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Amnesic shellfish poisoning biotoxin detection in seawater using pure or amino-functionalized Ag nanoparticles and SERS

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ABSTRACT

Domoic acid (DA) biotoxin responsible for the amnesic shellfish poisoning (ASP) has been unambiguously detected in seawater in a broad range of concentration, with both pure and amino-functionalized Ag nanoparticles employed for surface enhanced Raman scattering (SERS). To achieve this, a comprehensive SERS study on DA dissolved in distilled water has been conducted. SERS of DA dissolved in seawater in concentrations ranging from 3.3×10^{-4} to 3.3×10^{-8} mol l^{-1} exhibited specific signal, completely different to those of the corresponding DA aqueous solutions, due to the seawater interference in the overall SERS effect. In order to assess the capability of the technique as a cheaper alternative for rapid and unambiguous detection of the DA biotoxin in seawater, three detection schemes have been proposed. DA was detectable at 0.33 nmol l^{-1} concentration (0.33) dissolved in distilled water and 0.033 nmol l^{-1} (0.033 ppb) in seawater respectively, much lower than the admitted level by the current regulation. A solvent specific interaction of DA with the NPs was concluded, since DA aqueous solution added to Ag nanoparticles provided different SERS signal compared to that of DA directly dissolved in seawater. Employing amino-functionalized Ag nanoparticles with 4-aminothiophenol as SERS tag, SERS signal of DA on amino-AgNPs revealed significant specificity associated with the aromatic primary amine interaction of the SERS tag with DA, thus allowing DA detection in seawater at 4.16×10^{-4} mol l^{-1} concentration, much higher than in the case of pure NPs. To highlight the findings, a brief literature review to date on the DA biotoxin detection was also provided.

1. Introduction

Domoic acid (DA, $C_{15}H_{21}NO_6$, molecular weight 311.3303 g mol^{-1}) is a naturally occurring excitatory neurotoxin produced by certain marine organisms, such as the red alga *Chondria armata* and planktonic diatom of the genus *Pseudo-nitzschia* [1]. This acid is polar, soluble in water and insoluble in organic solvents [2]. Several reports mentioned that DA was used for centuries as an anthelminthic, a parasite remedy [3,4]. Later, it was found to be toxic to humans [5,6]. Domoic acid is the toxin responsible for amnesic shellfish poisoning (ASP). As a new disease entered the public health lexicon in 1987 [2], ASP symptoms include vomiting, nausea, diarrhea and abdominal cramps within 24 h of ingestion. In more severe cases, neurological symptoms develop within 48 h and include headache, dizziness, confusion, disorientation, and loss of short memory, motor weakness, seizures, profuse respiratory, secretions, cardiac arrhythmias, coma and possibly death [7–9]. The first case of human poisoning with ASP occurred in 1987 in Canada, in Prince Edward Island. The poisoning was caused by the ingestion of contaminated blue mussels [*Mytilus edulis*] [9,10], several people died and over 150 others people developed various toxic symptoms, after consuming contaminated shellfish [1]. DA has also resulted in the mortality of hundreds of marine birds, mammals and fish in several locations worldwide [2]. Since 1987, toxins monitoring programs have been successful in preventing other human incidents. However, documented reports as well as informal cases from seafood aquaculture areas on ASP intoxication as well as PSP (paralytic shellfish poisoning) or DSP (diarrheic shellfish poisoning) in wild animals and outbreaks of coastal seawater contamination in many regions world-wide still appeared [11]. Hence, such toxins continue to pose a global risk to the health and safety of humans and wildlife.

The mouse bioassay is the method most widely used to detect many of these toxins in shellfish samples, but animal welfare concerns have prompted researchers to seek alternative methods

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of detection [12]. Enzyme-linked immunosorbent assays (ELISAs) [12] or high performance liquid chromatography (HPLC) [13] usually employed to detect marine toxins, in spite of their high sensitivity and robustness, are very expensive, time consuming, laboratory-based, require significant technical expertise, therefore not applied as systematic control methods. Consequently, typical tests are not performed routinely on seafood products for consumption. The detection time is a very important issue, since such toxins could be present in perishable seafood.

Remote-sensing based techniques were also employed to monitor from aircraft the multispectral-radiance data while surface waters survey in the period of algae blooming [14]. A summarized review on the marine toxins detection is presented in Table 1. Most of the current methods applied in the detections of toxins or other pollutants in sea organisms were used for research purposes and were not intended for the monitoring programs. Marine biotoxins are poisonous substances that shellfish, echinoderms, tunicates and gastropods can accumulate, particularly as a result of feeding on plankton containing toxins. Biotoxins usually produced by Dinoflagellates are thermo stable and cannot be removed from the meat tissues during heating (culinary) treatment. Shellfish, echinoderms, tunicates and gastropods that accumulate toxins are completely healthy and without external changes or visual. In humans, throughout the food chain, they cause dangerous intoxication syndromes (ASP, PSP, DSP). Toxins produced by algae are held responsible for approximately 60,000 human intoxications yearly. Shellfish toxins also cause damage to wildlife and have a negative economic impact on recreation, tourism and shellfish industry [7].

Concerning DA, the most common method used for detection reported so far is HPLC, with a detection limit of 1 ppm (1000 ng ml^{-1}) [10,18]. The European Union regulations have established a permitted level of 20 mg DA/kg in shellfish or in mollusk [15,16].

Although crucial to develop sensitive techniques for toxins detection, little spectral information on them is available so far. Molecular structure of DA was first elucidated by Takemoto and Daigo [17] whereas Yao et al. [10] studied DA solutions by UV resonance Raman using 242 and 257 nm laser excitations. The detection limit was 1 ppm (1000 ng ml^{-1}) respectively 2.5 ppm (2500 ng ml^{-1}). Wu at al. [16] used a 251 nm laser excitation and the detection limit was 0.04 ppm (40 ng ml^{-1}) in D_2O water.

Djaoued et al. [1] studied DA solutions by non-resonance Raman spectroscopy using a 633 nm laser excitation. Non-resonance Raman was shown to generate a characteristic Raman band at 1650 cm^{-1}, and detection limit was 0.25 ppm (25 ng ml^{-1}).

Water security monitoring using surface enhanced Raman scattering (SERS) technique [19] was an important issue taken into consideration by US Environmental Protection Agency (EPA) for several years. SERS approach in detection would overcome most of the current disadvantages in toxin detection, by providing sensitivity down to the single-molecule level in particular experimental cases [19,20]. Metal nanoparticles, particularly silver and gold, due to their surface plasmon resonance in the visible electromagnetic range, provide a significant enhancement of the Raman signal from a molecule located in the close vicinity of nanoparticles surface [21,22]. Together with the new developments in controlled nanofabrications, SERS-based method in detection and monitoring became increasingly applied in various biological, medical or environmental fields [23]. SERS reached its maturity as a powerful analytical tool for detecting toxins at low concentration with molecular specificity and high sensitivity and

Table 1
List of the biotoxins causing poisoning syndrome and the detection methods described in the literature [7].

Toxin class	Toxin group	Syndrome	Genus	Species	Causal level of biotoxin	Detection methods
Hydrophilic toxins	DA	**ASP**	*Pseudo-nitzschia*	*australis, calliantha, cuspidata, delicatissima, fraudulenta, galaxiae, multiseries, multistriata, pseudodelicatissima, pungens, seriata, turgidula*	20 μg g^{-1} of shellfish meat tissue	MBA, LC-DAD LC-UV, LC–FLD, LC–MS (FAB-MS), LC–MS/MS, GC–MS ELISA
	STX	**PSP**	*Alexandrium*	*angustitabulatum, catenella, fundyense, lusitanicum, minutum, tamarense, tamiyavanichii*	80 μg/100 g of shellfish meat tissue	MBA, HPLC (LAWERENCE METHOD), LC–FLD, LC–MS (ESI-MS) HILIC ELISA
			Gymnodinium	*catenatum*		
			Pyrodinium	*bahamense*		
Lipophilic toxins	OA DTX PTX YTX	**DSP**	*Phalacroma* *Prorocentrum*	*rotundatum* *arenarium,* *belizeanum,* *concavem,* *lima*	160 μg/1000 g of shellfish meat tissue (1 mg YTX /kg of shellfish meat)	AOAC MBA, LC–FLD, LC–MS LC-MS/MS ELISA (VHPLC)–MS/MS CE
			Dinophysis	*acuminata, acuta, arenarium, caudate, fortii, mitra, norvegica, ovum, rotundata, sacculus, tripos*		

Abbreviations: TOXINS: DA=domoic acid, STX=saxitoxin, OA=okadaic acid, DTX=dinophysistoxins, PTX=pectenotoxins, YTX=yessotoxin. METHODS:MBA=mouse bioassay, RBA=rat bioassay; HPLC=high performance liquid chromatography, LC–FLD=liquid chromatography–fluometric detection, LC–MS=liquid chromatography–mass spectrometry, LC–MS/MS=liquid chromatography tandem mass spectrometry, VHPLC-MS/MS method=very high pressure liquid chromatography–mass spectrometry; ELISA=enzyme-linked immunosorbent assay; CE=capillary electrophoresis.

shows immense potential for fast sensing of toxins in various applications. Although EPA invested a lot in projects on water security monitoring programs using SERS [24] to create a portable echelle-based SERS system that would provide analytically reliable and reproducible information on toxins from seawater [25], the expected results on ASP, PSP or DSP SERS detection capability using such compact SERS-sensing equipment are absent in literature. Project reports mentioned however that the SERS sensors coupled to a high-resolution echelle spectrograph for precise mixture analysis could provide results directly at the water source in less than 1 min, whether the water source is treated or raw [26]. Concerning the report statement on "the potential for SERS as a cyanotoxin sensor, in which cylindrospermopsin, microcystins, anatoxin-a, saxitoxins, and domoic acid all were detected" [27], any reference paper is absent to date.

We investigated here the concentration dependence SERS spectra of DA in distilled water and seawater and propose a SERS sensing scheme for DA, using both pure and amino-functionalized Ag nanoparticles, aiming to set up a reliable SERS-based method suitable for portable Raman equipments for aquaculture monitoring programs.

2. Materials and methods

Domoic acid (~90%), silver nitrate ($AgNO_3$), sodium citrate and 4-aminothiophenol (~97%) were purchased from Sigma-Aldrich.

FT-Raman spectrum of the crystalline DA was recorded with a Bruker RAM II spectrometer, equipped with a Nd:YAG laser operating at 1064 nm with an output power of 1 W. Spectral resolution was 2 cm^{-1}, and 100 scans were co-added.

As SERS surface, a sodium citrate silver colloid prepared according to the standard procedure reported by Lee and Meisel [28] was employed. Briefly, 45 mg of silver nitrate was dissolved in 150 ml triply distilled water and brought to boiling. A solution of 1% sodium citrate (5 ml) and 100 ml pure water were added to the boiling solution, allowed to continue boiling for an hour.

SERS spectra were recorded with a Horiba Jobin-Yvon LabRam confocal Raman microscope (HR800) with a 300 groves mm^{-1} grating, using a Nd:YAG laser operating at 532 nm line, with an output power of 4 mW for excitation. The microscope was equipped with a 10× objective (NA 0.25). A Peltier cooled CCD was employed for signal detection.

Different stock solutions of DA in concentration ranging from 10^{-3} to 10^{-6} mol l^{-1} were prepared, dissolving DA powder in triply-distilled water. SERS samples were prepared by adding 10 μl of each stock solution to 100 μl colloidal Ag, resulting DA concentrations in the 3.3×10^{-4} to 3.3×10^{-7} mol l^{-1} range.

Seawater samples from 1 m depth, at 21 °C were randomly acquired from a wild area in Dubrovnik region, located in the South-Eastern coast of Adriatic Sea, in Dubrovacki-Neretvanska County, Croatia. The water samples were collected in small glass vials and transported to the laboratory for immediate SERS experiments. Same stock solutions of DA as above have been prepared in seawater in the 10^{-3} to 10^{-7} mol l^{-1} concentration range, and used for SERS measurements, where DA final concentration varied from 3.3×10^{-4} to 3.3×10^{-8} mol l^{-1}. The SERS samples were prepared by adding 50 μl from each DA stock solution to 100 μl colloidal Ag and measured immediately. All solutions were placed in a cuvette sealed with parafilm to prevent evaporation of the solutions and keep constant the toxin concentration during the SERS measurements. SERS samples of raw seawater were also prepared by adding from 10 to 40 μl seawater to 100 μl colloidal Ag and measured immediately.

We employed 4-aminothiophenol (4-ATP) to obtain amino-AgNPs conjugates [29] as SERS labels with available amino-functional group for tagging specific carboxylic groups of DA. We used 4.5×10^{-3} mol l^{-1} solution of 4-ATP in 3:2 ethanol:water solvent mixture. The amino-AgNPs conjugates were prepared by adding 10 μl of 4-ATP solution to 100 μl Ag colloidal nanoparticles. Finally, 50 μl DA solution was added to the AgNPs-4-ATP system and measured several times as well as after 24 h to check the signal stability.

SERS spectra were recorded in the 100–3300 cm^{-1} wavenumber range.

To concrete assess the reproducibility of the reported results here, every measurements was performed at least 3–5 times using three different stocks of Ag NPs. Two different domoic acid stock samples from Sigma-Aldrich with 90% purity were used to completely reproduce the experimental part. The 10% impurity was subject of raised queries since the producer indicated this matter as "non specified". Taking into account the very small amount of raw substance to prepare the solution and the microliter range volume used in SERS experiment, as well as the reproducibility of the results from one stock to another, we assumed that the impurity of the solid DA may have insignificant contribution in the overall background SERS signal.

3. Results and discussions

The FT-Raman spectrum of solid DA, showed in Fig. 1A revealed the most intense Raman band at 1649 cm^{-1} (1650 cm^{-1} in literature [1], DA Raman marker band) which is assigned to the stretching vibration of the conjugated C=C double bond [1].

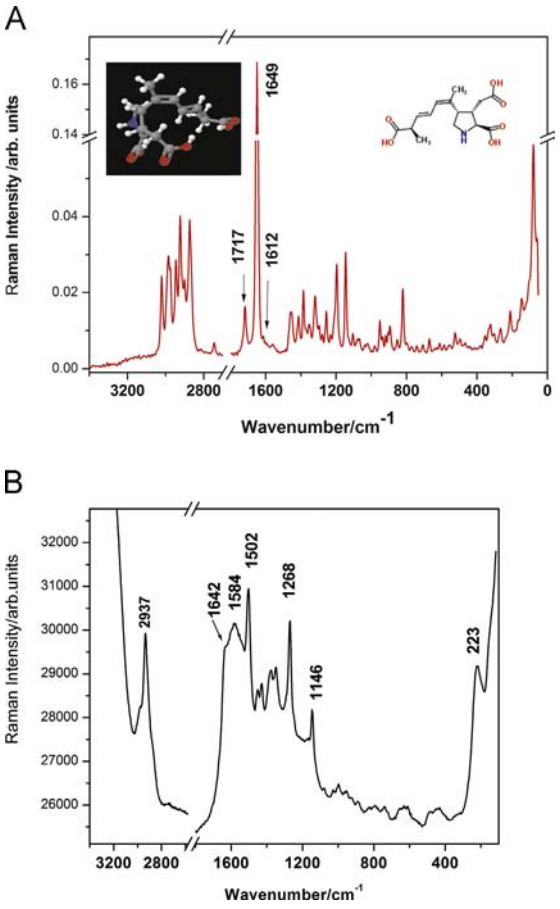

Fig. 1. (A) FT-Raman spectrum of solid DA and (B) SERS of DA dissolved in distilled water. Excitation laser line (A) 1064 nm, power 1 W, 100 scans. (B) 532 nm, 4 mW. Insertion: DA molecular structure.

Based on literature data [30] we proposed a complete assignment of the observed FT-Raman bands and compared to those previously reported by Djaoued et al. [1] on crystalline DA excited with 632.8 nm line. Although the bands positions are the same in the limit of resolution, their relative intensity was different, because of the different laser excitation line used. The observed Raman bands together with the proposed assignments as well as SERS bands are summarized in Table 2.

The SERS spectrum of DA 3.3×10^{-4} mol l^{-1} in pure water solution at pH 5.5 is presented in Fig. 1B. The most significant SERS bands are observed at 2937 (strong, broad), 1642, 1584, 1502 (very strong), 1376, 1347, 1268 cm^{-1} (very strong), 1146 and 223 cm^{-1}. Additional weak peaks are also observed as shown in Table 2. The SERS signal exhibits differences in band positions and relative intensities when compared to the FT-Raman spectrum of solid DA (Fig. 1A), suggesting a strong chemisorption process on the Ag nanoparticles. The presence of the SERS band at 223 cm^{-1} indicates the forming of the Ag–O [31,32] bond, with no counterpart for in the normal Raman spectrum. This is an indicative of the nearness of carbonyl group to the silver surface. Similar values are reported for the wavenumbers assigned to ν(Ag–O) SERS mode in literature [33].

The most prominent SERS band was observed at 1502 cm^{-1} with no counterpart in the normal Raman spectrum. Its SERS origin could be explained as a Raman forbidden band that became active in the adsorbed molecule and was assigned to the imine bending mode. The broadening of band assigned to C=C bond in SERS is likely due to a diverse set of molecular orientations with respect to the nanoparticles surface. The conjugated double bond from the skeletal structure with three distinct carboxylic groups (–COOH) at the molecule's periphery in a 3D geometry (as provided in ChemSpider ID: 4445428), converges in a C -letter molecular shape, as shown in the insertion of Fig. 1A. The imino group (NH) also competing to bind to the metal surface [34,35] is closer to one of the carboxylic group. Therefore, theoretically many possibilities of interaction with the metal surface exist, through one or two of the three carboxylic groups, imino group, through the π electrons of the penta-ring or through multiple binding sites, respectively. According to Falk et al. [36] DA exists in five distinct protonation states, whose proportion is strongly dependent on pH, where the imino >NH or the three carboxylate groups are sensitive in aqueous solutions. Taking into account that our stock DA solution has pH value of 5.5, where a single charged anion $-COO^-$ is present [36], the most probable adsorbed form would be

Table 2
Vibrational Raman and SERS wavenumbers/cm^{-1} of domoic acid and the proposed assignments.

Raman DA Djoued et al.[1]	**FT-Raman DA experimental**	**SERS DA (distilled water) 3.3×10^{-4} mol l^{-1}**	**SERS DA (seawater) 3.3×10^{-4} mol l^{-1}**	**SERS DA on amino-AgNPs in seawater 4.14×10^{-1} mol l^{-1}**	**Assignment**
3021	3020(m)	–	3062(vw)	3032(vw)	ν(CH) imino ring
2984	2985(m)	–		–	$\nu(CH_3)$
2947	2947(m)	2944 (m)	2954(s)	2963(vs)	ν(CH)
2924	2923(s)	2937 (s)	2920(vs)	2931(vs)	$\nu(CH_2)$
2900	2899(m)	–		–	ν(CH)
2875	2873(s)	–	2865(m)	2874(m)	ν(CH)
2747	2746	–		–	ν(CH) alifatic
1718	1717	–		–	>C=O
1650	1649 (vs), 1612 (w)	**1642(s)**	**1651(m), 1622(m)**	**1649, 1617(ms)**	**ν (–C=C–), (DA+amino-NPs) bending**
		1584(s)	1575(vw)	1575(m)	imino ring stretch
–	–	**1502(vs)**	**1505(s)**	**1505(s)**	**imine bending**
1451	1452	1450(w)	1450(s)	1450(m)	$\delta(CH_2, CH_3)$
1413	1413	1428(w)	1431(s)	1431(m)	ν(COO), δ(C–OH)
1387	1386(m)	1376(m)	1376(m)	1376(m)	$\tau(CH_2^+)$
1355	1352	1347(m)	1347(s)	1347(m)	ν(C–C), $\tau(CH_2)$
1320	1319(m)	–			δ(CH)
1255	1255	1268(vs)	1271(vs)	1271(vs)	–C–N– imino
			1209 (w)	1209(w)	C–N
1195	1196(s)	–	1172(m)	1184(w)	$\gamma(NH_2^+)$
1145	1145(s)	1146(m)	1146(s)	1146(m)	$\gamma(CH_2)$, ρ(COH)
1105	1105	–			$\sqrt{}$(CCN)
1067	1076		1072(w)	1072(w)	$\delta(CH_2)$
			1032(m)	1032(w)	Penyl stretch (organic from seawater)
1025	1019	998(vw)	991 (m)	991(s)	SO_4^{2-} $\sqrt{}$(CN)
952	951	954(vw)	957(vw)	919 (vw)	$\sqrt{}$(CCN), $\sqrt{}$(CC)
894	893	889(vw)	854(vw)	854 (w)	$\sqrt{}$(CC)
			802(vw)	802(w)	
821	820(m)	792(vw)	792(vw)	792(m)	$\rho(CH_2)$
742	742	736(vw)		744(w)	
671	669	652(vw)	642 (w)	642(m)	$\delta(COO^-)$
		612(vw)	610(w)	610(m)	
524	524	480(vw)	507(vw)	507(w)	γ(COO–)
323	324	431(vw)	455(vw)	445(w)	$\rho(CH_2)$
266	267	–		–	Skeletal bending
		223 (s)	220 (sh)		Ag–O
				219 (vs)	Ag–S (from 4-ATP)
211	210				Skeletal bending
	147(s)	–	152(vs)	152(vs)	Lattice vibration;
	79(s)	–		–	Lattice vibration

Abbreviations: s—strong, m—mediu, w—weak, v—very, $\sqrt{}$—stretching, δ—scissoring, bending, τ—twisting, out of plan bending, ρ—rocking, in plane bending, γ—wagging.

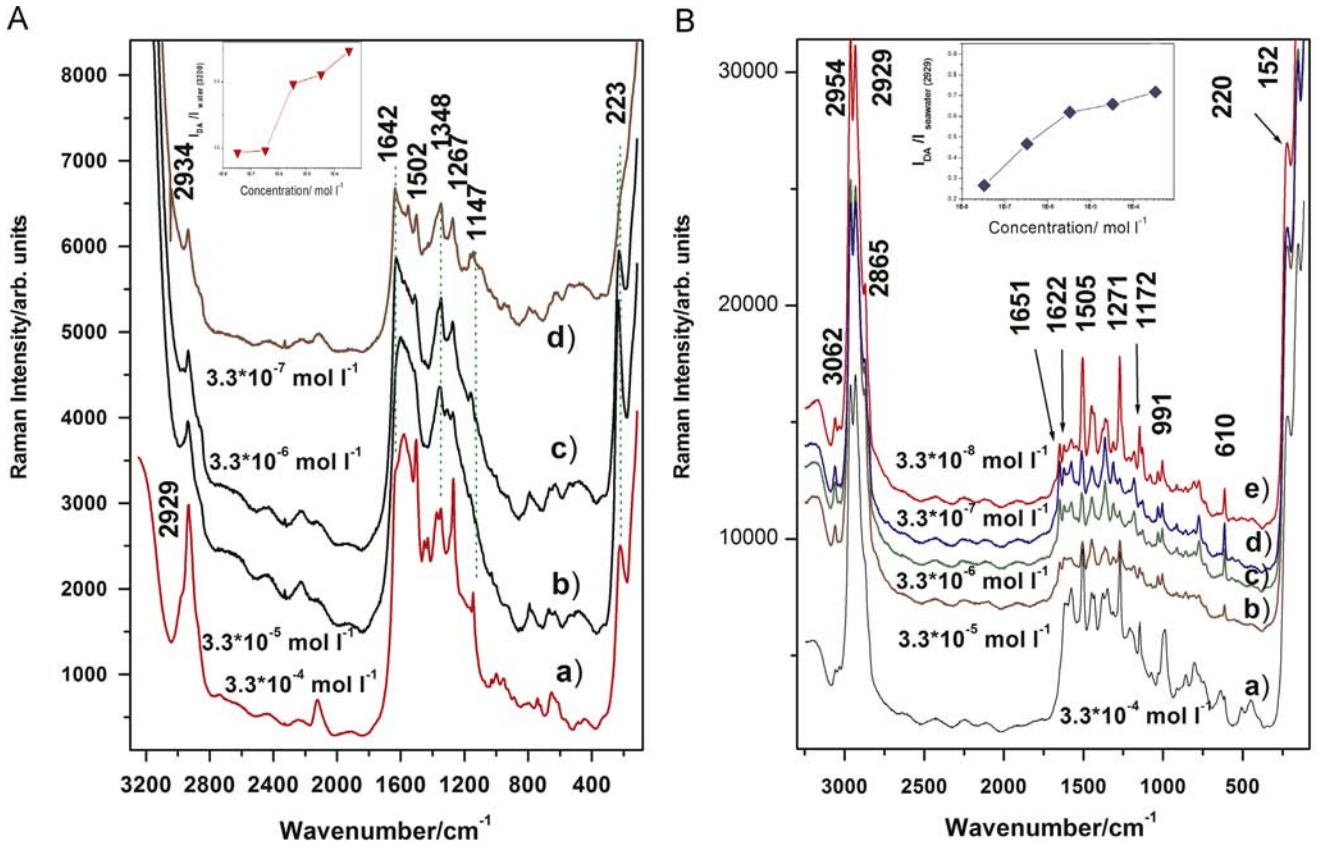

Fig. 2. SERS spectra of DA dissolved in distilled water (A) and in seawater (B) at different concentrations from 3.3×10^{-4} to 3.3×10^{-7} mol l^{-1} (from (a) to (d)), as indicated on each spectrum. In the case of seawater solution, an order of magnitude lower was achieved (3.3×10^{-8} mol l^{-1}). In seawater (B), the high wavenumber range is completely dominated by seawater signal. Excitation: 532 nm, 4 mW. The Insertion in (A) shows the relative intensity ratio $R=I_{1649}/I_{3200}$ of the DA SERS band at 1649 cm^{-1} and water band at 3200 cm^{-1} as a function of concentration whereas in (B), the relative intensity ratio $R_{sw}=I_{1651}/I_{2929}$ calculated using the DA band at 1651 cm^{-1} and the intense band observed at 2929 cm^{-1} (seawater contribution overlapped to the weak DA contribution).

through this functional group. Consequently, the observed SERS band corresponding to the DA–Ag conjugates at about 223 cm^{-1} (Fig. 1B), was assigned to Ag–O bond and confirmed the most probable adsorption supposition. This evidence is also supported by the absence of the C=O mode at 1717 cm^{-1} in the SERS signal, because of its parallel polarizability orientation with respect to the Ag nanoparticles surface. Because of the low symmetry of the molecular structure, the SERS bands observed in the high wavenumber range (2743–3021 cm^{-1}) corresponding to the C–H or $-CH_3$ modes of various aliphatic, aromatic or methyl groups respectively, could not be used to draw conclusions about the complex geometry of the adsorbed species.

3.1. Concentration dependence SERS spectra of DA in pure water and seawater

Concentration dependence SERS signal of DA is shown in Fig. 2A and B, both for distilled and seawater solutions, respectively. With decreasing concentration, slightly different relative intensity of the bands in fingerprint region (Fig. 2A) suggested a re-orientation of the adsorbed DA with respect to nanoparticles surface. In order to probe the SERS capability for lower detection limit, progressively diluted DA seawater solutions were prepared from stock. In the case of DA seawater solution (Fig. 2B), an order of magnitude lower was achieved (3.3×10^{-8} mol l^{-1}) in SERS detection. Large differences were observed between the SERS spectra of DA dissolved in seawater compared to that in pure water (Fig. 2A and B) mostly in the 1500–1650 cm^{-1} and in high wavenumber range (2850–3000 cm^{-1}). DA dissolved in pure water exhibited strong SERS bands in the 2929–2934 cm^{-1} range (Fig. 2A), slightly shifted to higher wavenumbers with decreasing concentration from 3.3×10^{-4} (bottom spectra) to 3.3×10^{-7} mol l^{-1} (upper), as indicated on each spectrum.

In the case of DA seawater solution the SERS signal is completely different to that of the distilled water solution, with prominent bands at 219 and 152 cm^{-1} in the low wavenumber range as well as in the fingerprint region at 1505, 1366, 1271 and 1172 cm^{-1} and in the high wavenumber range (Fig. 2B). Two distinct bands were also observed at 1651 and 1622 cm^{-1} whose intensity decrease with decreasing concentration from a) to e) spectra (3.3×10^{-4} to 10^{-8} mol l^{-1}) while the bands at 1505, 1366, 1271 and 1172 cm^{-1} exhibit different relative intensity with decreasing concentration, suggesting an interaction of DA with some component of seawater or a structural change of the molecule because of different seawater pH (7.5). A distinct band observed in the SERS of DA in seawater solution at 991 cm^{-1} was not observed in the pure water solution. Its origin was supposed to come from the sulfate from seawater [35], since the bulk seawater sample was large enough (50 μl) to itself provide bulk Raman signal. Pure sulfate solution usually exhibits medium SERS bands at 981 cm^{-1} (experimental results, not shown here). The high wavenumber range is completely dominated by seawater SERS signal.

A previous report on SERS on DA [36] in aqueous solution shows that the toxin was detectable at 2.5×10^{-5} mol l^{-1} concentration (7.8 μg ml^{-1}). With the current SERS method we demonstrated the possibility to detect the DA in pure water solution at 3.3×10^{-7} mol l^{-1} (0.1 μg ml^{-1}), and in seawater solution at 3.3×10^{-8} (0.01 μg ml^{-1}) mol l^{-1}. We reached a detection limit 78 times lower than previous SERS report, and even lower (780 times) when seawater was used as solvent.

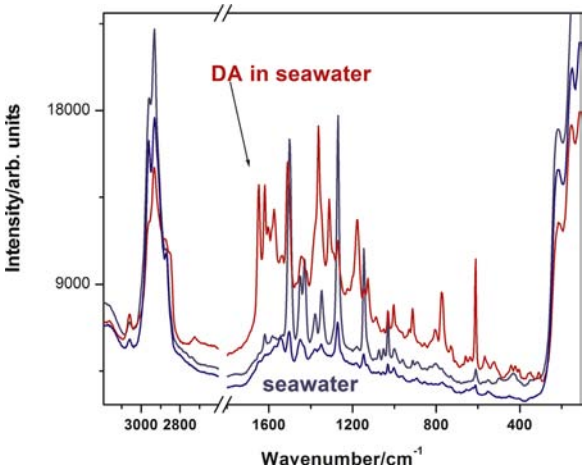

Fig. 3. SERS spectra of pure seawater (10 and 40 μl respectively in 100 μl Ag colloidal nanoparticles (bottom); and of 0.312×10^{-3} mol l^{-1} DA in seawater SERS sample (312 ppm), (top), equivalent to 833 μmol l^{-1}, in seawater only (833 ppm). Excitation laser line: 532 nm, 4 mW.

The relative intensity ratio $R = I_{1649}/I_{3200}$ of the DA SERS band at 1649 cm^{-1} and water band at 3200 cm^{-1} has been calculated and displayed as a function of concentration, as shown in the insertion in Fig. 2A. In the case of DA dissolved in seawater, the relative intensity ratio R_{sw} was calculated using the DA band at 1651 cm^{-1} and the intense band observed at 2929 cm^{-1} (seawater contribution overlapped to the weak DA contribution). The band at 2929 cm^{-1} although overlapped with the DA contribution in overall SERS, is believed to arise from the organic content of seawater. The insertion plot of calculated ratios $I_{DA}/I_{seawater}$ clearly revealed a different dependence of concentration (Fig. 2B). A similar calculation using the intensity of the water band at 3200 cm^{-1} as in the case of SERS with distilled water was considered irrelevant because of the SERS seawater interference in the overall signal. Seawater environment, a very complex system containing both organic and inorganic components, provides itself a significant SERS response, as shown in Fig. 3, for 10 and 40 μl seawater added to 100 μl Ag colloidal nanoparticles, respectively. Taking into account the pH dependence of DA molecular structure [35], we also prepared different SERS samples of DA, by adding DA already dissolved in distilled water to seawater solution, resulting in a concentration of DA in seawater of 0.312×10^{-3} mol l^{-1}. In this case the SERS signal of DA provided two sharp bands at 1649 and 1622 cm^{-1} that allowed to unambiguously detection of the toxin from seawater, as comparatively highlighted in Fig. 3.

SERS determination of the toxin in seawater opens an important issue for the real time monitoring of the role of dissolved DA and the distribution patterns across the trophic webs. Because of the extremely low concentration of biotoxins expected in seawater during harmful algae blooming events, analytical determinations have been preferably conducted in bivalve molluscs that accumulate these poisons by direct filtration of the plankton cells. Thus, previous analytical methods for determining domoic acid in phytoplankton, mussels and in general all kind of shellfish have been developed in an effort to limit the risk of widespread human intoxication by ASP [36–39]. Getting into comparative experimental details, the SERS technique prevails by far concerning the number of the experimental steps, the amount of reagents used, the required technique costs, the time of analysis and the suitability for conducting measurements outdoor, in situ. The extraction and concentration of DA from a water matrix required for example by the chromatography methods is a challenge because of the strong hydrophilicity of this compound. In an attempt to increase the volume of extracted sample (which is directly related to the final sensitivity of the HPLC method), and to keep the advantage of analyzing the whole sample extract, a sample preparation procedure combining off-line and on-line solid phase extraction was also developed [40] being strictly related to the lab-based operation. Moreover, DA has a rather high boiling point at 607.2 °C which is a major inconvenient for chromatography column and required additional steps (eventually methylation) to conduct the analysis.

The many steps and substantial amount of solvents used within a time span of several hours make the chromatography- combined with mass spectrometry technique generally recognized as expensive and suitable for the lab only, although a new generation of still rare and expensive mobile chromatography stations were commercially developed for specific routine analyses purpose. For example the chromatographic conditions included 10% aqueous acetonitrile containing 0.1% trifluoroacetic acid mobile phase [41]. Another mobile phase with pH 2–3 is needed in order to suppress ionization and subsequent tailing due to the characteristics of the three carboxyl groups and one secondary amino group. SERS method requiring just robust reproducible noble metal nanoparticles, a compact, even portable sensitive instrument to measure liquids and the expertise of the trained analyst would substantially decrease the cost of such analyses uniquely adding also the valuable promptness. A chromatography analysis although depending on the condition and the matrix for extraction could be hardly estimated to approximately several hundreds euros and a couple of days of work, while the price of one SERS analysis aiming to detect DA in aqueous solution the required reagents referees to the NPs cost that was estimated to 0.21 euro (21 euro cents) for 200 ml colloidal Ag sufficient for about 400 measurements of several seconds each.

3.2. SERS of DA on amino-functionalized Ag nanoparticles

Domoic acid appears to produce its neuroexcitatory effect by interaction with amino acid receptors [42]. Therefore, our approach aims to investigate the possibility to detect DA based on its affinity to amino-terminal group of a SERS label.

It is generally known that a SERS label comprised a SERS substrate (usually noble metal nanoparticles) and an adsorbed Raman reporter (a molecular species with high Raman scattering cross section [43].

In order to probe the capability of DA to bind to specific SERS tag, we employed 4-aminothiophenol (4-ATP) chemisorbed on Ag as a SERS label for detecting DA. It is well known that 4-ATP strongly interact with the Ag or Au nanostructure substrates and the Ag-ATP conjugates exhibits very strong SERS signals [43–46] of the chemisorbed ATP through the S atom, confirmed by the new SERS band of adsorbed ATP at about 212 cm^{-1} and assigned to Ag–S vibration mode [46].

Recent reports showed that p-ATP species could encount photo changes in molecular identity due to the incident laser photons [47], resulting new chemical species formation, like *p,p′*-dimercaptoazobenzene, with totally different SERS signal. The new "b2 modes' at 1142, 1388 and 1432 cm^{-1} reported in the SERS spectra of ATP were actually assigned to *p,p′*-dimercaptoazobenzene when laser power applied was high (ca 10 mW) [47].

Taking into account specific environmental conditions to obtain a reproducible SERS signal from 4-ATP on Ag, we recorded SERS spectra in the case of DA solution dropped into the Ag-ATP SERS conjugated complex. Indeed, after intermediary SERS measurement of amino-AgNPs system (meaning laser exposure) and further adding DA solution results in less availability of the Ag-ATP conjugates to provide free amino-group, because of the laser induced photoreaction between neighbor ATP molecules generating *p,p′*-dimercaptoazobenzene on the Ag surface. In the

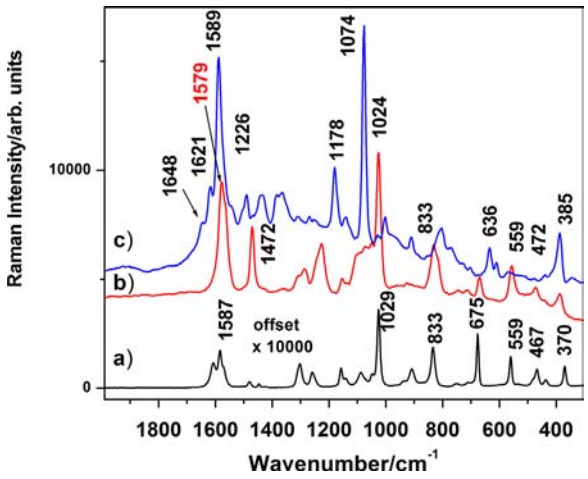

Fig. 4. FT-Raman spectrum (× 10,000 offset) of pure, solid 4-ATP (a), SERS of 4-ATP (b) and SERS of DA on amino-AgNPs (c). Toxin final concentration 4×10^{-4} mol l^{-1}. Excitation laser line: 1064 nm (a); 532 nm (b) and (c).

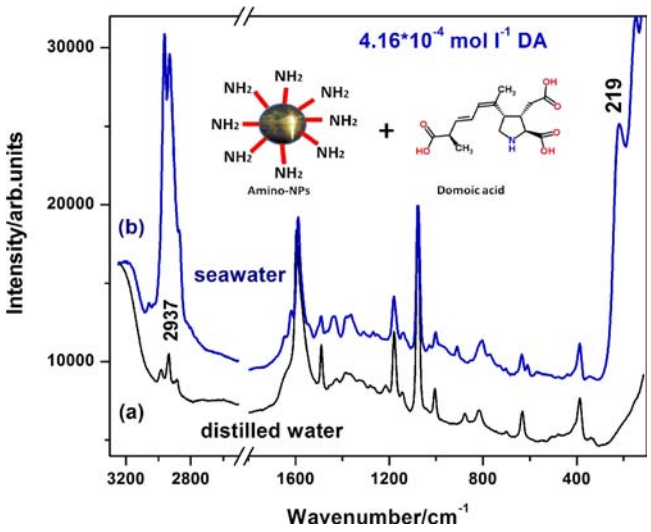

Fig. 5. SERS spectra of DA on amino-functionalized AgNPs with DA dissolved in distilled water (a) and in seawater (b). The final DA concentration in both cases was 4.16×10^{-4} mol l^{-1}.

case of chemical preparation of the SERS tag without laser exposure (no intermediary signal collection from SERS label only), when further adding DA solution results in specific SERS due to the capped DA on the label (Fig. 4).

The interaction of any carboxylate group with the amino-functionalized SERS labels is expected to be detectable as a change in the 1600–1700 cm^{-1} SERS range, where the amine bending (from 4-ATP) and the carboxylate groups exhibit significant bands.

In the SERS spectrum of 4-ATP absorbed on Ag surface, several very strong and medium bands were observed at 1024, 1472, 1597 cm^{-1} significantly depend on the experimental conditions (high laser power generating photoproducts versus low laser power). The spectral range between 1600 and 1700 cm^{-1} is free of bands (Fig. 4b). To achieve molar ratios of 1:1 for ATP:DA we prepared an ATP SERS sample from 100 μl colloidal Ag and 10 μl 4-ATP (4.5×10^{-3} mol l^{-1}), and finally we added 50 μl DA aqueous solution (seawater solution respectively), at 10^{-3} mol l^{-1} concentration. The SERS finally molar ratio was $C_{DA}/C_{4\text{-}ATP} = 0.99$. We recorded FT-Raman spectrum of pure 4-ATP and compared to the corresponding SERS signal on Ag nanoparticles, to check the identity of the absorbed 4-ATP species (SERS label). Upon adding DA, SERS spectrum from the label revealed strong differences in the 1580–1700 cm^{-1} wavenumber range (Fig. 4c). The band at 1589 cm^{-1} is broadening and at 1648 and 1621 cm^{-1} two distinct bands are additionally observed. Other new SERS bands were present at 1438 and 1362 cm^{-1}. The observed detailed differences clearly suggested the interaction of the DA toxin with the amino-functional group of the SERS label, thus, allowing to detect the toxin through the label.

In the seawater case (Fig. 5), the large differences were observed in the low and high wavenumber range, where the seawater contribution to the overall SERS signal remains significant. Appearance of new bands at 1645 and 1617 cm^{-1} as well as in the low wavenumber range at 219 and 149 cm^{-1}, demonstrated the possibility to identify the toxin species using the SERS specificity of the label. The specificity is raised not only from the margins of the spectral range, which is usually not well suited for portable equipments, but also in the fingerprint range, commonly used for Raman monitoring purpose.

4. Conclusions and perspectives

We demonstrated for the first time the possibility to detect the amnesic shellfish poisoning biotoxin from seawater in a wide range of concentration, up to the 0.033 ppb, much lower than the admitted level by the current regulations. To achieve this we used pure or amino-functionalized Ag nanoparticles and surface enhanced Raman scattering and proposed three detection schemes suitable for portable Raman equipments for aquaculture monitoring programs.

We obtained and characterized SERS signal of DA both in pure water and seawater and reached a detection limit of 0.33 nmol l^{-1} concentration (0.33 ppb) dissolved in distilled water and 0.033 nmol l^{-1} (0.033 ppb) in seawater respectively, much lower than admitted level by the current regulation. Three different detection scheme of DA in seawater were described, by dissolving the crystalline DA in seawater, by diluting pure water solution with seawater or by using animo-AgNPs, respectively. The signal specificity dependence on the method was discussed in all cases. Concentration dependence SERS spectra revealed significant differences in the adsorbed DA SERS signal, strongly dependent on environmental conditions. A concentration of 4.16×10^{-4} mol l^{-1}DA was detectable in the case of amino-functionalized NPs. These findings suggest more successful approach in toxin detection in seawater using pure Ag nanoparticles and an appropriate SERS spectral database. Further confirmation of the technique would be required when naturally toxin-contaminated seawater would be available.

The use of SERS for detection of domoic acid in seawater provide the first step to consolidate alternative real time in situ sensitive methods of high interest for the economy in the aquaculture sector. SERS approach demonstrate the possibility to provide alternative ways that could be further validated for aquaculture monitoring purpose according to the current regulations. SERS results suggests the possibility to replace the current expensive and time consuming methods in analyses for constant monitoring purpose. This work has demonstrated the orientation and the binding of domoic acid to the metal surface, and the detection of this marine toxin in seawater with SERS at lower concentration than current methods used. The specificity of the DA detection on amino-AgNPs was achieved not only on the margins of the spectral range, but also in the fingerprint range, which is usually well suited for any portable Raman equipment. Therefore, the results provided valuable information for implementing the method as rapid and cheap routine analysis in situ for marine programs monitoring using portable equipment, stock solutions of Ag colloidal nanoparticles and appropriate SERS spectral database.

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