20

Optical express monitoring of Internalin B, a protein of the pathogenic bacterium Listeria monocytogenes, using SERS-active silver-decorated silicon nanowires

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Surface-enhanced Raman Scattering (SERS) is a powerful vibrational optical spectroscopy method that allows highly sensitive detection of molecules at very low concentrations of matter by amplifying the electromagnetic fields created by excitation of localized surface plasmons on the surface of noble metal nanostructures. In the presented work a method of manufacturing composite nanostructures of silicon nanowires decorated with silver (AgSiNWs) has been developed. The SERS activity of AgSiNWs for protein detection was investigated using human serum albumin as an example. For the first time, the possibility of rapid diagnosis of internalin B (InlB) protein of pathogenic bacteria Listeria monocytogenes by SERS using the obtained nanostructures was shown. In the spectra of InlB adsorbed on AgSiNWs at different concentrations, distinct peaks corresponding to Raman scattering on protein molecules are observed. Based on the experimental data obtained, the detection limit of InlB was calculated to be $4.8 \cdot 10^{-9}$ M. The results presented in this work demonstrate the high potential of the obtained composite nanostructures for the diagnosis of various proteins by SERS.

Keywords: Surface-enhanced Raman scattering, silicon nanowires, composite nanostructures, albumin, listeria, internalin B.

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Introduction

Surface-enhanced Raman spectroscopy (SERS) is currently one of the high developing methods of optical spectroscopy, which provides highly sensitive detection of molecules at very low concentrations [1]. Due to its high sensitivity and specificity, SERS spectroscopy is widely used in many analytical applications, such as biomedical diagnostics, detection and identification of tracer quantities of substances, up to the detection of single molecules [1–3]. The SERS method consists in amplifying the intensity of the Raman scattering (RS) signal of molecules adsorbed on gold/silver/copper nanostructures. After SERS was first found for pyridine molecules adsorbed on a rough silver electrode in 1974 [4], chemical and electromagnetic mechanisms underlying it were proposed. The chemical mechanism in SERS is caused by charge transfer between adsorbed molecules and nanostructures and provides a 1000-fold amplification [5]. The electromagnetic mechanism is associated with localized surface plasmon resonance (LPR) in noble metal nanostructures, which provides an increase in signal intensity usually by 6−8 orders of magnitude [6,7]. It should be noted that the wavelength of light required for the occurrence of LPR depends on the material, size, shape, refraction index of the environment and the bond between neighboring nanoparticles [6,7]. In this case, a particularly strong amplification of RS up to 10^9-10^{10} times is observed in the region„of hot spots“ where the intense electric field is created in nanoscale gaps between plasmonic nanostructures and acts as a coupled optical resonator [8].

Compared to some other alternative methods for detecting biomolecules, SERS is regarded as an excellent choice for the quantification and structural characterization of proteins as well [9,10]. For example, in [11] the detection of C-reactive protein was demonstrated using silver nanoparticle agglomerates, and in [12] it were able to detect bovine serum albumin using gold nanoparticles.

For diagnostic purposes in SERS spectroscopy, composite nanostructures based on nanostructured silicon substrates are widely used, among which it is worth noting silicon nanowires (SNWs) coated with noble metal particles [13]. SNW is usually obtained using metal-stimulated chemical

etching (MSCE). This method is based on selective chemical etching of crystalline silicon wafers using a catalytic metal (Au, Ag, Pt, etc.) and makes it possible to obtain close packed arrays of silicon nanowires with a single crystallographic orientation and different doping level [13–16]. Previously, in our studies, using the SERS method using SNWs coated with silver and gold nanoparticles, one demonstrated the possibility of diagnosing pyocyanin, which is a specific metabolite of Pseudomonas aeruginosa, in artificial sputum at concentrations up to 6.25 μM, which is the lower limit of the clinically significant range [17], and of diagnosing bilirubin with a detection limit of 1 μM [18] was detected using SNWs coated with gold particles.

Despite significant progress in the fight against some infectious diseases, there are a number of infections, the diagnosis of which is still difficult. Among such diseases is listeriosis, the causative agent of which is the bacterium Listeria monocytogenes, a widespread food contaminant that causes a generalized infection induced in humans and animals [19]. In case of ingestion, Listeria can cause meningitis, encephalitis, septicemia, endocarditis, abscesses, and local purulent lesions [19]. Pathogenic Listeria differ from closely related non-pathogenic Listeria and other bacterial species by the presence of a surface protein, internalin B (InlB), which is responsible for the induction of phagocytosis and is necessary for active invasion of hepatocytes [20]. Thus, the presence of InlB in the body fluids (blood plasma, urine) may indicate the presence of the corresponding disease. In the practice of bacteriologists, serological methods for diagnosing listeria are often used, which, however, often give false results, have low sensitivity and specificity. Thus, the development of a reliable express method for diagnosing listeriosis is a problem number one at present.

The purpose of this work was to study the possibility of detecting InlB, a protein of L. monocytogenes bacteria, by SERS using SNWs coated with silver nanoparticles. To achieve the purpose of the work, the following tasks were assigned: to develop a methodology for preparing composite nanostructures of silicon nanowires decorated with silver, to study the morphological features of the obtained composite nanostructures using electron microscopy, to study the SERS activity of AgSNWs for protein detection using human serum albumin as an example; to study the possibility of detecting the InlB protein, based on the obtained data, to calculate the limit of detection of InlB in its diagnosis by SERS using the developed AgSNWs composite nanostructures.

Experiment procedure

SNW samples were fabricated by MSCE on *c*-Si wafers of *p*-type conductivity with resistivity 0.8–1.2 $\Omega\cdot$ cm with (100) crystallographic orientation. The preparation of the *c*-Si wafers for etching consisted of successively immersing them in acetone, ethanol, and 5 M HF for 2 min in the each compound. After that, the wafers were washed with distilled water and dried in air. Next, *c*-Si wafers were immersed in a solution of 0.02 M $AgNO_3$ and 5 M HF in the ratio 1 : 1 per 20 s. At this stage, the process of applying silver particles to the surface of the plates took place. After that, for the formation of SNW the plates were transferred into a solution of 5 M HF and 30% H_2O_2 in the ratio 10 : 1, in which the process took place for chemical etching of the *c*-Si wafer in places covered with silver particles. The etching time was 30 s. After that, the *c*-Si wafers were again washed with distilled water and dried in air. To impart SERS-active properties to SNWs, their tops were additionally coated with silver particles by immersing the samples in a solution of 0.02 M $AgNO_3$ and 5 M HF in the ratio 1 : 1 by 2 ,min. After that, the wafers were washed with distilled water and dried in air. The structural properties of SNWs were examined using a scanning electron microscope (SEM) Carl Zeiss SUPRA 40 FE-SEM.

To study the detection of proteins by SERS using the obtained AgSNWs, human serum albumin (HSA, Sigma Aldrich) was used first, and then recombinant purified InB protein. The recombinant InB protein was purified from the producer strain *Escherichia coli* BL21::pET28b(+)::InlBallele9 as described in [{]21. AgSNW samples were incubated for 60 min with HSA or InlB proteins diluted in phosphate buffer pH 7.0 at various concentrations. Then the samples were dried at room temperature in air. The finished dried samples were placed on an object table under a laser beam with a wavelength of 633 nm and a power of 1 mW. The spectra were recorded on a RS-spectrometer Confotec^{TM} MR350 for 10 s.

Results and discussion

Scanning electron microscopy (SEM) micrographs of the obtained samples of composite nanostructures of silicon nanowires decorated with silver nanoparticles (AgSNW) are shown in Fig. 1 (Fig. 1, *a* is top view of the sample, Fig. 1, *b* is side view of the sample).

It can be seen from Fig. 1 that the length of the SNW is 150–200 nm, the diameter and the distance between the nanowires are approximately the same and equal to 50 nm (for clarity, two separate SNW are marked with a yellow dash in Fig. 1, *b*). Silver nanoparticles, which are visible in SEM microphotographs at the base of the SNW, were used as a catalyst for the chemical reaction in the preparation of SNW by the MSCE method. The tops of the SNWs are also decorated with silver nanoparticles to impart SERS-active properties to such composite substrates [17]. It can be seen from Fig. 1, *b* that the thickness of the AgSNW layer is about 500 nm Silver particle diameter is 200–300 nm

The possibility of using the obtained composite substrates for the detection of proteins by SERS was studied. For this, the spectral data of the well-studied human serum albumin (HSA) protein were collected. Fig. 2 shows the SERS spectra of HSA adsorbed on AgSNW from their solutions

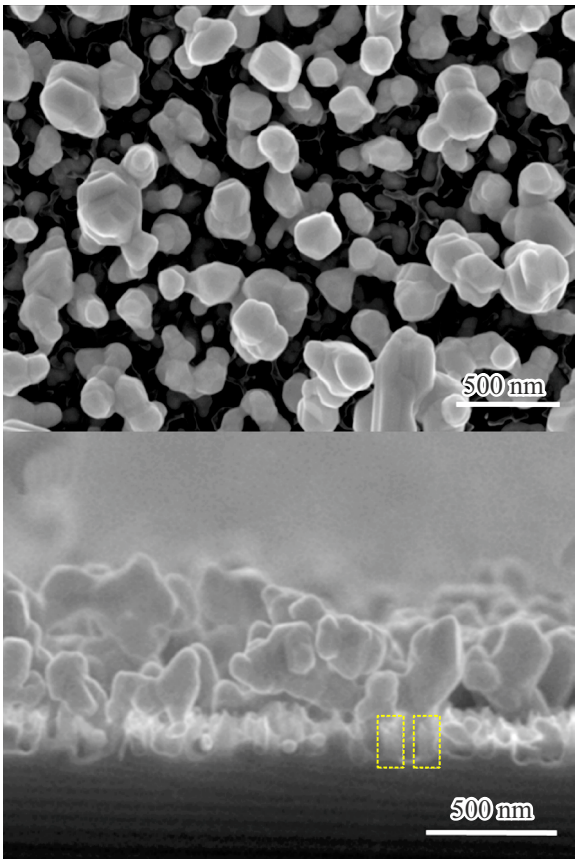

Figure 1. Scanning electron microscopy micrograph of AgSNW: top view (*a*), side view (*b*): two separate SNWs are marked with a yellow dash.

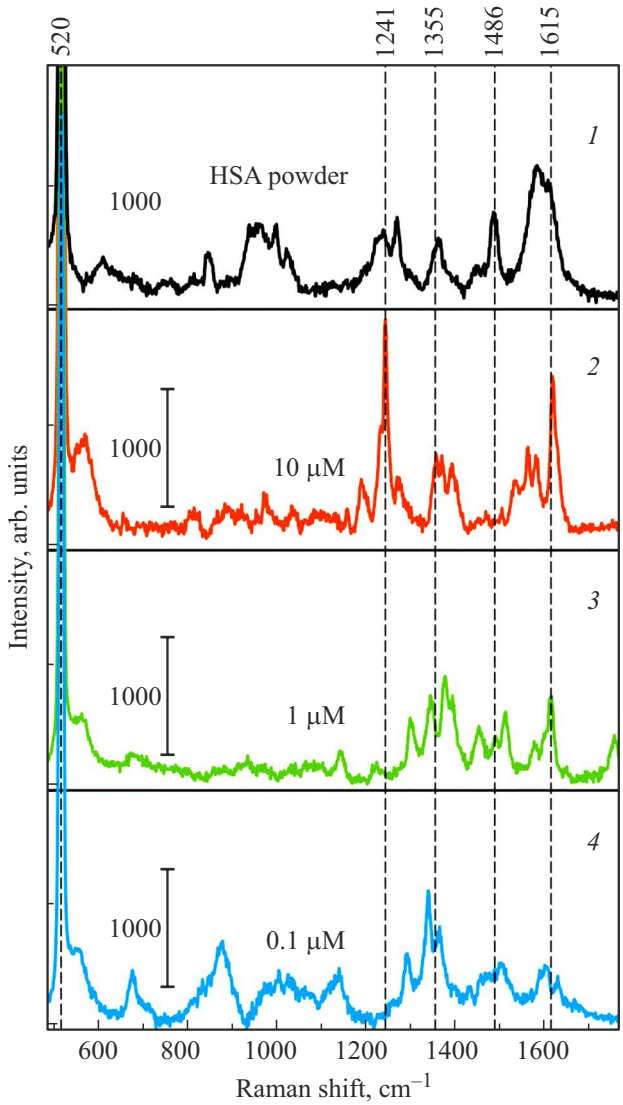

Figure 2. RS spectrum of HSA powder on a single-crystal silicon wafer (*1*); SERS spectra of HSA adsorbed on AgSNW with different concentrations: 10 (*2*), 1 (*3*) and 0.1 μM (*4*).

in phosphate buffer pH 7.0 with various concentrations: 10, 1 and 0.1 μM. The RS of HSA powder on a single-crystal silicon wafer is shown in Fig. 2 (*1*).

In the spectra shown in Fig. 2, the peak corresponding to 520 cm^{-1} is a characteristic RS signal of crystalline silicon [18]. It should be noted that this peak can be used for normalization when recording SERS spectra of molecules adsorbed on silicon substrates. This undoubtedly makes it convenient to use silicon-based composite materials in SERS diagnostics.

In the RS and SERS spectra of HSA shown in Fig. 2, vibrations of the amide group of polypeptides can be distinguished, the characteristic frequencies of which lie in the spectral regions near 1615, 1486 and 1240 cm^{-1} and correspond to changes in the length C=O of the peptide bonds for amide I, changes in the CNH angle for amide II, and stretching of C–N- and N–H-bonds for amide III, respectively [22,23]. The bands in the frequency range 1355 cm^{-1} can be attributed to deformation C–H-oscillations [22]. It should be noted that the shift of the frequencies of the bands in the SERS spectra of proteins from the known tabular values [22] is acceptable and is usually caused by changes in the orientation of the studied molecules or due to intermolecular interactions that occur between the amino acid residues of proteins [{]23 }.

The SERS spectra of the InlB protein adsorbed on AgSNW from their solutions in phosphate buffer pH 7.0 with concentrations of 10.1 and 0.1 μM, 10 and 1 nM are shown in Fig. 3.

The characteristic peak for crystalline silicon in the spectra in Fig. 3 is located at frequency of 520 cm^{-1}. The remaining peaks in the spectra can be assigned to InlB: the peak at a frequency of 1008 cm^{-1} corresponds to the symmetrical stretching of the phenyl group (most characteristic of the aromatic alpha-amino acid phenylalanine, which is part of all known proteins); bands in the region 1110–1260 cm^{-1} are attributed to vibrations of amide III; peaks at 1339 and 1439 cm^{-1} are characteristic of protein amino acids, tryptophan and histidine, respectively; the

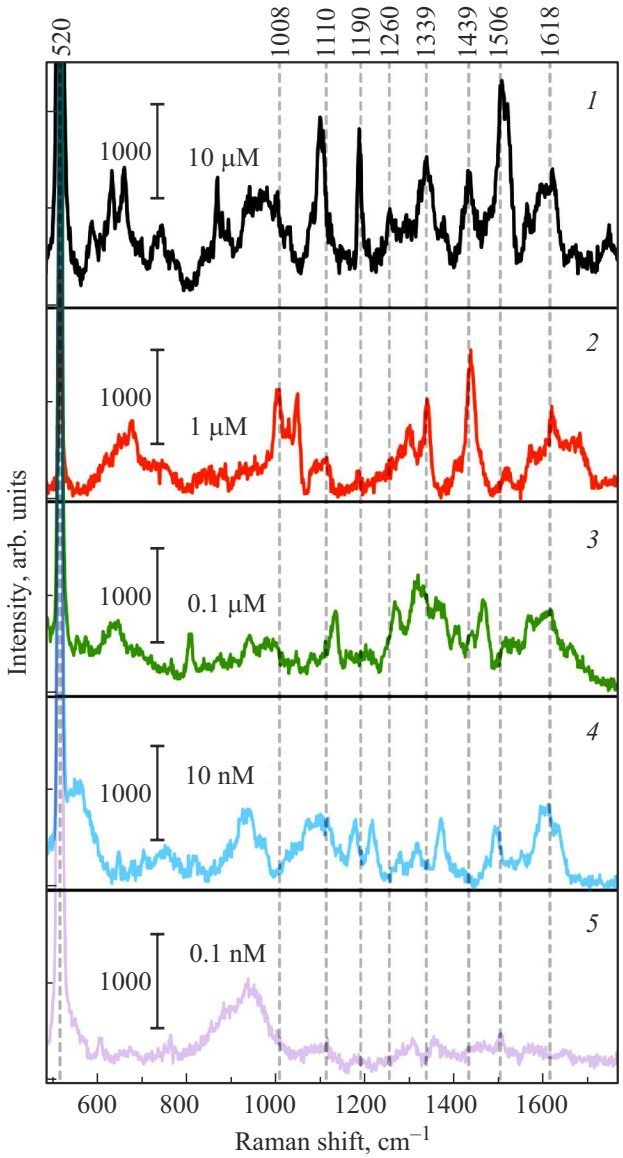

Figure 3. SERS spectra of the InlB protein adsorbed on AgKHN with a concentration of 10 (*1*), 1 (*2*), 0.1 μM (*3*) and 10 (*4*), 1 nM (*5*).

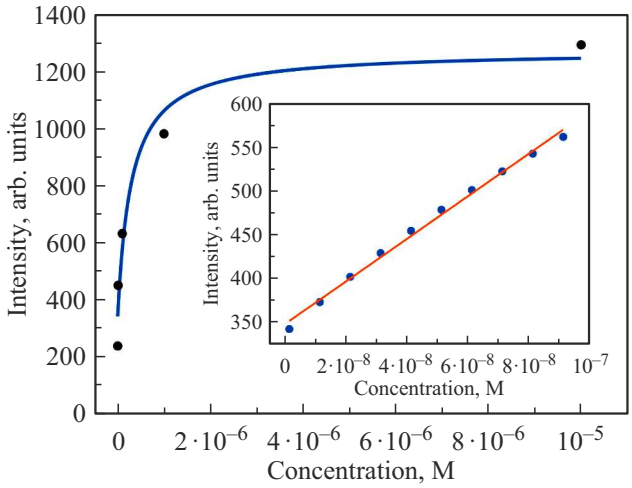

Figure 4. Dependence of the intensity of the SERS peak of amide I at frequency of 1618 cm^{-1} on the concentration of the InlB protein The blue line shows the result of approximation by the Langmuir adsorption isotherm. The inset shows the first 10 points obtained from the Langmuir isotherm at low concentrations of the InlB protein and the result of their approximation (red line) by the least squares method.

peak at 1506 cm^{-1} corresponds to the bend of the N–H bond of the peptide backbone and, finally, the peak at and 1618 cm^{-1} corresponds to the amide I [22]. Thus, in the presented spectra, the peaks characteristic of biomolecules of a protein nature are pronounced. This proves the possibility of efficient and fairly accurate detection of the InlB protein using the developed composite AgSNW substrates by SERS methods.

From the point of view of the sensitivity of the samples to a decrease in concentration, one can see a fairly clear repetition of the bands in the SERS spectra characteristic of InlB for all concentrations used in the experiment. The spectra show a change in the ratios between the heights of some peaks, as well as their shift, which can be explained by some changes in the orientation of the studied molecule on the sample surface and intermolecular interactions.

The limit of detection (LOD) of InB was calculated from the change in signal intensity from amide I. For this, the dependence of the signal intensity of the peak at 1618 cm^{-1} on the concentration of the InlB protein was plotted and the Langmuir adsorption isotherm was approximated (Fig. 4) [24].

The intensity values taken from the Langmuir adsorption isotherm at low concentrations were then approximated by the least squares method (insert in Fig. 3) to calculate the standard deviation ($\sigma = 3.514$) and curve slope ($slope = 2.42 \cdot 10^9$). After that, LOD was calculated according to the equation [25]

$$LOD = \frac{3.3\sigma}{slope} = \frac{3.3 \cdot 3.514}{2.42 \cdot 10^9} = 4.8 \cdot 10^{-9}\,\text{M}.$$

The obtained value of the limit of detection for InB, $\text{LOD} = 4.8 \cdot 10^{-9}$ M, indicates a high potential for the use of SERS-active nanostructures based on AgSNW for diagnosing proteins of Listeria monocytogenes bacteria.

Conclusion

Methodology has been developed for obtaining SERS-active nanostructures : the silver-decorated silicon nanowires AgSNW. In this case, SNWs were prepared by a simple and accessible method of metal-stimulated chemical etching of crystalline silicon substrates, and Ag was decorated by its chemical reduction from $AgNO_3$ in the presence of 5 M HF.

Using the human serum albumin protein as an example, the possibility of using the obtained AgCNW composite substrates for the label-free determination of proteins by the SERS method is shown.

The possibility of rapid diagnostics of the InlB protein of pathogenic bacteria Listeria monocytogenes by SERS using the obtained nanostructures was demonstrated for the first time. It was shown that the SERS spectra of InlB contain bands characteristic of biomolecules of a protein nature. The limit of detection of InlB calculated from the experimental spectra is $4.8 \cdot 10^{-9}$ M, and it is evidence that a high sensitivity of the developed SERS-active nanostructures.

Thus, it has been shown that the synthesized AgCNW composite nanostructures have a great potential for diagnosing various proteins, in particular the InlB protein, by the SERS method. The results obtained in the work are promising for the use of the proposed methodology in the diagnosis of Listeria monocytogenes bacteria in clinical practice.

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Conflict of interest

The authors declare that they have no conflict of interest.

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