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Nanomaterials and Nanotechnology

Enhanced Detection of Human Plasma Proteins on Nanostructured Silver Surfaces*

Regular Paper

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Received 3 December 2012; Accepted 6 June 2013

* An early version of this paper has been presented at The International Conference on Nanomaterials: Fundamentals and Applications - NFA2012, Slovakia

Abstract In chemical and medical research, recent methods combine the tools of nanotechnology, chemistry and biology in a way that introduces the most modern processes to current medical practice. The main blood plasma proteins – albumin and globulin and their amino acid sequences, are carriers of important information about human health. In this paper we employed silver nanostructured surfaces prepared by electrodeposition. Consequently, electrochemical deposition is introduced as a convenient, fast and cost-effective method for the preparation of metallic nanostructures with required morphology. Silver nanostructured surfaces were applied as the templates for Surface Enhanced Raman Spectroscopy (SERS) of albumin and globulin in the role of model analytes. We also studied the effect of a working electrode polishing process on electrodeposition and identification of proteins. The aqueous solutions of albumin and globulin were applied onto these Ag nanostructured substrates separately. An analytical signal enhancement factor of 3.6×10^2 was achieved for a band with a Raman shift of 2104cm^{-1} for globulin deposited onto silver nanostructured film on unpolished stainless steel substrate. The detection limit was 400μg/mL. Plasma or serum could present a preferable material for non-invasive cancer disease diagnosis using the SERS method.

Keywords Electrochemical Deposition, Nanostructured Surfaces, Albumin, Globulin, SERS

1. Introduction

There were an estimated 12.7 million cancer cases around the world in 2008, of these 6.6 million cases were in men and 6.0 million in women. This number is expected to increase to 21 million by 2030 [1]. It is therefore necessary to identify and develop improved, non-invasive methods suitable for early diagnosis and successful treatment. The

utilization of nanostructured surfaces in nanomedicine seems to be very useful in the diagnosis of cancerous diseases. Kwan at al. used nanostructured polymer surfaces for microfluidic label-free separation of human breast cancer cells by adhesion difference [2]. In this way, the application of nanostructured surfaces meets the basic requirements that are demanded for diagnosis i.e., speed [3], accuracy and cost-efficiency [4]. Nanostructured surfaces are very effective in the analysis of different samples and in combination with Surface Enhanced Raman Spectroscopy (SERS) and enhancements of the analytical signal of the order o 10^4 - 10^6 are routinely observed, while in some systems up to 10^{14} [5] can be obtained. Therefore, it has been shown to be a promising optical cancer diagnosing technique [6-8]. Several groups have applied SERS using gold nanoparticles [9] or colloidal silver nanoparticles for the diagnosis of gastric and nasopharyngeal cancer [10, 11]. It is also possible to discriminate normal and cancerous samples by this spectroscopic method together with statistical analysis [12, 13]. In comparison to nanoparticles, nanostructured surfaces have several important advantages. The morphology of nanostructured surfaces as identified on micrographs can be simply defined according to specific needs. Moreover, we can choose the best size and type of nanostructures, the cornerstones from which nanostructured surfaces are to be formed. An important feature is the stability of nanosurfaces with respect to gold nanoparticles, which tend to form clusters and/or aggregates [14]. For the application of new materials to everyday medical practice it is necessary to work with materials that are not subject to unwanted changes and whose physical characteristics vary depending on specific applications. Nanostructured surfaces definitely fulfil these requirements.

In our research we focused on Surface Enhanced Raman Spectroscopy (SERS) analysis and identification of the blood plasma proteins - albumin and globulin on silver nanostructured surfaces, which were prepared by electrodeposition.

Aromatic amino acids phenylalanine, tyrosine and tryptophane, present in both proteins are associated with specific vibrations and they all appear in the SERS spectra of both albumin and globulin. The major spectral difference between the purified proteins of normal and cancer blood plasma are in the relative intensities of the bands. These changes probably reflect variations in protein constituents and conformations when cancers develop. The detailed mechanisms for these spectral changes deserve further investigation [13].

SERS analysis can be widely applied in a multitude of interdisciplinary scientific investigations such as chemistry, biology, medicine, environmental sciences and archaeological applications, because of its high sensitivity [5].

1.1 Blood and blood serum

Blood, circulating through the bloodstream, is an ideal analyte for the diagnosis of cancer. Its continuous flow inter-connects all parts of the human body and thus blood is able to provide necessary information about on-going processes in various internal organs. Blood, as a viscous liquid, is a suspension formed from blood elements (red and white blood cells) and from platelets in the blood plasma Blood plasma is the liquid component of blood, which has a relatively constant composition. Blood plasma consists of three basic components: water (90 – 92%), inorganic (Na^+, Cl^-, HCO_3^-) and organic compounds (proteins). Plasma proteins can be generally divided into three groups: albumins (35 - 50g/L of plasma), globulins (25g/L of plasma) and fibrinogen (1.5 - 3.5g/L of plasma). Proteins have important transporting and immune functions, have nutritional importance in the blood and help the precipitating process [15].

Nowadays, the reproducibility of blood tests is limited by the presence of exogenous blood plasma impurities such as bacteria, viruses or drugs, whose presence in the plasma varies due to actual pathological conditions. SERS represents a sufficient method for the analysis of blood plasma serum samples since SERS detection is also highly sensitive to interference caused by these exogenous impurities [13].

1.2 Surface enhanced Raman spectroscopy

The SERS technique has become widely used for identifying and providing structural information about molecular species in low concentrations. There is an on-going interest in finding the optimum particle size, shape and spatial distribution for optimizing the SERS substrates and pushing the sensitivity toward the single-molecule detection limit [16]. Although the exact mechanisms behind SERS are still under discussion, it is widely accepted that the origin of SERS is closely correlated with the enhancement of the local electromagnetic field at the surface of small metallic nanoparticles and of the charge transfer between adsorbates and the metal particles [17-19]. It has been shown that the oscillation of electrons at the metal dielectric interface is strongly dependent on the size, symmetry and proximity of nanoparticles [20]. In addition, small metal particles of some metals (Ag, Cu, Au) have shown tremendous enhancement factors for Raman scattering, thus enabling Raman spectroscopy of single molecules [21]. Presently, the surface enhancement effect of metals is explained as the result of multiple cooperative mechanisms [17, 18, 22]. Their role and the contributions, however, have not yet been quantitatively

clarified. There seems to be agreement that SERS is a function of the roughness features of the enhancing surface. Therefore, the preparation of SERS active particles with a well-defined size and morphology can lead to a better theoretical understanding of SERS, thus enhancing the analytical value of this method.

SERS is one of the techniques capable of detecting a single molecule and simultaneously probing its chemical structure. It is possible to detect biomolecules such as proteins, DNA, RNA, pathogens [23] and also live cells [24]. The SERS spectra of proteins were first reported by the investigation of flavoproteins (proteins that contain a nucleic-acid derivative of riboflavin) [25]. There are two approaches available for SERS-based biomolecule detection: label-free and extrinsic SERS labelling. The label-free protocol aims at directly acquiring SERS spectra of biomolecules in the absence of Raman dyes and the extrinsic SERS labelling method employs Raman labels to detect biomolecules indirectly [25].

1.3 SERS and detection of cancerous diseases

SESR was developed for blood plasma biochemical analysis with the aim of developing a simple blood test for non-invasive nasopharyngeal cancer detection by Feng et al. for the first time in 2010 [11]. They used silver nanoparticles as SERS active nanostructures mixed with blood plasma to enhance the Raman scattering signals of various biomolecular constituents such as proteins, lipids and nucleic acids. SERS measurements were performed on two groups of blood plasma samples. One group from patients with pathologically confirmed nasopharyngeal carcinomas and the other group from healthy volunteers. Linear discriminate analysis based on the principal components analysis (PCA) differentiated the nasopharyngeal cancer SERS spectra from normal SERS spectra with high sensitivity (90.7%) and specificity (100%) [11]. Another blood plasma analysis combines membrane electrophoresis with nanoparticles-based SERS for cancer detection applications [13]. In this method, total serum proteins were isolated from blood plasma by membrane electrophoresis and mixed with silver nanoparticles to perform SERS spectral analysis. The obtained SERS spectra showed rich, fingerprint-type signatures of the biochemical constituents of whole proteins [13]. Lin at al. evaluated the usability of this method by analysing blood plasma samples from patients with gastric cancer and healthy volunteers [13]. The gastric cancer group could be unambiguously distinguished from the normal group in this initial PCA, i.e., with both diagnostic sensitivity and specificity of 100%. Results from these exploratory studies researches are very promising for developing a label-free, non-invasive clinical tool for cancer detection and screening [11, 13].

Our search has focused on the preparation of silver nanostructured surfaces that replace the silver nanoparticles used in the former studies of Feng et al. and Lin et al. Having a reliable, non-invasive method for early detection of cancer will dramatically improve the management and cure rate of this deadly disease. A blood test is a basic and quick examination and plasma or serum is a preferable material for non-invasive cancer diagnosis.

2. Experiments

2.1 Chemical reagents

All the chemicals for the silver nanoisland films preparation were of analytical grade and the solutions were freshly prepared. Other chemicals used within the study were purchased from Alfa Aesar GmbH (Germany) and were used without further purification.

2.2 Electropolishing of stainless steel targets

Stainless steel targets of 2.0cm × 1.0cm × 0.1cm were mechanically cut. They were degreased with acetone under ultrasonic vibrations for ten minutes at room temperature. The electropolishing process was electrochemically characterized through the use of chronopotentiometry. Initially, stainless steel targets were cathodically polarized in 1.0mol/L HNO_3 for 15 minutes. Then, stainless steel substrates were immersed in an aqueous solution of 5.0mol/L H_2SO_4 + 2.5mol/L CrO_3 at room temperature (20 ± 2 °C) and anodically polarized ($20\ Adm^{-2} \leq i \leq 40\ Adm^{-2}$) for 30 minutes. Two paraffin impregnated graphite electrodes (PIGE) were used as counter electrodes; no reference electrode was used [26].

2.3 Electrochemical preparation of Ag island films

Silver nanostructured surfaces were electrochemically synthesized by multiple scan cyclic voltammetry using an electrolyte containing 0.1mol/L KNO_3, 0.1mol/L KCN and 0.01mol/L $AgNO_3$ (pH = 10.25). Electrochemical deposition was performed using a conventional three-electrode cell controlled by an Autolab PGSTAT302N (Metrohm, Utrecht, Netherland) at room temperature and atmospheric pressure of 101.325kPa. As a working electrode, a sheet of stainless steel substrate with a bare surface area of $2cm^2$ was used, the counter electrode was a $0.56cm^2$ platinum target and an Ag|AgCl|3 M KCl electrode was used as a reference electrode. The working electrode was cycled 10, 15, 25, 30 and 40 times between –700 and –1550mV (vs. Ag|AgCl|3 mol/dm^3 KCl), beginning at –700mV, with a scan rate of 0.1V/s in order to affect electrodeposition [27].

2.4 The surface morphology of Ag island films

The morphology and homogeneity of the electrochemically prepared silver nanostructured films

were characterized ex situ using a scanning electron microscope JEOL JSM-7000F (Japan).

2.5 SERS analysis of Ag island films

For SERS analysis of silver nanostructured surfaces albumin and globulin (500μg/mL) were used as model analytes. Albumin (type A-7030 Bovine albumin) was purchased from Nack Sigma. Globulin was purchased from MANN RESEARCH LAB (type Alpha Globulin human IV fraction IV). The aqueous solution of 5μL proteins' volume was dropped onto each silver nanostructured surface and dried naturally.

The SERS identification of proteins deposited on silver nanostructured surfaces was performed by Raman spectrometer "Xplora" (Model BX41TF, HORIBA Jobin-Yvon, Japan) with a wavelength of 532nm.

3. Results and discussion

The measurements at electrochemically prepared silver nanoisland surfaces aimed to determine both albumin and globulin. The identification and analytical signal enhancement in the SERS analysis were strongly dependent on the conditions of Ag nanostructures preparation. We also investigated the influence of stainless steel target polishing for silver electrodeposition and subsequently for the proteins' estimation.

3.1 Preparation and morphology of Ag island films

Various types of silver nanostructured surfaces were electrochemically synthesized from electrolytes (0.1mol/L KNO_3, 0.1mol/L KCN and 0.01mol/L $AgNO_3$) by CV preparation. These silver surfaces differed one from another by number of cycles (10, 15, 25, 30 and 40). Other conditions selected for the CV, such as scan rate and range of potentials, remained unchanged [27].

To investigate the surface structure and morphology of the substrate types, SEM images of the substrates were acquired and examined. The image produced is a 2D-profile of the substrate at different magnifications, shown in Figure 1.

Scanning electron microscopy was used for investigation of the surface morphology and homogeneity of dynamically electrodeposited Ag nanostructured substrates. The micrographs obtained from the scanning electron microscope in Figure 1 show the details of an unpolished stainless steel working electrode coated with silver nanoislands.

The SEM images in Figure 1 are representative of many images taken in different regions of the substrate and at the one value of magnification. The number of CV scans has affected the morphology of the silver nanostructures electrolytically deposited onto the working electrode. The surface area of working electrode is partially covered by spherical silver nanoparticles and clusters with varying size distributions (ca. 90 – 500nm in diameter), which themselves consist of agglomerated smaller crystallites. It can be clearly seen that there is an increase in both size and density with increasing number of cycles.

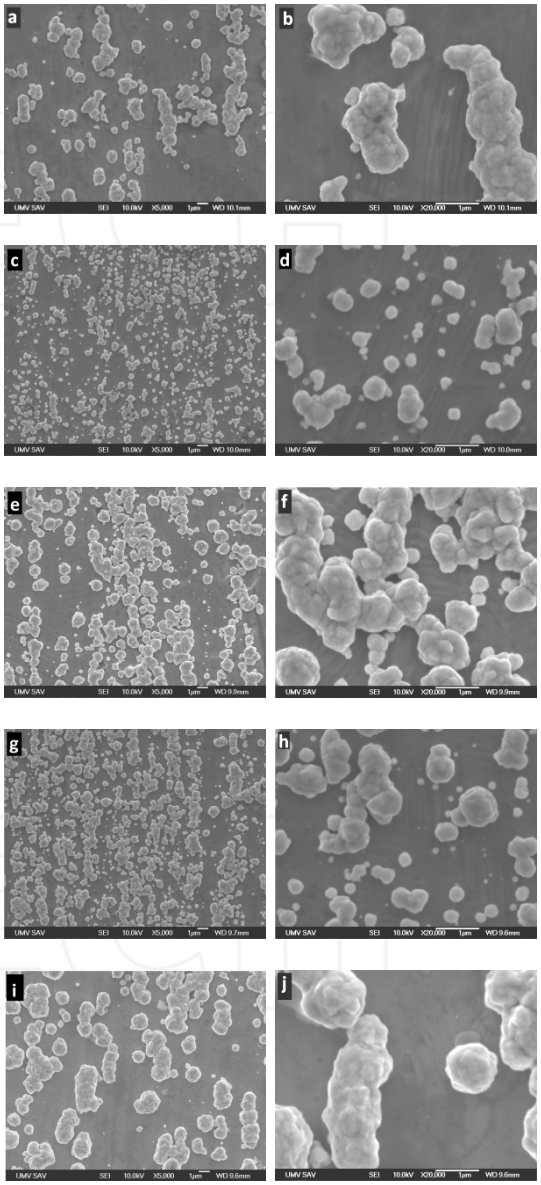

Figure 1. Representative SEM micrographs of Ag films deposited on unpolished stainless steel with different number of CV scans a),b) **10**, c), d) **15**,e) f) **25**, g) h) **30** and i) j) **40**. Magnifications: 5000x and 20 000x.

The SEM micrographs in Figure 2 show silver nanoisland surfaces deposited onto polished stainless steel substrates with Cyclic Voltammetry (CV) scan numbers of 25 and 30.

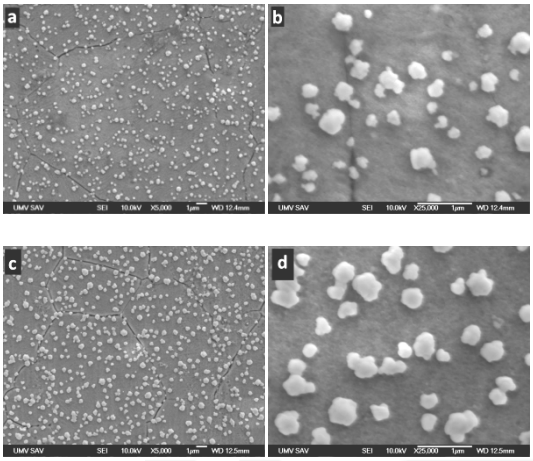

Figure 2. Representative SEM micrographs of Ag films deposited on polished stainless steel with different number of CV scans a), b) **25** and c), d) **30**. Magnification: 5000x and 25 000x.

The electrochemical deposition was controlled by diffusion process from the working electrolyte towards an electrode surface. Though we obtained the decrease in size of Ag nanoparticles and higher nanoparticles separation on the surface area of polished stainless steel substrate, there did not occur many surface defects corresponding to SERS active hot spots.

3.2 SERS analysis of Ag island films

Silver nanoisland formation and the study of their morphology were followed by SERS analysis of the model analytes. The aqueous solutions of albumin and globulin were applied onto Ag nanostructured substrates separately and after drying they subsequently were analysed by SERS. The representative resulting spectra with the highest intensities for proteins are shown in the following figures.

The analytical enhancement factor, F_e, was calculated according to the following formula:

$$F_e = \frac{c_{ref}}{c_{sample}} \cdot \frac{I_{sample}}{I_{ref}} \quad (1)$$

where c_{ref} and c_{sample} are the reference concentration and sample concentrations, respectively and I_{ref} the signal intensity of the respective Raman peak. As a reference a stainless steel substrate was used.

Figure 3 shows the spectrum with the highest values of intensity obtained for 5μL of albumin deposited onto a silver nanostructured surface.

The Ag nanostructured surface three prepared by 25 cycles, was selected as the most suitable surface for the identification and analysis of albumin. The bands of protein applied onto this silver surface demonstrated the highest intensities. The signal enhancement factor of albumin could not be evaluated since there was no measurable signal on the unpolished stainless steel substrate.

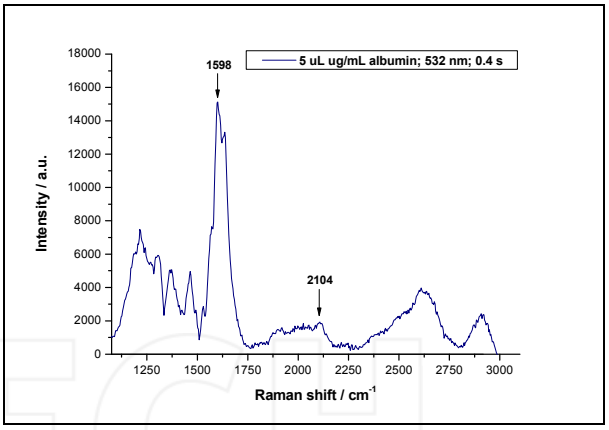

Figure 3. SERS spectrum of 5μL 500μg/mL albumin onto unpolished stainless steel targets deposited with Ag by CV scan number 25.

The SERS spectrum of 5μL globulin applied onto silver nanoislands film is demonstrated in Figure 4.

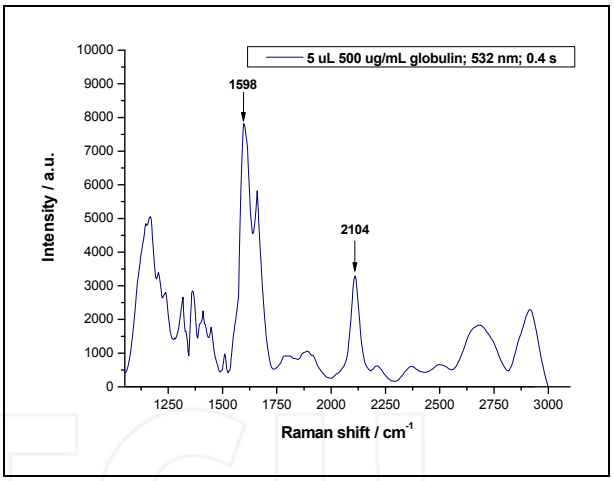

Figure 4. SERS spectrum of 5μL 500μg/mL globulin deposited onto unpolished stainless steel target covered by Ag film (CV scan number 30).

The silver nanostructured surface four, electrolytically prepared by 30 cycles, was selected as the most suitable for the identification and analysis of globulin. This surface was homogeneously and densely covered with Ag spherical nanoparticles. These nanoparticles were either scattered on the surface or they formed clusters, with their dimensions ranging from 70 to 360nm. The analytical signal enhancement factor was established at a value of 2.6×10^2 for a band with a Raman shift of 1598cm^{-1} and 3.6×10^2 for a band with a Raman shift of 2104 cm^{-1}. The results obtained for Ag modified unpolished stainless steel targets from SERS measurements are summarized in Table 1. The analytical enhancement factors for globulin were calculated according to Eq. 1.

Ag surface	CV scan number	Intensity for peak of globulin with Raman shift		Analytical signal enhancement factor for peak of globulin with Raman shift	
		1598 cm^{-1}	2104 cm^{-1}	1598 cm^{-1}	2104 cm^{-1}
1	10	5159.4	2673.7	1.7×10^2	3.1×10^2
2	15	4295.1	715.2	1.4×10^2	0.8×10^2
3	25	7811.1	1966.5	2.6×10^2	2.3×10^2
4	30	7816.9	3125.1	2.6×10^2	3.6×10^2
5	40	4988.1	1244.9	1.6×10^2	1.4×10^2

Table 1. The signal enhancement declared by Raman shift intensity values for unpolished working electrode modified with Ag nanoisland films in 500 μg/mL aqueous solution of globulin

Good SERS reproducibility with a maximum standard deviation of 25% was obtained for both proteins.

We observed from the higher values of analytical enhancement factors for silver films deposited on unpolished stainless steel substrates that Ag nanoparticles were capable of creating a great deal of SERS active hot spots. By these means, the isolated structures become interconnected to form uniformly distributed networks, providing many sites for analyte molecules.

In an effort to find out the most suitable electrodeposition conditions and to enhance the analytical signal we also identified plasma proteins for the case of polished stainless steel substrates. We also studied the influence of the polishing process on the surface homogeneity and signal enhancement factor. From the results gained from SERS analysis, the most appropriate silver nanosurfaces deposited onto polished substrates were chosen (Figure 2). The corresponding spectra are presented in the following figures for albumin on Ag surfaces three and four (Figure 5, Figure 6) and for globulin on Ag surfaces three and four (Figure 7, Figure 8).

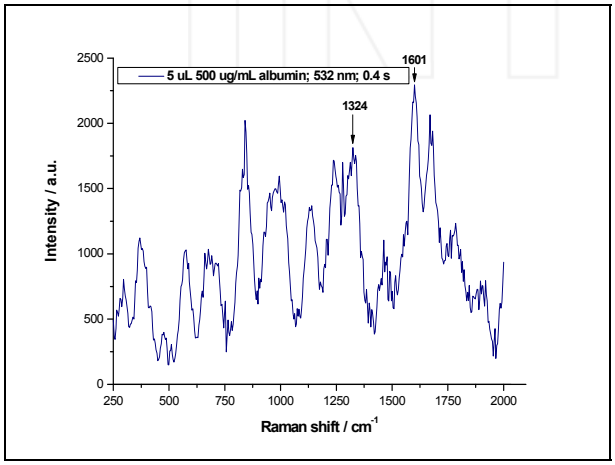

Figure 6. SERS spectrum of 5μL 500μg/mL albumin on a polished stainless steel target covered by Ag (CV scan number 30).

Although we aimed at a better distribution and surface density by depositing silver nanoislands on a polished working electrode, there was a clear decrease in the evaluated enhancement factor.

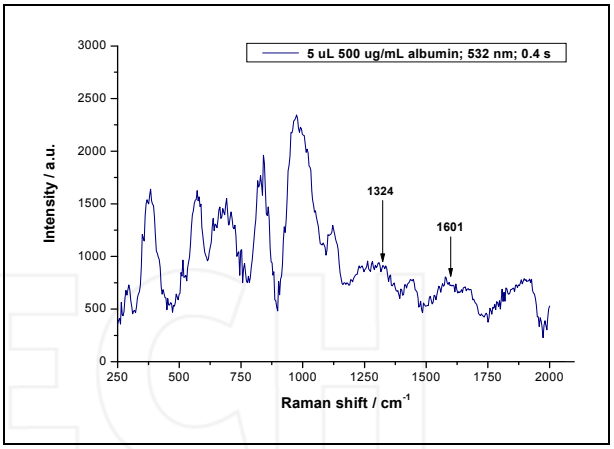

Figure 5. SERS spectrum of 5μL 500μg/mL albumin on polished stainless steel target covered Ag (CV scan number 25).

The enhancement factor for albumin applied onto the Ag surface deposited by CV scan number 25 was established for the peak with a Raman shift of 1324cm^{-1} at 0.1×10^2 and 0.1×10^2 for a band with a Raman shift of 1601cm^{-1}.
The same enhancement factor of 0.1×10^2 for a band with a Raman shift of 1324cm^{-1} and for a band with a Raman shift of 1601cm^{-1} was evaluated for albumin applied onto an Ag surface deposited by CV scan number 30.

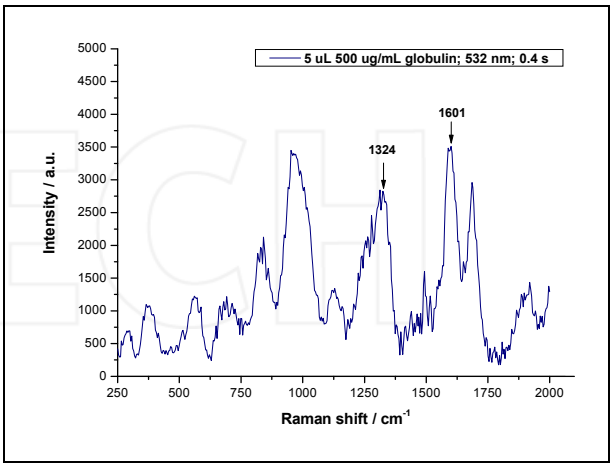

Figure 7. SERS spectrum of 5μL 500μg/mL globulin on a polished stainless steel target covered by Ag (CV scan number 25).

The analytical enhancement factor for globulin applied onto a silver nanostructured surface deposited by CV scan number 25 was established for the peak with a Raman shift of 1324cm^{-1} at 0.4×10^2 and 0.4×10^2 for a band with a Raman shift of 1601cm^{-1}.

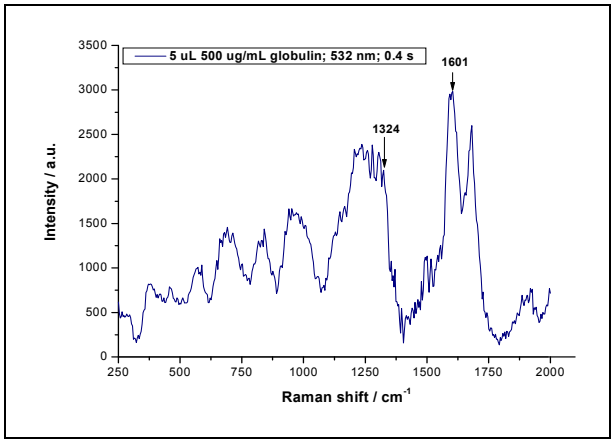

Figure 8. SERS spectrum of 5μL 500μg/mL globulin on a polished stainless steel target covered by Ag (CV scan number 30).

The analytical enhancement factor for globulin applied onto a silver nanostructured surface deposited by CV scan number 30 was established for the peak with a Raman shift of 1324cm^{-1} at 0.2×10^2 and 0.1×10^2 for a band with a Raman shift of 1601cm^{-1}.

Although there was a decrease in the size and aggregation of silver nanoparticles on a polished stainless steel substrate, Ag nanoparticles didn´t participate as much in hot spot creation.

4. Conclusions

The goal of this work is the preliminary application of silver nanoisland films for the detection of the main blood plasma proteins that present albumin and globulin. Cyclic voltammetry applied for metallic nanostructured surfaces preparation is a simple, fast and low-cost technique. In an effort to optimize the Ag nanoislands, electrodeposition conditions and to investigate these proteins, we also measured a more intense Raman signal employed on these silver nanoisland films. We calculated the higher values of analytical signal enhancement factors for both proteins deposited on an unpolished stainless steel substrate in comparison to polished substrate. The highest value of an analytical signal enhancement factor of 2.6×10^2 (1598cm^{-1}) and 3.6×10^2 (2104cm^{-1}) was achieved for globulin on the unpolished substrate and on the polished substrate the calculated value was 0.4×10^2 (1324cm^{-1}, 1601cm^{-1}) at the optimum electrodeposition conditions with scan numbers. The lower values of analytical enhancement factors of 0.1×10^2 (1324cm^{-1}, 1601cm^{-1}) were recorded for albumin on polished substrates. We were able to identify both plasma proteins, as well as achieving the highest enhancement of an analytical signal for SERS analysis on these surfaces. In our effort to test the suitability of Ag nanostructured surfaces for human plasma protein analysis we pointed toward possibilities for further application in non-invasive cancer diagnosis.

5. Acknowledgements

The authors wish to thank for financial support from MS SR grant VEGA 1/0211/12, APVV-0280-11, VEGA-1/0592/13 and CEEPM-ITMS 26220120067 Center of Excellency.

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