This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
Surface- and tip-enhanced Raman scattering of bradykinin onto the colloidal suspended Ag surface

D. Swiech, Y. Ozaki, Y. Kim and E. Proniewicz

In this paper, surface- (SERS) and tip-enhanced Raman scattering (TERS) techniques were used to determine the adsorption mode of bradykinin (BK), a small peptide implicated in, for example, carcinoma growth, onto colloidal suspended Ag surfaces under various environmental conditions, including: peptide concentrations (10^{-5} – 10^{-7} M), excitation wavelengths (514.5 and 785.0 nm), and pH of aqueous sol solutions (from pH = 3 to pH = 11). The metal surface plasmon and rheology of the colloidal suspended Ag surface were explored by ultraviolet-visible (UV-Vis) spectroscopy and atomic force/scanning electron microscope (AFM/SEM). The SERS results indicated that the peptide concentration of 10^{-5} M was the optimal peptide concentration for the monolayer colloidal coverage. The Phe^{8} and Arg^{9} residues of BK generally participated in the interactions with the colloidal suspended Ag surfaces. The amide group appeared to be arranged in the same manner to the Ag surface in the pH range of 3 to 11. At acidic pH of the solution (pH = 3 to 5), the BK –COOH terminal group binds to the Ag surface as a bidentate (at pH = 3) or monodentate (at pH = 5) chelating ligand. At pH = 11, the imino group of Arg^{8}, probably due to its –C=N^{+}H_{2} protonation state, was not involved in the interaction with Ag. The reduction in the solution alkalinity (pH = 9) produced the deprotonation of the –C=N^{+}H_{2} group followed by the group rearrangement in a way favoring the interaction between the lone electrons pair on N and Ag. The TERS studies confirmed the proposed, on the basis of SERS, behavior of BK onto the colloidal suspended Ag at pH = 7 and showed that in different points of the colloidal suspended Ag surface the same peptide fragments approximately having the same orientations with respect to this surface interact with it.

Introduction

Bradykinin (BK) is an endogenous hormone peptide with the peptide sequence, Arg^{1}-Pro^{2}-Pro^{3}-Gly^{4}-Phe^{5}-Ser^{6}-Pro^{7}-Phe^{8}-Arg^{9}COOH (all amino acids are in the L-conformation). This nanopeptide shows wide physiological and pathophysiological activities influencing the cellular and tissue responses. For example, it is involved in the regulation of the blood pressure and smooth muscle contractions. It increases the secretion of chloride ions by epithelial cells and endothelium prostaglandin secretion as well as induces sodium/water homeostasis. Also, due to activation of the endothelial cells BK promotes dilatation of the vessel, vascular permeability, and reproduces characteristic four inflammation symptoms (heat, redness, swelling, and pain). BK is also known as a factor of many diseases, such as: rhinitis, asthma, sepsis, rheumatoid arthritis, pancreatitis, epilepsy, and Alzheimer’s disease. It is suggested that BK is an important target for cancer growth; e.g., small-cell lung carcinoma (SCLC), non-SCLC, and prostate cancer.

The conformational analysis of native BK by molecular spectroscopy methods and computer simulations by molecular modeling methods have revealed that BK mainly exists in a flexible random coil conformation in an aqueous solution and in a solid state. However, when BK binds to its B₁ and B₂ receptors, belonging to the heptahelial transmembrane G-protein-coupled receptors (GPCRs), or when exists in lipidic or micellar environment, that is a good model for the natural environment of BK when interacting with GPCRs, it changes structure from random coil to a bioactive predominantly β-turn structure (at the peptide C-terminus). In this conformation the Phe residue of BK is shielded from aqueous environment. Lopez et al., based on the solid-state NMR results, have also proposed that the C-terminal fragment (Ser^{5}-Arg^{9}) of BK, essential for the interaction with the B₂ receptor, adopts “distinct double-S-shaped structure”. Similarly, Kyle et al. by homology modeling and docking simulations have suggested a twisted S-shaped model of BK bound to the rat B₂ receptor, where Phe^{5}, Phe^{8}, and Pro^{7} of BK are bound in the hydrophobic cavities. The removal of the BK C-terminal L-arginine (Arg^{9}), which drastically reduces the B₁ receptor affinity, has demonstrated that full-length BK is required for the B₂ receptor recognition and the receptor subtype selectivity depends on the presence of the charged terminal residues.

In our laboratory, we also investigated several environmental and structural factors that may help to clarify the biological
behavior observed for bradykinin. We have reported surface-enhanced Raman scattering (SERS) results for BK and its Phe-D$_3$ isotopically labeled analogues deposited onto the roughened in the oxidation-reduction cycles Ag, Au, and Cu electrodes (at different applied electrode potential)$^{17,29}$ and onto the Au colloidal nanoparticles surface. Briefly, our results have demonstrated that the Phe$^3$/Phe$^6$ residues (in tilted orientation of the rings) are mainly involved in the BK interaction with the colloidal Au surface with diameters of 20 nm and there is no preference of any of these two Phe residues to interact with the Ag, Au, and Cu surfaces. Together with the decrease of the Ag electrode charge successive reorientation of the Phe ring was detected, which was not observed in the case of the Ag and Cu electrodes. Based on the results for specifically mutated BK analogues we have also designated Arg$^9$ to be the one which interacts with the colloidal Au surface and Ag, Au, and Cu electrodes.

It is clear that the rheology of adsorbed species, at monolayer and submonolayer coverage, onto a metallic surface and the strength of competitive interaction of certain molecular fragments with this surface depend on analyte concentration, pH of aqueous solution, nanostructure of a metallic surface, and hence on controlled distribution of the metal surface plasmon. This is why it is necessary to investigate the SERS effect for molecule at different experimental conditions. Thus, to delineate a complete picture of the metal/molecule interaction different types of metals and structured metal surfaces should be used. In the case of colloidal structured metal surfaces, the chemical method used for colloid preparation can be chosen to obtain metal nanoparticle of proper size, shape, composition, morphology, and crystalline phase. The size and shape of these nanoparticles can be determined by SEM. Problem concerns only whether the SERS signal comes from molecules adsorbed on the surface of colloidal particles or from “hot spots”. However, the measurements at different analyte concentrations and colloidal particles should resolve this problem. Using AFM coupled with Raman microscope simplifies measurement and gives clear results. For these reasons, we performed this type of research.

Thus, presented in this work SERS and TERS (tip-enhanced Raman scattering) research focuses on the rheology of the monolayer and submonolayer coverage of native BK onto colloidal suspended Ag surfaces under various environmental conditions, including peptide concentrations ($10^{-5} - 10^{-7}$ M), excitation wavelengths (514.5 and 785.0 nm), and pH of aqueous colloidal solutions (from pH = 3 to pH = 11). Of note is the fact that there are only a limited number of TERS studies on biomolecules, including: nucleic acids, viruses and bacteria, lipids and human cell, oxidised glutathione, β-Amyloid(1–40) peptide fragments, histidine, di- (Tyr–Tyr and Phe–Phe) and tripeptides (Tyr–Tyr–Tyr), and bovine serum albumin.

SERS is the most common in situ technique in contexts involving determination of molecule structure, its orientation onto different metallic nanostructures, and complexation$^{17,40,44}$ because of its simplicity and ability to rapidly recognize the structures of peptides in aqueous solutions as well as its ability to probe different types of supramolecular architectures and to study the peptide-level adsorption phenomena at a metal/liquid interface. However, SERS is restricted by the relatively low spatial resolution. Thus, very important information about metal surface morphology cannot be obtained. Therefore, in the last several years, there is strong motivation to develop simple and rapid in vitro, or, if possible, in vivo, methods to study adsorption phenomena at the single-molecule level onto the nanostructure of the metallic surfaces with the controlled distribution of the metal surface plasmon. The TERS technique is believed to be one of such methods.$^{40,44,48}$ TERS combines Raman spectroscopy with scanning probe microscope (SPM) that collects images of metal surface using a physical probe that scans the specimen.$^{49}$ If a probe (tip) is coated with a SERS active metal, then the TERS effect occurs only within the immediate vicinity of the tip. Because the tip dimensions are typically <100 nm, the spatial resolution of this measurement would depend on the tip itself and would similarly be <100 nm.$^{50}$

**Experimental**

**Bradykinin synthesis**

BK (powder, ≥98% (HPLC)) was purchased from Sigma-Aldrich (Poland).

**UV-Vis measurements**

The UV-Vis spectra of the aqueous Ag sol and BK/Ag sol system (measured after 15 minutes of mixing) (see Fig. 1) were recorded on a Shimadzu UV-3100 spectrophotometer.

**Scanning electron microscope measurements**

The SEM images of the aqueous Ag sol were recorded on a SEM instrument, model S-5000 (Hitachi Ltd., Japan), operated at 20 kV (see Fig. 1, Inset A).

**Raman and SERS measurements in an Ag sol**

Preparation of an aqueous Ag sol was carried out according to the following procedure.$^{51}$ Silver nitrate (AgNO$_3$) and sodium borohydride (NaBH$_4$) were purchased from Sigma-Aldrich and used without further purification. Three batches of the aqueous colloidal Ag solutions were prepared by the simple borohydride reduction of silver nitrate. Briefly, 42.5 mg of AgNO$_3$ dissolved in 50 mL of deionized water at 4 °C was added drop-wise to 150 mL of 2 mM NaBH$_4$ immersed in an ice bath, while the mixture was stirred vigorously. When the addition of AgNO$_3$ was completed, the resulting dark-yellow solution (pH = 7) was stirred continuously at 4 °C for approximately 1 h.

Aqueous BK solutions were prepared by dissolution of BK in deionized water (18 MΩ-cm). The normal pM value of the BK solution was around 7. The concentration of the sample was adjusted to $10^{-5}$ M prior to its being mixed with the colloid. Then, ten microliters of peptide sample was mixed with 20 μL of the Ag sol. To investigate the effect of pH, 0.1 and 0.05 M HCl and 0.1 and 0.05 M NaOH solutions were prepared and added to the peptide/colloid mixtures to obtain the desired pH. The mixture was kept for 15 min before the SERS measurement. The peptide solutions at different peptide concentrations ($10^{-5}$ and $10^{-7}$) were prepared by mixing the Ag colloidal solution (20 μl) and peptide solution (10 μl) diluted ten and hundred times.

The Raman and SERS spectra of BK were collected with Renishaw spectrometer (model inVia) operating in confocal mode...
combined with a Peltier cooled CCD detector and a Leica microscope (50× long-distance objective). The 785.0 nm line of HP NIR diode laser (Invictus) and 514.5 nm line of Ar-ion laser (Renishaw) were used as excitation sources. The lasers power at the laser outputs was set at about 15 mW (SERS) and 20 mW (Raman). The typical exposure time for each the Raman and SERS measurement in this study was 40 s with four accumulations (series of 4 spectra, each accumulated 40 s = 160 s).

The SERS spectra were measured at eight spots on the surface of the colloidal Ag nanoparticles. The series of spectra were nearly identical (highly reproducible), except for small differences (up to 5%) in some band intensities.

**TERS measurements on an Ag surface**

Ten microliters of peptide sample was mixed with 20 μL of the Ag nanoparticles. After 15 minutes of incubation the mixture was deposited onto a glass plate and was dried in a vacuum dryer at 37°C for 30 minutes.

The TERS spectra were measured by an instrument consisting of a reflection-mode Raman microscope (Photon Design Inc.) equipped with a CCD detector (Princeton Instrument) and an AFM instrument (Photon Design, Nanostar NFRSM800). This system was arranged to introduce excitation light from the top of the sample and to collect backscattered signals using a 90x objective lens (numerical aperture, NA = 0.71). The 514.5 nm line from an Ar-ion laser (Spectra Physics, Stabilite 2017-06S) was used as the excitation source. The laser power at the sample point was 0.1 mW. A TERS needle coated with Ag (UNISOKU Co. Ltd.) was attached to the quartz tuning fork of a shear-force-based AFM at an angle of 45°. The tip radius of the probe was about 100 nm (spatial resolution of TERS experiments about 100 nm). The TERS tip approached the sample using the non-contact mode. A Raman signal was first collected under the tip-retracted conditions, and then, under the tip-approached conditions at the same point. The typical exposure time for each Raman measurement was 20 s with eight accumulations (series of 10 spectra each of 20 s = 200 s) under the tip-retracted and tip-approached conditions. TERS spectra were calculated by subtracting a spectrum measured under the tip-retracted conditions from that collected under the tip-approached conditions.

**Spectral analysis**

The spectral analysis was performed using the GRAMS/Al 8.0 program (Thermo Scientific).

Second-derivative of SERS spectrum was calculated in the range of 1700 – 600 cm⁻¹ using the Savitzky-Golay algorithm that produces the least squares best fit of the data to the selected polynomial. The number of convolution points can range from 5 to 10,000, although values greater than the number of points across a peak is not normally used. Only odd numbers are used for number of convolution points and even values are rounded up. The number of convolution points and degree adopted in this work was set at 10 and 5, respectively. These parameters did not distort the spectra. However, a larger number of convolution points provided to the more smoothing result.

**Results and discussion**

**Optical properties of Ag sol**

The surface plasmon absorption, sizes, shape, and distribution of Ag nanoparticles were examined by the excitation spectra (UV-Vis) and scanning electron microscope (SEM)/atomic force microscope (AFM) images (Fig. 1). As is evident from Fig. 1, the Ag colloidal solution has the surface plasmon absorption maximum at 392 nm. The SEM/AFM images (Fig. 1A and 1B) point out that this unaggregated colloid contained the statistical Ag spheres with diameters of approximately 20 nm. The addition of BK (10 μL of 10⁴ M) to the Ag colloid (20 μL) pronouncedly decreased the 392 nm band and caused a new band with the maximum at 500 nm. The aggregation produced large assemblies of apparently randomly adhering spheres, each of which with dimensions approximately equal to the original dimensions (Fig. 1C). These large assemblies are responsible for the color change (the metal particles become electronically coupled) that results from the broadness and red-shift to 500 nm of the plasmon resonance.

**pH of the solutions, excitation wavelength, and peptide concentration dependent SERS measurements**

By measuring the UV-Vis spectrum as a function of BK concentration, we observed that the maximum aggregation occurred at a concentration of 10⁴ M. This value was used as the optimal peptide concentration for monolayer colloidal coverage. The influence of the concentration of BK deposited onto the colloidal Ag surface in an aqueous solution at pH = 3 on the SERS spectrum is illustrated in Fig. 2. From this figure it is obvious that the BK SERS spectra for the peptide concentrations of 10⁴ M, 10⁵ M, and 10⁶ M showed the three pronounced spectral features (at 1396, 1239, and 1005 cm⁻¹) due to the carboxyl group, amide group(s), and Phe residue(s) vibrations (see Table 1 for detailed bands assignment). Although only in the SERS spectrum for the BK concentration of 10⁵ M, the medium-weak 1589, 1575, 1449, and 1278 cm⁻¹ bands due to Phe, the 1640 cm⁻¹ allocated to the amide
The SERS spectra of BK immobilized onto the colloidal Ag surface in aqueous solutions of pH = 3 and 7 obtained with the 785.0 and 514.5 nm laser excitations are presented in Fig. 3. It is noted that almost all bands observed in the SERS spectra excited with the 514.5 nm laser line (Fig. 3, traces: pH = 3 and 7, 785 nm) are also present in the spectra obtained upon the 785 nm laser excitation. However, the relative intensities of the bands in these spectra strongly depend on the excitation wavelength as is expected for resonance mechanism of enhancement. In general, the SERS spectra obtained with the 785.0 nm excitation wavelength are significantly strengthened compared to those obtained with the 514.5 nm laser excitation. This has to be associated with existing vibrational coupling often associated with the intervention of charge-transfer transition that was discussed in detail by Birke and Lombardi.56 Although there is no evidence of a charge-transfer transition in the UV-Vis spectrum (Fig. 1). At this peptide concentration (10^{-5} M), investigated molecules form a monolayer at
most. Thus, this thin layer will result in a weak charge-transfer transition, which would be most likely overwhelmed by the tail of UV-Vis coagulation peak. That is why it is not clearly observed in the UV-Vis spectrum.

As mentioned in the above paragraph, the BK SERS spectrum at pH = 3 upon the 785.0 nm excitation line (Fig. 3, the second trace from the bottom) is dominated by the 1396, 1239, and 1005 cm\(^{-1}\) bands due to the carboxyl group, amide group(s), and Phe residue(s) vibrations that are accompanied by other SERS signals of these molecular fragments (see Table 1). The models of the backbone structure of BK bound to the human B\(_2\) receptor, given by Lopez et al.,\(^{28}\) follow that Phe\(^{8/9}\) and Arg\(^{9}\)COO\(^-\) in the BK chain were those residues that interact with the colloidal suspended Ag surface and maintain the rather rigid structure of BK C-terminus. This is in agreement with the biological activity studies (see Introduction) that have demonstrated that the C-terminal fragment of BK is essential for the interaction with the B\(_2\) receptor. Based on the amide bands positions (1640 and 1239 cm\(^{-1}\)) this structure seems to correspond to the \(\beta\)-turn conformation. Again, this is in line with studies of the biological activity, which indicate that BK adopts this structure when binds to GPCRs (see Introduction).

The weaker 1005 cm\(^{-1}\) band in the discussed SERS spectrum (Fig. 3, the second trace from the bottom) in comparison to that in the corresponding Raman spectrum indicates that the Phe ring adopted a more or less tilted orientation with respect to the colloidal suspended Ag surface. The higher wavenumber (by 3 cm\(^{-1}\)) and band width (by 6 cm\(^{-1}\)) of the 1005 cm\(^{-1}\) SERS signal in comparison to those in the Raman spectrum support the above statement. This proposed orientation of Phe corresponds to its behaviour in an aqueous colloidal Au sol.\(^{30}\) On the other hand, the strong enhancement of the 1396 cm\(^{-1}\) spectral feature (fwhm = 20 cm\(^{-1}\); fwhm – full width at half maximum), that is weakly scattered at ~1405 cm\(^{-1}\) (fwhm = 14 cm\(^{-1}\)) in the BK Raman spectrum, leads to the conclusion that the C-terminal deprotonated carbonyl moiety (pK\(_{a1}\) = 1.8) remained in close contact with the surface of colloidal Ag at pH = 3 in a manner that favors the interaction of the lone pair of electrons on oxygen(s) with this surface. The 1520 cm\(^{-1}\) band in the BK SERS spectrum at pH = 3 upon the 514.5 nm irradiation due

![Fig. 3 The SERS spectra of BK in the aqueous Ag sol (nanoparticles with diameter ~20 nm) at different pH of solution and excitation wavelengths. Measurement conditions: peptide concentration: 10\(^{-5}\) M; excitation wavelengths: 785.0 and 514.5 nm; pH values between 3 and 11; spectral range: 1800 – 200 cm\(^{-1}\); Inset A: proposed adsorption modes of BK at different pH of solutions.](image-url)
to antisymmetric stretching vibrations of the –COO’ group confirms the –COO’...Ag interactions and sheds some light on a carboxylate-binding mode. This is because the wavenumbers of the antisymmetric (ν_{as}(COO')) and symmetric (ν_{s}(COO')) stretching vibrations of the carboxylate group have been shown to be indicative of the strength of coordination and the mode of binding in the carboxylate compounds, which can be monodentate or bidentate. The carboxylate group has the C_{3v} point symmetry group when acts as a bidentate chelating ligand. In such case, the wavenumbers of –COO’ are expected to be similar as those seen in the free ion spectrum. Although coordination to a heavy metal atom may cause shift in these wavenumbers, these changes are small. On the other hand, for the monodentate coordination the carboxylate ligand shows a lower symmetry than that of the bidentate form and therefore should exhibit similarities to the spectrum of the monomeric undissociated carboxylic acid itself. For monodentate carboxylate bonding it has been reported that ν_{as}(COO') increases, whereas ν_{s}(COO') decreases in wavenumbers and the frequencies of these modes should differ by 105 – 140 cm⁻¹. Based on the above and on the noticed 124 and 154 cm⁻¹ separations in the wavenumbers of the carboxylate group vibrations at pH = 3 and 5, respectively, it seems that in an aqueous solution at pH = 3 the BK –COO’ terminal moiety binds to the Ag nanoparticles surface through the both oxygen atoms of the carboxylate group, whereas at pH = 5 the carboxylate moiety serves rather as the monodentate chelating ligand.

Fig. 3 presents the pH-dependent changes (from pH = 3 to pH = 11) in the SERS profile of BK adsorbed onto the Ag nanoparticles in an aqueous solution at the concentration of 10⁻⁸ M. An examination of this figure showed that the SERS spectra at pH = 5 and greater pH levels, in contrast to the previously discussed SERS spectrum of BK at the pH solution of 3 (Fig. 3, trace: pH = 3, 785.0 nm), have very similar spectral patterns and consist of bands mainly due to the Phe terminal moiety assisted in the BK -Ag interaction, whereas at pH = 5 the carboxylate moiety serves rather as the monodentate chelating ligand. Consequently, this suggests that, at the pH solutions level ranging from 11 to 9 there is no contact between the carboxylate group of the terminal Arg residues and the Ag surface. Although at pH = 5, the –COO’ moiety assisted in the BK –Ag interaction, whereas at pH = 3 it strongly binds to the Ag nanoparticles surface.

The SERS spectra of BK in an aqueous solution at pH = 3 (Figs. 3 and 4) show also the strong 245 cm⁻¹ band due to the Ag-O stretching vibrations, whereas the BK SERS spectrum at pH = 5 has the ν(Ag-O) mode exhibiting only the weak intensity. This phenomenon implies that at this pH the Phe ring tilted towards the substrate. On the other hand, the weak intensity of the 1640 cm⁻¹ band at pH = 11 indicates that the lone electrons pair on nitrogen of the Arg –N=H group was not involved in the interaction with Ag at this pH, probably due to the protonation state of this group (the presence of –C=N=H₂, pK=12; pK values depend on temperature, ionic strength, and the microenvironment of the ionicizable group). The decrease in pH from 11 to 9 has caused deprotonation of the –C=N=H₂ group followed by the group rearrangement in a way favoring the interaction between the lone electrons pair on N and Ag. Additionally, at the lowest two pH of solutions, the new band (at 1386 cm⁻¹ at pH = 5 and at 1396 cm⁻¹ at pH = 3) due to the carboxylate group vibrations appeared. This band is weak at pH = 5, whereas it is the strongest SERS signal in the spectrum at pH = 3. Consequently, this suggests that, at the pH solutions level ranging from 11 to 7 there is no contact between the carboxylate group of the terminal Arg residues and the Ag surface. Although at pH = 5, the –COO’ moiety assisted in the BK –Ag interaction, whereas at pH = 3 it strongly binds to the Ag nanoparticles surface.

**TERS measurements**
Fig. 4 presents the AFM image of the contact Ag surface (scale bar 1000 nm) with the nine randomly chosen probing points where the TERS spectra were measured and the surface characteristic diagrams: A (for points from A1 to A5) and B (for points from B6 to B9). As is evident from this figure, colloid drying produced the Ag film with randomly distributed different-sized aggregates (high up to ~50 nm, length 80 – 500 nm) of the 20 nm spherical Ag nanoparticles. For the nine probing points, Fig. 5 shows the TERS spectra measured under the tip-approached conditions. Fig. 5 also presents the TERS spectrum (red dashed line) collected under the tip-retracted conditions at the probing point no. A1. The comparison of the BK spectra at the A1 point evidences the same set of bands; however, the TERS signals are significantly enhanced in comparison to those in the SERS spectra. To enhance resolution of the spectrum measured under tip-approached conditions at the A1 probing point (complex spectrum containing overlapped bands) the second-derivative (in the range 1700 – 600 cm\(^{-1}\)) spectrum was calculated (Fig. 5A). Also, a TERS spectrum was calculated (Fig. 5B) by subtracting the spectrum collected under the tip-retracted conditions from that measured under the tip-approached conditions. In general, there is good agreement between the read and calculated bands positions (Table 1). The TERS wavenumbers (the 1645, 1584, 1537, 1500, 1366, 1317, 1278, 1225, 1199, 1122, 1089, 1001, 962, 699, and 624 cm\(^{-1}\)) (Fig. 6B) demonstrated also close similarity to the bands frequencies in the SERS spectra of BK in an aqueous Ag silver sol (Fig. 3). Therefore, the TERS spectra support predicted mode of BK adsorption, through Phe\(^{5/8}\) and Arg\(^9\), drawn based on the SERS spectra. However, there are some changes in the bands relative intensities among the TERS spectra recorded at the different probing points. These changes are mainly caused by molecules adsorbed onto the heterogeneous colloidal suspended Ag surface (the changes in the morphology of the surface presents the diagram A and B) (see Fig. 4) than by molecules adsorbed on “hot spots”. The main difference denotes to the strong broad band with maximum centered at 912 cm\(^{-1}\), due to superposition of the \(\nu (\text{C–C}) + \nu (\text{C–N})\) (~899 cm\(^{-1}\)), \(\delta (\text{CH}_2)\) (~920 cm\(^{-1}\), and \(\nu (\text{CCC}) + \rho (\text{oopw(CH)})_{\text{Phe}}\) (~950 cm\(^{-1}\)) modes, in the TERS spectra at the A probing spots.

A noteworthy fact is that the strong and broad 1210 – 1450 and 1450 – 1580 cm\(^{-1}\) TERS bands due to the ‘D peak’ and ‘G peak’, respectively, of the hydrogenated amorphous carbon contamination, which is formed on a metal surface during rapid photodecomposition (by the highly enhanced electromagnetic field in the “hot” tip-sample nanogap) of the adsorbed organic compound influence the TERS spectra.

---

**Fig. 5** The SERS (red dashed line; at sampling point no. A1) and TERS spectra measured upon tip-approached conditions (black (at A sampling points) and blue (at B sampling points) solid lines) of BK adsorbed onto the Ag nanoparticles. Measurement conditions: peptide concentration: \(10^{-5}\) M; pH = 7; excitation wavelength: 514.5 nm; spectral range: 3200 – 2800 and 1800 – 500 cm\(^{-1}\). Insets: the second-derivative TERS spectrum (A) and the TERS spectrum obtained by subtracting the spectrum collected upon tip-retracted conditions from that measured upon tip-approached conditions at the A1 sampling point (B).
Conclusions

The adsorption mode of BK onto the colloidal Ag surfaces (with statistical Ag nanoparticle diameters of 20 nm) and the changes of this mode, which are influenced by peptide concentrations (10⁵ – 10⁷ M), excitation wavelengths (514.5 and 785.0 nm), and pH levels of the aqueous sol solutions (from pH = 3 to pH = 11), were determined by SERS. The TERS studies of BK behavior onto the colloidal suspended Ag surface were also carried out and the results were compared with the corresponding SERS and Raman, of bulk BK, data. By observing the spectral profiles, the following conclusions were drawn:

i) The BK concentration of 10⁵ M was the optimal peptide concentration for monolayer colloidal coverage.

ii) The relative intensities of the SERS bands in the spectra strongly depend on the excitation wavelength as is expected for resonance mechanism of enhancement.

iii) The Phe⁹/ eight and Arg⁹ fragments of BK generally participated in the interactions with the colloidal suspended Ag nanoparticles. This result together with the earlier data indicates that BK shows similar mode of adsorption onto different nanostructured Ag, Au, and Cu surfaces²¹,³²,3³ and the same molecular fragments are responsible for both the metal binding and receptor binding (see Introduction).

iv) With the change of pH of the colloidal Ag solution from 3 to 11, distinct change in the protonation state of BK adsorbed onto the Ag colloidal surface was observed, which was clearly evident in the reorientation of the Phe⁹/ and Arg⁹ COO⁻ onto the Ag surface.

v) The TERS studies of BK showed that the bands of the same peptide fragments approximately having the same orientations with respect the colloidal suspended Ag film, with randomly distributed different-sized aggregates (high up to ~50 nm, length 80 – 500 nm) of the 20 nm spherical Ag nanoparticles, arise from the different probing points on the Ag film.

vi) The TERS experiments demonstrated clearly the feasibility of the TERS technique for studying complex biological molecules.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

This work was supported by the National Research Center of the Polish Ministry of Science and Higher Education (Grant No. N N204 354840 to E.P. and Grant No. 2012/05/N/ST4/00175 to D.S.). We thanks to Prof. C. Glaubitz (Department of Biophysical Chemistry, Goethe University, Frankfurt am Main, Germany) for the low energy model structure of BK bound to the human B₂ receptor.

Notes and references

32. A. Rasmussen and V. Deckert, J. Raman Spectrosc., 2006, 37, 311.