MOLECULAR ORIGIN OF SURFACE-ENHANCED RAMAN SPECTRA OF *E. COLI* SUSPENSIONS EXCITED AT 532 AND 785 NM USING SILVER NANOPARTICLE SOLS AS SERS SUBSTRATES

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Research into the molecular origin of surface-enhanced Raman spectra (SERS) of bacteria is a crucial step in assessing the future of SERS-based discrimination and identification of bacteria in clinical analysis, food quality control, etc. Previous studies have revealed that at 785 nm excitation wavelength SERS of bacterial cells placed on a solid surface functionalized with *in-situ* grown aggregated gold nanoparticles covered with SiO_2 originate from a mixture of 6 purine derivatives (adenine, guanine, AMP, hypoxanthine, xanthine, and uric acid) that are released by the cells into the medium. The aim of the present work was to investigate whether such interpretation is possible with a different class of SERS substrates: silver nanoparticle sols at excitation wavelengths of 785 and 532 nm. The suspension of the *Escherichia coli* DH5α strain was used as a model bacterium. Sols of silver nanoparticles were obtained by reducing silver nitrate in the presence of alkaline hydroxylamine hydrochloride. Number-weighted mean hydrodynamic diameter of the particles was 43±2 nm. We confirm that at both excitation wavelengths the spectra can be best described as a superposition of 4 purine derivatives: adenine, guanine, hypoxanthine, and xanthine. Importantly, we have discovered that 1) the spectra of the purine mixture are characteristic of viable cells only; 2) due to the variations in the concentrations of purine metabolites released by the cells into the surrounding medium the spectra of a bacterial strain can vary significantly when a silver nanoparticle sol is used as a SERS substrate.

Keywords: SERS of bacteria, *E. coli*, silver nanoparticles, purines

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МОЛЕКУЛЯРНАЯ ПРИРОДА ГКР-СПЕКТРОВ СУСПЕНЗИИ *E. COLI* ПРИ ДЛИНАХ ВОЛН ВОЗБУЖДЕНИЯ 532 И 785 НМ С ИСПОЛЬЗОВАНИЕМ ЗОЛЕЙ НАНОЧАСТИЦ СЕРЕБРА В КАЧЕСТВЕ ГКР-СУБСТРАТОВ

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Вопрос о молекулярной природе спектров гигантского комбинационного рассеяния (ГКР) бактерий является ключевым для оценки перспектив их дискриминации и идентификации данным методом в целях клинической диагностики, обеспечения безопасности пищевых продуктов и др. Ранее было показано, что при использовании в качестве ГКР-субстрата агрегированных и покрытых слоем SiO_2 золотых наночастиц на твердой поверхности источником спектра при длине волны возбуждения 785 нм является смесь шести пуриновых производных (аденина, гуанина, АМФ, гипоксантина, ксантина и мочевой кислоты), выделяемая клетками в раствор. Целью настоящей работы было показать применимость данной интерпретации спектров на примере суспензии клеток *Escherichia coli* штамма DH5α для другого класса ГКР-субстратов — золей наночастиц серебра при длинах волн возбуждения 785 и 532 нм. Золи получали восстановлением нитрата серебра хлоридом гидроксиламина в щелочной среде, среднечисловой размер частиц составил 43 ± 2 нм. Выявлены две важные особенности: во-первых, спектр пуриновых метаболитов регистрируется только при наличии живых клеток; во-вторых, при использовании золей наночастиц серебра в качестве ГКР-субстрата спектрам даже одного и того же штамма присуща значительная вариативность вследствие изменения соотношения концентраций пуриновых метаболитов, выделяемых клетками в раствор.

Ключевые слова: ГКР-спектры бактерий, *E. coli*, наночастицы серебра, пуриновые производные

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Surface-enhanced Raman spectroscopy (SERS) is an optical technique that relies on the amplification of the weak Raman signal emitted by a molecule located in close proximity to a metal surface with nanoscale roughness. Dating back a few decades, SERS still has not lost its appeal as a powerful detection technique. It is rapid and simple in instrumentation; it can be optimized to achieve a very high sensitivity and measure multiple analytes. SERS can also be employed for local analysis. Metal nanostructures used for signal enhancement are referred to as SERS substrates and fall into two major categories: nanostructures on solid supports and colloidal sols of metal nanoparticles.

SER spectra of bacteria were first recorded at a 514.5 nm laser excitation wavelength (EW) from *Escherichia coli* and *Bacillus megaterium* [1]. It was soon discovered that excitation at 488 or 514.5 nm results in almost identical SER spectra from gram-positive and gram-negative bacteria, as well as their isolated cell membranes: all acquired spectra originated from reduced or oxidized riboflavin (RF) [2–4]. The RF extinction band overlaps with 488 and 514.5 nm EWs, which induces resonant enhancement of the RF spectrum [3]. RF is a component of cofactors of redox enzymes and electron transport proteins found in cell membranes. If a bacterial cell is applied onto the surface of a solid SERS substrate or, alternatively, metal nanoparticles are synthesized or absorbed onto the cell, RF will come to occur in close proximity to the metal surface [3, 4]. From a bioanalytical standpoint, it means that laser sources with short wavelengths of 488 and 514.5 nm cannot be used for the identification of or discrimination between different bacteria.

At the same time, bacterial cells excited at long incident EW (785 nm) have SER spectra that do not contain the bands characteristic of riboflavin. Moreover, SER spectra vary between bacterial species and sometimes strains, not to mention intact and inactivated samples of the same strain [5–7]. This inspired a hypothesis in the early 21st century about the feasibility of SERS for the identification of bacterial species and strains excited at 785 nm wavelength. If adopted, this approach would have sped up pathogen detection in patients' samples, food products, and environmental objects. However, the molecular origin of bacterial SER spectra at long EWs was vague. According to a proposed hypothesis, such spectra could originate from the molecules localized in the bacterial glycocalyx (a slime layer or a capsule) or bacterial envelope (a cell wall or a membrane), as was the case with short wavelength lasers. Some authors speculated that the spectra might originate from N-acetyl-D-glucosamine [6], amino acid residues, peptides, protein prosthetic groups, phospholipids, metabolites (such as glucose or acetoacetic acid), or DNA and its constituents (guanine and adenine) [5, 7–13]. However, the proposed hypotheses lacked substance: they interpreted the origin of individual bands only ignoring the full spectra. As a result, this area of science was long dominated by a formal mathematical approach that combined the method of principal component analysis (PCA) used to reduce the dimensionality of experimental data and discriminant or cluster analysis aimed to prove the feasibility of discrimination between bacterial genera, species and strains based on their SER spectra [6, 12–15].

But then a study published in 2016 demonstrated convincingly that it was 6 purine metabolites released into the medium by the bacterial cell that were the source of SER spectra at 785 nm EW for 10 investigated samples [16]. The purine derivatives included adenine, guanine, adenosine monophosphate, hypoxanthine, xanthine, and uric acid. The suggested interpretation imposes a dramatic limitation on the use of bacterial SER spectra for the identification/discrimination of pathogens because the differences between their spectra are caused by only 6 secreted purine derivatives and not the whole diversity of molecules on the cell surface. For example, a hypoxanthine-free *E. coli* mutant with a silent adenosine deaminase gene was closer in its SER spectrum to *Staphylococcus aureus* than to the parent strain. The EW used in that experiment was 785 nm. Aggregated gold nanoparticles grown on a solid surface and coated with a thin silica layer were used as a SERS substrate. The aim of our study was to verify the authors' conclusions using a principally different type of SERS substrates (silver nanoparticle sols) and to investigate the molecular origin of bacterial SER spectra at 532 nm EW lying between riboflavin-dominated (488 and 514.5 nm) and infrared (785 and 1,064 nm) spectral regions.

METHODS

E. coli DH5α (Thermo Fisher Scientific; USA) was used as a model strain. The cells were cultivated in a liquid culture medium consisting of 10.0 g/l tryptone (Difco; USA), 5.0 g/l yeast extract (Difco; USA) and 10.0 g/l chemically pure NaCl (pH 6.8) (Chimmed; Russia) at 37 °C for 14–16 h until the stationary phase was reached. According to the literature [17], the cultured cells should be washed thoroughly to remove the residual components of the culture medium. Bearing that in mind, we applied the following protocol. Briefly, the cells were pelleted in the Beckman J-2-21 centrifuge (Beckman Coulter; USA) at 8,000 rpm for 7 min. The pellet was washed in an equivalent volume of 0.9% NaCl. The procedure was repeated twice. The obtained biomass was diluted with 0.9% NaCl taken at a volume sufficient for obtaining a suspension of $1 \cdot 10^8$ cells per ml. The final concentration was determined spectrophotometrically at 540 nm.

For the experiments with partially inactivated bacteria, the suspensions were placed into a water bath preheated to 70 °C or 90 °C and kept at this temperature for 1 h. The degree of inactivation was inferred from the concentration of intracellular ATP measured by the luciferase-luciferin assay using the reagent kit and calibration standards by Lumtek; Russia.

A sol of Ag nanoparticles (AgNPs) was used as SERS substrate. The sol was prepared by reducing silver nitrate with hydroxylamine hydrochloride in the presence of sodium hydroxide using $AgNO_3$ (ASC reagent, ≥ 99.0%; Sigma-Aldrich; USA), $NH_2OH \cdot HCl$ (purified; Prime Chemicals Group; Russia), and NaOH (reagent grade; Mosreaktiv; Russia). Following the original protocol [18], the silver nitrate solution was poured into the alkaline hydroxylamine solution. The final concentrations of the reagents in the mixture were 1 mM $AgNO_3$, 1.5 mM $NH_2OH \cdot HCl$, and 3 mM NaOH. Sols older than 3 days were not used in the experiment.

The absorption spectra of the synthesized nanoparticles were measured in a UV-visible region (300–750 nm) with the cuvette spectrophotometer UV-1800 (Shimadzu; Japan). The size and concentration of AgNPs were measured by nanoparticle tracking analysis (NTA) using the Nanosight LM10 HS-BF system (Nanosight Ltd; UK).

The SER spectra of both intact and inactivated bacteria were recorded on the day of sample preparation. Until then, the samples were stored at +4 °C. Immediately before the measurement, a sample aliquot was centrifuged twice at 3,700 rpm for 5 min in the Biofuge A centrifuge (Heraeus Sepatech; Germany) and washed in an equivalent volume of deionized water. The obtained cell suspension in water was mixed with the AgNP sol at a ratio of 1:1 and incubated for 1 min. Then, the NaCl solution taken at a final concentration of 40 mM was introduced into the mixture to stimulate particle aggregation and enhance the signal. An aliquot of this mixture (260 µl) was transferred into a well of an aluminum well-plate to minimize the background signal and improve heat dissipation. The spectra were measured in 3 to 4 replicates per sample; the samples were stirred by pipetting between measurements.

To study the changes in the SER spectra over time, 5 ml of the *E. coli* suspension were transferred to deionized water following the procedure described above. The obtained water suspension was stored at +4 °C and its aliquots were picked

to register SER spectra over the course of 4 h.The filtrate was prepared by filtering the *E. coli* water suspension slowly using a syringe filter SFNY030022S (Membrane Solutions; USA) with a diameter of 30 mm and a pore size of 0.22 μm.

At 785 nm EW, SER spectra were recorded using the innoRam BWS445(B)-785S spectrometer (BWTek; USA) with a 785 nm diode laser source and a ×20 PL L 20/0.40 objective. The instrument was operated at a measuring range of 64–3,011 cm^{-1} and resolution of 4 cm^{-1}. The spectra were recorded using the incident beam power of 42 mW, 5 s signal accumulation time, and averaging over 20 repeated scans. At 532 nm EW, SER spectra were recorded using the iRaman BWS415-532S spectrometer (BWTek; USA) with a 532 nm diode laser source and a ×20 PL L 20/0.40 objective. The instrument was operated at a measuring range of 174–4,001 cm^{-1} and resolution of 4 cm^{-1}. The spectra were recorded using the incident beam power of 20 mW; 5 s signal accumulation time, and averaging over 20 repeated scans.

Table. The table shows all spectral bands observed in the SER spectra of *E. coli* including the filtrates of cell suspensions and their assignment to the purine metabolites whose spectra were characterized in [16]. A — adenine, G — guanine, Hx — hypoxanthine, X — xanthine

785 nm		532 nm		Assignment
Peak position, cm^{-1}	Band frequency in the spectra, %	Peak position, cm^{-1}	Band frequency in the spectra, %	
502–515	75	502–512	50	X, G
522–540	100	526	75	G
		549–550	25	Hx, A
561–574	100			X, G, A
621–633	50	617–623 (sh)	100	Hx, A, G
653–667	100	648–651	100	G, X, A
680–683	38			X, A
724–735	100	721–728	100	A, Hx
780–792	63	770	25	Hx
		788	25	A
838–842	38	833	25	Tyrosine (?)
		848–850	50	
867–883	63	875–878	25	G, X
925–930	38			Hx
958–966	100	952–955	100	X, G, Hx, A
1002–1008	88	1000–1006	100	Phenylalanine (?), A+Hx+X interaction (?)
1027–1033	25	1024–1027	100	G, A, Hx
		1043–1045	75	G, X
1084–1096	50	1085–1095	100	Hx
1115–1130	38	1129–1140	100	X, G, A
1157–1160	25	1154	25	Hx
1175–1189	63			G, A
1213–1215	25	1215–1233	100	G, Hx, A
1245–1251	50	1242	25	X
1267–1276	25	1276	25	G, A
1310–1315	25			X, A
1324–1334	63	1322–1325	75	Hx
		1330–1331	50	Hx
		1341	25	A
1362–1380	63	1371–1379	100	G, X, Hx, A
1389–1390	13	1399	25	X, Hx
1444–1453	88	1444	25	Hx, G, A
1464–1473	75	1457–1468	100	Hx, G, X, A
1508	13	1506	25	A+Hx+X interaction (?)
1528–1534	25	1532–1538	100	G, Hx, K
1568–1578	63	1567–1575	50	G, A
1582–1591	25	1584–1595	25	X, Hx
1630–1721 (broad)	75	1646	25	G, A
		1692–1698	100	X, Hx, G

The recorded spectra were processed in OPUS 7.0 (Bruker Optik GmbH; Germany). The data outside the 500–1,800 cm^{-1} range was discarded, and the baseline was subtracted using the Background correction tool. Smoothing was not applied to determine peak positions and intensity. However, SERS data for plots was smoothened using the Smooth tool with a frame width of 9 cm^{-1}. The spectra were processed using vector normalization for a clear visual representation of qualitative differences. Normalization was not performed when the intensities of the spectra were compared.

RESULTS

The popular hydroxylamine technique for the synthesis of AgNP sols [18] is simple and reproducible; the sols it yields significantly enhance the spectra emitted by various analytes, including bacterial cells [19–21]. The AgNP sols we prepared were transparent, deep yellowish-brown in color and did not contain any precipitate. They had a broad and intense absorption band in the near UV-blue region corresponding to the localized surface plasmon resonance of AgNPs with a maximum at 407–409 nm and absorption at this wavelength ranging from 16.5 to 18 (this accounts for 30-fold dilution with deionized water). The number-weighted mean hydrodynamic diameter of the particles measured by nanoparticle tracking analysis was 43 ± 2 nm in three independent AgNP batches. The total particle concentration was (8.0 ± 1.7) • 10^{11} particles per ml. The synthesized AgNS sols aggregated in 40 mM NaCl did not have their own SER spectra at both EW except for a broad low-intensity band contributed by aluminum (the material of the plate) in the region between 1,200 and 1,700 cm^{-1}. This band can be totally subtracted during data processing.

The reproducibility of intact *E. coli* SER spectra at 785 nm EW was tested in a series of different experiment. First, we repeatedly measured the spectra of the same mixture of *E. coli* + AgNPs + NaCl. Second, we measured the spectra of different aliquots of bacterial sample using the same and different AgNP batches. Third, we measured the spectra of independently cultured and isolated *E. coli* applied onto one and the same AgNP substrate. Repeatedly measured bacterial samples demonstrated good repeatability (Fig. 1A). The SER spectra of independently cultured bacterial samples varied considerably (Fig. 1B). The most significant variations were observed in the following spectral regions: 508–532; 655; 730–734; 958; 1,450; 1,570–1,576 cm^{-1}.

E. coli stored in water at +4 °C for 4 h (Fig. 1C) demonstrated a gradual increase in the total intensity of the SER spectrum over time accompanied by a change in the intensity ratio of its individual bands. For example, the ratio I_{730} / I_{655} = 1.2 remained constant at all time points, but the ratio I_{1325} / I_{655} monotonously declined from its initial value of 1.6 to 1.0 over the course of 4 h.

The spectra of the intact *E. coli* suspension in water were compared to its filtrate (0.22 μm) in order to locate the molecules giving rise to the SER spectra (Fig. 1D). Considering the slow dynamics, the spectra of aliquots of the initial bacterial suspension were recorded before and after filtration. All spectral

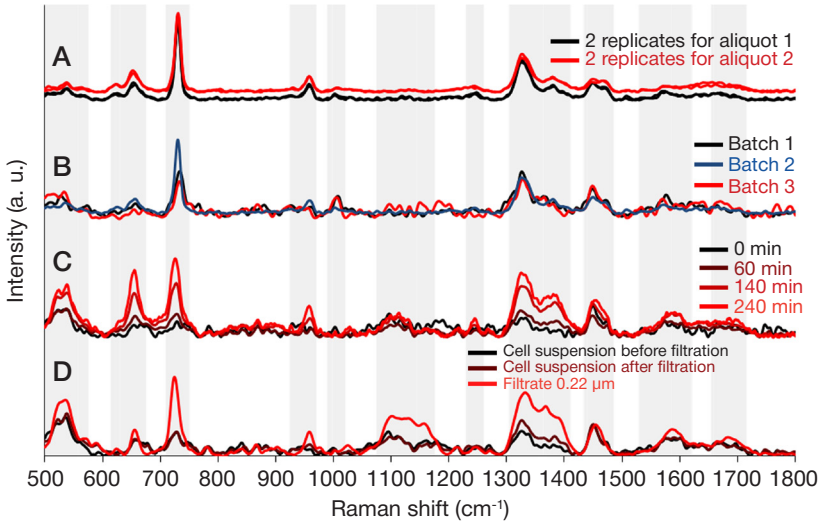

Fig. 1. SER spectra of *E. coli* suspensions at 785 nm excitation wavelength. **A**. Repeatability of measurements for one aliquot and one cell batch. **B**. Reproducibility of the spectra for different batches of cell suspensions. **C**. Dynamics of SER spectra over time observed in cells stored in water at +4 °C. **D**. Comparison of the spectra of the cell suspension and the filtrate (0.22 μm) of the same suspension. Vector normalization was applied to the spectra (**A**, **B**); the spectra (**C**, **D**) were not normalized to demonstrate the difference in their intensity. Ranges of spectral differences are shown in gray

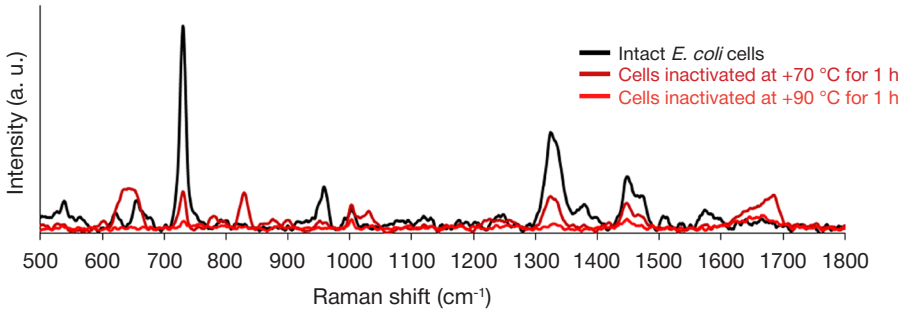

Fig. 2. Changes in the SER spectra of *E. coli* suspensions (785 nm) following inactivation by heating. The spectra were not normalized to demonstrate the difference in their intensity

bands observed for the cell suspension were present in the spectra of the filtrate. Moreover, the total intensity of the filtrate spectrum was significantly higher.

The SER spectra of intact cells and those inactivated at 70 °C or 90 °C for 60 min were compared in an attempt to understand whether the observed SER spectra can indicate the presence of viable *E. coli* or whether they come from an inactivated bacterial biomass (Fig. 2). The residual concentration of intracellular ATP was also measured in all three sample types (intact bacteria and cells inactivated at 70 °C and 90 °C) as it is indicative of cell viability. The ATP concentrations were $1 \cdot 10^{-9}$, $5.6 \cdot 10^{-12}$ and $4.1 \cdot 10^{-12}$ mol per 1 ml of cell suspension, respectively. On the whole, considerable variability was observed in the number and position of spectral bands. However, the total intensity of the spectrum tended to decrease. The spectra of inactivated bacteria (90 °C) contained only 4 very low-intensity bands characteristic of intact *E. coli* (730; 1,002; 1,325, and 1,450 cm^{-1}) and two low-intensity bands of the amide III (1,230–1,270 cm^{-1}) and amide I (1,640–1.680 cm^{-1}) regions.

The reproducibility of the SER spectra of intact *E. coli* was also tested at 532 nm EW. In this case, the intensity of the spectra was twice as high as that observed at 785 nm, resulting in a higher number of informative spectral bands and a better accuracy in locating their position. Similar to 785 nm EW, at 532 nm the high repeatability of the spectra was observed for one and the same aliquot of one and the same bacterial sample (Fig. 3A). But the SER spectra of independently cultured and isolated bacteria varied considerably (Fig. 3B).

DISCUSSION

Measurements conducted at EW 785 nm demonstrate that the SER spectra of *E. coli* stored in water are not determined by a single compound, but rather by a mixture of a few different components. This becomes clear when we look at the array of all recorded spectra that contains a fixed set of spectral bands (see the Table). The ratios of the mixture components slowly change over time when cells are stored in water (Fig. 1C) and differ significantly between independently cultured batches of intact cells (Fig. 1B). Comparison of the spectra of the intact bacterial preparation and its filtrate (Fig. 1D) shows that the components of the mixture do not originate from the cell surface but are present in the solution. Moreover, the cell itself can be seen as interfering with the recording of SER spectra, as it adsorbs particles on its surface. This is suggested by a significant increase in the total intensity of the filtrate spectrum in comparison with that of the cell suspension.

Inactivation of bacterial cells demonstrates that the mixture of the compounds in question bears connection to cell viability (Fig. 2) but is not a product of passive desorption from the surface of inactivated cells.

The Table features a list of spectral bands observed in all acquired SER spectra of intact *E. coli*, including the filtrates.

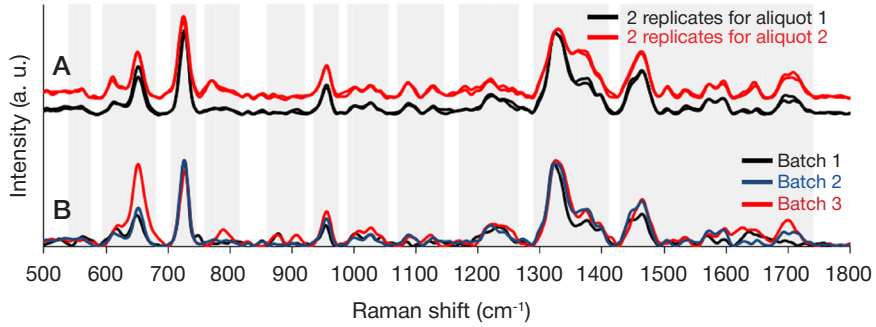

Fig. 3. SER spectra of *E. coli* suspensions at 532 nm excitation wavelength **A**. Repeatability of measurements for one aliquot and one cell batch. **B**. Reproducibility of the spectra for different batches of cell suspensions. Vector normalization was applied to the spectra. Ranges of spectral differences are shown in gray

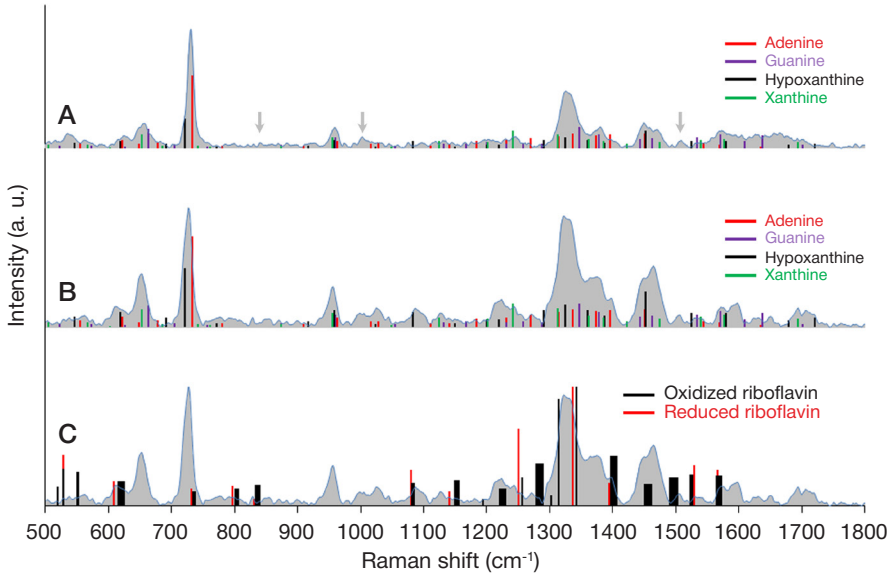

Fig. 4. Overlay of SER spectra corresponding to purine derivatives [16] and riboflavin [4, 24, 25] on the SER spectra of *E. coli* suspension **A**. Spectral bands of purine derivatives in the spectra of *E. coli* at 785 nm EW. Arrows mark uncharacterized low-intensity bands. **B**. Spectral bands of purine derivatives in the spectra of *E. coli* at 532 nm EW. **C**. Overlay of spectral bands of riboflavin on the spectra of *E. coli* at 532 nm EW. The width of riboflavin bands on the graph reflects the variability of their positions in literature sources

Upon analyzing the literature, we concluded that at 785 nm EW almost all spectral bands are a product of superposition of spectra originating from 4 purine derivatives (adenine, guanine, hypoxanthine, and xanthine). This conclusion is consistent with [16]. Besides, for every individual SER spectrum, the intensity and positions of bands correspond to such superposition as well. (Fig. 4A). Unlike the authors of [16] who exploited aggregated gold nanoparticles on a solid surface, we used sols of silver nanoparticles. Considering the possibility of slight variations in the relative intensity of the spectral bands associated with the use of different SER substrates and an increase in intensity following overlay of spectral bands of individual compounds, our description is quite accurate.

Only 3 low-intensity bands remain uncharacterized: 838–842, 1,002–1,008 and 1,508 cm^{-1}. On the one hand, there is a chance that low-intensity bands can be lost during digital conversion of the spectra from literature sources. On the other hands, it is possible that those bands have never been present in the spectra of individual purine derivatives. Then, their origin can be explained by two hypotheses. First, the bands can result from the interactions between the components of the mixture. For example, a SER spectrum of a mixture consisting of adenine, hypoxanthine and xanthine contains two bands (1,000 and 1,510 cm^{-1}) absent in the individual spectra of its constituents [22]. Besides, the presence of 838–842 and 1,002 cm^{-1} bands can be explained by minor presence of tyrosine (the most intense spectral bands are 824; 847; 928; 1,046; 1,389 and 1,583 cm^{-1} [23]) and phenylalanine (the most intense spectral bands are 930; 1,002; 1,031; 1,394; and 1,602 cm^{-1} [23]).

The suggested origin of bacterial SER spectra explains a considerable variation in the position of peaks observed for some bands within *E. coli* spectra. Thus, the variability in the position of the peak of a broad multicomponent band ranging from 502 to 574 cm^{-1} can be explained by an overlap of the following bands: xanthine (508 cm^{-1}), guanine (526 cm^{-1}), hypoxanthine (550 cm^{-1}), adenine (558 cm^{-1}), and guanine (577 cm^{-1}). For the band in the region between 653 and 667 cm^{-1}, the contributing bands are 652 cm^{-1} (adenine), 657 cm^{-1} (xanthine) and 667 cm^{-1} (guanine); for the 724–735 cm^{-1} band, 725 cm^{-1} (hypoxanthine) and 734 cm^{-1} (adenine); for the broad double band with peaks at 1,444–1,452 cm^{-1} and 1,464–1,473 cm^{-1}, the contribution is made by guanine (1447 cm^{-1}), adenine (1455 cm^{-1}), hypoxanthine (1456 cm^{-1}), guanine (1466 cm^{-1}), and xanthine (1478 cm^{-1}).

The SER spectra of *E. coli* are very similar at 785 and 532 nm EW (Fig. 4A, 4B) with regards to the position and intensity of some of their constituting bands (Fig. 1B, 3B). This finding encouraged us to describe the acquired SER spectra at EW 532 nm as representing a mixture of 4 purine metabolites (adenine, guanine, hypoxanthine, and xanthine) as well. The reference spectra characterized in [16] differ from the acquired *E. coli* spectra in the type of the SER substrate used and EW, resulting in slight shifts in band positions. Considering that, our description of the experimental SER spectra of *E.coli* at EW 532 nm can be characterized as satisfactory.

We also explored a possibility of ascribing the bands in the SER spectra of *E. coli* at 532 nm EW to reduced or oxidized riboflavin and FAD whose reference spectra were borrowed from some early works [4, 24, 25] (Fig. 4C). A few high and medium intensity bands of *E. coli* are absent in the SER spectra of RF including 650, 725–733, 955, and 1,365 cm^{-1}. In turn, the SER spectra of *E. coli* either miss a number of RF bands or include the bands with a strongly different intensity: 528–529, 834–839, 1,149–1,156, 1,279–1,289, 1,491–1,502, and 1,523–1,527 cm^{-1} for oxidized RF and 528, 1,251, 1,501 and 1,530 cm^{-1} for reduced RF. Nevertheless, the contribution of the RF to the *E. coli* spectra at 532 nm EW cannot be ruled out. It could additionally increase the intensity of a broad band in the region between 1,300 and 1,350 cm^{-1} in comparison with the mixture of purine derivatives. However, RF does not dominate the spectrum. This somewhat contradicts the early conclusions about its dominance in the spectra of *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Geobacillus stearothermophilus* at 532 nm [25]. Such discrepancy can be explained by the difference in the used bacteria species.To sum up, the SER spectra of *E. coli* at 532 nm EW can be best described as a superposition of the spectra of purine derivatives. Similarly to 785 EW, the variability of the spectra of bacterial samples from different batches at 532 nm EW results from the difference in the concentrations of these compounds released by the cells into the solution.

CONCLUSIONS

The SER spectra of *E. coli* excited at 785 and 532 nm originate from a mixture of purine derivatives released by the cells into the solution, given that a silver nanoparticle sol synthesized following the hydroxylamine technique is used as a SERS substrate. For both excitation wavelengths, the acquired spectra are best described as originating from adenine, guanine, hypoxanthine, and xanthine. Riboflavin may slightly contribute to the spectra excited at 532 nm. The acquired spectra are characteristic of viable bacteria cells only. Their variability results from the differences in the ratio of the contributing components. Such molecular origin of bacterial SER spectra imposes serious limitations on the use of SERS for bacterial identification and discrimination.

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