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New substrate sample handling and micro-Raman sensing methods for biomolecular analysis

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The paper discusses a novel sample deposition method and a new substrate for micro-Raman applications, together with some applications for pharmaceutical and fluorescent compound analysis. Teophylline and indigo carmine azo-dye were used as testing molecules. They were able to be detected in trace amounts, compatible with the applications at physiological level. SERS samples of Ag colloid containing small amounts of tested molecule solutions were drop-coated onto distinct small regions of the SpectRIM plate and characterized using AFM and micro-Raman spectroscopy. An enhancement mechanism from the first monolayer of drop coated deposition SERS sample was concluded.

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1. Introduction

Raman spectroscopy is a powerful tool for the analytical purpose of biological materials because of its high structural information content, high sensitivity and selectivity, rapid detection and minimal sample preparation requirements.

The intensity of normal Raman scattering being low, it may be increased by resonance and/or surface-enhancement, but these techniques introduce spectral variations and greater susceptibility to photochemical damage especially for biological molecular species. For biomedical or environmental analytical purpose, the sensitivity of Raman spectroscopy techniques has to be increased and fast detection is required. The recently developed SpectRIM^{TM} [1] slides show ideal substrate properties for measuring small quantities of material, especially from diluted solutions in polar solvents [1, 2]. These slides consist of a 3.0X1.0X0.1 inch plastic carrier with an optically flat 0.9X0.9 inch stainless-steel plate coated with an ultra-thin (<50 nm) hydrophobic layer. If solutions containing bio-molecules are applied to this substrate, the sample will bead up and dry with a much smaller diameter than when applied to an untreated surface, i.e. glass plate. Moreover, the substrate allows the segregation of proteins or other macromolecules from buffers and fluorescent contaminants, before or after lyophyllization.

Recent reports (3- 6) demonstrated that drop coated deposition Raman spectroscopy (DCDR) has the sensitivity, both in terms of detection limit and in terms of chemical information content, to distinguish proteins of very similar structure under conditions that are compatible with biomedical research and diagnostics.

DCDR that facilitates microanalysis of biological and pharmaceutical compounds using normal Raman scattering (1) is compatible with other analytical methods including light microscopy, infrared (IR) spectroscopy and Matrix Assisted Laser Adsorption Ionization (MALDI) mass spectrometry [5].

Within the pharmaceutical applications, aalthough different extraction methods can be used to extract drug active components from the pharmaceutical matrices, the drugs detection concentration of the dried extract can be problematic. This is especially true when using traditional glass slides and/or IR transmission windows (i.e. BaF2). The dried material will disperse rather than dry down into a concentrated area. As a result, larger volumes may be needed to build up a significant concentration of the analyte of interest to be able to detect it using Raman spectroscopy.

In order to test and get insight into the optical and spectroscopic properties of these new substrates, the Raman behaviour of different molecular species were tested on SpectRIM^{TM} slide. Micro-Raman and surface enhanced Raman scattering (SERS) tests of drop-coated deposition samples [2] have been performed after carrying out AFM [6] of the dried droplets.

2. Experimental

Micro-Raman

Teophylline (TF) and indigo carmine (IC) aqueous solutions of different concentrations were prepared using triple distilled water and dropped on the SpectRIM plate. After drying, the Raman measurements were performed using a Dilor-Jobin Yvon Spex spectrometer with an 1800 grating and a back-scattering geometry using a microscope equipped with an Olympus LMPlanFL 50x objective. The slit was 100μm and the spectral resolution was 2 cm^{-1}. For the excitation of the Raman spectra, the 632.8 nm line from a He-Ne laser or the 514.5 nm line from an Ar ion laser Spectra Physics Model 2016 was employed, respectively. The detection of Raman signal from tested molecular species was carried out with a CCD camera (Photometric model 9000) and for the signal acquisition the analysing software package LabSpec 3.1 was employed. The laser output power was 3.5 mW. The spectra were obtained by averaging 5 cycles of 30 seconds under the red excitation and 10 cycles of 10 seconds under the green excitation, respectively. The concentration ranged between 10^{-3} to 10^{-5} $moll^{-1}$ for the tested IC whereas in the case of teophylline, $2.3x10^{-2}$ $moll^{-1}$ solution concentration was used.

SERS

Two different Ag colloids obtained by citrate (Lee-Meisel) [7] or hydroxylamine reduction [8] were prepared and used as SERS substrate; in conjunction with a classical thermally evaporated Ag island film [9]. SERS sample was obtained mixing 3 ml colloid with 3 μl from TF or IC tested solutions. A small amount of each mixture was drop coated on the SpectRIM slide.

AFM

Mercury 100 AFM from ***WITec*** [6] was used for high resolution imaging of drop-coated SpectRIM, operating in AC-mode with *Arrows*™ cantilevers from ***Nanoworld.*** Spring constant was 42 N/m and the resonance frequency of the cantilevers of 280 kHz.

3. Results and discussions

The pure SpectRIM^{TM} slide was firstly tested for the Raman background signal (Fig. 1). The absolute intensity counts were monitored upon incident laser power increasing. For the maximum output power of 250 mW, the micro-Raman signal obtained using two microscope objective (x50, x100) is rather similar, not exceeding 500 counts in both cases. Several weak intensity bands were observed at 2176, 1546, 1072, 769 and around 500 cm^{-1}, being related to the Teflon coated stainless steel.

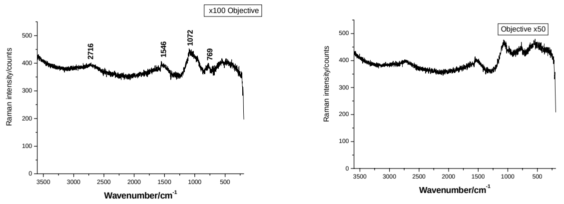

Fig. 1. Micro-Raman signal (absolute intensity) obtained from the pure SpectRIM^{TM} slide using two different objectives (x100, x50) as indicated. Excitation 250 mW output power, 514.5 nm.

Teophylline tests

The benefit and toxicity of teophylline, a largely used pharmaceutical compound for the treatment of asthma, for its anti-inflammatory properties and immunomodulatory effects, are closely related to serum concentration. Its clinical usage therefore requires careful consideration of product absorption characteristics at physiological concentration of about 10 μg/mL [10]. In order to test the ability of SpectRIN slide to detect such trace amount of teophylline in diluted solution, prior micro-Raman tests on a normal glass slide have been performed. When a liquid microdroplet of teophylline aqueous solution is applied to the SpectRIM substrate it beads up and dries to a smaller diameter, thus concentrating the analyte deposit (Fig. 2). The deposition volume was 4 μL and the concentration of the deposited solution was 2.3 $x10^{-2}moll^{-1}$. Positioning the slide with the deposited sample on the microscope stage and focusing on an area of the outer ring results in high quality Raman spectrum (Fig. 3). The absolute intensity counts recorded exceeded about 50 times the background intensity.

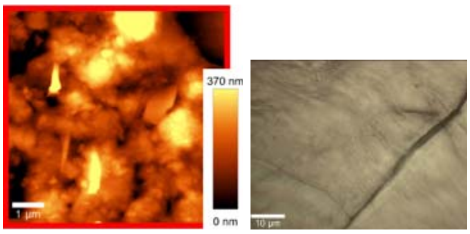

Fig. 2. AFM topography and optical microscopy image and of teophylline on the SpectRIM substrate after solvent evaporation.

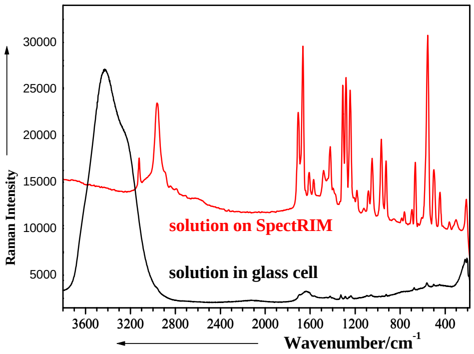

Fig. 3. Micro-Raman spectra of $2.3.10^{-2}$ $moll^{-1}$ teophylline aqueous solution collected from a glass cell and from a drop coated SpectRIM slide.

Spectra taken from inside the ring for comparison have not been able, no signal attributable to the species being found inside the ring.

Indigo carmine tests. Upon red excitation, the IC aqueous solution presents strong fluorescence that completely hides any Raman signal (Fig. 4).

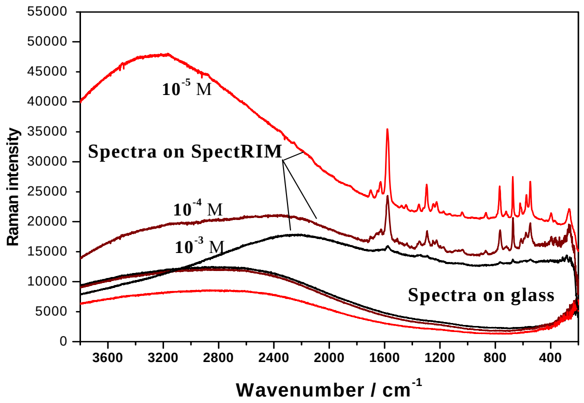

Fig. 4. Micro-Raman spectra of IC solution concentration ranging from 10^{-3} to 10^{-5} moll^{-1} as indicated, collected from glass and SpectRIM plate. Laser line 632.8 nm.

The fluorescence of this species was reduced by simply dissolving the IC in water and then depositing the solution on the Tienta SpectRIM^{TM} slide prior to taking the micro-Raman spectra. Excitation with the green laser line results in background decreasing with very well-resolved Raman bands even at 10 μM concentration, whereas the same concentration exhibit poor Raman signal on glass plate (Fig. 5).

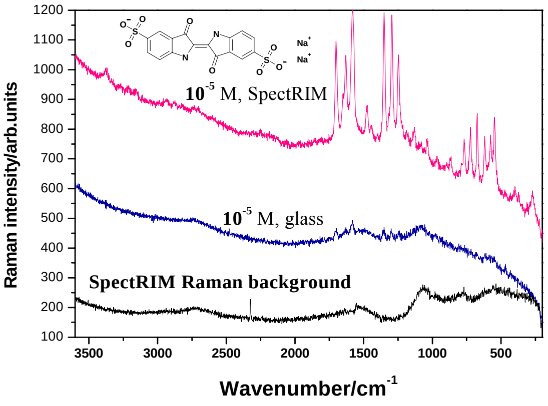

Fig. 5. Micro-Raman spectra of IC 10^{-5} moll^{-1} aqueous solution from SpectRIM slide compared to that from glass plate. Raman background of the slide is bottom inserted for comparison. Excitation 514.5 nm.

SERS tests. On a separate SpectRIM^{TM} slide area, two deposits of 5 μL volume of the Ag colloid obtained under Lee-Meissel [7] or hydroxylamine reduction procedure [8] respectively, have been dropped and their Raman spectra collected (Fig. 6).

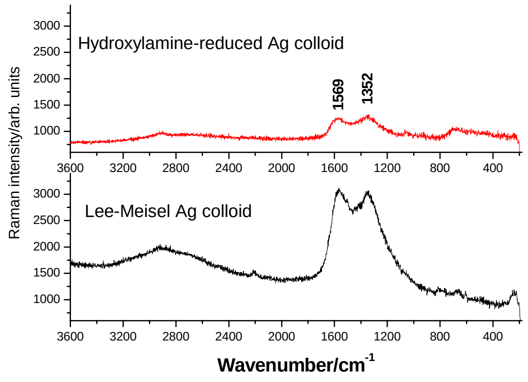

Fig. 6. Micro-Raman signal from different chemically reduced Ag colloids drop-coated on the SpectRIM slide. Original intensity counts of acquisition are given. Excitation 514.5 nm.

Depositing the Ag colloid drops 1 cm apart, allowed the sample to dry approximately 20 minutes, clear rings being observed under the light microscopy. Their AFM images (not given here) indicated a much uniform Ag particle dispersion with an average clustering dimension of of 200 nm for the hydroxylamine reduced colloid deposition, whereas the Lee-Meisel one exhibits larger average size (about 600 nm) of self-assembled Ag particles upon drop coated deposition and dehydration on SpectRIM slide. Unexpected Raman signal enhancement [9, 11] was obtained in the case of the TF and IC molecule. Fig. 7 gives several comparative SERS spectra for the IC molecule. Since the band positions and relative intensities in the SERS spectra collected on SpectRIM and on Ag film are similar, a similar enhancement mechanism was supposed.

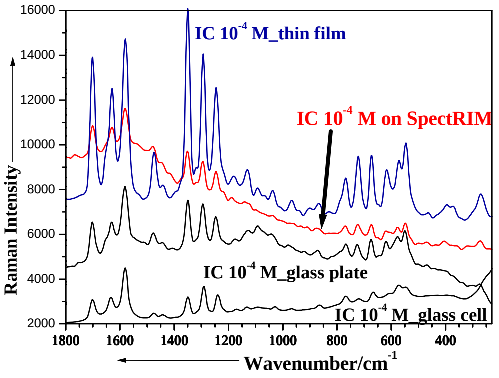

Fig. 7. SERS spectra of IC on Ag island film compared to the corresponding SERS signal of IC dropped on SpectRIM slide, glass plate, and glass cell, as indicated on each spectrum. Excitation line 514.5 nm.

The increasing signal intensity with the concentration decrease was observed upon red or green laser excitation. This anomalous behaviour should be explained in terms of surface enhancement of the Raman signal from the first monolayer (SERS) [12]. From these preliminary studies we conclude a surface enhancement Raman mechanism on the SpectRIM surface. Since the Ag nanoparticles dimension after drying the colloid droplet is rather larger than the usual SERS required Ag particles for visible excitation of plasmon resonance, the enhancement mechanism is subject of further investigations. The major advantages of using SpectRIM as substrate for drop coated sampling of molecular species with low Raman cross section make it an attractive sampling method for biological or pharmaceutical Raman analysis. However, the results may vary depending on instrumentation, illumination wavelength, integration time and the thickness and purity of the sample.

4. Conclusions

The ultra-thin hydrophobic layer tested with almost no appreciable Raman or fluorescence signal allowed trace amount of teophylline and indigo carmine detection from sample solution analyte spectra. The hydrophobic surface enables fast drying of the sample. It has been demonstrated that even after drying its solution structure is preserved and Raman spectra taken from the dried deposit and (concentrated) solution are virtually identical. Upon red excitation, the fluorescence signal of IC aqueous solutions has been quenched on SpecTRIM, allowing high quality Raman recognition at micromole concentration. The increasing signal intensity with the concentration decrease was observed upon red or green laser excitation. This anomalous behaviour should be explained in terms of surface enhancement of the Raman signal from the first monolayer (SERS). Therefore, we conclude an enhancement mechanism of the nanometer structured hydrophobic layer of the SpecTRIM surface which is subject of further studies.

No spectral changes were observed in the Raman spectra after drying periods as long as several weeks. Therefore, promising high selective and ultra sensitive Raman applications using this substrate could be performed.

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