

Organometallic nanoprobe to enhance optical response on the polycyclic aromatic hydrocarbon benzo[a]pyrene immunoassay using SERS technology

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Abstract We demonstrated the use of a new organometallic nanoprobe for competitive surface-enhanced Raman scattering (SERS) immunoassay devoted to the detection of polycyclic aromatic hydrocarbons (PAH) such as benzo[a]pyrene (BaP) in seawater. The nanoprobe is a gold nanoparticle (GNP) labeled by a Raman reporter, the 5,5'-dithiobis(succinimidyl-2-nitrobenzoate) (DSNB) and functionalized with monoclonal antibodies anti-BaP. The antibodies are bound with a high specificity to the analyte while the GNPs enhanced the Raman scattering of the DSNB. This type of immunoassay involved the grafting of BaP onto a sensing surface. Thus, NH₂-terminated self-assembled monolayer is formed on the surface of gold substrate using cysteamine. Amines finally reacted with 6-formylbenzo[a]pyrene. So, this SERS detection involves four steps: (i) the nanoprobe is incubated with the sample; (ii) a drop of the mixture is then put onto the substrate; (iii) the surface is rinsed; and (iv) the surface is analyzed by Raman spectroscopy. To synthesize the nanoprobe, firstly, we prepared GNPs according to Frens'

method. Then, GNPs were spontaneously labeled by the DSNB Raman reporter, thanks to a strong gold-sulfur interaction. Thereafter, BaP antibodies were cross-linked to the DSNB labeled GNPs by reaction of proteins primary amino groups with *N*-hydroxyl succinimide (NHS). Before use in SERS detection, their activity was controlled by surface plasmon resonance technique. The present method allows us to detect BaP at trace concentration (2 nmol/L). The results demonstrate that the proposed method has a great potential for application in the monitoring of seawater.

Keywords Biosensor · Surface-enhanced Raman scattering · Polycyclic aromatic hydrocarbons · Benzo[a]pyrene antibody · Organometallic nanoprobe

Introduction

In the investigation of chemical pollutions, benzo[a]pyrene (BaP) has been identified as a relevant tracer of polycyclic aromatic hydrocarbons (PAHs) contamination in the environment by the European Commission (Donata 2010). Standard methods such as GC/MS are commonly used to detect and quantify BaP in compliance with the 0.05 µg/L concentration fixed by the European Commission (2008/105/CE). However, it is not possible to carry out these experiments in situ. Nowadays, there is a lack of relevant technique that allows qualitative, quantitative, fast, and in situ analysis of PAHs in aqueous media. Therefore, a detection method which is suitable to detect BaP in situ will be very useful for chemical pollution investigations even if the limit of detection is higher.

Concerning PAHs detection at trace concentration (nmol/L), surface-enhanced Raman scattering (SERS) effect reveals an immense potential regarding the environment as shown by the recent review made by Li et al. (2014). Since the

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discovery by Fleischmann et al. in 1974 of a particularly intense enhancement of the Raman signal of pyridine molecules adsorbed on the roughened surfaces of silver electrodes, the scientific community has shown an increasing interest in SERS effect (Fleischmann et al. 1974). Very huge Raman enhancements, up to 10^{10} -fold the normal Raman signal of molecules in bulk, have been observed when these molecules are adsorbed on active metallic surfaces (Fleischmann et al. 1976; Albrecht and Creighton 1977; Jeanmaire and Van Duyne 1977; Kneipp et al. 1997; Nie and Emory 1997; Guillot and Lamy de la Chapelle 2012). Concomitantly, the low solubility of BaP in the aqueous media requires a method which is not only sensitive but also selective sensors takes into consideration the variety of the encountered molecules in the media. Hydrophobic SERS-active substrates could offer the expected solution for PAHs as shown in pioneer works (Bao et al. 2012; Murphy et al. 1999; Péron et al. 2009a, b, 2010; Pfannkuche et al. 2012; Schmidt et al. 2004; Lin et al. 2014; Gu et al. 2013). But, in order to ensure selectivity and sensitivity to the sensor towards BaP, immunoassay is a powerful technique based on the specific interaction between the antigen and the complementary antibody. Coupled to SERS, immunoassay is already applied for medical diagnosis, biochemical analyses, and environmental monitoring for macromolecules such as proteins and peptides (Xu et al. 2005).

Although silver presents a higher Raman enhancement factor compared to gold (10–100-fold greater), Au was chosen in this study to allow measurements in seawater on account of AgCl formation on silver surfaces (Murphy et al. 1997). The method of detection by competitiveness on a surface grafted with molecules of BaP is chosen. An organometallic complex assembling gold nanoparticles and antibodies is used. This complex is chemically designed using 5,5'-dithiobis(succinimidyl-2-nitrobenzoate) (DSNB) as a bifunctional coupling agent. DSNB plays also a role of Raman reporter due to its particularly intense Raman signal (Grubisha et al. 2003). Grafted antigen on the sensor competes with free antigen in the sample for nanoprobe antibody binding sites. The more antigens there are in the sample the less organometallic nanoprobe are retained on the sensor surface resulting in a weaker signal. This article demonstrates the interest of developing sensitive sensors for marine analyses with the aim of detecting BaP. As far as we know and as it is confirmed in a review on recent progress in surface enhanced Raman spectroscopy for the detection of environmental pollutants, this paper relates for the first time BaP detection coupling immunoassay and SERS (Li et al. 2014).

For this reason, we present in this preliminary study, in addition to the methodology employed, techniques and test results of the various elements designed for the development of immuno-SERS system for the detection of BaP. An example of analysis is presented at the end of this work to point out the feasibility of the method.

Materials and methods

Chemicals

Benzo[a]pyrene (96 %), cysteamine (98 %), 2-mercaptoethanol (99 %), *N*-methylformanilide (99 %), phosphorus oxychloride (99 %), sodium carbonate (99 %), sodium cyanoborohydride (95 %), methanol (99.8 %), 5,5'-dithiobis(2-nitrobenzoic acid) (DNBA) (99 %), 1,3-dicyclohexylcarbodiimide (99 %), albumin from bovine serum (BSA) (98 %), and *N*-hydroxysuccinimide (NHS) (98 %) were obtained from Sigma-Aldrich. *N,N*-dimethylformamide (99.8 %), sulfuric acid (95–98 %), tetrahydrofuran (p.a.), and boric acid (p.a.) were from Merck. Dimethyl ether (99.9 %) and sodium hydroxide solution at 10 N (9.8–10.2 N) were from Fluka. Hydrogen peroxide solution (30 wt% in H₂O), dimethyl sulfoxide (99.99 %), and PBS were from Fisher. Acetonitrile (HPLC grade) and sodium tricitrate (reagent grade) were purchased from Prolabo. Antibody anti-benzo[a]pyrene (BaP13) was purchased from Exbio Antibodies. The relative sensitivity of the immunoassay using BaP13 towards 21 parents PAHs was determined by competition ELISA. Highest affinity was found for four- to six-ring PAHs. The greatest specificity among the tested compounds was that toward benzo[a]pyrene (Scharnweber et al. 2001). This compound is considered as a marker for PAHs by European Commission (Donata 2010).

Gold III chloride trihydrate (HAuCl₄·3H₂O) (>49 % Au basis) was obtained from Acros organics. Water used in all experiments was purified using a Milli-Q ultrapure water purification system (Merck Millipore) with resistivity of 18 MΩ/cm. One micrometer-filtered seawater was collected from experimental mollusks hatchery of Ifremer in Argenton (France).

Instruments

A homemade surface plasmon resonance system was used. It is based on wavelength modulation in a configuration similar to the one designed by Cahill et al. (1997). The system was developed for laboratory and underwater experiments (Colas et al. 2010).

A NanoDrop 2000 Spectrophotometer (Thermo Scientific) was used to record UV-Vis spectra of colloidal solutions in the spectral region between 350 and 800 nm (resolution ≤ 1.8 nm). Analyzed solutions were diluted in distilled water (10 %, v/v). Water spectrum was used as the baseline. The light source is a xenon flash lamp.

A Horiba Jobin Yvon Labram HR800 spectrometer was used for recording the Raman and SERS spectra. Spectra were acquired with a 785 nm laser diode using a 300-groove per millimeter grating and a $\times 100$ -long working distance immersion microscope objective from

Olympus (NA=1). Using a 800-nm spectrograph, the resolution was 2 cm^{-1} . The laser power was estimated at 0.1 mW focused on an area of $1\text{ }\mu\text{m}^2$.

Synthesis procedures of organometallic nanoprobe

To quantify benzo[a]pyrene contamination in seawater, we chose the method of detection by competitiveness. An organometallic complex assembling gold nanoparticles and antibodies BaP13 is used as a nanoprobe (Scheme 1a). This assembly is performed chemically using a bifunctional coupling agent called DSNB (5,5'-dithiobis (succinimidyl-2-nitrobenzoate). This molecule also plays the role of the reporter due to its particularly intense Raman signal around $1,330\text{ cm}^{-1}$ (Grubisha et al. 2003). This nanoprobe reacts with the BaP antigen of water sample (Scheme 1b). The free antibody binding sites of the nanoprobe are then available to react with grafted BaP on a gold-coated quartz substrate (Scheme 1c). In this configuration, the grafted BaP competes with sample free antigen for nanoprobe antibody binding sites. The more antigens there are in the sample the less organometallic nanoprobe are retained in the surface sensing resulting in a weaker signal.

Synthesis of 6-benzo[a]pyrenecarboxaldehyde

Direct synthesis of 6-benzo[a]pyrenecarboxaldehyde from BaP was accomplished via Vilsmeier-Haack reaction using *N*-methylformanilide and POCl_3 . POCl_3 (1.2 g) and *N*-methylformanilide (1.2 g) were added to a solution of benzo[a]pyrene (1 g) in DMF (10 mL) in a 90-mL Pyrex bottle. The screw cap was then sealed, and the reaction bottle was heated at $90\text{ }^\circ\text{C}$ overnight. The reaction mixture was cooled and washed with 40 mL of diethyl ether. The red slurry was recovered and mixed with Na_2CO_3 (2.5 g/50 mL in water) in a 150-mL beaker. The green precipitate was then filtered, washed copiously with water, and then dried off at room temperature (Dai et al. 2007).

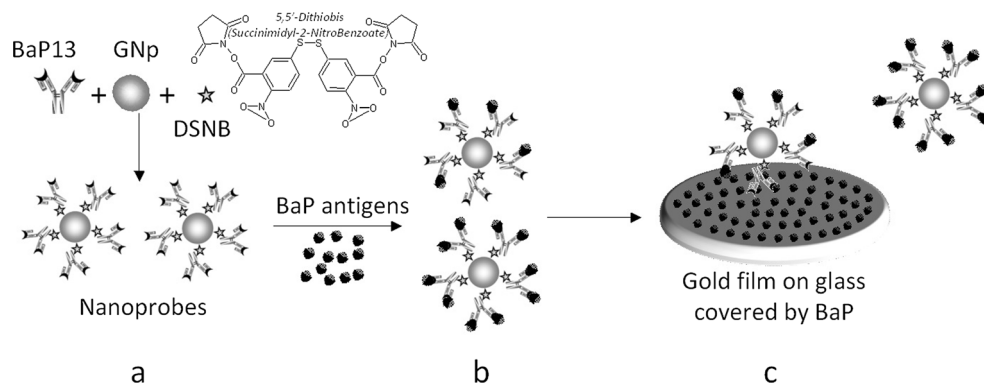
Functionalization of the sensor surface with a SAM presenting BaP head groups

Grafting of BaP on the gold surface begins with a self-assembled mixed monolayer formation of cysteamine on it. This step was assured through the gold-alkanethiolate self-assembly process using cysteamine and 2-mercaptoethanol. The substrate was cleaned in a piranha solution ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$ 30 %–3:1 v/v) at $80\text{ }^\circ\text{C}$ for 4 h to remove organic contaminants. Next, the gold surface of the sensor chip was immersed in methanol solution of 5 mM cysteamine and 5 mM 2-mercaptoethanol for 4 h. The sensor chip is then washed with methanol to remove any adhered/loosely bound alkanethiol molecules from the sensor chip. Afterwards, the substrate was rinsed in DMSO and treated in DMSO solution of 6-benzo[a]pyrenecarboxaldehyde (0.68 mg/mL) for 2 h. This forms a Schiff base which was then reduced by reaction of $14\text{ }\mu\text{L/mL}$ of DMSO of a sodium cyanoborohydride solution 5 M in NaOH 1 M for 1 h. Finally, grafted gold substrate was rinsed in distilled water and dried under a stream of nitrogen gas.

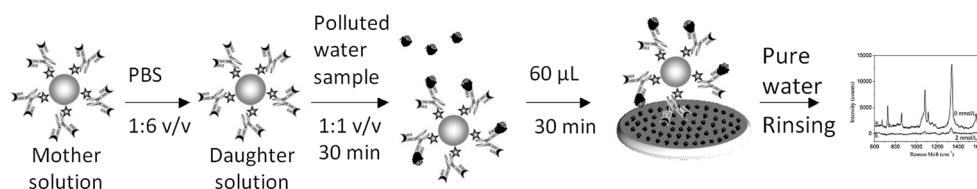
Synthesis of 5,5'-dithiobis(succinimidyl-2-nitrobenzoate)

DSNB is one of derivatives of dithiobis(benzoic acid), which could easily be converted to the corresponding succinimide ester with NHS. The formula of DSNB is shown on Scheme 1. DSNB is a particularly attractive example because of the strong scattering cross-section of its symmetric NO_2 stretch. To obtain this compound, we activated carboxylic functions of DNBA using a reagent mixture carbodiimide/succinimide. So, 50 mL of dried tetrahydrofuran were added to 0.50 g of DNBA, 0.52 g of DCCD, and 0.29 g of NHS in a 100-mL round-bottom flask equipped with a drying tube. The mixture was magnetically stirred at $25\text{ }^\circ\text{C}$ for 12 h, filtered, and then rotaevaporated to remove solvent. The crude product was dissolved in 100 mL of acetonitrile and filtered yielding a yellow solution. We denote by C_0 , the DSNB concentration of this solution. This concentration is equal to 2.5 mM if the yield of the DNBA activation was equal to 100 %.

Scheme 1 Description of the procedure undertaken for the SERS detection of benzo[a]pyrene



Scheme 2 Description of the measurement protocol



Preparation of Raman reporter-labeled immunogold colloids

Suspensions of gold nanoparticles were prepared according to the method described by Frens (1973). Our preparation technique has been used in different studies and consists in the reduction of HAuCl_4 by sodium tricitrate (Péron et al. 2009b, 2010). Here, 10 mL of sodium tricitrate at 8×10^{-3} M was slowly added to 10 mL of boiling tetrachloroauric acid at 2×10^{-3} M while stirring. The reactor was placed on a hot plate and magnetically stirred. The mixture of both solutions was refluxed for 1 h. After 1 h of boiling, the mixture was cooled at room temperature while stirring.

The gold colloidal nanoparticles are coated by the DSNB derivative thanks to its thiolate. The primary amines of an antibody can be then linked by the formation of an amide linkage. The particle synthesis is conducted in two steps. First, 100 μL of a 2.5 mM DSNB solution in acetonitrile was added to 1 mL of the unconjugated colloidal gold suspension and the mixture reacted during 3–5 h. The reporter-labeled colloids were then separated from the solution by centrifugation at 10,000g during 7 min. The clear supernatant was discarded, and the loose red sediment was resuspended in 1 mL of borate buffer (2 mM, pH 9). In step two, BaP13 was coupled to the gold particles via the succinimidyl terminal of the DSNB-derived coating. As such, 35 μg of detection antibody (35 μL of 1 mg/mL BaP13 solution) was added to the 1 mL suspension of the reporter-labeled colloid. The mixture was then incubated at room temperature for 1 h. After centrifugation at 10,000g for 7 min and the supernatant removed, the red sediment was resuspended in 1 mL of PBS (pH 7.0, 1 % BSA) (Grubisha et al. 2003).

SERS immunoassay procedure

Raman analysis is performed using the following steps: firstly, we diluted the probe suspension obtained according to the protocol described above with six volumes of PBS. After that, we mixed one volume of the suspension with one volume of seawater containing benzo[a]pyrene at a predefined concentration and incubate it for 30 min. Therefore, the molecules of BaP present in the sample to analyze will block the antigenic sites of antibodies in the organometallic probes. Then we placed a microdrop (60 μL) of this mixture on the surface of a substrate previously covered with benzo[a]pyrene. We rinsed the substrate with water 30 min after the deposit, and

finally, we analyzed it with the Raman spectrometer using $\times 100$ water immersion objective (Scheme 2).

Results and discussion

Surface grafting control of BaP-coated substrate

In order to control the surface grafting of benzo[a]pyrene, we have measured the binding ability of anti-BaP antibodies to the BaP-functionalized substrate. So, we used surface plasmon resonance (SPR) spectroscopy. Thus, we have studied two types of surface, functionalized and nonfunctionalized as controlled surface. The analysis started with the injection of phosphate buffer at pH 7. The BaP13 antibodies at 50 ppm in PBS was then injected. With the two surfaces, we observed an increase in the SPR signal just after injection of antibodies in the measuring cell (Fig. 1). However, we have noted that the increase obtained with the functionalized surface is considerably higher. Indeed, after PBS rinsing, the SPR profile obtained with the functionalized surface is characteristic for specific ligand-receptor interaction. Moreover, the measured offset is much higher with the functionalized surface ($\Delta\lambda = 7.8$ nm) compared with the uncoated surface ($\Delta\lambda = 0.4$ nm) (Fig. 1). Thus, we can maintain the presence of BaP molecules on the surface of functionalized substrate and therefore the effectiveness of our protocol.

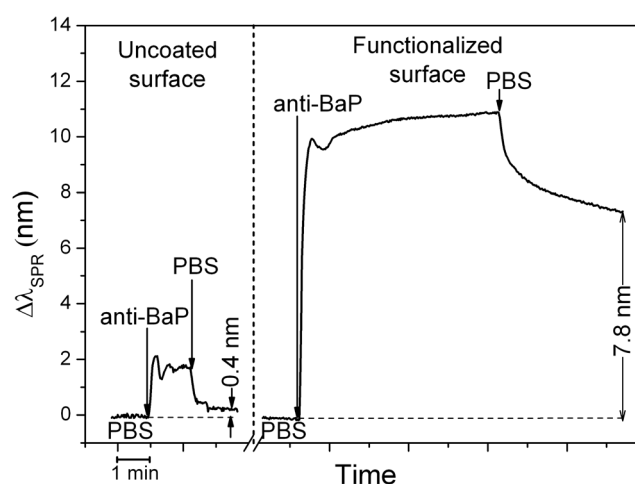


Fig. 1 SPR responses of the BaP13 antibodies adsorption onto the uncoated and functionalized surfaces

Determination of size and concentration of gold nanoparticles from UV-Vis spectra

Size

We have determined the size of nanoparticles by measuring the wavelength of plasmon resonance. Indeed, a relationship was demonstrated between the optical absorption spectrum (in particular, the plasmon resonance) and the size of the spherical gold nanoparticles (Link and El-Sayed 2000). Thus, we have analyzed with the spectrophotometer the solution of gold nanoparticles obtained using the protocol described above. The resonance wavelength of this solution is equal to 525 nm (Fig. 2). The displaying of spectra of nanoparticles solutions with sizes ranging from 9 to 99 nm allows us to determine that the diameter of our nanoparticles is between 22 and 48 nm.

The dependence of the optical properties of spherical gold nanoparticles on particle size were analyzed by Haiss et al. (Haiss et al. 2007). They have succeeded in establishing a mathematical relationship between the diameter (d) of gold nanoparticles and the absorption values of the corresponding solution at defined wavelengths.

$$d = \exp(B_1 \times A_{\text{SPR}} / A_{450} - B_2) \quad (\text{Haiss et al. 2007})$$

with A_{SPR} the absorbance at the surface plasmon resonance peak and A_{450} the absorbance at 450 nm. B_1 and B_2 values are 3 and 2.2, respectively (Haiss et al. 2007).

With the absorbance of 0.734 and 0.386 at respective wavelengths of 525 and 450 nm (thick line in Fig. 2), the average diameter of our nanoparticles calculated is equal to 33 nm.

Number density of the GNPs

To estimate the concentration of nanoparticles of our nanoparticles solution, we have established a relationship between

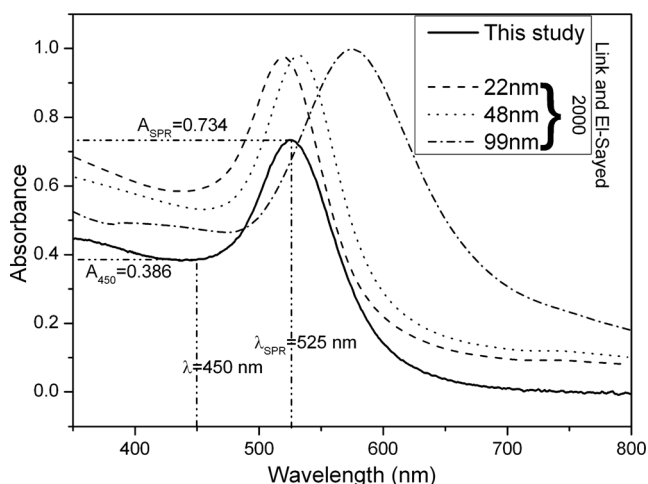


Fig. 2 Correlation between size and surface plasmon absorption of spherical gold nanoparticles. Thin lines: literature data (Link and El-Sayed, 2000). Thick line: our experiment

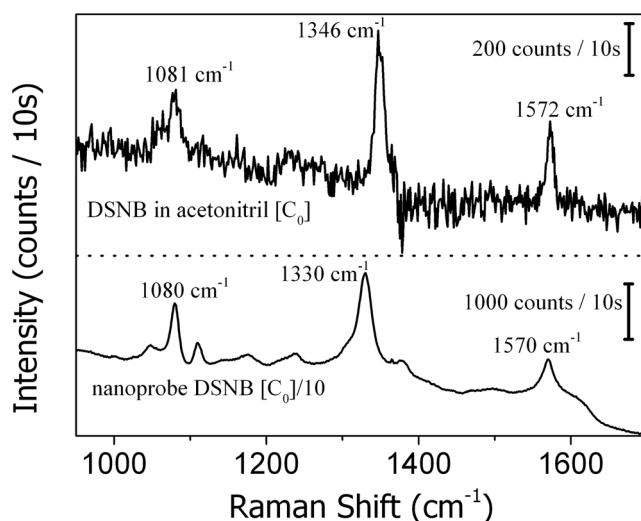


Fig. 3 Raman spectra of DSNB: (a) Spectrum of DSNB in acetonitrile solution at C_0 concentration (10s); (b) SERS spectrum of gold nanoprobes solution with DSNB at $C_0/10$ concentration (10s)

the number density of the GNPs and their average size. Indeed, if we consider that the whole quantity of gold used for the preparation of nanoparticles has been consumed Pluchery and Carriere (2011), we can express the number density n of nanoparticles in solution using the following equation:

$$n = V_T / V_P = 3 \times m / (4 \times r \times \rho \times r^3 \times V_S)$$

with V_T the volume of all gold nanoparticles, V_P the average volume of one nanoparticle, m the total mass of used gold, ρ the density of gold (19.28 g cm^{-3}), and V_S the solution volume.

Thus, with an average diameter of about 33 nm, the nanoparticles prepared are dispersed with a number density of about 5.4×10^{11} particles/mL.

SERS assay of nanoprobes

Prepared DSNB and the nanoprobes made of DSNB and antibodies were analyzed by Raman spectroscopy. The spectra of the range 600 and $1,800 \text{ cm}^{-1}$ are presented in Fig. 3. The free DSNB spectrum reported in Fig. 3a is obtained by subtracting the spectrum of the pure solvent from the acetonitrile solution. This spectrum is dominated by the symmetric nitro stretch at $1,346 \text{ cm}^{-1}$. The band at $1,572 \text{ cm}^{-1}$ is assigned to an aromatic ring mode, and the band at $1,081 \text{ cm}^{-1}$ is a succinimidyl N–C–O stretch overlapping with aromatic ring modes (Grubisha et al. 2003). The same bands in the solution spectrum are also observable in the spectrum of the DSNB nanoprobes (Fig. 3b), although NO_2 signal has shift which is probably assigned to the solvent effect due to the $\text{C}\equiv\text{N}$ group of acetonitrile. We were especially interested in the higher intensity bands to estimate the enhancement factor obtained with the Raman probes that we have prepared. Taking into

account the areas of the symmetric nitro stretch bands and the concentration ratio of DSNB for each sample, an enhancement factor equal to about 36,000 is calculated. In order to stick to this calculation, the experimental conditions were proceed the same way for each (exposition time, laser power, ...). This estimation assumes that the recovery rate of gold nanoparticles by reporter molecules is equal to 1 molecule/nm².

Nanoprobe activity by surface plasmon resonance sensor

We have studied the nanoprobe activity by surface plasmon resonance (SPR) spectroscopy using a gold surface covered with BaP. We observed that the coupling of BaP13 antibodies to gold nanoparticles did not affect their affinity for the molecules of benzo[a]pyrene.

BaP detection

Figure 4 presents the results obtained with concentrations of BaP 0 and 2 nmol/L in natural 1 μ m-filtered seawater from Argenton, France. When there is no BaP in the sample, the grafted antibodies onto the nanoparticles recognize and adsorb the BaP molecules immobilized at the surface of the sensor. Hence, the signal of reporter on spectrum (a.) in Fig. 4 is important. On this spectrum, we observe the Raman bands related to C–H (from 700 to 1,000 cm⁻¹), C–N (from 1,000 to 1,200 cm⁻¹), N–O (from 1,300 to 1,400 cm⁻¹), and C=C (from 1,500 to 1,600 cm⁻¹) bonds of DSNB. These bands are nearly extinct in the spectrum (b.) in Fig. 4 obtained in the presence of BaP at the concentration of 2 nmol/L. This is perfectly in accordance with the sensing method. Indeed the BaP molecules in the analyzed sample inhibit Raman probes to adsorb on the sensor surface by blocking the antigenic sites free of antibodies at the surface of these probes. Hence, a significant attenuation of the SERS effect was observed.

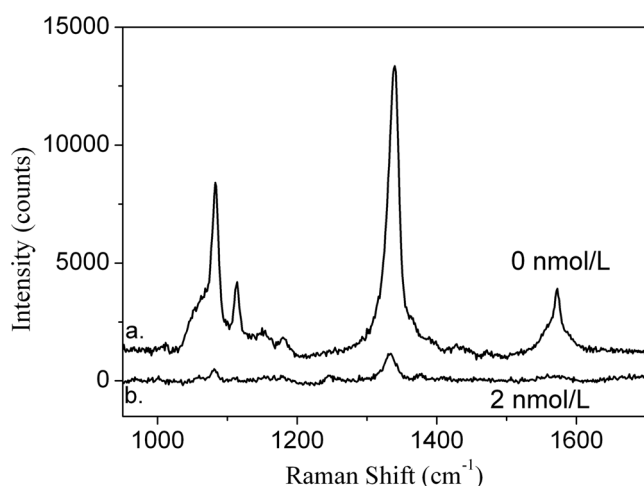


Fig. 4 Results of SERS immunoassay for BaP at 0 nmol/L (a) and 2 nmol/L (b)

However, it is important to note that this result suffers of a lack of reproducibility. Indeed, a more advanced work on the effectiveness of the analysis is underway to solve this problem.

Conclusions

The present work has demonstrated an efficient method to prepare surface sensor and SERS nanoprobe for specific detection of benzo[a]pyrene in seawater. The 6-benzo[a]pyrenecarboxaldehyde was used to modify the sensor surface for competitive assay. The SERS enhancement is observed through organometallic nanoprobe that were obtained by coupling of GNPs, DSNB, and BaP13. Detection of BaP at nanomolar level was achieved (2 nmol/L). However, improving the reproducibility are necessary in order to confirm the effectiveness of this detection method. This method has already the advantages of an approach that does not require extraction steps or large sample volumes. This should have great applications in rapid quantification of BaP in complex media such as environmental waters, as BaP is considered as a PAHs tracer. Furthermore, SERS detection of PAHs would be most suitable for in situ applications than standard method as GC/MS as shown by the pioneering works (Murphy et al. 1999; Schmidt et al. 2004; Péron et al. 2009a, b, 2010; Pfannkuche et al. 2012). Moreover, previous works carried out by Pfannkuche et al. show the good agreement between SERS in situ measurements and laboratory GC/MS analyses. In our work, by coupling SERS detection to immunoassay, the limit of detection is reduced. However, the specificity of our immunoassay towards another derivative of PAHs has to be tested to open new ways and generalized the use of our technique, not only in the laboratory but also in field assays with a portable Raman spectrometer.

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