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SE(R)RS microspectroscopy of porphyrins on immobilized Au nanoparticles: Testing spectral sensitivity and reproducibility

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ABSTRACT

Although surface-enhanced (resonance) Raman scattering SE(R)RS spectroscopy is currently employed as a (bio)analytical technique, designing and optimizing of cheaply and easily available SERS-active substrates are still in progress. In this paper, we tested SERS substrates prepared by immobilization of Au colloidal nanoparticles via aminosilane on glass from the point of view of their spectral sensitivity and reproducibility. This preparation procedure is easy, cheap and provides uniform SERS substrates on a large scale. We measured SE(R)RS spectra of free-base cationic 5,10,15,20-tetrakis(1-methyl-4-pyridyl)porphyrin (TMPyP) as a testing biomolecule using a confocal Raman microspectrometer and two excitation wavelengths (514.5 and 632.8 nm). SERS microspectroscopy had several advantages 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 support. Analytical enhancement factor (AEF) of Au substrates between 10^5 and 10^6 and limits of detection (LOD) of TMPyP $\sim 5 \times 10^{-8}$ M were determined. Excellent spectral reproducibility of Au substrates (relative standard deviation of signal ~15%) was proved. Immobilized Au colloidal nanoparticles can be therefore considered as suitable substrates for SE(R)RS (bio)analytical applications.

1. Introduction

Interaction of metal nanostructures with electromagnetic radiation of different wavelengths can enhance electromagnetic field depending on the nanostructure and its resonance. In the case of surface-enhanced Raman scattering (SERS), interaction of light with noble metal nanostructures produces large amplification of the electromagnetic field through excitations generally known as plasmon resonances and Raman scattering of adsorbed molecule is enhanced by a factor above 10^5 [1]. Besides this electromagnetic enhancement, chemical enhancement originating from charge transfer between the molecule and the surface takes place in specific cases. The most commonly used metals are silver and gold because both have suitable plasmon resonance frequencies to give effective enhancement with visible excitation [1]. Although gold nanoparticles provide generally lower SERS enhancement than silver ones, they are particularly attractive for biological and biomedical applications because of their long-term stability and biocompatibility [2].

Although recent advances have turned SERS into an extremely sensitive analytical technique for small quantities of molecules (even single molecule) [3], the employed SERS-active substrates are often a serious drawback for routine analytical applications. For these purposes, the substrate should not only be uniform, stable and providing reproducible spectroscopic results but also easily available. Metal colloidal nanoparticles are the most frequent SERS substrates due to their cheap and simple preparation [4], but they suffer from chemical instability and spectral irreproducibility. This problem can be overcome by assembling the nanoparticles into two-dimensional array structures using a self-assembled monolayer (SAM) of bifunctional silane [5,6]. 3-Aminopropyltrimethoxysilane (APTMS) is routinely used to produce stable and highly SERS-active surfaces with regular and uniform distribution of Au nanoparticles [7–12]. Although SERS spectra of different molecules, including pyridylethylen [5], mercaptoundecanol [6], aminothiophenol [9], hydroxythiophenol [10], benzoic acid [11], and crystal violet [8], have been reported, biomolecular applications of these substrates are rare. We previously studied cationic and anionic porphyrins on such substrates using a classical (macro) Raman spectrometer [13,14]. Although manipulation with samples is not easy (reflection geometry has to be used) and SERS spectra of porphyrins are overlapped by a strong Raman signal from the glass support, low limits of detection (LOD) of porphyrins including TMPyP ($3–5 \times 10^{-8}$ M) and excellent spectral reproducibility were obtained [14].

In this paper, we turned our attention to SERS microspectroscopy of free-base 5,10,15,20-tetrakis(1-methyl-4-pyridyl)porphyrin (TMPyP) adsorbed on immobilized Au

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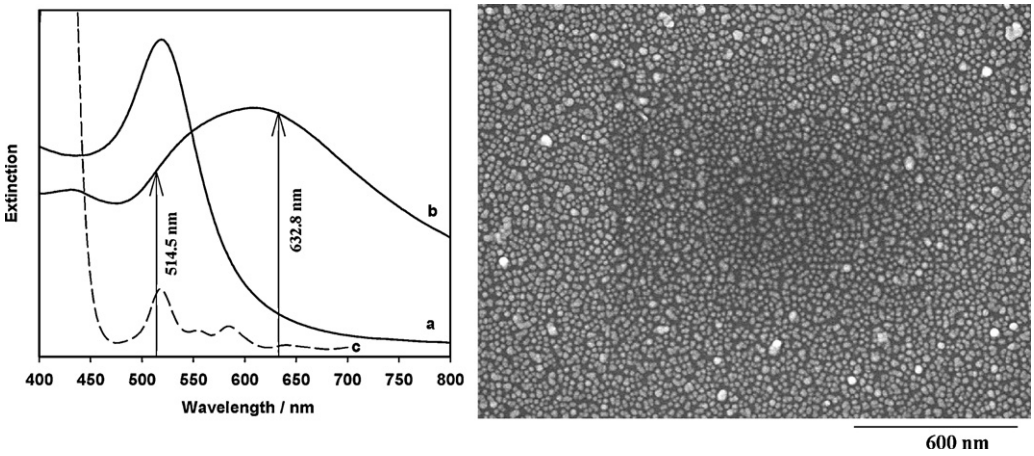

Fig. 1. Left: SPE spectra of the parent Au colloidal solution (a), Au substrate (b) and electronic absorption spectrum of TMPyP solution (c). Excitation wavelengths are marked by arrows. Right: Typical SEM image of Au substrate.

nanoparticles using an integrated Raman microspectrometer with two excitation wavelengths (514.5 nm and 632.8 nm). The 514.5 nm excitation falls into the maximum of electronic absorption Q-band of TMPyP at ~518 nm (Fig. 1) and thus, in this case, spectra of TMPyP are surface-enhanced resonance Raman scattering (SERRS) spectra. We determined the analytical enhancement factor (AEF) of our Au substrates and the limit of detection (LOD) of TMPyP for both excitation wavelengths and compared the LODs with our macro-SERS results [13,14]. Confocal Raman microspectrometer provides the possibility of spectral mapping over the surface giving us information about uniformity and spectral reproducibility of the SERS substrate as well as allowing us to selectively detect the signal from highly enhancing sites ("hot spots"). On the other hand, the focused laser beam in the case of micro-Raman set-up can cause decomposition of the adsorbate and/or of another surface species and consequently "spurious" Raman bands can appear in the spectra [15].

2. Experimental

2.1. Chemicals and glassware cleaning

Deionized water of a specific resistance of 18 MΩcm was used for all preparations. H_2SO_4 (96%), H_2O_2 (30%), HCl (35%) and HNO_3 (65%) were obtained from Lachema. $HAuCl_4$, sodium citrate, methanol (99.8%), 3-aminopropyltrimethoxysilane (APTMS, 97%) and free-base 5,10,15,20-tetrakis(1-methyl-4-pyridyl)porphyrin (TMPyP, tetra-p-tosylate salt) were purchased from Sigma–Aldrich.

All glassware was cleaned using "piranha" solution (4 parts H_2SO_4, 1 part H_2O_2) to remove organics and then aqua regia (3 parts HCl, 1 part HNO_3) to remove metal particles.

2.2. Substrate and sample preparation

Au colloidal nanoparticles were prepared by reduction of $HAuCl_4$ by sodium citrate: 250 ml of 1 mM solution of $HAuCl_4$ was brought to a boil and then 25 ml of 38.8 mM solution of sodium citrate was added. Boiling continued for 15 min and then the solution was left to cool.

Preparation of Au silanized substrates is described in our previous paper [12]. Clean borosilicate glass slides (1 cm × 2 cm strips) were immersed into 10% solution of APTMS in methanol for 30 min. After silanization, the substrates were rinsed several times with methanol and then with water to remove unbound silane that could cause colloid aggregation when the substrate is put into it. Then each silanized substrate was dipped separately in a vertical position into a vial containing 1 ml of the colloidal suspension for 3–4 h. After that, they were rinsed again with water and left to dry at 100 °C for 10 min. Au nanoparticles are attached to glass slides by electrostatic interactions between the anions covering their surface and the positively charged amino groups of APTMS [7,8].

For SERS measurements, the Au substrate was placed into porphyrin aqueous solution for 20 min, then it was rinsed with deionized water and SE(R)RS spectrum was measured. 20 min soaking time was found to be optimal to obtain maximal SE(R)RS signal of TMPyP [13].

2.3. Instrumentation

Scanning electron microscopy (SEM) images of Au substrates were obtained using a Hitachi S5000 device.

Surface plasmon extinction (SPE) spectra of Au substrates and electronic absorption spectrum of TMPyP were recorded with a UV/VIS Perkin Elmer Lambda 12 spectrometer. The light spot at the sample was a line of ~9 mm length and ~1 mm width.

SE(R)RS spectra of TMPyP were recorded at room temperature with an integrated confocal Raman microscopic system LabRam HR800 (Horiba Jobin-Yvon) equipped with a 800-mm single spectrograph (600 g/mm holographic grating) and a nitrogen cooled CCD. The laser beam was focused using 100× objective to a spot of a diameter of ~1.2 μm. 632.8 nm excitation line of an internal He–Ne laser (laser power 0.065 mW at sample) and 514.5 nm excitation line of an Ar^+ laser (Melles Griot, laser power 0.19 mW at sample) were used. Accumulation times were 1 × 60 s for all spectra.

3. Results and discussion

Prepared Au substrates were characterized by SPE and SEM techniques. Typical SPE spectra of the parent Au colloidal solution and immobilized Au colloidal nanoparticles on glass are shown in Fig. 1, left. Au surfaces are grey-violet with a broad SPE spectrum between 500 and 800 nm. SPE spectrum is formed by two overlapping bands with maxima at 520–530 nm and 600–650 nm, but their ratio is slightly different from preparation to preparation [12,14]. The 520–530 nm extinction band is close to the extinction band of the parent colloidal solution and thus corresponds to isolated gold nanoparticles. The 600–650 nm one indicates an interaction between the neighbouring nanoparticles manifested by the red shift of the SPE maximum caused by plasmon resonance coupling. No change in the SPE spectrum was observed during storage or after adsorption of TMPyP. SEM image of Au substrates (see Fig. 1, right)

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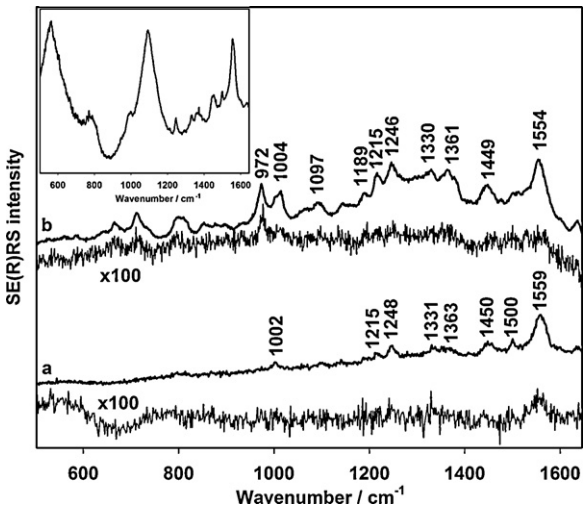

Fig. 2. SE(R)RS spectra of TMPyP adsorbed on Au substrate excited by 514.5 nm (a) and 632.8 nm (b) laser line. Top spectra are of 1×10^{-6} M TMPyP soaking concentration, bottom spectra of 6×10^{-8} M TMPyP soaking concentration. Inset: Rough SERRS spectrum of TMPyP (1×10^{-6} M soaking concentration) measured using macro-Raman spectrometer (see details in [14]), 514.5 nm excitation, 5×60 s accumulation time.

shows a compact coverage of glass by Au nanoparticles of diameters varying from ~30 to 100 nm and by small aggregates. Scanning the whole surface and comparing several samples indicate that our Au substrates are uniform and the preparation is highly reproducible.

Typical SE(R)RS spectra of TMPyP (1×10^{-6} M soaking concentration) adsorbed on Au substrate excited by 514.5 nm (a, top spectrum) and 632.8 nm (b, top spectrum) are shown in Fig. 2. Comparison of these spectra with Raman spectra of 1×10^{-3} M TMPyP measured without Au nanoparticles indicates that we obtained SE(R)RS spectra of TMPyP in the unperturbed free-base form (see doublet at ~1330 + 1360 cm^{-1}) [16,17]. This result is in agreement with the fact that TMPyP cannot be metalated by Au atoms [14]. Spectral changes in SE(R)RS spectra obtained using two different excitation lines originate from resonance effect in the case of 514.5 nm laser line. The 514.5 nm excitation falls into the maximum of electronic absorption Q-band of TMPyP at ~518 nm while the 632.8 nm excitation is out of electronic absorption of TMPyP (Fig. 1). Decomposition of the adsorbate on the metal surface has not been observed in our experimental conditions. However, if laser power higher than 0.2 mW is used, strong bands of amorphous carbon at ~1300–1600 cm^{-1} are seen for both excitation wavelengths.

Our results demonstrate significant advantages of confocal micro-Raman spectrometer in comparison to macro-Raman one. Crucial advantage is an absence of strong Raman spectrum of glass support which interferes with macro-SE(R)RS spectrum of TMPyP. Inset of Fig. 2 shows an example of rough SERRS spectrum of TMPyP (1×10^{-6} M soaking concentration) measured using macro-Raman set-up (see [14] for details) and 514.5 nm excitation and 5×60 s accumulation time. Lower part of the spectrum (500–1200 cm^{-1}) is overlapped by normal Raman spectrum of glass. Other advantages of micro-Raman set-up include at least 5 times shorter collection times (under our experimental conditions), easier manipulation with samples (reflection geometry has to be used in the case of macro-Raman experiment) and the possibility of spectral mapping over the substrate.

We tried to estimate analytical enhancement factor (AEF), a parameter suitable to display the analytical capability of a particular SERS substrate. The AEF is defined as the following equation considering the intensity ratio of SE(R)RS (I_{SERS}) and normal (resonance) Raman (I_{RS}) spectrum of TMPyP and the ratio of TMPyP concentrations in each experiment [1,18]:

$$\mathrm{AEF} = \frac{I_{SERS}/c_{SERS}}{I_{RS}/c_{RS}}$$

In our calculation, we used integral intensities of the strongest band at ~1555 cm^{-1} in SE(R)RS spectra of TMPyP (1×10^{-6} M soaking concentration) measured from Au substrates and in normal (resonance) Raman spectra of 1×10^{-3} M TMPyP measured without Au nanoparticles under the same experimental conditions. Since SE(R)RS intensities measured from different spectral points of Au substrates slightly differ, the intensity ratio I_{SERS}/I_{RS} varies between 10–20 and 50–250 for 514.5 nm and 632.8 nm excitations, respectively. Concentration c_{SERS} is the most critical approximation. It should be the concentration corresponding to the amount of TMPyP molecules adsorbed on Au substrate and providing the SE(R)RS signal. Using absorption spectroscopy it was possible to measure the decrease of TMPyP molecules from the soaking solution (after taking out the Au substrate) and thus determine the amount of TMPyP molecules adsorbed on the Au substrate. In our calculation, when 1×10^{-6} M TMPyP soaking concentration is used, the amount of adsorbed TMPyP is ~10^{14} molecules/cm^2. It represents ~1×10^{-7} M concentration in solution which corresponds to submonolayer coverage of metal colloidal nanoparticles [17]. Finally, the AEF values for TMPyP on our Au substrates were estimated as ~10^5 and ~10^6 for 514.5 nm and 632.8 nm excitation, respectively. This result is consistent with theoretically predicted and experimentally proved SERS enhancement of Au nanoparticles [1,9].

SERS sensitivity of the Au substrates for TMPyP was characterized by limit of detection (LOD) of TMPyP. We measured SE(R)RS spectra of TMPyP of soaking concentrations between 1×10^{-5} M and 6×10^{-8} M. Typical SE(R)RS spectra of TMPyP (6×10^{-8} M soaking concentration) adsorbed on Au substrate excited by 514.5 nm (a, bottom spectrum) and 632.8 nm (b, bottom spectrum) are shown in Fig. 2. The LOD was determined by extrapolation to the concentration for which the intensity of the strongest porphyrin band at ~1555 cm^{-1} exceeds the triple of the blank signal standard deviation [19]. The LODs of TMPyP on our Au substrates are 6.5×10^{-8} M and 4.0×10^{-8} M soaking concentrations for 514.5 nm and 632.8 nm excitation, respectively. The LODs are comparable to the values obtained previously for TMPyP on the same Au substrates but using a classical macro-Raman spectrometer (LOD is 5×10^{-8} M at 514.5 nm excitation) [14].

Uniformity and spectral reproducibility of Au substrates in mm-scale was studied by measurement of TMPyP spectra from several

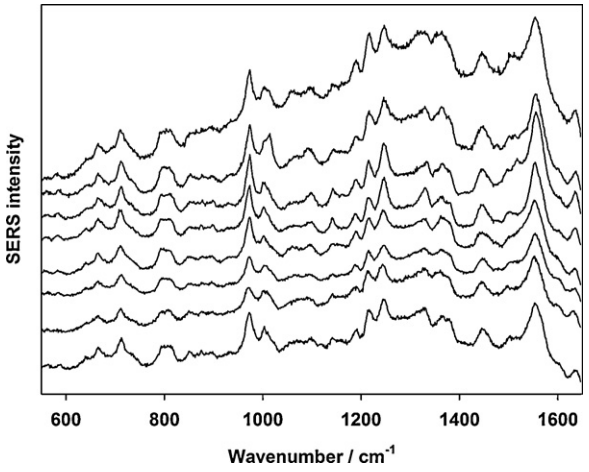

Fig. 3. Eight spectra of TMPyP measured from several random places of one Au substrate, 5×10^{-6} M TMPyP soaking concentration, 632.8 nm excitation.

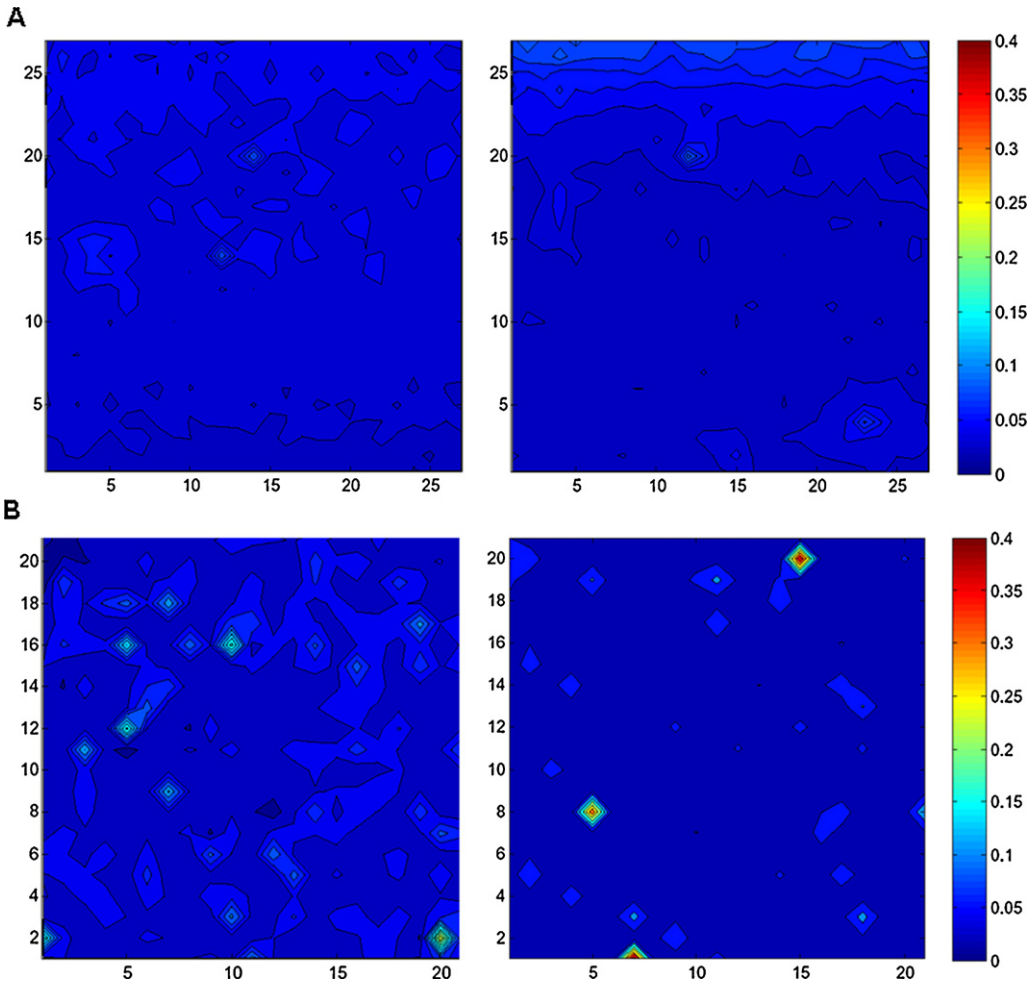

Fig. 4. Spectral maps of TMPyP (1×10^{-6} M soaking concentration) on Au substrate: excitation 514.5 nm (A) and 632.8 nm (B), 20 μm (left) and 2 μm (right) steps between mapping points. Numbers on the axes represent the mapping points; the scale represents the relative intensity. (Color figure is available on the web version of the article.)

random places of the substrate. Fig. 3 shows such eight spectra measured from one substrate with 632.8 nm excitation. Relative standard deviation (RSD) of signal is 15.3%. In the μm-scale, we carried out spectral mapping of the Au surface. Fig. 4 shows examples of spectral maps of SE(R)RS spectra of TMPyP (1×10^{-6} M soaking concentration) with 20 and 2 μm steps between mapping points for both excitations. The obtained sets of spectra were treated by an in-house developed software including baseline correction and factor analysis (FA) [17,20]. "Singular value decomposition" FA algorithm provides a set of orthonormal subspectra S_j and weights W_j, and a set of scores V_{ij} representing the relative presence of the S_j subspectrum in each experimental spectrum Y_i. A particular experimental spectrum $Y_i(\nu)$ can then be approximated as:

$$Y_i(\nu) = \sum_{j=1}^{M} W_j V_{ij} S_j(\nu)$$

The first subspectrum S_1 is a weighted average of the experimental spectra reflecting changes of the overall intensity, while the second subspectrum S_2 can be considered as a difference spectrum reflecting the spectral changes. In our maps, the SE(R)RS intensity in each mapping point is represented by the corresponding coefficient of the first subspectrum of the FA. The advantage of this approach is that it is a rapid and precise way to analyze intensity of huge set of spectra. The results demonstrate that only slight intensity variation is observed for 514.5 nm excitation. In the case of 632.8 nm excitation, spectral reproducibility is not so good, the intensity jumps 5–10 times in a few points. We suggest that by using the 632.8 nm excitation, some aggregates of Au nanoparticles containing "hot spots" are excited.

We can summarize that spectral mapping over the surface of our Au substrates shows excellent spectral reproducibility in mm-scale (relative standard deviation of signal ~15%). Worse spectral reproducibility in μm-scale is caused by selective excitation of "hot spots" in some spectral points. 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. Moreover, 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. Thus, such substrates can be considered as suitable for SE(R)RS (bio)analytical applications.

4. Conclusions

SERS-active substrates were prepared by immobilization of Au colloidal nanoparticles via aminosilane on glass and characterized by SPE and SEM techniques. This preparation procedure leads to easy and cheap fabrication of uniform SERS substrates on a large scale. SERS sensitivity and spectral reproducibility of Au substrates were investigated using free-base 5,10,15,20-tetrakis (1-methyl-4-pyridyl)porphyrin (TMPyP). SE(R)RS spectra of TMPyP were measured by a confocal Raman microspectrometer with two

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different excitation wavelengths (514.5 nm and 632.8 nm). Both excitations provide good SE(R)RS spectra of TMPyP, the analytical enhancement factor (AEF) of Au substrates was found to be between 10^5 and 10^6 and the limits of detection (LOD) of TMPyP $\sim 5 \times 10^{-8}$ M in soaking solution. Spectral measurement over the Au surface proved their excellent spectral reproducibility (relative standard deviation of signal ~15%). Confocal micro-Raman spectrometer shows significant advantages in comparison to macro-Raman one including shorter collection times and the absence of strong Raman bands of the glass support. Thus, SERS microspectroscopy using immobilized Au colloidal nanoparticles can be considered as suitable approach for SE(R)RS (bio)analytical applications.

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