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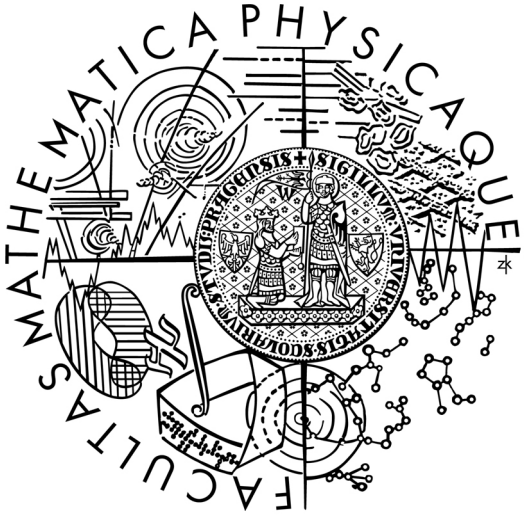
DOCTORAL THESIS



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Optimization of surface-enhanced Raman scattering spectroscopy for study of biologically important biomolecules and their interactions

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Supervisor: Doc. RNDr. Marek Procházka, PhD.

Study programme: Physics
Specialization: Biophysics, chemical and macromolecular physics

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I would like to specifically thank my supervisor Doc. RNDr. Marek Procházka, PhD. without whose endless willingness I would have never finished this thesis.
On a personal note, I would like to express my deepest gratitude to my parents and sister whose love and sacrifice are the biggest reasons behind all my success. A special thank you goes to my dearest son whose mere presence has given me the strength and inspiration to be myself.

I declare that I carried out this doctoral thesis independently, and only with the cited sources, literature and other professional sources.
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In Nouzov, April 15, 2012

Natália Šmídová

Abstrakt

Název práce:	Optimalizace spektroskopie povrchem zesíleného Ramanova rozptylu ke studiu biologicky významných molekul a jejich interakcí
Autor:	Natália Šmídová
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Abstrakt:

Hlavním cílem práce byla optimalizace spektroskopie povrchem zesíleného Ramanova rozptylu (SERS) pro studium významných biomolekul. K tomuto účelu byly vybrány povrchy na bázi zlatých koloidních nanočástic imobilizovaných na silanizované skleněné podložky. Stabilní, homogenní a reprodukovatelné povrchy vhodné pro SERS spektroskopii byly připraveny použitím aminopropyl-trimetoxysilanu a citrátem redukovaných zlatých koloidních nanočástic tepelně upravených po jejich imobilizaci. Na těchto površích byly studovány modelové biomolekuly 5,10,15,20-tetrakis(1-metyl-4-pyridyl)porfyrin (TMPyP) a 5,10,15,20-tetrakis(4-sulfonatofenyl)porfyrin (TSPP) pomocí klasického Ramanova spektrometru v makro-módu a konfokálního Ramanova mikrospektrometru. Podmínky pro SERS měření porfyrinů byly optimalizovány s ohledem na citlivost a reprodukovatelnost. SERS mikrospektroskopie ukázala řadu výhod oproti SERS měření v makro-módu: možnost spektrálního mapování povrchu, snadnější manipulace se vzorkem, kratší akumulační časy a absence silného Ramanova signálu ze skleněné podložky. Obě techniky vykazují detekční limit (LOD) porfyrinů okolo 5×10^{-8} M a potvrzují výbornou spektrální reprodukovatelnost povrchů jak v mm-, tak μm- škále. SERS zesílení bylo optimalizováno měřením SERS spekter TMPyP pomocí šesti excitačních vlnových délek (excitační profil). Výsledky ukazují, že SERS intenzita TMPyP je korelována s extinkčním spektrem systému Au povrch/TMPyP, ačkoliv se pozice intenzitního maxima pro určité vibrační módy liší v závislosti na molekulární resonanci. Největší zesílení je dosaženo pro excitaci 568.2 nm, což poskytuje LOD porfyrinu 2×10^{-8} M.

Klíčová slova: SERS, SERS mikrospektroskopie, imobilizované zlaté nanočástice, porfyriny, excitační profil

Abstract

Title:	Optimization of surface-enhanced Raman scattering spectroscopy for study of biologically important biomolecules and their interactions
Author:	Natália Šmídová
Department:	Institute of Physics of Charles University
Supervisor of the doctoral thesis:	Doc. RNDr. Marek Procházka, PhD.

Abstract:

The main goal of this thesis was to optimize surface-enhanced Raman scattering (SERS) spectroscopy for study of biologically important biomolecules. For that purpose we focused on substrates based on gold colloidal nanoparticles immobilized to silanized glass plates. Stable, uniform and highly reproducible SERS-active substrates have been prepared by using aminopropyltrimethoxysilane and citrate-reduced gold nanoparticles thermally stabilized after their immobilization. Model biomolecules 5,10,15,20-tetrakis(1-methyl-4-pyridyl)porphyrin (TMPyP) and 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin (TSPP) were studied on these substrates by using a classical Raman spectrometer in macro-mode and a confocal Raman microspectrometer. Conditions for SERS spectroscopy of porphyrins were optimized with respect to sensitivity and reproducibility. SERS microspectroscopy showed several advantages over SERS measurements in macromode: possibility of surface spectral mapping, easier manipulation with samples, shorter collection times and absence of strong Raman signal from glass support. Both techniques show limit of detection (LOD) of porphyrins ~ 5×10^{-8} M and prove very good spectral reproducibility of substrates in both mm- and μm- scale. SERS enhancement was optimized by measuring of TMPyP SERS spectra using six excitation wavelengths (excitation profile). Results show that SERS intensity of TMPyP is correlated with the extinction spectrum of the system Au surface/TMPyP although the position of the maximum of intensity differs for particular vibrational modes depending on the molecular resonance. Maximal enhancement is obtained for 568.2 nm excitation that provides LOD of TMPyP 2×10^{-8} M.

Keywords: SERS, SERS microspectroscopy, immobilized gold nanoparticles, porphyrins, excitation profile

CONTENT

1. INTRODUCTION

Identification and structural characterization of molecules play an important role in many biophysical and biochemical applications. Therefore, vibrational spectroscopic techniques are of particular interest because they provide a high degree of molecular structural information. One of them is Raman spectroscopy based on inelastic scattering (Raman scattering, RS) of light by molecule. Although RS is very weak, a strong signal enhancement can be observed if the analyte molecule is adsorbed to a metal surface consisting of structures of subwavelength (nanometer) dimensions. This effect known as surface-enhanced Raman scattering (SERS) was discovered in 1974 by Fleischmann et al. [1] who observed intense Raman spectra of pyridine adsorbed to a roughened silver electrode. Two independent groups of researchers explained observed enormous RS enhancement (10^5 to 10^6): Jeanmaire and Van Duyne [2] proposed an electric field enhancement mechanism whereas Albrecht and Creighton [3] suggested that interaction of the molecule with the surface might be responsible. Nowadays we know that both mechanisms account for SERS effect.
After almost 40 years of SERS research, SERS has been used for detection of a large number of molecules in low concentration and became even suitable technique in bioanalytical and biomedical research [4, 5].

1.1 SERS mechanisms

SERS enhancement is the product of two contributions: electromagnetic and chemical (molecular) enhancement mechanism. As their names imply, electromagnetic mechanism is related to the enhancement of local electromagnetic field whereas chemical (molecular) mechanism is related to the changes in the electronic structure of the molecule upon adsorption. For more details of theory of both mechanisms see e.g. ref. 4 – 10.

Electromagnetic mechanism

When an electromagnetic field interacts with a metal surface, it may excite localized surface plasmons (LSP) on the metal. LSP are understood as collective charge density oscillations which are not propagating (are localized e.g. on the surface of a spherical particle). Resonant excitation of LSP (localized surface plasmon resonance, LSPR) results in a strong enhancement of the electromagnetic fields near to the surface. In the case of SERS, resonant condition is fulfilled for both the incident and scattered field and consequently enormous enhancement (above 10^5) of RS intensity can be reached.

The simplest model to understand the concept of electromagnetic enhancement is to consider a spherical nanoparticle in an external environment. Using the Mie scattering theory for the small sphere, many features of electromagnetic mechanism can be explained including (i) the need of a metal roughened (5 to 200 nm scale) surface, (ii) the observation that different metals have their LSPR in different regions (e.g. the noble metals have the LSPR conditions satisfied at the visible region), (iii) not necessarily a direct contact of the molecule with the surface (even if the intensity decays as r^{-12} with the increasing distance r of the molecule from the surface), (iv) existence of surface selection rules leading to different SERS intensities of vibration modes according to their orientation relative to the metal surface.

LSPR and, hence, the electromagnetic field enhancement depends on the size and shape of the metal nanostructures. SERS is generally measured from assemblies of different nanostructures (such as nanoparticles aggregates) and thus enhancement factor (EF) is an average of EFs for huge number of molecules at different situations (adsorption state, surface morphology, etc.). Typical experimental values of EFs are in the range of 10^4 to 10^6 but theory predicts much stronger EFs in some cases. For example, when two particles are brought together close enough (~ 1 to 2 nm), EF of ~ 10^{11} can be obtained for molecules residing between the particles due to coupled plasmon resonance at the gap. Location with such enhancement is usually referred to a “hot spot”. Only selective excitation of such “hot spots” leads to experimental EF ~ 10^7 to 10^{11} allowing spectral detection in single molecular levels (single-molecule SERS). EF value of 10^4 to 10^6 in ordinary SERS systems is obtained as an average from a few highly enhancing “hot spots” and many weak enhancing sites.

Chemical (molecular) mechanism

Although the electromagnetic mechanism is dominant SERS enhancement effect, other weaker enhancement mechanism (contributing by factor 10^1 to 10^2 to total enhancement) should be considered in some cases. It is known that this mechanism is based on the modification of the polarizability of adsorbed molecule interacting with the metal surface (even flat one) but its origin is still controversial. It involves various explications such as perturbation of electronic structure of adsorbate, formation of new electronic states, indirect transitions, charge transfer (CT) between metal and adsorbate due to adsorbate-metal bonding interactions, etc. The most popular model is CT process when the exciting light is in resonance with a CT transition of adsorbate-surface complex. Due to the interaction between molecule and metal, SERS lines can be slightly shifted in frequency and changed in line width compared to a free molecule.

It is necessary to emphasize that CT mechanism is a resonance effect and should be distinguished from surface-enhanced resonance Raman scattering (SERRS). SERRS is considered as a case when the exciting light is in resonance with LSP of metal and simultaneously in resonance with electron transition of adsorbed molecule (which is considered to be a free one).

1.2 SERS-active substrates

Since the discovery of SERS, a broad variety of substrates have been prepared and tested for SERS spectroscopy. Silver and gold are the dominant metals but SERS has been reported on a few others (e.g. alkali metals, platinum and transition metals) as well [11]. Although gold provides generally lower SERS enhancement than silver, the former one is particularly attractive for biological and biomedical applications because of its long-term stability and biocompatibility. SERS-active substrates commonly employed are metal electrodes, island films, colloidal nanoparticles (NP) and highly ordered nanostructures (prepared by self-assembling, lithography and nanoimprint and other methods) [5, 6, 10, 12, 13]. Ideal SERS-active substrate for bioanalytical applications should (i) possess high Raman enhancement, (ii) be uniform so that the SERS signal does not deviate remarkably over the whole surface, (iii) provide good spectral stability and reproducibility, (iv) be clean enough to study

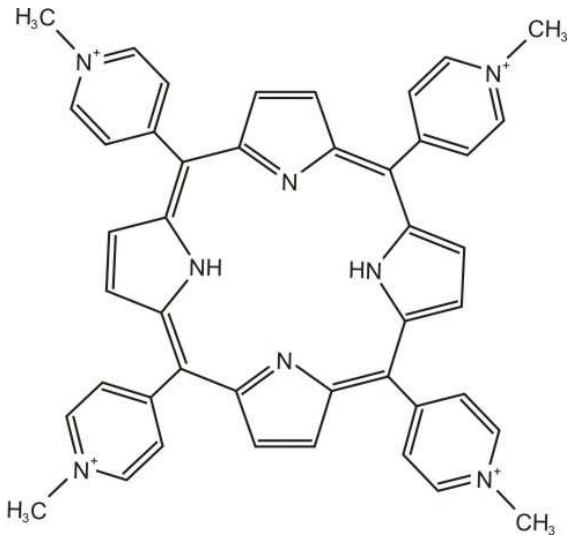
even weak and/or unknown adsorbates. It is difficult to obtain SERS-active substrates that simultaneously meet all these requirements.

In this thesis, we used metal colloidal NP immobilized onto glass plates. Colloidal NP (usually silver or gold) of the 10 to 80 nm size range provide large SERS enhancement. They are simply prepared by chemical reduction or laser ablation and used either in a colloidal solution or "dry" on a solid support. Size and shape of colloidal NP, and so the surface plasmon resonance, depend on a variety of factors, such as used metal, reduction agent, temperature, stabilizing agents and concentration of metal ions. We can prepare, by appropriate procedure, a colloid consisting of NP of narrow and homogeneous size distribution (determined by surface plasmon extinction (SPE) or electron microscopy). The serious drawback of all metal colloidal solutions is their instability because they suffer from hardly controlled aggregation (spontaneous or after addition of an adsorbate) which leads to SERS spectral irreproducibility. Therefore colloidal NP immobilized to different surfaces started to be fabricated. They connect advantages of metal colloids with stability and reproducibility of solid substrates. The first mention of these substrates appeared in [14, 15]. According to [16], a glass plate is dipped into a solution of organosilane that binds through siloxane bonds to the plate and forms a self-assembled monolayer (SAM) on it. The silanized substrate is then able to bind metal NP when immersed in a colloid. Preparation procedures may differ in many factors, e.g. in used metal colloid, in used organosilane and its concentration, in duration of particular steps of preparation and in other special treatments of substrates. By using a colloid with narrow distribution of NP sizes we obtain a homogeneous and reproducible SERS-substrate suitable for analytical applications and quantitative measurements. Usually silver or gold NP immobilized to silanized glass plates are used (hereafter ImAgNP or ImAuNP). Although SERS spectra on ImAuNP and/or ImAgNP of different molecules, including pyridylethylen [14], mercaptoundecanol [15], benzoic acid [17], aminothiophenol [18], hydroxythiophenol [19] and crystal violet [20] have been obtained, their application to biologically important molecules are rare.

1.3 SERS of biomolecules

Biomolecular and biomedical applications of SERS and SERRS are summarized in several books and reviews [e.g. 4, 5, 21, 22, 23, 24]. Numerous biomolecules provide sufficiently good SERS signal that can serve for their identification, quantification and/or determination of their state, like conformation or complex formation. The problem to detect weakly or non SERS-active species can be resolved by labeling or derivatized chemistry. An important advantage of SERS is its high sensitivity. SERS spectroscopy allows even single molecular detection in some special cases but it suffers from strong spectral irreproducibility, decomposition of studied molecule and simultaneous detection of surface contaminants. On the other hand, for quantitative chemical analysis or biosensing, highly reproducible substrates formed by regular nanostructures with lower average sensitivity should be used.
Interesting advantages brings also SERRS. In addition to higher intensity, resonance Raman scattering (RRS) spectroscopy allows selective excitation of chromophoric molecules which are in resonance with exciting field. Thus, SERRS allows study of chromophors (e.g. porphyrins) in complexes with large biomolecules (proteins). As a further advantage, fluorescence, which can make RRS spectroscopy extremely difficult, is in SERRS quenched by nonradiative energy transfer from molecule to metal. However, a disadvantage of SERS arises from the fact that molecular conformation and structure can be changed upon the adsorption onto the metal surface. For instance denaturation of DNA and proteins or metalation of porphyrins (incorporation of a metal ion into the porphyrine core) occurs.

In this theses, we used porphyrins as model biomolecules. Porphyrins, substituted derivatives of porphins, heterocyclic macrocycles consisting of four pyrrole rings joined together by four methine groups, are biomolecules essential for life. The most important among them are porphyrins with central metal atom, referred to as metaloporphyrins (e.g. heme with central Fe atom, chlorophyll with Mg atom). A porphyrin without central metal atom is called free-base (see Scheme 1 for an example of chemical structure of a free-base porphyrin). Synthetic porphyrins are being used in various branches of molecular biology and medicine. Their applications include photodynamic therapy of cancer, antiviral therapy, specific sensing of DNA sequences, selective cleavage of nucleic acids and transport of oligonucleotides into the cells [25].



Scheme 1: Chemical structure of 5,10,15,20-tetrakis(1-methyl-4-pyridyl) porphyrin.

In ultraviolet-visible (UV-Vis) absorption spectroscopy porphyrins exhibit a strong absorption at 400 nm (Soret band) and several weaker maxima in the region from 450 to 700 nm (Q-bands). Strong fluorescence of porphyrins makes difficult to apply RRS spectroscopy and furthermore, many of them aggregate under high concentrations which do not correspond to conditions of their natural incidence and applications in medicine. Hence, sensitive SERRS spectroscopy that quenches potential fluorescence seems to be convenient to study porphyrins.

However, in the case of free-base porphyrins studied usually on silver colloids, the metalation occurs and two porphyrin forms, free-base and metalated, contribute to SERS spectrum. Proportion of both forms is time dependent and thus leads to irreproducibility of SERS spectra. Although it is possible to prevent free-base anionic porphyrins from metalation by using molecular spacers [26], it seems to be difficult for cationic porphyrins [27]. On the other hand, metalation kinetics serves as a sensitive characteristic of the SERS-active system [28, 29] and this fact can be exploited to study porphyrin aggregates, stability of Ag colloid [30] and interaction of porphyrins with nucleic acids [31].

2. AIMS OF THESIS

The main goal of this thesis was to optimize SERS spectroscopy for study of biologically important molecules.

We focused on ImAuNP which combine advantages of metal NP (narrow distribution of NP sizes) and solid substrates (stability and reproducibility of the substrates and subsequently of SERS spectra) and in addition to that they are attractive for biosensing applications. The aim was to optimize the preparation procedure to provide stable, uniform and highly reproducible SERS-active substrates. We studied the influence of the used metal NP, used organosilane and "drying treatment" of substrates.

Porphyrins have been chosen as model biomolecules because they are suitable for SERS study and are commonly used in medical applications. In the first part of experiments, conducted by using classical Raman spectrometer in macro-mode, we tested cationic 5,10,15,20-tetrakis(1-methyl-4-pyridyl) porphyrin (TMPyP) and anionic 5,10,15,20-tetrakis(4-sulfonatophenyl) porphyrin (TSPP) on ImAuNP. The aim was to characterize the systems by detection limits as well as by time and concentration dependences of SER(R)S spectra.

In the second part of experiments we turned our attention to SERS microspectroscopy of TMPyP on ImAuNP. Higher spatial resolution of confocal Raman microspectrometer gave us the opportunity to characterize the system by surface mapping. The aim was to optimize SERS spectroscopy from the point of view of the optimal soaking time and concentration of TMPyP for further SERS studies. This optimization was based on the measuring of soaking time and concentration dependences of SERS spectra. Dependence of SERS intensity on excitation wavelength is a key point for optimizing electromagnetic enhancement of particular SE(R)RS system. Thus, further study included measuring of SE(R)RS and (R)RS excitation profile of TMPyP and their correlation with SPE spectra.

3. RESULTS AND DISCUSSION

This chapter summarizes results from five papers (3 published, 1 accepted and 1 submitted) in international impacted journals. All of them are attached as a part of this thesis (supplements [I – V]).

3.1 Preparation and characterization of ImAuNP

First let us briefly remind the preparation of ImAuNP. A clean glass substrate is dipped into a solution of organosilane that forms a SAM on it. Each silanized substrate is then separately immersed into Au colloidal suspension that results in binding of Au nanoparticles (AuNP) to terminal functional groups of organosilane. Some of the substrates can be left to dry in an oven during different steps of the preparation (discussed below).

To optimize the substrates preparation we studied influence of the used organosilane, used metal colloid and drying treatment on the substrates properties. We tested two types of organosilanes: 3-mercaptopropyltrimethoxysilane (MPTMS) and 3-aminopropyltrimethoxysilane (APTMS), and three types of metal colloids: citrate-reduced (c.-r.), borohydride-reduced (b.-r.) and laser-ablated (l.-a.) ones. The substrates were characterized by their SPE spectra, Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM) images. Results summarized in this section are described in detail in [II] (a SEM image is taken from [III]).

For tested organosilanes we estimated that the efficiency to bind AuNP from metal colloid is almost 100% for APTMS whereas it is less than 20% for MPTMS. It can be explained by a strong tendency of MPTMS to be air-oxidized leading to decrease of the number of their functional –SH groups which can bind AuNP. On the contrary, protonated functional $–NH_2$ group of APTMS is stable and can electrostatically bind AuNP covered by surface anions (in the case of chemically-reduced colloids). Therefore, we used APTMS for preparation of all ImAuNP for further SERS study.
Our results show that all three types of AuNP (c.-r., b.-r. and l.-a.) can be attached to silanized substrates. While both chemically-reduced colloids were very stable during

immobilization, l.-a. colloid was extremely unstable. The reason is the absence of stabilizing anions on the l.-a. AuNP surface leading to strong aggregation of the colloid. Therefore, l.-a. colloid was excluded from routine preparation of Au substrates.

Stability and reproducibility of chemically-reduced ImAuNP were monitored by their SPE spectra. Although SPE spectra are nearly same when measured from various spots of particular substrate, they are often different from substrate to substrate (Fig. 1). Au substrates prepared from b.-r. colloid exhibit one SPE maximum at 520 – 530 nm and vary mostly in the extinction value (Fig. 1, left). On the other hand, two types of substrates were prepared from c.-r. colloid (Fig. 1, right): (i) the first type with single SPE maximum at 520 – 530 nm was of red colour, (ii) the second type consisting of two overlapping bands with SPE maxima at 520 – 530 nm and 600 – 650 nm were substrates of grey-violet colour. The extinction band at 520 – 530 nm corresponds to the band of the initial nonaggregated colloid and thus can be attributed to the extinction of isolated AuNP while the band at 600 – 650 nm results from the interaction of AuNP forming aggregates. Moreover, the substrates prepared from c.-r. colloid characterized by single extinction band convert spontaneously into the latter ones within short periods (minutes or hours).

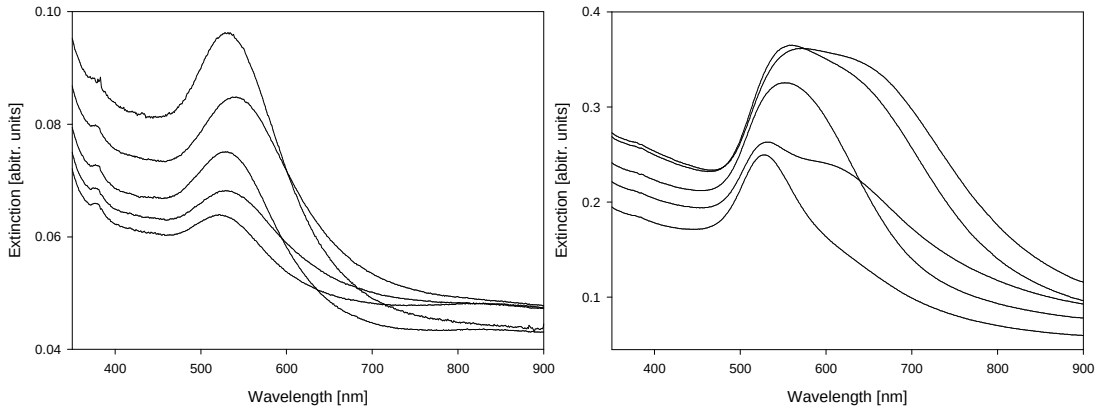


Fig. 1: Typical SPE spectra of substrates prepared from b.-r. AuNP (left) and c.-r. AuNP (right).

Au substrates prepared from both chemical colloids were characterized also by AFM (typical images are shown in Fig. 2). In the case of substrates prepared from c.-r. colloid we obtained images only for the second type of substrates due to time instability of the first type mentioned above. Scanning of the whole surface and comparing several substrates confirmed results from SPE, i.e. very good uniformity

of each substrate. AFM images show compact coverage of both surfaces by AuNP of varying diameter from ~ 30 to 100 nm and presence of small aggregates of hundred nanometers sizes. In the case of c.-r. AuNP the amount of larger nanoparticles and aggregates is though higher. It is also obvious that while b.-r. AuNP are rather isolated, c.-r. AuNP are more closely spaced leading to stronger interaction of AuNP and thus to formation of aggregates. As a result, the second extinction band in SPE spectra measured from c.-r. ImAuNP occurs.

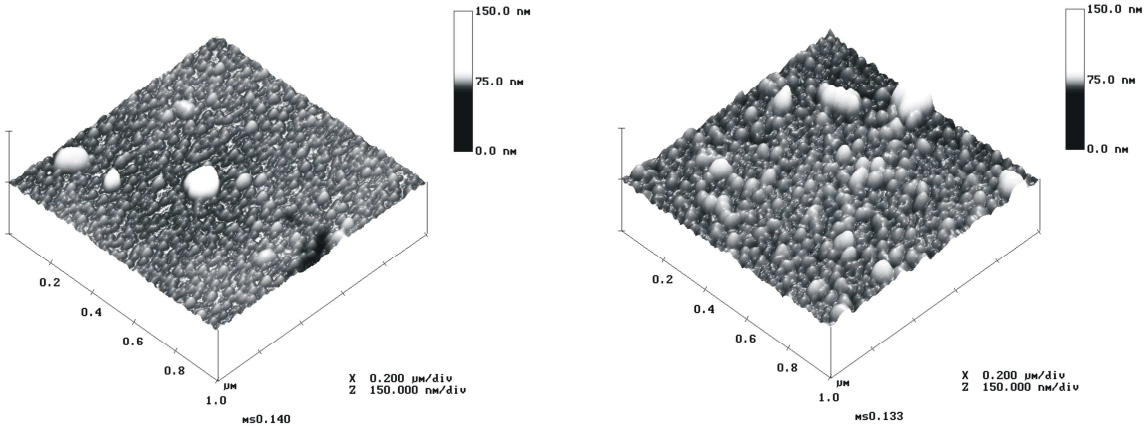


Fig. 2: Typical AFM images of substrates prepared from b.-r. AuNP (left) and c.-r. AuNP (right).

Results obtained from AFM for substrates prepared from c.-r. AuNP were confirmed also by SEM. Typical SEM image (again of the second substrate type, Fig. 3) shows very good uniformity and compact coverage of the substrate by c.-r. AuNP of ~ 30 to 100 nm size and by small aggregates.

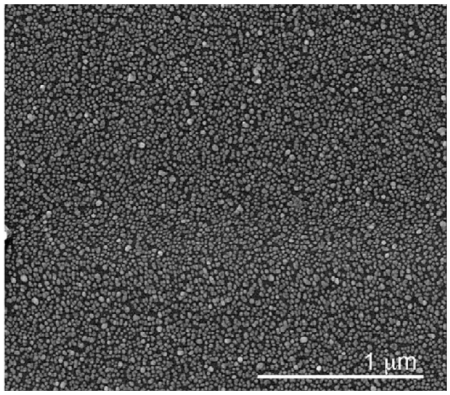


Fig. 3: Typical SEM image of a substrate prepared from c.-r. AuNP.

Due to the bad reproducibility of substrates prepared from c.-r. colloid (two types of substrates and instability of the first type) we tried to improve their preparation in order to increase their stability and reproducibility. We studied the influence of drying treatment on the substrates properties and tested four preparation procedures: (a) without any drying, (b) drying after immobilization of AuNP, (c) drying after silanization and (d) both drying steps (b and c). As mentioned above, two substrates types can be fabricated by procedure (a). Procedure (c) causes broadening of the extinction band at 520 – 530 nm and finally, procedures (b) and (d) always lead to appearance of the second extinction band at 600 – 650 nm and to good stability of substrates during their aging. We found out that the most important step is thus drying after immobilization of AuNP. Very probably high temperature increases mobility of $–NH_2$ groups of the silane that allows movement of AuNP and simultaneously reduces hydration of the citrate anions adsorbed on the AuNP surface. It results in closer interaction of neighboring AuNP leading to stabilization of the structure. However, exact positions, widths and intensities of the longer-wavelength bands as well as the overall extinction can differ from preparation to preparation like it is known for colloids themselves.

The preparation procedure of c.-r. ImAuNP has been optimized to produce stable SERS-active surfaces with uniform distribution of AuNP. The main advantage of this method is that AuNP are not strongly aggregated and thus provide reproducible SERS signal (discussed below). Immobilization of Au colloidal nanoparticles *via* aminosilane to glass support is an easy and cheap way to fabricate SERS-active substrates on a large scale.

3.2 Optimization of experimental conditions, sensitivity and reproducibility of ImAuNP studied by using macro-Raman spectrometer

Prior to SERS study of porphyrins, all three kinds of ImAuNP (c.-r., b.-r. and l.-a.) were tested as SERS-active substrates [II]. For this purpose, cationic TMPyP was chosen due to its known easy adsorption on both chemically-reduced colloids. Following experiments were done by using classical Raman spectrometer with common 514.5 nm excitation wavelength; results are summarized from [I, II, III].

The 514.5 nm laser line falls into the Q-band of TMPyP at ~ 518 nm and therefore we obtained its SERRS spectra.

Good SERRS spectra of TMPyP were obtained from all three types of ImAuNP showing the same positions and relative intensities of Raman bands. SERRS nature of the spectra was demonstrated by an experiment in which no Raman signal was obtained from a drop of TMPyP on a pure glass slide without AuNP keeping the same SERS experimental conditions. From the spectra exhibiting typical Raman bands corresponding to vibrations of the free-base porphyrin macrocycle at 965, 1000, 1332, 1362 and 1556 cm^{-1} is evident that gold does not metalate free-base TMPyP.

Even though SERRS spectra obtained from l.-a. AuNP are of a good quality, the l.-a. colloid is extremely unstable and therefore these substrates were excluded from further SERS study. Concerning ImAuNP prepared from chemically-reduced AuNP, we decided to use the c.-r. ones due to their better SERS enhancement resulting probably from higher surface coverage and due to broader region of suitable excitation wavelengths. Thus, all further SERS study was done on the c.-r. ImAuNP.

Further, two kinds of porphyrins were studied: cationic TMPyP and anionic TSPP. Both porphyrins showed very good adsorption on ImAuNP. We again were interested in time stability and reproducibility of the SERRS signal and in estimation of detection limits. We also measured dependences of the spectra on the soaking time and soaking concentration of the porphyrins from which we could estimate their optimal adsorption time and covering concentration limit. Following results are summarized from [I] and [III].

Excellent spectra of high intensity and good signal-to-noise ratio were obtained for both TMPyP and TSPP (Fig. 4, spectra a) in wide concentration range although strong Raman signal of glass should be subtracted from low-wavenumber part of spectra. Comparison of our SERRS spectra with the well-known free-base (~ 330, 960 + 1000, 1330 + 1360, 1550 cm^{-1}) and metalated form (~ 395, 1010, 1340, 1540 cm^{-1}) markers, as well as with the RRS spectra (Fig. 4, spectra b) clearly proves that we obtained spectra from the free-base forms of both porphyrins. It means that Au substrates do not change their structure and are thus suitable for their SERS spectroscopy. Tens of measurements revealed again excellent uniformity and time

stability of the Au substrates as well as very good reproducibility of the SERRS spectra for both porphyrins.

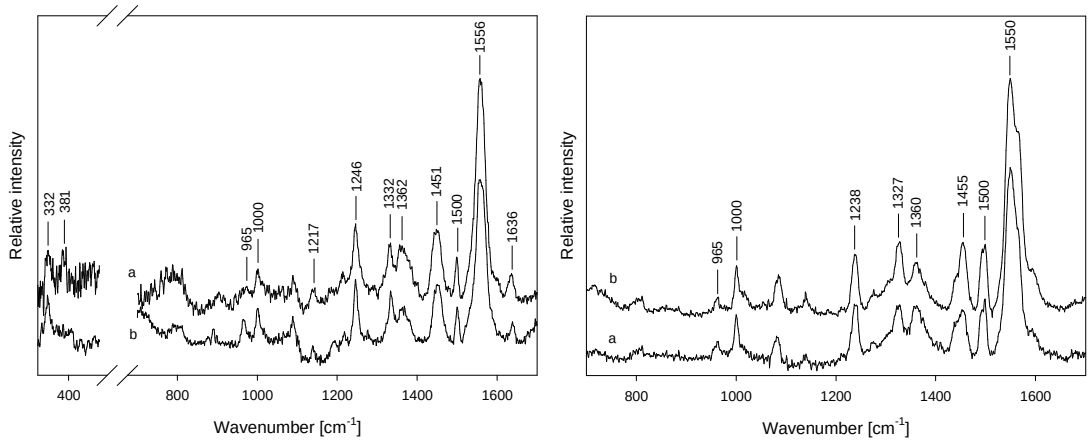


Fig. 4: (Left) SERRS spectrum of 1μM TMPyP (a) and RRS spectrum of 1 mM TMPyP (b). (Right) The same for TSPP. The baseline was corrected and the Raman signal of glass subtracted.

Dependences of the SERRS signal on the soaking time were measured for 1μM soaking concentration of both porphyrins from 1 to 60 minutes. Dependences on the soaking concentration were measured for 20 minutes soaking time in 1×10^{-7} to 1×10^{-4} M range for both porphyrins. Intensity was determined as the first coefficient of factor analysis (FA) of the spectral set. Results from FA (Fig. 5, the soaking time dependences on the left, the concentration ones on the right) show that the SERRS signal increases up to value of 15 to 20 minutes in time dependences, resp. of ~ 1×10^{-5} M concentration in concentration dependences for both porphyrins. These values should correspond to an optimal soaking time and to a covering limit of the porphyrin molecules on the Au substrate, although we expect lower real porphyrin concentration at the surface. However, while TMPyP shows typical saturation behaviour of both dependences, the SERRS signal of TSPP substantially decreases for longer soaking times and higher concentrations than the optimal values. This decrease can be interpreted as a depolarization effect of TSPP molecules or as a result of adsorption-desorption equilibrium on the Au substrate. Absence of this effect for TMPyP may indicate different adsorption behaviour very probably due to the different charge.

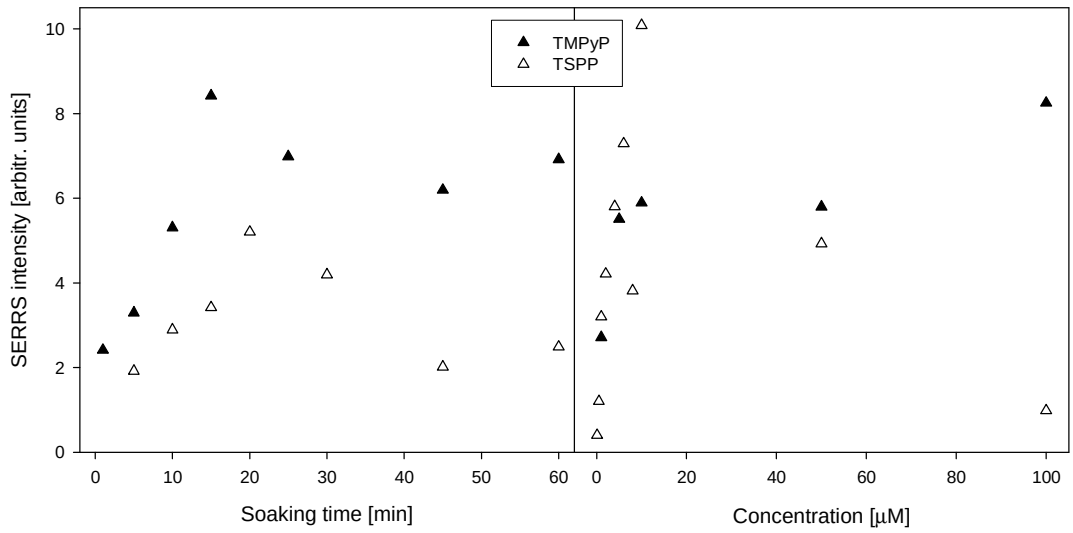


Fig. 5: (Left) SERRS intensity of TMPyP and TSPP versus soaking time (1 μM porphyrin concentration). (Right) SERRS intensity of TMPyP and TSPP versus soaking concentration (20 min soaking time).

SERS sensitivity of Au substrates can be precisely characterized by limit of detection (LOD). LOD was estimated by extrapolation to concentration when intensity of the strongest porphyrin band at ~ 1550 cm^{-1} exceeds triple of the blank signal standard deviation. In our case, the LODs are 5×10^{-8} M and 3×10^{-8} M soaking concentration for TMPyP and TSPP, respectively.

Comparing of SERRS spectra obtained from tens of measurements showed their very good reproducibility, uniformity over the surface (in mm-scale) and stability in time. Relative standard deviation (RSD) of signal from tens of experiments is 4.2%. Time stability was proved by measurement of SERS spectra from fresh samples and 3 days-old samples, RSD of signal is 6.9%. Both RSDs demonstrate that the Au substrates are very uniform and can be stored with adsorbed TMPyP for several days without losing the SERRS signal.

3.3 Optimization of experimental conditions, sensitivity and reproducibility of ImAuNP studied by using micro-Raman spectrometer

In previous section we discussed sensitivity and reproducibility of ImAuNP tested by TMPyP and TSPP and detected by using macro-Raman setup. In this part we turned our attention to SERS study of TMPyP by using a confocal Raman microspectrometer and two excitations (514.5 and 632.8 nm) [IV, V]. Since 632.8

nm excitation falls out of the electronic absorption of TMPyP, we used the SE(R)RS nomenclature to name both SERRS spectra measured with 514.5 nm and SERS spectra measured with 632.8 nm excitation wavelength.

Optimal experimental conditions have been determined using 514.5 nm excitation like in the case of macro-Raman measurements. Soaking time dependences of SERRS signal were monitored for four fixed TMPyP concentrations (1, 5, 10, 30 μM), each from 1 to 60 minutes soaking time (Fig. 6, left). Dependences of SERRS intensity on soaking concentration were measured for four fixed soaking times (15, 20, 25, 30 min), each in range from 1×10^{-7} M to 3×10^{-5} M TMPyP concentration (Fig. 6, right). SERRS intensity was determined by an integral intensity of the strongest Raman band at ~ 1550 cm^{-1}. From the dependences we managed to determine an optimal soaking time of 25 to 30 minutes and a covering limit of ~ 1×10^{-5} M soaking concentration of TMPyP molecules on the surface. The value of the covering limit is roughly the same like we detected in previous macro-Raman measurements mentioned above, only the optimal soaking time is slightly longer than the previous one.

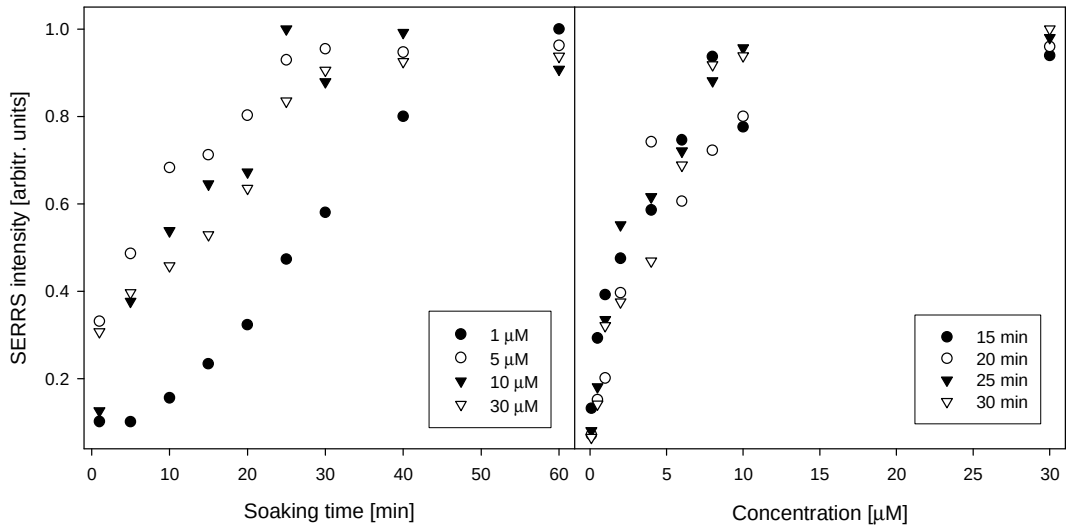


Fig. 6: Optimization of SE(R)RS measurements: dependences of TMPyP SE(R)RS intensity on soaking time (left) and TMPyP soaking concentration (right).

Thus, under changed experimental conditions, the optimal values for further SERS study of TMPyP on our Au substrates are 30 min soaking time and 1×10^{-5} M soaking concentration.

We determined the LOD of TMPyP from SE(R)RS spectra measured in range from ~ 6×10^{-8} M to 1×10^{-5} M concentration of TMPyP soaking solution by the same method mentioned above. The LODs are 6.5×10^{-8} M and 4×10^{-8} M TMPyP soaking concentration for 514.5 nm and 632.8 nm, respectively. They are comparable to each other and also to the LOD of TMPyP obtained from macro-Raman measurements mentioned in the previous section.

Good uniformity and spectral reproducibility was revealed in mm-scale by measurements of TMPyP spectra from various random places of the Au substrate (Fig. 7). RSD of signal is 15.3 %.

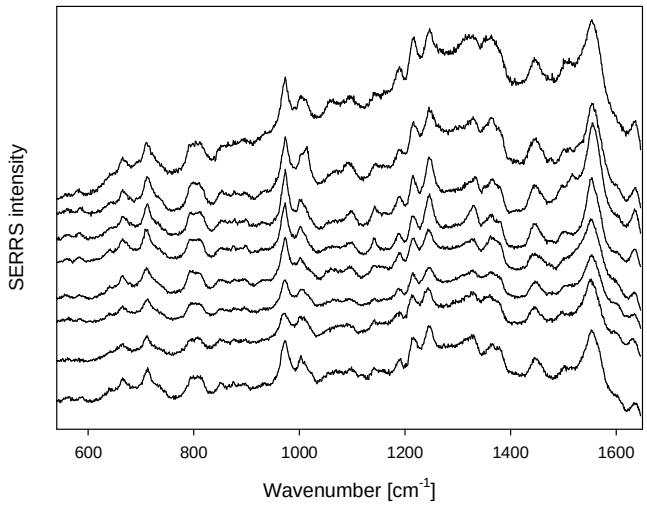


Fig. 7: Eight SERS spectra of TMPyP measured from various random places of the particular Au substrate, 5×10^{-6} M TMPyP, 632.8 nm excitation.

The study in μm-scale was done by spectral mapping of the Au surface with 2 and 20 μm steps between mapping points. After treatment by FA, SE(R)RS spectral maps of TMPyP (Fig. 8, 2 μm steps) were obtained for 514.5 nm (left) and 632.8 nm (right) excitations. Excellent spectral reproducibility was detected for 514.5 nm excitation when only slight intensity variation is observed. However, by using the 632.8 nm excitation, the AuNP aggregates very probably containing hot spots are excited because the intensity jumps 5 to 10 times in a few points.

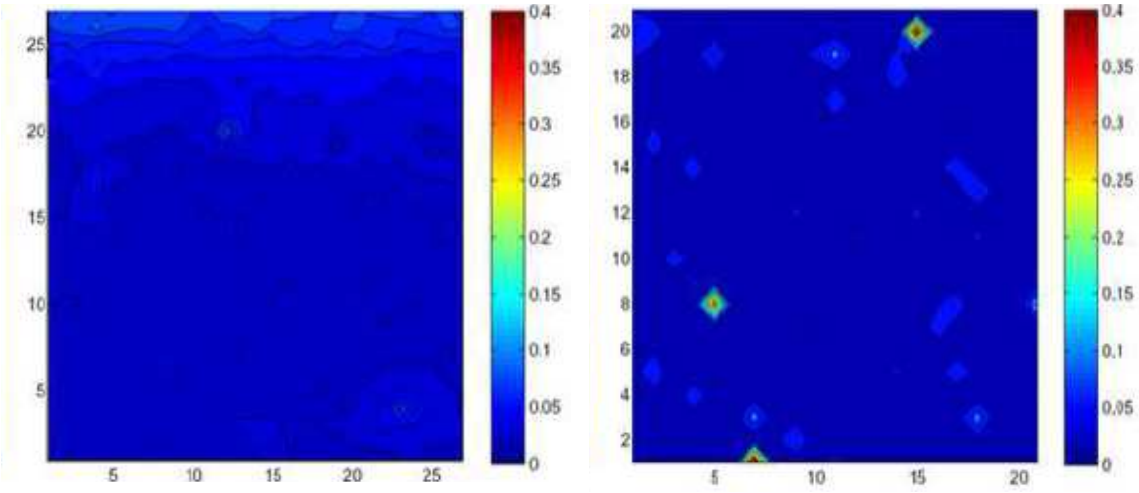


Fig. 8: Spectral maps of TMPyP (1μM soaking concentration) on Au substrate, 2 μm steps between mapping points: excitation 514.5 nm (left) and 632.8 nm (right). Numbers on the axes represent the mapping points; the scale represents the relative intensity.

Comparable LODs of TMPyP and spectral reproducibility of Au substrates were found out by both macro- and micro-Raman measurements. Although macroscopic approach allows better quantitative analysis due to limitation of the effects of hot spots, micro-Raman technique has some advantages as well including shorter collection times and the absence of strong Raman bands of the glass support. Practical implementations of SE(R)RS detection are expected to rely on substrate architectures which are uniform over at least square millimeter-sized areas. Uniformity of SERS substrates given by RSD ~ 15% is sufficient for this purpose and thus ImAuNP can be considered as suitable for SE(R)RS (bio)analytical applications.

3.4 Excitation profile of free-base TMPyP on ImAuNP

Our further study included measuring of SE(R)RS and (R)RS excitation profile (dependence of the spectral intensity on excitation wavelength) of TMPyP using a confocal Raman microspectrometer with six excitation wavelengths (457.9, 488, 514.5, 530.9, 568.2 and 647.1 nm) [V]. Optimal soaking time and porphyrin soaking concentration are very important for SERS excitation profile measurement from the point of view of the maximal SERS signal of adsorbed porphyrin on the Au substrate. Thus, we prepared TMPyP on ImAuNP under detected optimal conditions (section 3.3), i.e. 30 min soaking time and 1×10^{-5} M soaking concentration of the

porphyrin. (R)RS excitation profile was measured from a drop of TMPyP of 1×10^{-3} M concentration dried on a pure glass.

We were also interested in relation between the SE(R)RS excitation profile and corresponding SPE spectrum of the system.SE(R)RS excitation profile was measured under all six excitations. However, we were not able to obtain (R)RS spectrum of TMPyP for 647.1 nm excitation line due to strong fluorescence background. Comparing of (R)RS and SE(R)RS spectra measured for particular excitation indicates mostly flat orientation of the porphyrin core on the surface as well as some deformation of the porphyrin macrocycle during adsorption. (R)RS excitation profile of TMPyP together with electronic absorption spectrum of TMPyP solution is shown in Fig. 9, left. SPE spectrum of TMPyP on ImAuNP and corresponding SE(R)RS excitation profile of TMPyP are shown in Fig. 9, right. In this case, we determined (R)RS and SE(R)RS intensities as integral intensity of whole spectra in 300 – 1700 cm^{-1} region. (R)RS excitation profile shows that the highest intensity is obtained for 457.9 nm (falling into the absorption Soret band of TMPyP at 424 nm) while substantially lower intensity is observed for other excitations (falling into the absorption Q-bands of TMPyP). SE(R)RS excitation profile is correlated with the corresponding SPE extinction spectrum (LSPR): the highest SE(R)RS intensity is obtained for 568.2 nm excitation line. On the other hand, the maximum of SE(R)RS intensity (~ 570 nm) is slightly shifted from the maximum of SPE (~ 545 nm).

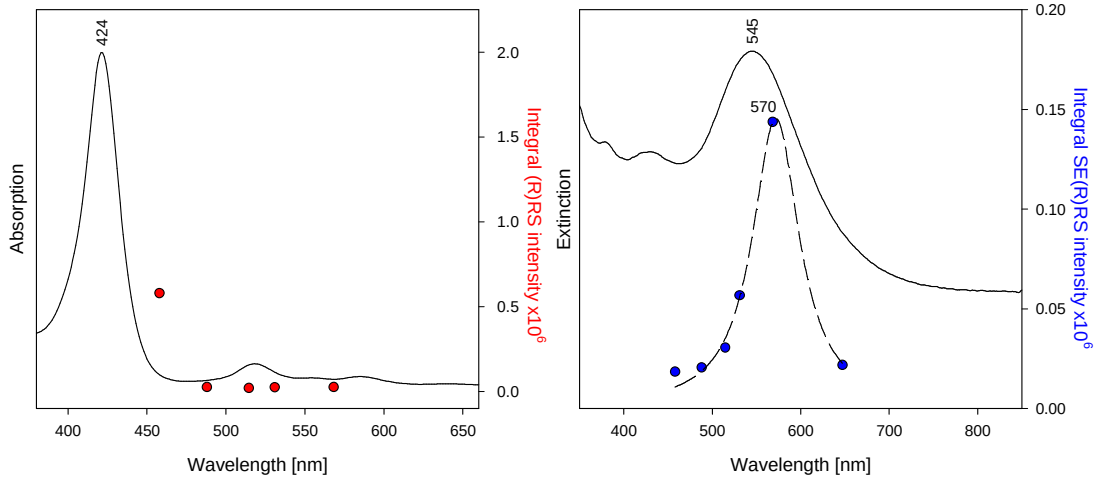


Fig. 9: (R)RS excitation profile of TMPyP and electronic absorption spectrum of TMPyP solution (left). SPE spectrum of TMPyP on ImAuNP and corresponding SE(R)RS excitation profile of TMPyP (right).

Both (R)RS and SE(R)RS spectra show significant spectral changes as a function of excitation wavelength. SE(R)RS excitation profile of TMPyP for particular spectral bands is shown in Fig. 10 (normalized to maximal intensity). There are three groups of spectral bands with different excitation profile: (i) 331, 1245, 1332, 1552 cm^{-1} with excitation profile correlated with SPE, maximum at ~ 570 nm (red shift), (ii) 1000, 1215, 1635 cm^{-1} with excitation profile correlated with SPE, maximum at ~ 530 nm (blue shift) and (iii) 965 cm^{-1} with excitation profile correlated with (R)RS excitation profile of TMPyP. On the basis of vibrational assignment [V], one can see that the spectral bands of particular group with the same behavior (i-iii) include the same vibrational modes: stretching and bending deformations of porphyrin core, all very sensitive to metalation and/or stacking, (ii) stretching and bending deformations of N-methylpyridyl group. The 965 cm^{-1} band (iii) is assigned to stretching deformation of $C_\alpha - C_\beta$ or $C_\alpha - C_m$. We suggest that differences in SE(R)RS excitation profile for three groups of vibrational modes are caused by different contribution of molecular resonance of particular vibrational mode of TMPyP for particular excitation wavelength.

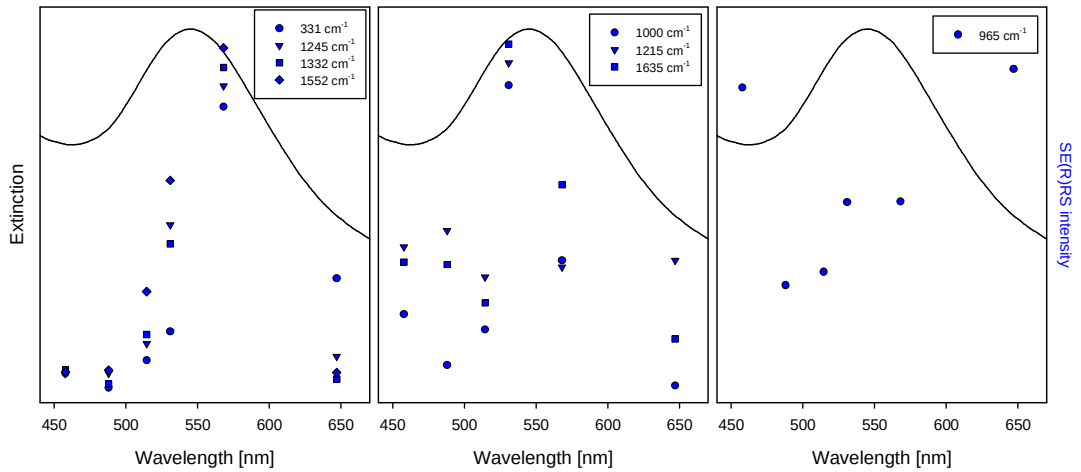


Fig. 10: SE(R)RS excitation profile of TMPyP for particular vibrational modes.

SE(R)RS excitation profile shows that the best SERS enhancement is obtained for 568.2 nm excitation that provides LOD of TMPyP 2×10^{-8} M in soaking solution. This LOD is at least two times smaller than the previous LODs obtained with 514.5 nm and 632.8 nm excitations (see section 3.3).

4. CONCLUSIONS

In the first part of the work we studied influence of different parameters on the preparation of ImAuNP. We proved that immobilization of Au colloidal nanoparticles *via* aminosilane to glass support is an easy and cheap way to fabricate SERS-active substrates on a large scale. We obtained the following results:

1. Aminopropyltrimethoxysilane (APTMS) is more efficient in the process of immobilization of AuNP than mercaptopropyltrimethoxysilane (MPTMS).
2. Laser-ablated colloids are due to their poor stability unsuitable for routine preparation of the substrates.
3. Both chemically-reduced AuNP form stable, uniform and reproducible substrates suitable for SERS spectroscopy. However, in the case of citrate-reduced AuNP, drying of substrates after AuNP immobilization is a needful step to stabilize morphological properties of the substrates.
4. Citrate-reduced ImAuNP are more suitable for SERS spectroscopy than borohydride-reduced ones due to their higher surface coverage and broader region of suitable excitation wavelengths.

In the second part of the work, free-base cationic TMPyP and anionic TSPP porphyrins on c.-r. ImAuNP were studied. Macro-Raman technique with 514.5 nm excitation was used to characterize both systems whereas in the case of micro-Raman setup with different excitations we focused on TMPyP. Main results can be summarized as follows:

1. Macroscopic approach allowed us to obtain excellent SERRS spectra of both porphyrins in wide concentration range in their unperturbed free-base forms. Measurements revealed very good time stability and uniformity of the Au substrates as well as reproducibility of SERS spectra (RSD ~ 4%). For both porphyrins we estimated limits of detection (LOD) ~ 5×10^{-8} M soaking concentration, optimal soaking time ~ 20 minutes and covering limit ~ 1×10^{-5} M soaking concentration.
2. In the case of TMPyP, comparable results were obtained also by using micro-Raman setup with 514.5 nm excitation, except slightly longer optimal soaking time (~ 30 minutes). Our results demonstrated several advantages of

SERS microspectroscopy in comparison to macro-Raman technique: namely the possibility of spectral mapping over the surface, shorter collection times and the absence of strong Raman bands from the glass. Excellent reproducibility of SERS signal was revealed in mm-scale (RSD ~ 15%) as well as in μm-scale done by spectral mapping.

3. SE(R)RS excitation profile of TMPyP measured under the optimal conditions (30 min soaking time, 1×10^{-5} M soaking concentration) and six excitation wavelengths (457.9, 488, 514.5, 530.9, 568.2 and 647.1 nm) is correlated with SPE spectrum of ImAuNP. SE(R)RS excitation profile peak position for particular vibrational mode is slightly shifted from SPE maximum position depending on its molecular resonance contribution (red or blue shift). Only vibrational mode at 965 cm^{-1} fits (R)RS excitation profile of TMPyP but not SPE spectrum of Au surface. The best SERS enhancement is obtained for 568.2 nm excitation that provides LOD of TMPyP 2×10^{-8} M in soaking solution.

Finally, ImAuNP can be considered as suitable substrates for SE(R)RS (bio)analytical applications. Our ImAuNP substrates have been used in studies of photoactive organic molecules (namely zinc and copper phtalocyanines) [32, 33] and adsorption/desorption processes of porphyrins [34]. Interesting results were obtained on ImAgNP which were employed to study TMPyP and water insoluble 5,10,15,20-tetraphenyl porphyrin [35, 36, 37, 38]. It was found out that these substrates, in contrast with silver colloids, do not metalate porphyrins due to presence of silane.

Another application of the Au substrates is shown in [VI] where magneto-optical activity of ferromagnetic thin films near the Au surface was studied. A great magneto-optical response is observed when localized surface plasmons of the substrate are in resonance with electronic transition in the films compared to films on a bare glass substrate.

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[I] M. Procházka, **N. Hajduková**, J. Štěpánek: Surface-enhanced resonance Raman scattering of porphyrins on gold nanoparticles attached to silanized glass plates, *Biopolymers* **82** (2006), 390-393.
Author's contribution: majority of experimental work and participation on writing of paper.

[II] **N. Hajduková**, M. Procházka, J. Štěpánek, M. Špírková: Chemically reduced and laser-ablated gold nanoparticles immobilized to silanized glass plates: Preparation, characterization and SERS spectral testing, *Colloids and Surfaces A: Physicochem. Eng. Aspects* **301** (2007), 264-270.
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Author's contribution: part of experimental work.

[V] **N. Hajduková-Šmídová**, M. Procházka, M. Osada: SE(R)RS excitation profile of free-base 5,10,15,20-tetrakis(1-methyl-4-pyridyl)porphyrin on immobilized gold nanoparticles, *submitted to Vibrational Spectroscopy.*
Author's contribution: majority of experimental work and writing of paper.

[VI] M. Osada, **N. Hajduková**, K. Akatsuka, S. Yoguchi, T. Sasaki: Gigantic plasmon resonance effects on magneto-optical activity of molecularly-thin ferromagnets near gold surface, *submitted to Journal of the American Chemical Society.*
Author's contribution: part of experimental work (preparing of the Au substrates).

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1. M. Procházka, **N. Hajduková**, E. Kočišová, J. Štěpánek „SERS of biomolecules on gold nanoparticles attached to silanized glass plates“, poster, *XXVII European Congress on Molecular Spectroscopy,* Krakow, Poland, 5-10 September 2004, abstract in Proceedings (M. Handke, M. Hasik, C. Paluszkiewicz, Eds.), Wydawnictvo Naukowe „Akapit“, Krakow, p. 304.

2. M. Procházka, **N. Hajduková**, E. Kočišová, J. Štěpánek „Surface-enhanced Raman scattering (SERS) of biomolecules on gold nanoparticles attached to silanized glass plates“, poster, *International Conference Nano'04*, 21-23 October 2004, Brno University of Technology, Czech Republic, Book of Abstracts (P. Šandera, Ed.) p. 55, Proceedings (P. Šandera, Ed.), p. 228-231.

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