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ARTICLE *in* ANALYTICAL CHEMISTRY · NOVEMBER 2007

Impact Factor: 5.64 · DOI: 10.1021/ac071121l · Source: PubMed

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Ultrasonic Trapping of Microparticles in Suspension and Reaction Monitoring Using Raman Microspectroscopy

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An ultrasonic standing wave around 2 MHz has been used for trapping and concentration of suspended micrometer-size particles in a flow cell, whereas Raman microspectroscopy was used as a nondestructive technique to provide molecular information about the trapped particles. With this approach, detection and discrimination of different polymer microparticles based on their characteristic Raman spectra was performed. Dextran, poly(vinyl alcohol), and melamine resin-based beads, with and without functionalization, were used for this purpose. Furthermore, taking advantage of the flow-through characteristics of the cell and the versatility of the employed flow system, full control over the media surrounding the trapped particles was achieved. This allowed us to perform chemical reactions on the trapped particles and to monitor spectral changes in real time. Here retention of cation-exchanger beads loaded with silver ions and subsequent reduction of the silver ions was demonstrated. In this way, surface-enhanced Raman (SER) active beads were prepared and retained in the focus of the Raman microscope by means of the ultrasonic field. Injection of analytes in the flow system thus allowed recording of their SER spectra. Using 9-aminoacridine, a linear dependence of the found SER signal in the range from 1 to 10 μM has been achieved. The repeatability in the recorded SER intensities was on the order of 4−5%. This included bead retention, surface-enhanced Raman layer synthesis, and analyte detection.

The possibility of handling particles (e.g., microsized beads, cells) and extracting information about molecular processes within them is relevant in life sciences.[1,2] A large number of different techniques have been developed for this purpose in the last years. Most of them are based on approaches that use optical, electrical, and magnetic properties to handle the particles. Optical methods are based on the principle that dielectric objects are drawn to regions of high optical intensity through a gradient force, which, if the light is focused tightly enough, is sufficient to confine objects in three dimensions.[3] Dielectrophoresis refers to the force exerted on the induced dipole moment on an uncharged dielectric or conductive particle by a nonuniform electric field.[4] Magnetic manipulation have also been used successfully in particle and cell applications.[5]

Ultrasound-based immobilization techniques also allow manipulation and positioning of particles or cells. By applying an ultrasonic standing wave field of selected frequency and intensity to an initially homogeneous particle suspension, the particles are forced to move along the direction of sound propagation into areas of vanishing acoustic pressure (pressure nodes).[6] A number of studies indicated that the acoustic fields used caused no variations in particle integrity. Therefore, this approach of particle manipulation has been applied in numerous biotechnological applications where unchanged cell viability was also demonstrated.[7] An advantage of ultrasonic trapping in comparison with the above-mentioned approaches is that the latter technique is able to handle particles without previous knowledge of their position.[8] Furthermore, ultrasound-based techniques allow the simultaneous manipulation of all particles in a suspension, in contrast to optical tweezers and dielectrophoresis that can only be used on a limited number of particles.[9]

Raman microspectroscopy is a powerful technique to provide spatially resolved information about chemical composition of materials in many fields, including the study of biological structures. The spatial resolution of Raman microspectroscopy in the low-micrometer scale and its ability to probe samples even under in vivo conditions providing direct structure-sensitive information allow new insights into living single cells without the need for fixatives, markers, or stains.[10]

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10.1021/ac071121l CCC: $37.00
Published on Web 09/18/2007

Since the first example of combination of optical trapping and Raman microspectroscopy, where single micrometer-sized solid particles were measured,[11] several applications have been developed. They include the identification and characterization of single optically trapped microparticles,[12] yeast, bacteria and red blood cells,[13,14] liposomal membranes,[15] and bacterial spores.[16] The technique has also proven to be useful for probing chemical reactions on small particles, such as photoinduced conjugation of polystyrene[17] and chemical synthesis reactions on solid-phase support particles.[18] In the former study, the reaction was initiated by the laser itself, whereas in the latter, the reagent had to be added to the particle suspension manually before placing it on the microscope stage. Consequently, a considerable time delay occurred between mixing and the acquisition of the first Raman spectrum. Recently, the combination of a microfluidic system with optical trapping and Raman microspectroscopic detection has been reported.[19] This approach fulfills better the demand for monitoring chemical processes in real time with good control over the addition of reagents. However, in order to optimize the experimental conditions for optical trapping and Raman measurement, two different lasers and microscope objectives were needed.

In contrast, acoustic trapping can provide an experimentally simpler approach to manipulate and immobilize microparticles, while they are exposed to a flow of a variety of reactants, and the chemical changes are monitored by Raman micro-spectroscopy. Acoustic trapping has been previously reported for the stable positioning of specimens in air, enabling the study of different physical and chemical processes such as crystallization[20] and evaporation.[21] Raman spectroscopy has also been used as a detection technique for levitating samples.[22−24] Furthermore, the possibility of monitoring containerless chemical reactions in ultrasonically levitated nanodroplets has been demonstrated using flow-through microdispensers to dose picoliter droplets into the pressure node of the ultrasonic trap.[25,26]

Here, ultrasonic trapping of particles in suspension combined with confocal Raman microspectroscopy is presented as a powerful tool for capture and identification of micrometer-sized particles in a liquid. Furthermore, the strategy of trapping the particles in a flow cell allows the execution of a given set of chemical reactions that can be continuously monitored by means of Raman microscopy, avoiding drawbacks related to levitation in air, such as solvent evaporation. The working principle of the presented approach will be illustrated with an example of automated generation of a surface-enhanced Raman spectroscopy (SERS)-active layer on ultrasonic trapped beads.

EXPERIMENTAL SECTION

Chemicals. All experiments were performed with reagents of analytical grade. Sephadex SP C-25 cation exchanger on dextran beads (40−120 μm), Sephadex G-25 dextran-type gel without exchangeable group beads (40−120 μm), and microparticles based on melamine resin (12 μm) were purchased from Sigma (Steinheim, Germany). Poly(vinyl alcohol) (PVA-N13), primary amine-functionalized beads (5−8 μm) were obtained from Chemagen (Baesweiler, Germany). All of them were used without any previous treatment.

Standard solutions of silver nitrate (Merck, Darmstadt, Germany), hydroxylamine (Fluka, Steinheim, Germany), and 9-aminoacridine (Merck) were prepared in distilled water. Working solutions were prepared daily by appropriate dilution with distilled water.

Apparatus. Raman spectra were recorded with a confocal Raman microspectrometer LabRam HR800 (Jobin Ybon GmbH, Bensheim, Germany) equipped with an intensified charge-coupled device detector using a 20× magnification objective with a working distance of 20.5 mm. A 500-μm pinhole was used. A HeNe laser emitting at 632.8 nm with power set to 14.5 mW was employed for recording of the Raman spectra. Using a 20× microscope objective, the delivered power was 7.3 mW. Acquisition time for each spectrum was 10 s. Spectra were recorded and processed (for smoothing, baseline subtraction, and peak detection) by means of the Labspec software (Jobin Ybon GmbH).

A demountable flow cell for IR spectroscopy was adapted to trap the beads (Figure 1a). A 5-mm spacer containing a circular hole with a diameter of 1.8 cm was machined out of PMMA. Connections to the flow system was achieved via holes (1.4-mm diameter) through which the PTFE tubings were inserted. In order to reduce the inner volume of the flow cell without changing the acoustic properties of the cell cavity, part of the cell was filled with agarose. In doing so, a rectangular channel with a width of 3 mm, a height of 5 mm, and a length of 18 mm was achieved. Furthermore, one CaF_2 and one quartz window were used to complete the flow cell. A circular ultrasonic transducer (PZT ceramics, 1-mm thickness and 15-mm diameter) was glued on the quartz window for the excitement of the ultrasonic standing wave. The PZT was connected to a FPS 2540 frequency power synthesizer (PSI Systems). This device has a scale for the phase angle between current and voltage. Resonance was estimated by minimizing the angle, thus maximizing through electrical power input by changing the frequency. A resonance of the system of ∼2 MHz was used. The upper-side slide acted as an acoustic reflector and provided optical access to the trapping sites. The flow cell was placed horizontally under the Raman microscope with the CaF_2 window directed to the microscope objective.

The flow system (Figure 1b) was built up using a Gilson Miniplus 3 peristaltic pump (Villers le Bell, France) and three Rheodyne (model 5041) rotary valves (Cotati), one of them connected as selection valve and the others as injection valves. Teflon tubing with an inner diameter of 0.8 mm was also used.

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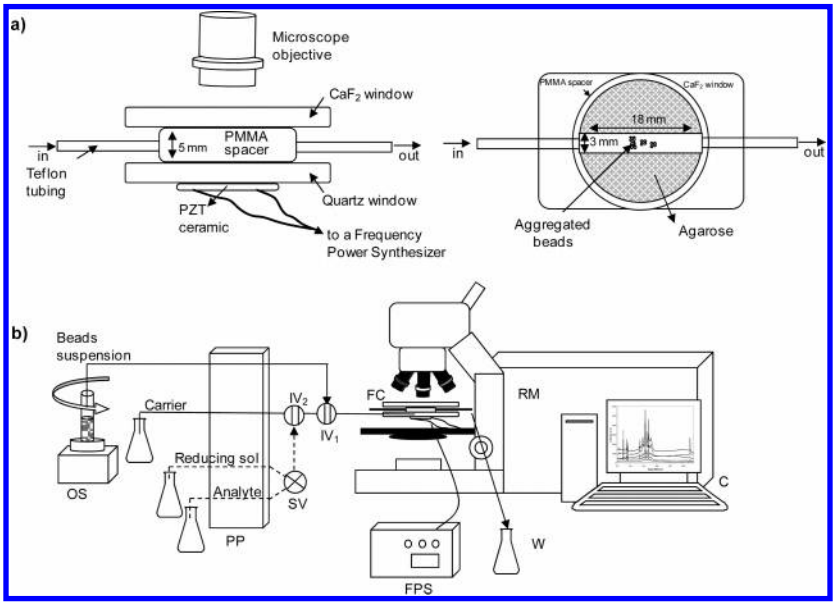

Figure 1. (a) Diagram of the developed flow cell holding the ultrasonic transducer: side and front views. (b) Flow injection system employed for ultrasonic trapping of beads (solid lines) and SERS (dashed lines). PP, peristaltic pump; OS, orbital shaker; IV_1, IV_2, injection valves; SV, selection valve; RM, Raman microscope; FC, flow cell; W, waste; C, computer; FPS, frequency power synthesizer.

An orbital shaker was used to maintain the bead suspension homogenized.

Procedure. (1) Ultrasonic Trapping. A suspension of 5 mg mL^{-1} of each type of bead was prepared and maintained homogenized with the help of an orbital shaker. A volume of 200 μL from this suspension was inserted through the injection valve (IV_1) in a carrier of distilled water and transported to the flow cell at a flow rate of 0.3 mL min^{-1}. When the beads arrived in the flow cell, the ultrasound field trapped the beads in the pressure nodes. Then the laser beam was focused on the beads and Raman spectra were recorded.

(2) Surface-Enhanced Raman Spectroscopy. A 25-mg sample of cation-exchanger Sephadex SP C-25 beads was added to 5 mL of a 0.01 M $AgNO_3$ solution. A volume of 200 μL of homogeneous bead suspension was inserted in the carrier stream (distilled water). As described above, the beads were trapped in the pressure nodes inside the flow cell and the laser focused on them through the microscope. Next, 200 μL of hydroxylamine solution (1% v/v) was injected into the carrier by means of the injection valve (IV_2). Then the selection valve (SV) was turned to fill the injection loop with the sample solution. By acting IV_2, 200 μL of sample was injected in the carrier stream. Raman spectra were continuously measured and registered throughout one experiment. Finally, to discard the beads from the flow cell, the ultrasound field was turned off and the flow rate increased to 1 mL min^{-1}. Prior to the next measurement, the whole flow system was rinsed with distilled water.

RESULTS AND DISCUSSION

Raman Spectra from Ultrasonically Trapped Beads. Different types of particles and ion-exchanger beads with different functional groups and sizes (between 5 and 120 μm) were measured. The ultrasonic field was continuously applied; therefore, the beads were trapped when reaching the cell whereas the carrier stream of distilled water passed. The acoustic nodal planes were oriented perpendicular to the incident laser beam. By varying the excitation frequency (centered at ~2 MHz), it was possible to control their position with respect to the focus of the microscope objective. The ultrasound field provided enough stability to keep the aggregates in position while the flow was on. Thus, it was possible to focus the laser on different aggregates with the recorded Raman spectra always being similar in shape and intensities. Bead aggregation in the flow cell remained stable throughout one experiment (~10 min).

Figure 2a shows Raman spectra of PVA primary amine-functionalized beads recorded with and without applying the ultrasound field. As a result of the aggregation and the immobilization under the laser beam, spectra were much more intense than those obtained without ultrasonic field, thus allowing the identification of some peaks that were negligible when the US field was off and the beads were in suspension. In this way, the region between 1200 and 1400 cm^{-1}, attributed to CH_2 and CH wag, twist stretch, as well as a characteristic band at 1615 cm^{-1}, can be investigated.

The comparison between Raman spectra of different types of beads recorded using the described ultrasonic trap and Raman spectra of the beads that were measured in very concentrated slurries was performed. Figure 2b shows good concordance between the spectra for melamine resin-based beads. These beads can be characterized by the bands at 675 and 977 cm^{-1}, which are assigned to the triazine ring deformation and breathing vibration, respectively. The latter band is used for quantification of the total melamine of a melamine resin.[27]

With the proposed system, the distinction between beads with the same skeleton and different functionalization can also be achieved. Figure 2c shows, as an example, the spectra for two Sephadex-type beads, a cation-exchanger gel (SP C-25), and a

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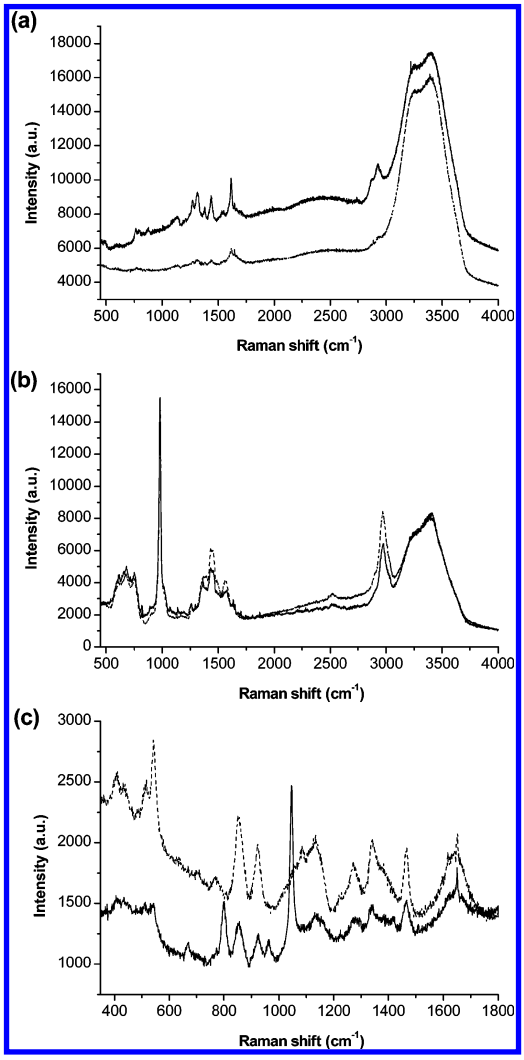

Figure 2. Raman spectra of (a) PVA primary amine functionalized beads with: ultrasonic field on (solid line) and off (dashed line) (b) Melamine resin based beads using the flow system with ultrasonic field (solid line) or very concentrated slurries (dotted line) and (c) Sephadex beads using the flow system with ultrasonic field. SP-C25 (solid line) and G25 (dotted line).

filtration gel (G-25) in the region of 300−1800 cm^{-1}. The skeleton of Sephadex beads is derived from dextran, which shows up in the spectra with a characteristic marker band at 543 cm^{-1}. [28] In the spectra of the cation-exchanger beads, the O═S═O stretching of the sulfopropyl group can be clearly identified at 1044 cm^{-1}. Additionally, other bands (853 and 922 cm^{-1}) of the polymer split up as a consequence of the asymmetry due to the functionalization.

Monitoring Reactions on Ultrasound Trapped Beads. Surface-Enhanced Raman Spectroscopy. The potential of the ultrasonic trapping methodology to monitor on-bead chemical reactions is demonstrated on the example of on-line synthesis of a SER-active silver on the retained beads and their subsequent use to record SER spectra of different analytes.

The Ag^+ loaded bead suspension was inserted in the flow system with IV_1 and transported to the cell where the beads were trapped in the focus of the laser beam. The retained beads were perfused with hydroxylamine to reduce silver ions, achieving a complete and homogeneous coverage of the beads with a rough silver layer. The analytical performance of the SER substrate was tested using 9-aminoacridine as model compound.

Figure 3 shows spectra corresponding to the different steps of the described process during the measurement of 9-aminoacridine (3 μmol L^{-1}).

The first spectrum (t = 0 s) corresponds to the aggregated beads with Ag^+ retained on their surface. Then, silver was reduced with hydroxylamine solution (t = 120 s). When the analyte was injected in the flow system and interacted with the silver layer, a strong SER signal could be observed (t = 180 s). Sample pH was adjusted to 2, to favor the imino form of the analyte that is reported to be stronger attached to the active surface.[29] The main bands correspond to 9-aminoacridine and are located at 3063 (C−H stretching), 1501 (C═N and C═C stretching), 1371 (ring stretching), 707, 656, and 538 cm^{-1} (skeletal vibrations).[29] After recording the analyte SER spectra, fast bead discharge from the cell was achieved by turning off the acoustic field and by increasing the flow rate. Now the spectrum of the clear solution can be seen (t = 240 s). Upon repeating this sequence, it was possible to repeat SER measurements without any carryover effects.

The dependence of the SER intensities on the concentration of the model compound was studied. Linearity was found in the concentration range of 1−10 μM according to the equation: $I = 1599.9c + 34.7$ ($R^2 = 0.9915$), where I is the peak height at 1501 cm^{-1} and c the concentration of 9-aminoacridine expressed in micromolar. Repeated measurement of a 3 μM 9-aminoacridine standard produced a relative standard deviation of 4.5%.

The proposed methodology for SER measurement circumvents many problems related to the difficulty of producing reproducible SER substrates. For example, degradation of substrates and memory effects as a consequence of adhesion of nanoparticles to the walls of the cell are a common problem in flow systems. Recently, Strehle et al.[30] described a method to avoid these effects, by conducting the aqueous droplet, which contained the analyte solution, in a stream of lipophilic solvent. Here the use of a fresh, unused SER-active substrate for each measurement is also presented as a strategy to avoid both degradation and memory effects. Furthermore, the proposed technique is advantageous in comparison to previously described procedures that also employed beads as carrier for SER substrates.[31,32] These procedures used reduced cross sections in the flow cell to retain beads. In these procedures, it was necessary to remove the SERS-active silver layer with strong oxidants like nitric acid prior being able to discard the beads.[32] In the here presented ultrasound-based approach, discharge of silver-loaded beads is simply achieved by switching off the ultrasonic field.

CONCLUSIONS

The feasibility to trap microparticles of an aqueous suspension in an ultrasonic trap to collect their Raman spectra has been

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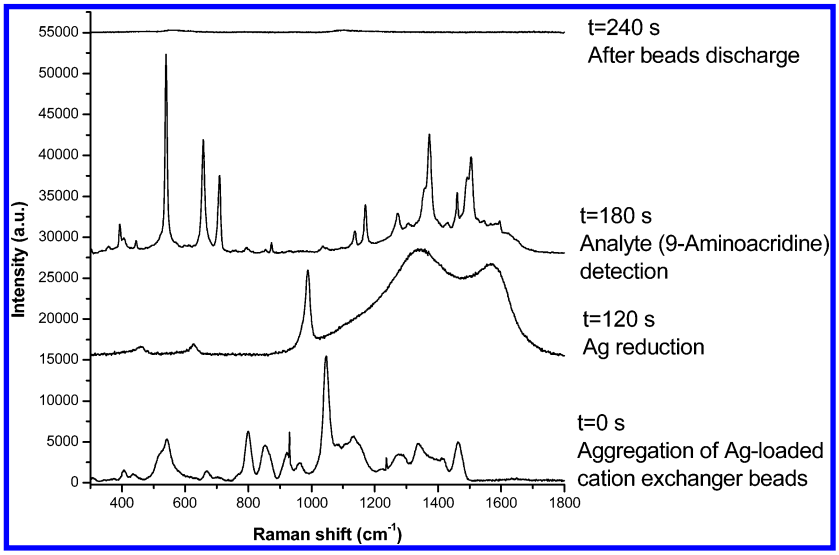

Figure 3. Selected spectra recorded during SER substrate synthesis and and from the SERS monitoring.

demonstrated. Furthermore, it has been shown that chemical reactions carried out on the trapped beads can be monitored. Due to the flexibility of the proposed system, a broad variety of assays can be performed with application in many fields, such as solid-phase synthesis and bead-based bioassays. In a future parallel analysis, employing trapped beads carrying different functionalities can be envisioned. This could be achieved in a microfluidic device allowing fast profiling of the spatial and temporal changes in an array of trapped beads. Furthermore, the proposed trapping approach can be extended, allowing bead manipulation by ultrasound. Finally, it should be mentioned that due to the robustness of ultrasonic bead retention also prolonged studies on beads as well as living cells can be performed.

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ACKNOWLEDGMENT

The work was financially supported by the Austrian Science Fund within the project FWF L416-N17. M.J.R.-R. and A.D.-V. also acknowledge the Spanish Ministry of Education and Science for mobility and postdoctoral grants, respectively.

Received for review May 29, 2007. Accepted July 18, 2007.

AC071121L