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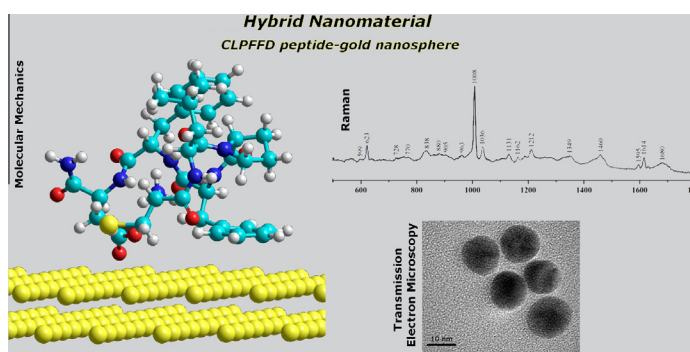
Interaction of the CLPFFD peptide with gold nanospheres. A Raman, surface enhanced Raman scattering and theoretical study

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HIGHLIGHTS

- The SERS spectrum of the CLPFFD peptide as anti-aggregation of β -amyloid has been registered.
- Spectral SERS reproducibility is obtained by using spherical gold nanoparticles.
- Theoretical calculations predict the orientation of hydrophobic amino acidic residue onto the Au surface.

GRAPHICAL ABSTRACT



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ABSTRACT

In a previous work we demonstrated that toxic aggregates of the protein β -amyloid (ATA β) involved in the Alzheimer's disease (AD) can be destabilized upon electromagnetic irradiation of the peptide Cys-Leu-Pro-Phe-Phe-Asp (CLPFFD) adsorbed on gold nanospheres (AuNSs). For a selective recognition of the therapeutic target (i.e. ATA β) of AD by the conjugates peptide-nanoparticle it is relevant to understand how the interaction between attached ligands and nanoparticles occurs. In this work a surface enhanced Raman scattering spectroscopy (SERS) study of the interactions of CLPFFD with AuNSs of 10 nm average diameter was carried out. The SERS data suggest that phenylalanine displays its aromatic ring coplanar to the surface which is supported by theoretical data obtained from molecular mechanics (MM) and Extended Hückel Theory (EHT) calculations.

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Introduction

The interaction of biomolecules (especially proteins and peptides) with metal nanoparticles is a subject of great interest in

biomedicine and nanotechnology [1]. Gold nanoparticles are a special platform to obtain organic-inorganic hybrid nanomaterials [2]. The hybrid nanomaterials are obtained by functionalization of metal surface with diverse biomolecules of biological and pharmacological interest. Gold nanoparticles have interesting optical properties [3]. These nanoparticles can be irradiated with a visible or near infrared light of appropriate wavelength inducing a great intensification of the electromagnetic field on the metallic surface through localized surface plasmon resonance; thus, a great

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enhancement of Raman signal from biomolecules adsorbed on particular metal nanoparticles is verified [4]. SERS is a highly sensitive technique which uses metal nanoparticles for detection of hundreds of biomolecules and processes at the molecular levels [5]. SERS based on gold nanoparticles functionalized with peptides is a useful and powerful analytical tool which can be used for the specific recognition of proteins disease-causing [6]. In this sense, great efforts have been focused into obtain stable gold nanoparticles peptide systems which could be used as potentials therapeutic tools for the diagnosis, prevention or cure of diseases [7], such as the Alzheimer's disease (AD). AD mainly affects people over 65 years old and is one of the top ten causes of death in this age group [8]. It is a neurodegenerative disease that is manifested through behavioral and cognitive impairment [9]. The causes of AD are unknown, however the development of senile plaques in the brain of patients seems to be one of its causes [10]. These insoluble senile plaques are extracellular deposits of the β -amyloid protein; this protein is composed by peptides having 39–43 amino acids and it is synthesized from the amyloid precursor protein [11]. In pathological conditions, the β -amyloid protein changes its conformation from a α -helix to a β -sheet, forming aggregates that eventually could be transformed into fibers [12]. Recently, it has been proposed that the key to an early pathogenic event in the onset of AD is the presence of toxic aggregates of β -amyloid as oligomers, amylospheroids, protofibrils, pores, and short fibrils. A strategy for treating the disease could be reducing the amyloidogenicity and toxicity of the mentioned species [13]. In order to develop a potential therapeutic tool for AD, we have functionalized gold nanospheres (AuNSs) with the Cys-Leu-Pro-Phe-Phe-Asp (CLPFFD) peptide [7,14]; this peptide selectively recognizes toxic aggregates of the β -amyloid protein (AT-A β) involved in AD. The chosen peptide mimics a hydrophobic core (residues 17–20, LVFF) of the β -amyloid protein of 42 amino acids [15]. In the design of this peptide, keys amino acids are preserved (leucine and phenylalanine) for recognition of AT-A β . The amino acid valine (V) was replaced by proline (P) conferring the disruptive ability of the β -sheet, a secondary structure found in fibers and mostly toxic protein aggregates [10,15]. The cysteine residue was incorporated to anchor the peptide to the Au surface by using the thiol group, as already reported [7]. However, the process of functionalization of the metallic surface may induce drastic effects on the conformation of the peptide affecting its biological activity and the delivery toward the therapeutic target [16]. In a previous work, we conjugated gold nanoparticles with the peptide CLPFFD-NH₂ and demonstrated that the peptide sequence influences the degree of conjugation, the stability, and the interaction of gold nanoparticles peptide conjugates with β -amyloid [7]. In the present work, to infer about the orientation and organization of the CLPFFD peptide on the gold surface, a vibrational study is performed by using the Raman and SERS techniques. AuNSs of 10 nm average diameters have been functionalized with the peptide CLPFFD and the hybrid nanomaterial has been characterized by high-resolution transmission electron microscopy (HR-TEM), dynamic light scattering (DLS), zeta potential (ξ -potential) and UV-visible spectroscopy (UV-vis). The spectral assignment of the SERS data was supported on our Raman data; the ligand-metal surface interaction nature was analyzed from the SERS band analysis of the amino acid groups along with theoretical calculations of a molecular model for the peptide interacting with the gold surface. Molecular Mechanics with Optimized Potentials for Liquid Simulations force field (MM-OPLS) was employed to optimize the geometry of the peptide-Au surface interacting system keeping constant the gold surface. Extended Hückel Theory (EHT) was used to calculate the wave function of the peptide as an isolated system and interacting with the metal surface to describe the possible bond formation.

Experimental

Sample preparation

CLPFFD peptide was synthesized following protocols described by our working group [7,17]. The AuNSs of 10 nm average diameter were synthesized following the method developed by Turkevich [18]; the pH was adjusted to 7.4 using dilute NaOH solution, and the filtrate was stored at 4 °C. The functionalization of the AuNSs was performed by adding a stock solution of the peptide (1 mg/mL) to 20 mL of AuNSs in a ratio of 10:1, under constant stirring during 15 min. The hybrid nanomaterial obtained was dialyzed against a sodium citrate solution (1.2 mM) for 3 days; the citrate solution was removed every day. The pH was adjusted to 7.4 using concentrated NaOH. Raman spectra were obtained by deposition of the solid peptide on a quartz slice. SERS spectra were obtained after deposition of 10 μ L of the hybrid nanomaterial solution on a quartz slide and dried at room temperature.

Instrumentation

The AuNSs were characterized by HR-TEM using a microscope JEOL 2010F instrument with an energy resolution of 1.2 eV. The software Digital Micrograph was used for gauge the average diameter of the particle.

The hybrid nanomaterial was characterized through TEM using a microscope JEOL JEM-1010 apparatus operating with an acceleration voltage of 120 keV. For observation of the sample, a drop (20 μ L) was deposited on a copper grid coated with a formvar polymer.

The ξ -potential and DLS measurements were performed using a Malvern Instrument Zetasizer ZS; disposable plastic cuvettes 1 cm were used. Nanomaterials were dispersed in milli-Q water (18.2 M Ω /cm resistivity).

The UV-vis spectra were recorded using a Lambda 25 spectrophotometer and disposable plastic cuvettes of 1 cm optical path length.

The Raman and SERS spectra were recorded with a micro-Raman Renishaw 1000 (RM 1000) spectrometer, equipped with a Leica microscope and a CCD sensor. The available laser excitation lines were 514, 633 and 785 nm. The signal was calibrated using as standard the spectral line at 520 cm^{-1} of silicon and a 50 \times optical lens. The intensity of the 785 nm laser liner on the sample was around 2 mW. The spectra were obtained using optical 50X. The spectral resolution was 4 cm^{-1} ; 5–10 spectral scans of 10 s were registered. Raman and SERS spectra were recorded in the 450–4000 cm^{-1} region. The spectral scanning conditions were chosen to avoid sample degradation.

Molecular model, methods and calculations

The gold surface was simulated by building an Au cube with a face centered-cubic structure ($a = 0.408$ nm) and following the same methodology used in our previous works with Ag [19,20]. The resulting structure was trimmed to get a double Au planar layer composed by 1024 atoms. MM-OPLS was employed to optimize the peptide-Au system keeping constant the metallic layer geometry (Fig. 1). EHT was used to calculate the wave function of CLPFFD as a fully ionized isolated system (isoelectric point structure) and interacting with the metal surface. Hyperchem program was used [21]. The most extended conformation was employed as a starting point. EHT calculations produce qualitative or semi quantitative descriptions of molecular orbital and electronic properties [22]. The combination of EHT with MM provided qualitative explanations of our previous SERS results in other peptides

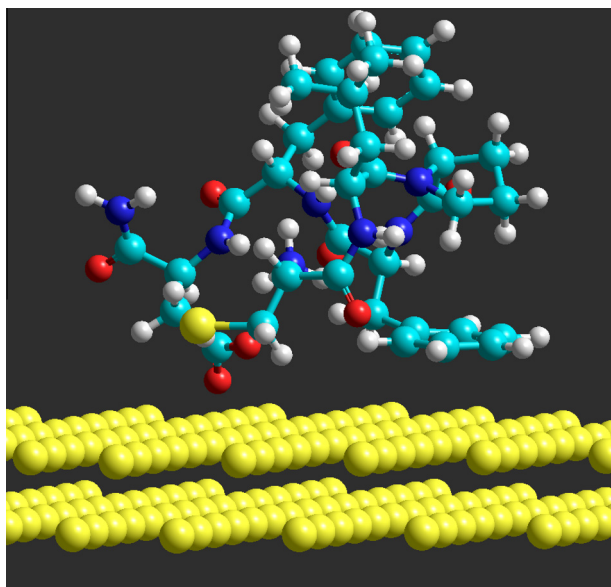


Fig. 1. Molecular model for the CLPFFD–Au layer interaction.

[4,20,23], interacting with Ag surfaces. EHT was chosen because it was shown that, within the Hartree–Fock–Rüdenberg picture, it is compatible with the nonempirical Hartree–Fock method in Root-haan’s form [24].

Results and discussion

Preparation and characterization of AuNSs

An averaged diameter of AuNSs of 10 nm was obtained using TEM micrographs from a statistical distribution of sizes performed on 133 nanoparticles. Fig. 2a shows the HR-TEM micrographs. A large homogeneity of shape and size was observed. UV–vis data of the AuNSs allow determining that the surface plasmon is centered at 520 nm (see Fig. 2b).

Functionalization of AuNSs with the CLPFFD peptide

The surface-adsorbed peptide system was confirmed by ζ -potential and UV–vis data. The ζ -potential decreases after the peptide functionalization; this is due to a charge modification

occurring in the metal surface (see Table 1). We attribute this modification to the replacement of ion citrates (ion citrates have three dissociated negatively charged carboxylic groups at pH 7.4) by peptide molecules (which bear one formal negative charge at pH 7.4, see Table 2). In fact, the thiol group of the cysteine residue could induce a peptide–metal interaction due to a chemisorption process [25]; the thiol group should interact with the positive residual charge of the AuNSs [26]. At the same time, the interaction between the surrounding citrate ions (negatively charged) and the negative net charge of the peptide mainly imposed by the aspartic acid residues that are negatively charged at pH = 7.4 should induce a charge redistribution around the interface. If this phenomenon occurs then the organization and orientation of the peptide on the metal surface are modified. Moreover, once the functionalization occurs a spectral shifting to higher wavelengths (from 520 to 527 nm) of the absorption maximum is verified (Fig. 2b). In previous work we demonstrated that the number of peptide molecules per nanoparticle is approximately 460 [7].

Raman and SERS spectra

The Raman and SERS spectra were analyzed in the region 500–1800 cm^{-1} . The bands assignment of the peptide was performed from the spectral analysis of each amino acid constituent. The relative contribution of each amino acid residue determines the peptide spectral profile. Fig. 3 shows the Raman and SERS spectra of CLPFFD. Table 3 contains the most probable band assignment, specifying each amino acid residue. The band assignment is based on published data on related molecular systems [4,20,23]. Raman bands (Fig. 3a) located at 1615, 1595, 1213, 1165, 1131 and 1009 cm^{-1} , are ascribed to vibrations of phenylalanine. The bands at 1461 and 1349 cm^{-1} are assigned to CH_2 deformations and carboxylate symmetric stretching modes of leucine and aspartic acid, respectively. Vibrational signal at 905 cm^{-1} corresponds to proline while the band at 963 cm^{-1} is a vCC mode; the band at 624 cm^{-1} is ascribed to a vCS mode. The cysteine Raman spectra were studied at pH 6.0 and 9.0 in aqueous solution [27]. Typical bands of the aspartic acid fragment corresponding to the carboxylic moiety are not possible to distinguish. The bands at 1681 and 963 cm^{-1} are attributed to the amide I and vCC peptide backbone modes, respectively. The most intense band (1009 cm^{-1}) is due to Phe. The vSH mode of cysteine located around 2500 cm^{-1} [28] is not clearly identified in the present Raman spectra. The SERS spectrum of the peptide anchored to the AuNS, shown in Fig. 3b, is analyzed in the range 500–1800 cm^{-1} . The strong surface plasmon signal avoids any SERS band analysis in the region below 500 cm^{-1} . Most

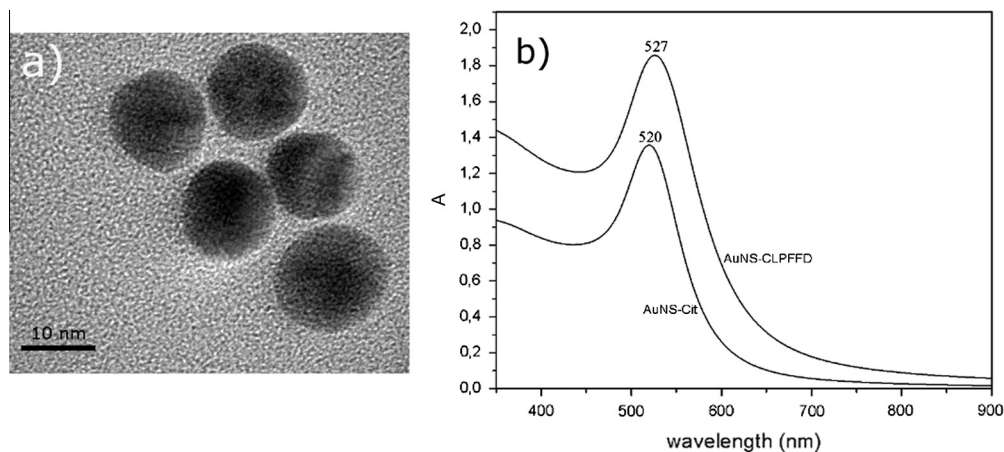
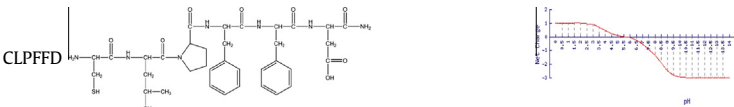


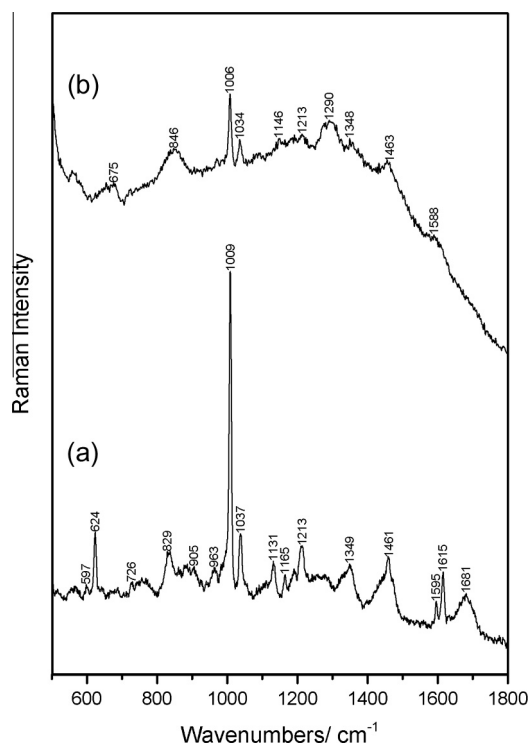
Fig. 2. (a) HR-TEM of AuNSs (bar corresponds to 10 nm) and (b) UV–visible spectra of AuNSs (dotted line) and AuNS-CLPFFD (solid line).

Table 1
Characterization of the AuNSs functionalization.

Molecular system	λ_{\max} (nm)	Averaged diameter (TEM) (nm)	Hydrodynamic diameter (DLS) (nm)	ζ Potential (mV) pH = 7.4
AuNS-CLPFFD	527	13 ± 4	16.4 ± 0.5	-15 ± 5
AuNSs	520	10 ± 3	9.9 ± 0.3	-44 ± 6

Table 2
Physicochemical parameters of the CLPFFD peptide.

Molecular system	Isoelectric point (5.4)	Net charge ^a	Hydrophobicity index ^b
		-1	1.1

^a At pH 7.4.^b Calculated according to the scale of Kyte–Doolittle hydrophobicity [37].**Fig. 3.** (a) Raman of CLPFFD in solid and (b) SERS spectra of AuNP-CLPFFD 10⁻⁵ M.

of the Raman bands decrease in intensity by surface effect. A comprehensive analysis allows a specific assignment of the vibrations (see Table 3). An Au–S bonding cannot be discarded in the present case; this mode is normally observed near 390 cm⁻¹, as described in SERS experiments with AuNSs [29]. If this interaction is verified, then the adjacent CS bond vibration should be sensitive to the surface ligand bonding [30]. In fact, a strong intensity decreasing of the vCS band at 624 cm⁻¹ is verified by surface effect. Due to the low relative intensity of this band and the spectral noise is not possible to identify a probable wavenumber shifting by surface effect. The intensity variation is explained on the basis of the SERS selection rules [31]; on this basis it is inferred that the CS bond α_{zz} polarizability component is not parallel to the laser beam. The spectral shifting suggests a CS bond weakening due the chemisorption process which is in agreement with XPS data reported by us [7]. Due to the metal ligand interaction, a conformational rearrangement of the peptide on the surface is expected, as already

Table 3
Raman and SERS wavenumbers (cm⁻¹) and the most probable bands assignment of the CLPFFD peptide.

Raman	SERS	Assignment
1681 wb		Amide I, δNH_2^+
1615 mw		F, δNH
1595 w	1588 wb	F, $\nu_{\text{as}}\text{COO}^-$
1461 mw	1463 wb	L, ωCH_2 , $\nu_{\text{s}}\text{COO}^-$
1349 mw	1348 vw	L, ωCH_2 , $\nu_{\text{s}}\text{COO}^-$
	1290 wb	Amide III, tCH ₂
	1273 wb	Amide III, rCH ₂ , tCH ₂
1213 m	1213 vw	F, δCH , δNH
1165 vw		F, ωCH_2
1131 w	1146 wb	F, L, D, δCNH
1037 m	1034 m	F, vCCN
1009 s	1006 s	F, ring breathing
963 w		vCC
905 mw		P, vC-COO ⁻
838 wb	846 wb	tCH ₂
770 vw		δCOO^-
726 vw		$\delta, \omega\text{COO}^-$
624 m	618 vw	C, vCS, COO ⁻ def.

Bands intensity description: w, weak; vw, very weak; b, broad; mw, medium weak; s, strong, m, medium. Amino acids: C, cysteine, L, leucine, P, proline, F, phenylalanine, and D, aspartic acid. Symbols: ν , stretching, δ deformation, ω wagging, r, rocking, t, twisting.

concluded from the functionalization data and predicted from the physicochemical data discussion.

The most intense SERS bands of Phe are observed at 1006 and 1034 cm⁻¹. This spectral behavior is consistent with a phenyl fragment of the phenylalanine residue close to the surface and oriented plane parallel to the metal surface, in agreement with the SERS selection rules [31]. No a formal surface adsorption is verified for the phenyl group. On the other hand, the spectral behavior of the band at 1034 cm⁻¹ and the CH₂ one at 846 cm⁻¹, consistent with the aromatic rings in hydrophobic microenvironment [32], suggests structural conformational modifications of the peptide by surface effect.

SERS bands of the aspartic acid on Ag colloids were observed by Stewart and Fredericks [33] at 1372 cm⁻¹ assigned to stretching modes of the carboxylic group. This band is observed in our SERS spectrum at 1348 cm⁻¹, thus suggesting that the corresponding group is close to the surface. According to the SERS selection rules [31] one could expect that the COO⁻ symmetric stretching band should be enhanced by surface effect if the α_{zz} component of the vibration is perpendicular to the surface. As this band is observed in SERS with a small intensity it is proposed that the carboxylic group is rather tilted on the surface, as predicted by the present calculations, Fig. 1.

It has been demonstrated [34] that the hydrophilicity and hydrophobicity nature of the amino acid residues of a peptide is highly sensitive to pH variations. On this basis we could expect that the interaction of CLPFFD with the metal surface be affected by pH variations. Thus, the peptide–AuNSs interaction was studied under different pH conditions in the range 5.0–9.0; as predicted, the spectra collected show variations, in particular the relative intensity of the phenylalanine signals, see Fig. 4. The band at 1007 cm^{-1} displays the highest intensity at pH 7.0 suggesting a stable preferential orientation of the peptide on the surface. Concerning the quality of the present SERS spectra, we realize that some physicochemical difficulties arise when working with the SERS sample at pH close to 5 that is the aggregation and sedimentation of the citrate Au colloid; moreover, at this pH the sample displays a net charge equal to -1 . At higher pH, close to 9, the net charge of the peptide is now -2 ; this means that the analyte surface interaction is not favored related to the pH 7 SERS spectrum. Under such conditions, the sulfur atom is negative charged, the observed SERS spectra resulted mainly from the driven effect of the S–Au interaction.

At pH 5.5 and 9.0 the metal ligand interaction through the Phe groups is less favored than in the pH = 7 condition. This could be related to the structural modifications of the hydrophobic micro-environment into the folded peptide on the surface; this is in agreement with our previous work dealing with the pH effect on AuNSs functionalized with the peptide CLPFFD by using force spectroscopy [35].

Other interesting features are the spectral modifications on the vCS band at about 675 cm^{-1} at pH 9.0 and on the coupled carboxylic band at 1458 cm^{-1} at pH 7.0, thereby suggesting that the whole structure of the peptide has not the same organization and orientation on the surface by changing the pH. The presence of the vCS band at pH 9.0 suggests a S–Au binding, being the CS

fragment perpendicular to the gold surface. In the same sense, it is expected that the aspartic residue, in particular the carboxylic group, be sensitive to the increasing of the alkalinity. The aspartic residue, or citrate ions, interacts at pH 7.0 through both an ionic interaction and unidentate binding as deduced from the wavenumber separation of the $\nu_s(\text{COO})$ and $\nu_{as}(\text{COO})$ signals [36]. In fact, the SERS spectral behavior of the symmetric stretching COO^- band at 1354 cm^{-1} is modified by pH change see Fig. 4, thus suggesting an ionic interaction. Only a unidentate binding is predicted at pH 9.0 from the observed weak SERS intensity of the 1458 cm^{-1} band”.

Theoretical aspects

CLPFFD–metal interaction

For the case of the isolated gold bilayer, the valence and conduction bands overlap, indicating that the present microscopic model is a good representation of a metallic cluster. EHT results for CLPFFD peptide (confirmed by B3LYP/6-31G* and B3LYP/6-311G*** calculations with full geometry optimization) indicate that the HOMO and LUMO are located mainly on one of the phenyl aromatic ring. In the final CLPFFD–Au geometry (see Fig. 1), the aromatic ring of phenylalanine is almost coplanar to the surface. CLPFFD is located at the center of the Au layer in which the electronic densities of the HOMO and LUMO (that can be considered respectively as being closer to the upper part of the valence band and the lower part of the conduction band of the cluster) are zero. As the occupied and empty MOs of the peptide do not overlap with the HOMO and/or LUMO of the Au layer a charge transfer is ruled out.

The necessary conditions to obtain a SERS spectrum are mainly two: a direct peptide–surface interaction and the necessity that this interaction occurs on a hot spot. The direct interaction occurs when thermal agitation carries out the peptide molecules relatively close to the surface without any solvent component between them. This last condition allowed us to model what happens to a peptide molecule arriving closely to the metal surface without needing to include solvent components. By accepting that the CLPFFD molecule is oriented as in Fig. 1, but on a hot spot and the laser line is perpendicular to the surface, then the breathing mode band at 1007 cm^{-1} , should be enhanced which is in agreement with the SERS experimental result. Moreover, the model predicting that the fragment containing the thiol group of the cysteine residue is close to the surface, the corresponding Raman signals should be influenced by surface effect, which is in agreement with the SERS data for the vCS band at 623 cm^{-1} .

Conclusions

The AuNSs were synthesized and characterized; the functionalization of AuNSs with CLPFFD peptide was confirmed by the ζ -potential and UV–vis data. The analysis of the net charge values and hydrophilic characteristics of peptide makes possible to infer about the SERS activity in the colloidal solution. The SERS spectrum shows that most of the vibrational signals disappear or drastically decrease its intensity by surface effect. The intensity of the phenylalanine SERS band at 1007 cm^{-1} ascribed to a breathing mode increases by surface effect suggesting that the corresponding aromatic ring is close and almost coplanar to the metal surface. The Raman vCS band of the cysteine residue is modified by surface effect. The CLPFFD–AuNS interaction studied under different pH conditions show variations on the breathing aromatic ring phenylalanine signal, suggesting that a new conformation of the peptide on the surface is verified. No pH effect on the aspartic signals was observed. MM and EHT calculations for a molecular model of

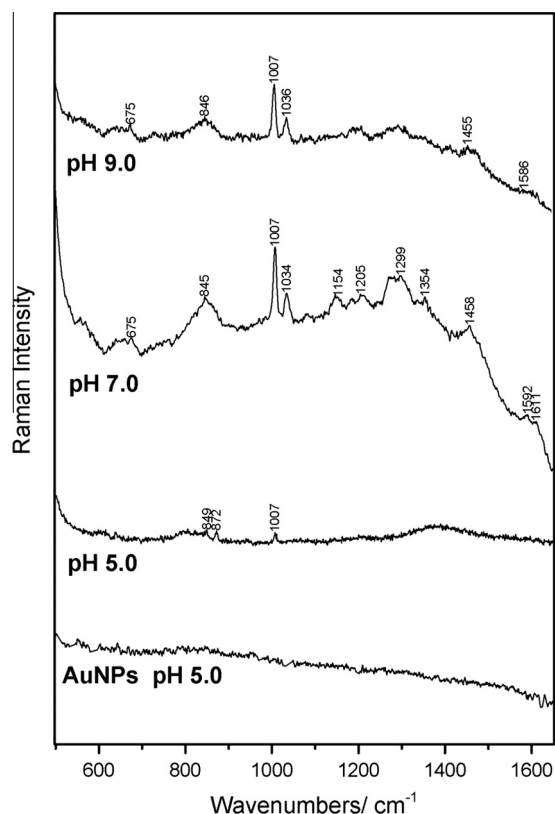


Fig. 4. SERS spectra of AuNSs and AuNP–CLPFFD at different pH.

CLPFFD interacting with a gold surface predict, in agreement with the SERS data, a nearly coplanar orientation of the phenyl moiety of the phenylalanine residue on the metal surface and a close distance between the surface and the cysteine thiol group.

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