 cm^{-1} νCN and at 1449 cm^{-1} δNH, were ascribed to the lysine residue (see Fig. 2). The general spectral characteristics of these SERS bands, their intensity and frequencies, were compared with the Raman and SERS spectra of lysine (see Fig.2) and arginine [5], suggesting that the analyte surface interaction occurs through the amino group (NH_3^+) moiety of lysine. Although this amino acid displays a high hydrophilic index (3.0), it seems that the charge effect (+1) drives its interaction with the negatively charged environment surrounding the metal surface. The medium SERS band of the peptide at 728 cm^{-1}, is assigned to a coupled vibration involving the ρCH_2 and COO^- deformation modes [6]. This band characterizes most of amino acids containing the CH_2 fragment, in particular lysine. Strong characteristic guanidinium Raman bands of arginine [5], observed at 1046 and 1069 cm^{-1}, are not identified either in the Raman spectrum or in the SERS spectrum of the peptide. However,

we find a large and medium band in SERS at 1449 cm^{-1} ascribed to the NH_3^+ group of lysine, and another medium and large band at 844 cm^{-1} composed by two shoulders contains information of tyrosine modes. In both cases the large bands could contain vibrational information of the guanidinium group of arginine according to that observed in MRKDV [13]. Thus, the guanidinium moiety interacts weakly with the metal surface or it is far from the surface. This seems to be related to the negatively charged and highly hydrophilic neighbourhood imposed by the amino acids E and D.

Figure 1. (a) Raman and (b) SERS spectra of $PCC_{87\text{-}104}$ in the 300-1800 cm^{-1} spectral region.

Figure 2. (a) SERS spectrum of $PCC_{87\text{-}104}$ (b) SERS spectrum of lysine and (c) Raman spectrum of lysine in the 350-1800 cm^{-1} spectral region.

Aromatic amino acids display characteristic Raman and SERS bands. The maximum of the large SERS band observed at 844 cm^{-1} is assigned to the stretching C-C mode of the aliphatic fragment; this vibrational mode is attributable to several amino acids in the peptide sequence. Two shoulders of this broad band are observed at 828 and 848 cm^{-1}, which correspond to the in-plane ring breathing and out-of-plane ring bending modes of tyrosine, according to Fang Wei et al. [12] and Deckert et al. [21]. In addition, tyrosine has a characteristic band at 643 cm^{-1}, which corresponds to a symmetric ring breathing mode [12]. This band is observed at identical frequency in the Raman and SERS spectra of the peptide. This spectral region is displayed in figure 3. The experimental data suggest that the corresponding aromatic ring interacts with the metal surface. According to the definition of the ring breathing mode and the SERS selection rules [39,40] indicating that modes having their Raman polarizability z-component perpendicular to the surface are likely to become

more enhanced than the parallel ones, it is possible to suggest a tilted orientation of the aromatic ring of tyrosine with regard to the surface. Thus, it can be proposed that the ring fragment is not oriented parallel to the surface as predicted by our calculations. Therefore, tyrosine with a neutral net charge but with a negative hydrophilic index could be close to the metal surface.

The Raman bands of the peptide at 1367 and 598 cm^{-1} are ascribed to alanine [10,14]; these bands are not observed in the SERS suggesting that its interaction with the metal surface is weak or non-existent. The hydrophilic index -0.5 and the neutral charge of alanine support this idea.

From the above results it is possible to propose that the net charges on the amino acids in the peptide and not their hydrophilic indices drive the orientation of the peptide towards the negatively charged metal surface.

Figure 3. (a) SERS spectrum of $PCC_{87\text{-}104}$ and (b) Raman spectrum of tyrosine expanded in the 600-900 cm^{-1} spectral region.

Table 2. Raman, SERS wavenumbers and the most likely band assignment of $PCC_{87\text{-}104}$.

4.3. Theoretical discussion

EHT results for the isolated Ag monolayer show that the occupied and virtual eigenvalues (HOMO and LUMO have an energy of about -7.03 eV) are sufficiently packed to form valence and conduction bands, indicating that the proposed surface structure is an acceptable model of a metallic surface. The isolated peptide has a HOMO energy of -12.15 eV and a LUMO energy of -9.20 eV. Within this model, if a charge transfer occurs it will be from the peptide's HOMO to the conduction band of the Ag layer. Figure 4 shows the final geometry of the peptide-Ag layer. The peptide-Ag layer interaction occurs through

several sites. Ammonium groups of Lys-87 (positively charged) are about 3.7 Å from some Ag atoms and the ammonium group of Lys-104 (negatively charged) is about 3.0 Å away. The carbonyl groups (negatively charged) between Lys-87 and Lys-88, between Lys-88 and Ala-89, between Tyr-97 and Leu-98, between Lys-99 and Gln-100, between Ala-101 and Thr-102 and between Thr-102 and Ala-103, are about 3.0-3.2 Å from the layer. The negatively charged O atoms of the carboxylate moieties of Glu-90 and Ala-92 are about 3.1-3.3 Å from the Ag layer. The phenyl ring of Tyr-97 (negatively charged and including the OH group) is almost coplanar to the silver surface, its atoms being 3.1-3.3 Å from that surface. The N and O atoms of the amide group of Gln-100 are about 3.2 Å from the Ag layer.

The peptide-metallic layer interaction occurs therefore at intermediate intermolecular distances [41] and its main component is an electrostatic one. We cannot preclude a charge transfer from the HOMO that is located on the phenyl ring of Tyr-97 and is of π nature, to the conduction band of the Ag layer. These theoretical results largely support the observed SERS experimental data.

Figure 4. Predicted molecular model for the $PCC_{87\text{-}104}$ -Ag interaction

5. Conclusions

The analysis of the net charge values and hydrophilic characteristics of $PCC_{87\text{-}104}$ allowed us to make inferences about the SERS activity, and then to propose an idea about how this peptide interacts with a metal surface. Here, we have not observed an arginine-metal surface interaction normally expected through the guanidinium group. This is mainly due to the negatively charged environment generated by the aspartic and glutamic acid moieties which induce a conformational change that places the guanidinium group far from the nanoparticle surface. These ideas are confirmed by experimental SERS data. SERS spectra were observed for the peptide by coating it with silver nanoparticles; this also allows reproducible spectra to be obtained. The SERS spectrum of the peptide is dominated by signals coming from the amino acid residues, mainly lysine in positions 87-88-99 and 104; some tyrosine 97 signals also appear in the SERS spectrum.

Theoretical results confirm the inductive effect imposed by the ammonium group of lysine and the effect that the aromatic moiety of tyrosine could have on the peptide-metal interaction. However, the proposed orientation of the tyrosine aromatic ring, almost coplanar to the silver surface, is not completely consistent with the experimental results which suggest that the ring fragment is not oriented parallel to the surface. The proposed structural model for the peptide-Ag surface system suggests that the $PCC_{87\text{-}104}$-Ag interaction is almost totally electrostatic.

The SERS spectral profile of the peptide in the amide I and III regions along with the skeletal bands does not allow us to propose, under the present experimental conditions, a definitive conformational structure of the peptide on the surface [42,43].

FIGURE CAPTIONS

1. (a) Raman and (b) SERS spectra of PCC_{87-104} in the 300-1800 cm^{-1} spectral region.

2. (a) SERS spectrum of PCC_{87-104} (b) SERS spectrum of lysine and (c) Raman spectrum of lysine in the 350-1800 cm^{-1} spectral region.

3. (a) SERS spectrum of PCC_{87-104} and (b) Raman spectrum of tyrosine expanded in the 600-900 cm^{-1} spectral region.

4. Predicted molecular model for the PCC_{87-104}-Ag interaction

TABLE CAPTIONS

Table 1. Structural amino acid components, hydrophilic index and net charges of PCC_{87-104}.

Table 2. Raman and SERS wavenumbers and the most likely band assignment of PCC_{87-104}.

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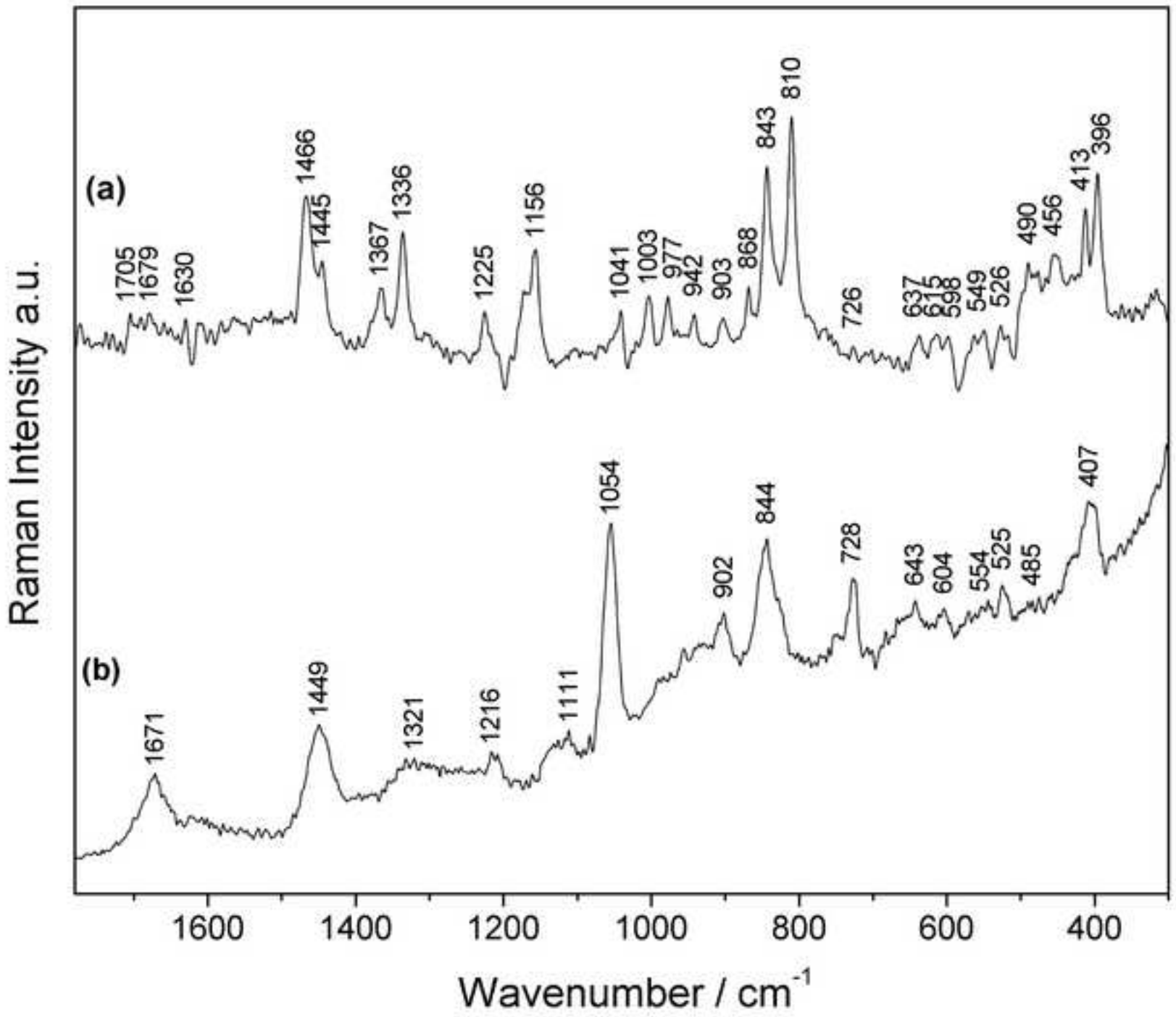
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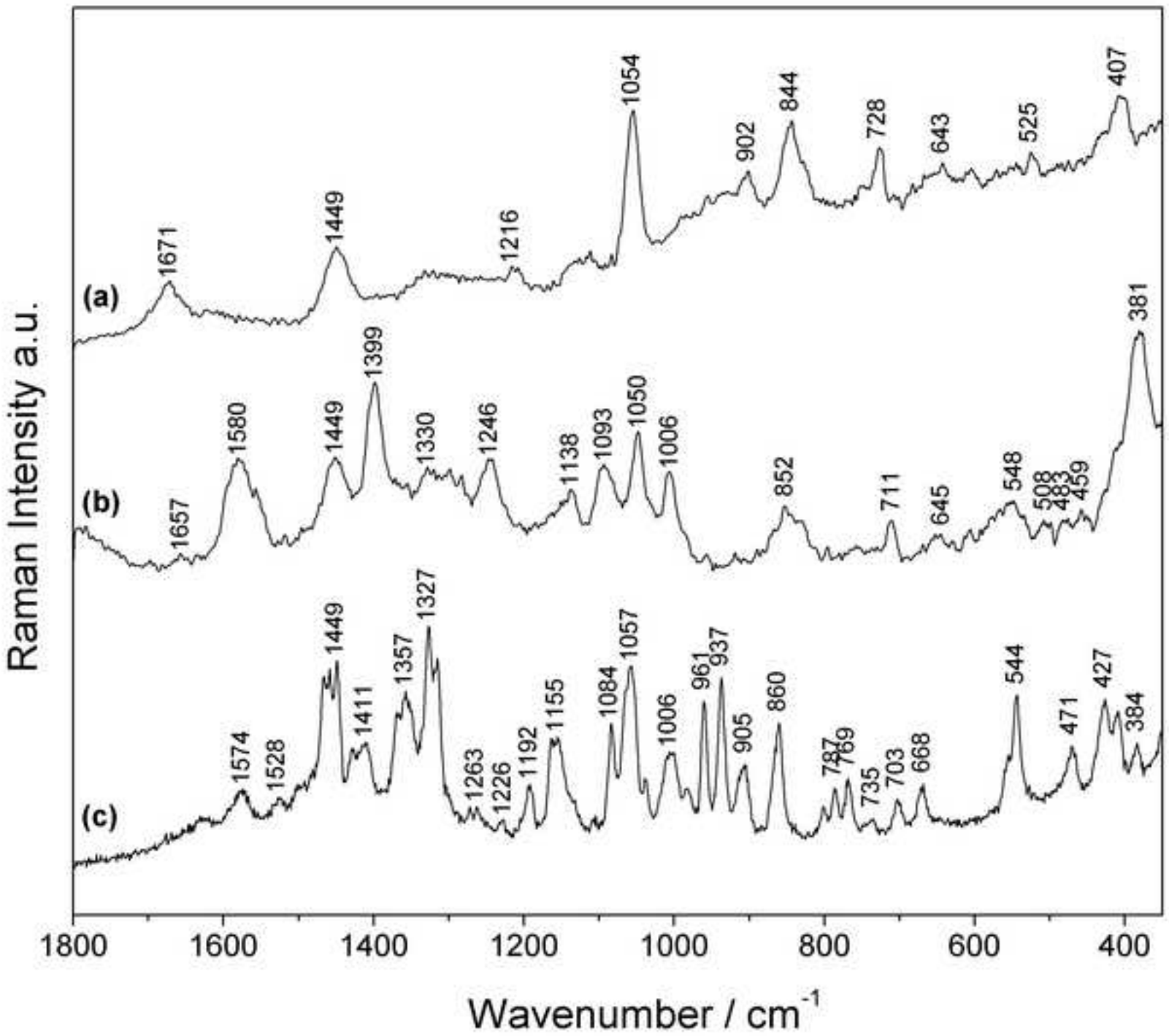
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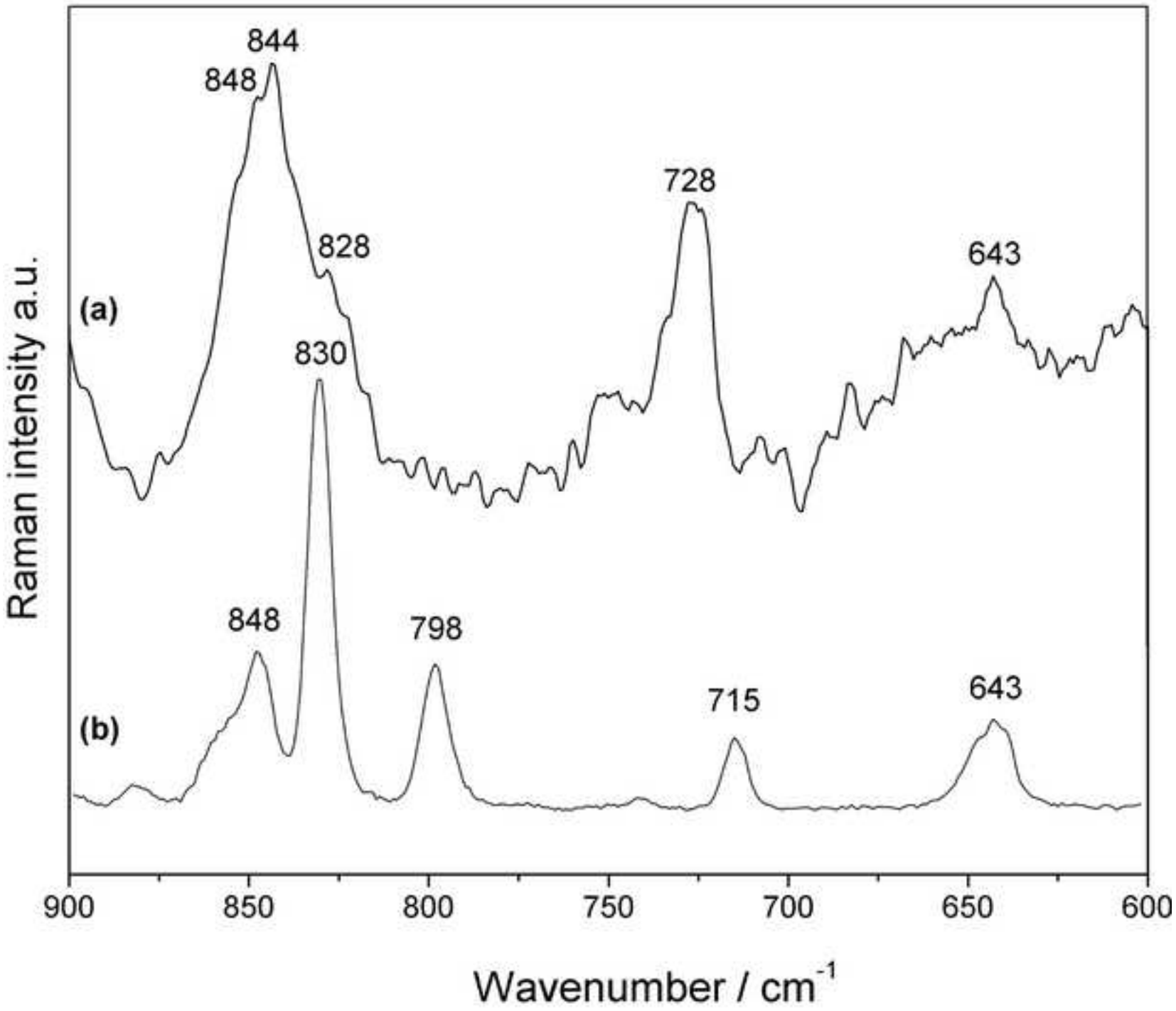
728
643
525
407
1657
1580
1449
1399
1330
1246
1138
1093
1050
1006
852
711
645
548
508
483
459
381
1574
1528
1449
1411
1357
1327
1263
1226
1192
1155
1084
1057
1006
961
937
905
860
787
769
735
703
668
544
471
427
384
1800
1600
1400
1200
1000
800
600
400
Wavenumber / cm-1

Figure(s)


844
848
828
728
643
(a)
830
848
798
715
643
(b)
Raman intensity a.u.
900
850
800
750
700
650
600
Wavenumber / cm-1

Figure(s)

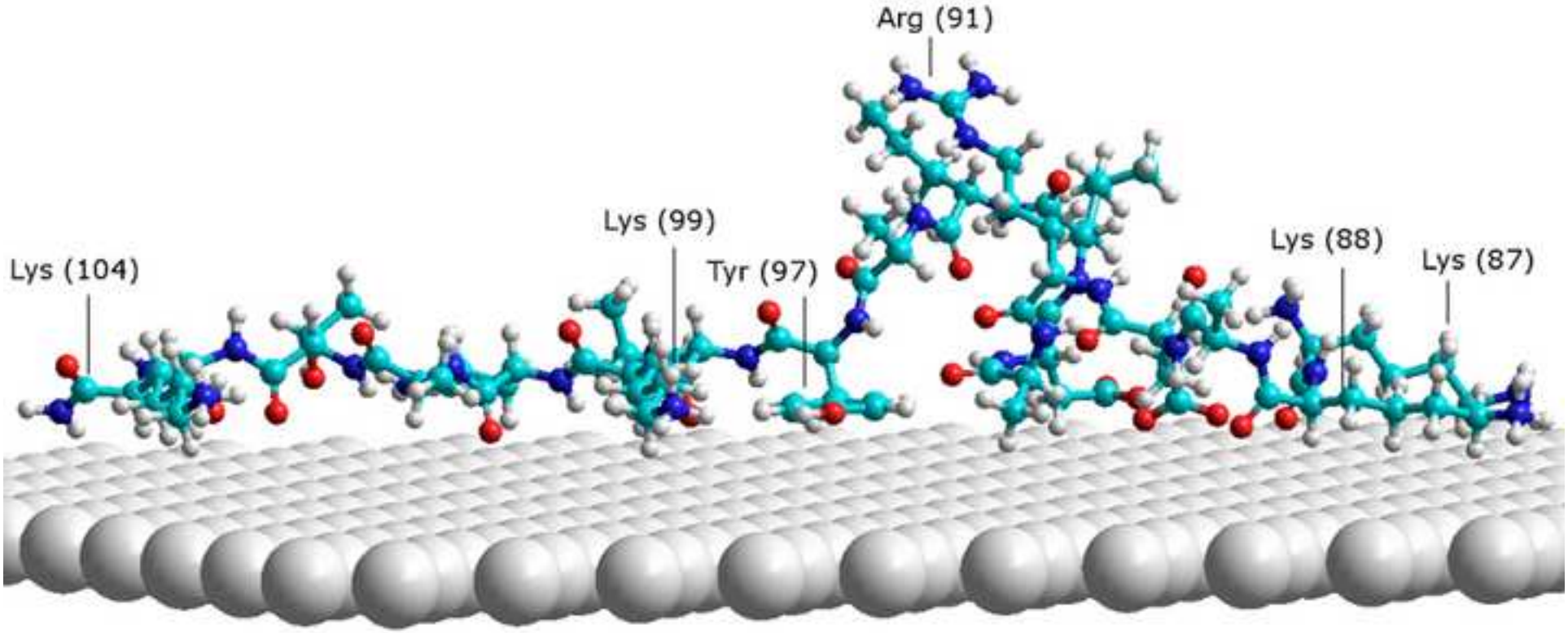


Table 1. Structural amino acid components, hydrophilic index [31,37] and net charges of PCC_{87-104}

Amino acids position	Peptide and amino acids	Hydrophilic index	Net charge*
	PCC_{87-104}	0.6	+ 4
87	Lys K	3.0	+ 2
88	Lys K	3.0	+ 1
89	Ala A	-0.5	0
90	Glu E	3.0	- 1
91	Arg R	3.0	+ 1
92	Ala A	-0.5	0
93	Asp D	3.0	- 1
94	Leu L	-1.8	0
95	Ile I	-1.8	0
96	Ala A	-0.5	0
97	Tyr Y	-2.3	0
98	Leu L	-1.8	0
99	Lys K	3.0	+ 1
100	Gln Q	0.2	0
101	Ala A	-0.5	0
102	Thr T	-0.4	0
103	Ala A	-0.5	0
104	Lys K	3.0	+ 1

**Estimated at pH 7.*

Table 2. Raman, SERS wavenumbers and the most likely band assignment of PCC_{87-104}

Raman PCC_{87-104} cm^{-1}	**SERS PCC_{87-104} cm^{-1}**	**Proposed bands assignment**
1679 vw	1671 m	Amide I
1466 s		$\delta_a CH_3$ (A)
1445 m	1449 m	sccis.CH_2, δNH (K)
1367 w		$\delta_s CH_3$ (A)
1336 s	1321 vw	ωCH_2(K)
1225 m	1216 w	Amide III
1156 s	1111 w	δNH_3^+ (K)
1041 w	1054 vs	νCN(K)
1003 w		(K)
977 w		νCC
942 w		νCC
903 w	902 m	νCC (K)
868 w		νCC
843 m	844 bmult.	Ring breath., bend. (Y), νCC
810 vs		νCC
726 vw	728 ms	ρCH_2, COO^- def. (K)
637 vw	643 w	ring δ (Y)
615 vw	604 w	δCOO^-
598 vw		(A)
526 vw	525 w	tNH_3^+ (K,R)
456 vw		(K)
413 m	407 m	δCN (K)

Relative intensity: s, strong; m, medium; w, weak; vw, very weak; b, broad; mult., multiple. Proposed band assignments in one-letter code for the amino acid: A, alanine; K, lysine; Y, tyrosine; E, glutamic acid. For more information about the specific normal modes involved in the vibrations, please see References 10 and 14.

Highlights

> Net charge values and hydrophilic characteristics of the peptide $PCC_{87\text{-}104}$ allowed to infer about your SERS activity.

> Lysine residues play an important role in the peptide-AgNPs interaction.

> Theoretical results confirm the inductive effect imposed by the lysine in the electrostatic peptide-AgNPs interaction.