Comparative analysis of SERS-active colloidal silver solutions of various type and prospects of their applications

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Abstract. Raman spectroscopy is a promising method for optical vibrational spectroscopy. Nowadays, Raman spectroscopy finds many applications, in particular, in biological and medical diagnostics. However, the Raman scattering can be enhanced using the Surface-enhanced Raman scattering method. Colloidal solutions of noble metals are used as SERS-active systems. In this work, the enhancing factors were estimated for colloidal silver solutions of three different types (citrate, borohydride, chloride) with two substances (phenylalanine, cytochrome C). Phenylalanine is a widely used model substance for Raman and Surface-enhanced Raman spectroscopy. Cytochrome C is one of the most researched proteins. It involves in the electron transport chain of the mitochondrial inner membrane and provides cellular respiration. Borohydride, citrate and chloride sols with phenylalanine gave an enhancement about 50, 200 and 30 times, respectively, and with cytochrome C about 30, 160 and 20, respectively. A comparative analysis of active and inactive sols by SERS and absorption spectroscopy was also performed. The absorption spectra of active sols have characteristic maxima in the region of 400 nm. Both the SERS method of model substances and absorption spectroscopy can be used to assess the enhancing properties.

1. Introduction
Spectral research methods are widely used to study the qualitative and quantitative composition of substances [1, 2]. These methods are inherently related to the processes of absorption or emission of electromagnetic radiation as a result of transitions between quantized energy levels. Therefore, these methods provide information on the interaction processes at the molecular level. Vibrational spectroscopy, which includes Raman spectroscopy (RS), occupies an important place among such methods. RS is based on the effect of inelastic scattering of optical radiation on matter molecules. One of the applications of this method is biological and medical diagnostics [3].

Surface-enhanced Raman scattering (SERS) method is used to enhance the Raman scattering [4-7]. This method gives the ability to view the spectra if the analyte in the solution is present at a low concentration or its own Raman spectrum is too weak. The SERS occurs due to the multiple enhancement of the local field near the metal surface and is caused by the excitation of surface plasmons at the interface between the metal and the dielectric (also called surface plasmon resonances) [8, 9]. One of the advantages of this method is the ability to view small amounts of the substance. Colloidal solutions of noble metals are used as SERS-active systems. The most common SERS substrate is the silver colloid obtained by silver cation(I) reduction with citrate, proposed by Lee and Meisel in 1982 [10]. Another often used SERS substrate is the silver colloid obtained by silver cation(I) reduction with sodium borohydride [11, 12], or with hydroxylamine hydrochloride [13]. Three different types of

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colloidal silver solutions were synthesized: citrate, borohydride and chloride. As analytes were chosen phenylalanine and cytochrome C.

Phenylalanine (Phe) is a widely used model substance for Raman and Surface-enhanced Raman spectroscopy. The structural formula of this compound is shown in figure 1. The spectra of phenylalanine have the band at 1000 cm\(^{-1}\), which corresponds to the breathing vibration of the benzene ring stretch [14, 15]. The peak at 1000 cm\(^{-1}\) is the most intensive, therefore it is effectively used to calculate the enhancement of SERS-active systems.

![Phenylalanine structural formula.](figure1)

Cytochrome C is one of the most researched proteins. It involves in the electron transport chain of the mitochondrial inner membrane and provides cellular respiration [16]. Moreover, cytochrome C is required for apoptotic process. All members in the C-type cytochrome superfamily contain a heme prosthetic group that is covalently attached to the protein via two thioether bonds to cysteine residues. Most cytochromes occur in a where the histidine residue is one of the two axial ligands of the heme iron. Figure 2 shows the structure formula of heme. A comparative analysis of enhancement can performed of intensive cytochrome peaks at 750 cm\(^{-1}\), 1172 cm\(^{-1}\) and 1374 cm\(^{-1}\) that is originated from the normal group vibrations of pyrrole rings (bonds C\(_2\)N, C\(_{\alpha}\)NC\(_{\alpha}\), C\(_{\alpha}\)C\(_{\beta}\)).

![Heme structural formula and position of heme in the protein.](figure2)

In this work, three types of colloidal silver solutions were tested with these analytes to estimated enhancing factors and investigate how they interact with different type of substances such as phenylalanine and cytochrome C.

2. Experimental

2.1. Colloidal silver solutions

Three different types of colloidal silver solutions were synthesized as SERS-active systems: citrate, borohydride, and chloride. For control experiments, each of the three types of sols was deactivated by shock freezing followed by thawing. The mixtures obtained in this way contained the initial agents, but
were devoid of enhancing properties, which made it possible to obtain additional data on the enhancing factor of sols.

2.1.1 Citrate colloidal solution
To obtain the citrate sols, 90 mg of silver nitrate was solved in 500 ml bidistilled water and heated to boiling. Then, 114 mg of sodium citrate solved in 10 ml bidistilled water was added dropwise. The mixture was further boiled under vigorous stirring for 90 min.

2.1.2 Borohydride colloidal solution
To obtain the borohydride sol, 0.69 mg of silver nitrate was solved in 4 ml bidistilled water. 0.91 mg of sodium borohydride was solved in 12 ml bidistilled water. Solution of silver nitrate is added to a solution of sodium borohydride. The synthesis should be carried out at a temperature of about 0 °C. Store the sol in the refrigerator after mixing.

2.1.3 Hydroxylamine hydrochloride colloidal solution
To prepare the chloride sol, 10 ml of a solution of silver nitrate (10^{-2} M) in bidistilled water. In a separate container, 0.5 ml of solution of hydroxylamine hydrochloride (0.1 M) in bidistilled water and 90 ml of sodium hydroxide solution (3.33 mM) in bidistilled water. The silver colloid was obtained at room temperature by rapidly adding the hydroxylamine hydrochloride mixture to the silver nitrate solution under vigorous stirring. The resulting colloid was further stirred for 5 min.

2.2 Measurements

2.2.1 Measurements of Raman and SERS spectra of phenylalanine and Cytochrome C with colloidal silver solutions of three different types
To check the enhancing factor of sols, an aqueous solution of phenylalanine in bidistilled water at a concentration of 1 mM was used as sample. A solution of cytochrome C in phosphate buffer at a concentration of 10^{-5} M was used as sample too. The RS spectra were obtained using the Renishaw inVia confocal Raman microscope at an excitation wavelength of 532 nm and a sample laser power on the sample of 3.15 mW with an exposure time of 100 s.

For measurements, 50 μl of model substance was mixed with 50 μl of sols. In the case of citrate sol, 10 μl of NaClO₄ at a concentration of 0.6 M was added.

2.2.2 Measurements of absorptions spectra of colloidal silver solutions of three different types
The absorption spectra of sols were measured on a UV/VIS SPECTROMETER T90+ PG Instruments limited spectrophotometer.

3. Results

3.1 A comparative analysis of two substances for studies by the SERS method
In this study, a comparative analysis of enhancing properties of sols using the phenylalanine and cytochrome C as analytes were executed. The figure 3 shows the Raman and SERS spectra of phenylalanine with colloidal silver solutions of three different types which were recorded at the same parameters.

Note that the Raman spectrum of phenylalanine is recorded at the limit of sensitivity, therefore, only the vibration lines of the phenyl ring at 1003 cm⁻¹ and 622 cm⁻¹ are reliably recorded. The dominant line in this Raman spectrum is the line of bending vibrations of water molecules, about 1650 cm⁻¹, which overlap the natural vibrations of Phe, namely, the lines arisen on phenyl ring vibrations of the: 1586 and 1606 cm⁻¹.

The smallest enhancement among the studied sols was shown by the chloride sol (enhancement factor of about 30). The spectrum shows well both the lines corresponding to the vibrations of the phenyl ring (622, 750-770, 1003, 1032, and 1600) cm⁻¹, and the lines of the side chain: bend mode δ(NCH) – 1360
cm\(^{-1}\), stretching mode \(\nu(OCO)\) - 1260 cm\(^{-1}\), deformation \(\delta(CCH)\) - 1160 cm\(^{-1}\), stretching \(\nu(C-C)\) - 922 cm\(^{-1}\) [14].

The borohydride sol showed a slightly higher enhancement - about 50. The nature of the spectrum practically repeats the features of the spectrum of the chloride sol.

The citrate sol showed the greatest enhancement (the enhancement factor is 200). Moreover, the Phe spectrum obtained using citrate sol differs markedly from the spectra obtained with borohydride and chloride sols. The difference is a significant enhancement of the lines associated with vibrations in which the nitrogen atom participates: \(\delta(NCH)\) -1360 cm\(^{-1}\) and \(\nu(NC)\) - 850 cm\(^{-1}\) In addition, the Raman spectrum clearly shows the 1700 cm\(^{-1}\) line corresponding to asymmetric bending vibrations of NH\(_3^+\), which indicates that Phe molecules bind to silver sol particles through the amino group of the Phe molecule.

We also note that in all cases (all three sols), the SERS spectra completely lack lines corresponding to the bending vibrations of CH\(_2\) groups (the region around 1450-1480 cm\(^{-1}\)).

The enhancement factors of the sols were estimated. Borohydride, citrate and chloride sols on phenylalanine gave an enhancement about 50, 200 and 30 times, respectively.

![Figure 3](image_url)

**Figure 3.** Raman spectra of phenylalanine (a) and SERS spectra of phenylalanine with colloidal silver solutions of three different types: (b) chloride sol, (c) borohydride sol, (d) citrate sol.

The figure 4 shows the Raman and SERS spectra of cytochrome C with colloidal silver solutions of three different types.

Characteristic of the interaction of cytochrome with the electromagnetic radiation by is determined by its chromophore group, heme. Absorption band of the cytochrome c chromophore is 550 nm. Because of we use a 532 nm laser, the resonant excitation of the chromophore is realized. Therefore, heme spectral lines are dominant in the spectrum (similar to the chromophore retinal which is define Raman spectrum of the bacteriorhodopsin [12]). Heme molecule is a symmetric conjugated system of four pyrrole rings [2] The symmetry of this system is somewhat broken by side chains containing cysteine residues. The vibrations of the system are mainly collective, involving all the atoms of the pyrrole rings.
and methine bridges. Nevertheless, it is possible to distinguish groups that make a larger contribution to defined modes of vibrations, in particular, the 1570 and 1638 cm\(^{-1}\) modes are determined by the large contribution of vibrations of the bonds of the atoms (-C-C-) of the methine bridges connecting the pyrrole rings; the modes 1313, 1378, 1371 cm\(^{-1}\) are symmetric vibrations of pyrrole rings, and the mode 1170 cm\(^{-1}\) is asymmetric one. Mode 750 cm\(^{-1}\) is the stretching vibrations of ν(CN). The region 500 - 800 cm\(^{-1}\) includes stretching mode with the participation of iron and sulfur atoms (in the molecular groups of cysteine) [2].

![Figure 4](image.png)

**Figure 4.** Raman spectra of cytochrome C (a) and SERS spectra of cytochrome C with colloidal silver solutions of three different types: (b) chloride sol, (c) borohydride sol, (d) citrate sol.

The SERS spectra of cytochrome c, recorded using different sols, differ significantly and significantly differ from the Raman spectrum of this protein. In this case, the positions of the spectral lines are preserved, but significant differences in their intensities are observed. All this indicates that the enhancement of the Raman signal is carried out mainly by the electromagnetic mechanism [12]. The differences in intensities are due to the different orientation of the protein and the proximity of one or another fragment of the chromophore to the SERS of the active surface [17-19]. In particular, the Raman signal in the cases of using chloride and borohydride sols is determined by the vibrations of the pyrrole ring system. In contrast to this, it can be assumed that when cytochrome c interacts with citrate sol particles, cysteine fragments appear close to the particle surface, which makes a significant contribution to the low-frequency part of the Raman spectrum. Of course, all this requires additional investigations. Most important that by varying the type of sol, it is possible to obtain selective information on the structure of molecules and to reveal specific signals, which make it possible to obtain additional information on the functional properties of the compounds under study.

To estimate the overall enhancement factor, we used the averaged values of the Raman signal intensities of three spectral lines corresponding to vibrations originated from the normal group vibrations of pyrrole rings (bonds C,N, C,N,C, C,C). Thus, it was found that the enhancement factors for borohydride, citrate, and chloride sols determined using cytochrome c as a model substance were approximately 30, 160, and 20, respectively.
3.2. A comparative analysis of active and inactive sols by the SERS method and absorption spectroscopy

Colloidal solutions are not stable and require special conditions for storage and measurements. If this condition is not satisfied, they become inactive. To confirm this assumption, the sols were subjected to shock freezing and subsequent defrosting. In the course of the experiment, the SERS spectra of phenylalanine with active colloidal silver solutions of three different types and Raman spectra of phenylalanine in a similar concentration with inactive sols were obtained. It was shown that inactive sols not only almost completely lose their SERS-activity, but their absorption dramatically decreases.

Colloidal solutions are unstable and require special storage and measurement conditions. If this condition is not met, they become inactive. To confirm this assumption, the sols were subjected to shock freezing and subsequent thawing. In the course of the experiment, the SERS spectra of phenylalanine were recorded with active solutions of colloidal silver of three different types and the Raman spectra of phenylalanine in a similar concentration with inactive sols.

The absorption spectra of active sols have characteristic maxima in the region of 400 nm, while the absorption spectra of inactive sols do not show pronounced peaks. The enhancing factors of sols correlate with the absorption intensity (figure 5). Thus, adsorption spectroscopy allows a quick assessment of the SERS activity of sols.

![Absorption and SERS spectra](image)

**Figure 5.** (a) Absorption spectra of active (1) and inactive (2) chloride sol; (b) SERS spectra of phenylalanine with active chloride sol (2) Raman spectra of phenylalanine with inactive sol (2).

4. Conclusion

In this work, colloidal silver solutions of three different types were synthesized: citrate, borohydride and chloride. The enhancing factors were estimated for colloidal silver solutions with phenylalanine and cytochrome C. Borohydride, citrate and chloride sols with phenylalanine gave an enhancement about 50, 200 and 30 times, respectively, and with cytochrome C about 30, 160 and 20, respectively, which indicates that during the study of complex organic molecules by the SERS method for analyzing the enhancement factor more effective use of cytochrome C. Note that for the cytochrome c protein molecule, different sols selectively enhance various vibration modes. This can be used to selectively obtain Raman signals from different parts of the molecule and to assess changes in the conformation of the molecule under different influences.

Each of these colloidal silver solutions was devoid of enhancing properties for comparing their enhancing factors and absorption spectra with the initial ones. SERS spectra of phenylalanine with active colloidal solutions of each type and Raman spectra with inactive sols were obtained, which suggests that the initial colloidal silver solutions lost their enhancing properties after freezing and subsequent defrosting.

The absorption spectra of active and inactive sols were also obtained. The absorption spectra of active sols have characteristic maxima in the region of 400 nm. However, the intensity of these maxima differs,
in agreement with the estimated enhancing factors. The absorption spectra of inactive colloidal silver solutions of each type did not have pronounced peaks, which confirms the deprivation of the absorbing capacity of the soils. Thus, the performed comparative analysis of various types of active and inactive soils demonstrates that both the SERS method of model substances and absorption spectroscopy can be used to assess the enhancing properties.

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