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Development of a dual-analyte fluorescent sensor for the determination of bioactive nitrite and selenite in water samples

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ABSTRACT

Nitrite and selenium are two bioactive compounds found in the environment which show beneficial effects for health at low levels but have toxic effects at higher doses. Consequently, quantification of both analytes in water samples results of great interest in areas such as biomedicine, food technology and environmental analysis. In a recent paper, we immobilized the inclusion complex formed between 2,3-diaminonaphthalene (DAN) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) in a sol–gel matrix, in order to prepare a highly sensitive reagentless fluorescence-based sensor for the specific measurement of nitrite. Here we have explored the possibility of using the sol–gel immobilized complex to quantify selenite (Se (IV)), the more toxic form of selenium, as well as to act as a dual-analyte chemical sensor for simultaneous quantification of both nitrite and selenite in aqueous samples. Results show that (a) inclusion of DAN in HP-β-CD and its subsequent immobilization in a sol–gel matrix do not modify the reactivity of DAN against selenite, (b) the reaction product formed (4,5-benzopiazselenol) remains into the cyclodextrin increasing considerably its fluorescence quantum yield and avoiding, therefore, its extraction into organic solvents, (c) the developed sensor can detect selenite concentrations at submicromolar level with a minimum detection limit of 13 nM, (d) the immobilized system is able to simultaneously quantify nitrite and selenite at submicromolar concentrations in natural water samples with no further sample pre-treatment.

1. Introduction

The development of new sensor devices is presently the subject of extensive research in areas such as clinical diagnostic, food technology and environmental analysis. One of the major trends in the current research of optical chemical sensor technology is the multi-parameter functionality on a single platform (so-called multi-analyte sensing) in order to provide analytical devices that are rapid, sensitive, specific, non-expensive, and suitable for real-time on-site detection [1–7]. Fluorescent sensors have great potential in this field due to their high sensitivity, specificity and versatility [8–10]. Most of these sensors include multiple fluorophores immobilized either in separate polymer matrices [6,11] or in the same single-layer polymer matrix [12]. Other type of multi-analyte sensor, much less frequent, consists of a single fluorophore immobilized on a solid support able to interact with multiple analytes, producing different spectral responses. In this work, we have developed a dual-analyte fluorescent sensor platform for selenite and nitrite detection, based on this last scheme, through the immobilization of the fluorescent compound 2,3-diaminonaphthalene (DAN) in an inorganic polymeric matrix, made using a sol–gel process.

Selenium and nitrite are two natural bioactive compounds which behave as a double-edged sword, since show beneficial effects for health at low levels but are toxic at higher doses. In the case of selenium, it is an essential nutrient of fundamental importance for animals and humans, however, it is highly toxic at levels above a narrow range of that considered a health level in the human diet; daily consumption of less than 0.05–0.1 mg/kg of body weight will result in selenium deficiency while levels that exceed about 1 mg/kg are considered toxic [13]. The bioavailability and toxicity of selenium depend to a large extent on its chemical form. Selenate (Se (VI)) and selenite (Se (IV)) appear to be the dominant species in natural waters, being selenite more bioavailable and approximately 5–10 times more toxic than selenate [14]. On the other hand, nitrite has emerged as a molecule with potential therapeutic implications for cardiovascular disease [15,16], however, at high levels (300 mg/kg body weight), nitrite is toxic if consumed, since it binds with the hemoglobin in red blood cells and prevents the transfer

Abbreviations: DAN, 2,3-diaminonaphthalene; NATH, 1-(*H*)-naphthotriazole; NAT, 2,3-naphthotriazole anion; TEOS, tetraethyl orthosilicate; β-CD, β-cyclodextrin; HP-β-CD, 2-hydroxypropyl-β-cyclodextrin.

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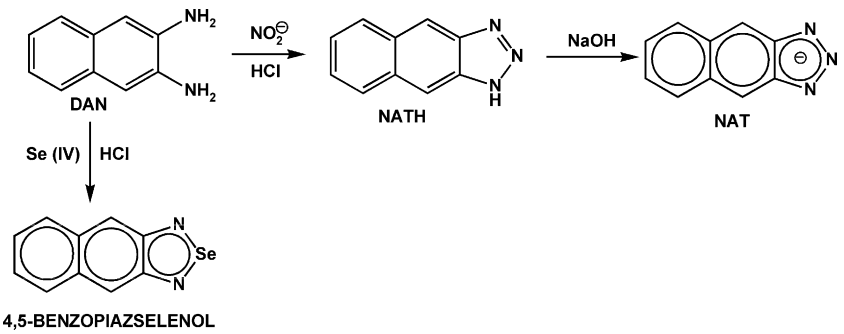

Fig. 1. Generation of NAT from the nitrosation of DAN via nitrite and generation of 4,5-benzopiazselenol from the reaction of DAN with selenite (Se (IV)).

of oxygen within the body [17]. Because of this dual role there is a need to monitor amounts of selenium and nitrite in biological and environmental systems, particularly in natural waters, where the "window" of useful concentrations, especially in the case of selenium, is very narrow. Consequently, during these last years many efforts have been carried out to develop accurate, precise and sensitive analytical methods, which are economical and at the same time adaptable for purposes of *in situ* or remote applications [18–21].

The fluorescent compound DAN reacts with nitrite in acidic conditions to yield the fluorescent product 1-(*H*)-naphthotriazole (NATH). Fluorescence of NATH is in fact rather low and usually interferes with that from DAN, however it is considerably increased in an alkaline medium due to the formation of the 2,3-naphthotriazole anion (NAT), which has a high quantum yield around 415 nm [22]. On the other hand, DAN also reacts in acid medium with selenite, the more toxic form of selenium, to form the fluorescent compound 4,5-benzopiazselenol, which fluoresces around 550 nm [23] (see Fig. 1 for both reactions). Thanks to this reactivity, the compound has been successfully used in numerous occasions to detect and quantify either nitrite or selenite in biological and environmental samples [24–31]. However, despite these assays offer great sensitivity and versatility, they show several drawbacks such as the poor solubility of DAN in water and its toxicity. Moreover, there is a great difficulty in employing the method to detect traces of nitrite and selenite in certain aqueous samples because of high blank values, as well as the fluorescence quenching, and interference by inherent biological substances and colorimetric chemicals, which often complicate the accurate detection of the analytes [32–34]. In addition, for the selenium determination, the product formed in aqueous medium must be extracted with organic solvents, such as cyclohexane, in order to improve the sensitivity of the method, since 4,5-benzopiazselenol shows a very low fluorescent quantum yield in water [23,33,35]. Consequently, all these drawbacks restrict the practicability of both assays and make the detection of selenite and nitrite using DAN somewhat time-consuming and laborious.

Most of the above problems could be overcome by using an efficient procedure for immobilization of the indicator on appropriate polymer matrix. Sol–gel technology provides a useful and versatile process for making stable, optically transparent host matrices for the design of materials for fluorescent sensors [10,36–40]. Encapsulation of indicators within sol–gel materials is currently obtained from hydrolysis of alkoxide precursors, resulting in a colloidal sol solution. Then, an aqueous solution containing the indicator is added to the sol producing a polycondensation reaction, which leads to the formation of a transparent highly porous gel, which encloses the dyes within their pores. In order to minimize the leaching of the dye from the sol–gel matrix different strategies can be used, such us covalent immobilization or previous incorporation of the dye within a molecular assembly [39–41]. Recently our group has incorporated DAN in β-cyclodextrin (β-CD) and 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), a derivative of β-CD which greatly increases the solubility of the compound in water [42,43]. Results showed that an important part of the DAN molecule is spontaneously inserted into the cyclodextrin cavity through hydrophobic interactions, forming stable 1:1 complexes which continue to be reactive against nitrite. These complexes were encapsulated in a sol–gel matrix, in order to prepare a highly sensitive reagentless fluorescence-based sensor for the specific measurement of nitrite ions at submicromolar concentrations with no sample pre-treatment [43].

Given the good results obtained for the nitrite sensor, we have decided to investigate the possibility of using the sol–gel immobilized DAN/HP-β-CD complex to quantify selenite, as well as to explore the capacity of this system to act as a dual-analyte chemical sensor for simultaneous quantification of both nitrite and selenite in aqueous samples. With this goal changes in the fluorescence spectrum of the immobilized complex were monitored at different concentrations of selenite in absence and in presence of nitrite anion. Results show that incorporation of DAN in HP-β-CD and its subsequent immobilization in a sol–gel matrix do not modify the reactivity of DAN against selenite and that the immobilized system is able to simultaneously quantify nitrite and selenite at submicromolar concentrations in a single sample with no further sample pre-treatment. The developed sensor was applied to determine nitrite and selenite species in natural water samples (tap, well and spring water).

2. Materials and methods

2.1. Reagents and standard solutions

All reagents used were of analytical or spectroscopic grade and deionised doubly distilled water was used throughout. 2-Hydroxypropyl-β-cyclodextrin and tetraethyl orthosilicate (TEOS) were purchased from Sigma–Aldrich (Spain). 2,3-Diaminonaphthalene was provided by Molecular Probes Inc. (Eugene, OR, USA). A stock solution 7.4 mM of DAN in *N*,*N*′-dimethylformamide was prepared and stored in the dark at −20 °C before use. Standard sodium selenite (25 μM) and standard sodium nitrite (1 mM) stock solutions as well as sodium phosphate buffers (50 mM, pH 7.3) were prepared.

2.2. Fluorescence spectra

Fluorescence measurements were done using a PTI-QuantaMaster spectrofluorometer. For fluorescence spectra, samples containing DAN, 4,5-benzopiazselenol and naphthotriazole anion (NAT) were excited at 340, 380 and 382 nm, respectively. The experimental samples (sol–gel monoliths and aqueous solution) were placed in 10 mm × 10 mm path length quartz cuvettes. For all experiments, appropriate blanks were subtracted from each sample.

2.3. Incorporation of DAN in HP-β-CD and immobilization of the complex in a sol–gel matrix

DAN/HP-β-CD inclusion complexes were prepared and immobilized in sol–gel matrix as described in Ref. [43]. Briefly, solutions containing DAN (74 μM) and HP-β-CD (20 mM) were magnetically stirred for 48 h at room temperature in the dark before use. 0.7 mL of this solution were placed in a disposable polymethylmethacrylate cuvette and mixed with the same volume of TEOS, which was previously hydrolyzed with a 0.62 M HCl solution and rotaevaporated to eliminate the alcohol generated during the hydrolysis process, following the protocol described by Ferrer et al. [44]. Gelation occurred readily after mixing. The freshly formed monoliths (∼9 mm × 9 mm × 12 mm) containing the immobilized dye were rinsed with phosphate buffer solution 3 times and were wet aged in 0.5 mL of the same buffer at 4 °C during 24 h. For selenite and nitrite determination phosphate buffer was substituted by a 0.31 M HCl solution. Cuvettes were sealed with parafilm and stored in the dark at 4 °C temperature before use, which was never more than 4 days, with exception of the stability experiments.

2.4. Nitrite assay

Samples containing nitrite were analysed with the fluorescent sensor described by Martínez-Tomé et al. [43]. Briefly, monoliths containing the DAN/HP-β-CD complex were submerged in 2.5 mL of sample and incubated at the appropriate temperature and time. To ensure the formation of the fluorescent NAT, which needs an alkaline environment, monoliths were placed in quartz cuvettes containing 2 mL of 2.8 M NaOH solution. The contact of the monolith with the NaOH solution modified its appearance in the first minutes, making the matrix more turbid and reducing its size. In order to avoid artefacts due to the scatter and to correctly measure the fluorescence signal, we decided not to measure this signal directly from the monolith but dissolve completely the matrix in the NaOH solution. Fluorescence measurements were then performed.

3. Results and discussion

3.1. Immobilization and stability of DAN/HP-β-CD complex in sol–gel matrix

Incorporation of DAN in HP-β-CD and subsequent immobilization of the complex in sol–gel matrix was previously demonstrated in Ref. [43] from the fluorescence emission spectra of DAN. The inclusion of DAN into the cyclodextrin cavity was carried out with the aim of reducing the leaching of the dye from the sol–gel matrix. However, the size of the DAN/HP-β-CD complex is still small comparing with that of the sol–gel pore, so the complex could also be able to diffuse between the interconnected pores and escape into the surrounding buffer. In order to investigate this possibility, we explored the stability of the immobilized dye in presence and in absence of HP-β-CD. Fluorescence spectra of DAN were collected over a period of several days (see Fig. 2 and inset). Results show that the inclusion of DAN into the cyclodextrin does not prevent completely the leaching of the dye from the matrix but largely decreases the leak speed, especially during the first days.

3.2. Ability of the sol–gel immobilized DAN/HP-β-CD complex to quantify selenite (Se (IV))

As was mentioned in Section 1 of this article, the fluorimetric method using DAN is widely accepted as a technique for the routine determination of selenite in biological materials and water. During the assay, selenite reacts quantitatively with DAN in acidic solution to yield 4,5-benzopiazselenol, which is measured fluorimetrically following extraction in organic solvents. Incorporation of DAN into the cyclodextrin cavities could modify the reactivity of the molecules against selenite preventing its analytical use as selenite sensor. On the other hand, if the reaction takes place within the cyclodextrin cavity, detection of the analyte could be possible without successive extraction with organic solvent. For this reason, before assessing the ability of the immobilized inclusion complex to quantify selenite we explored the reactivity of the DAN/HP-β-CD complex in solution. Samples containing DAN, in absence and in presence of HP-β-CD, were incubated with selenite (0.5 μM) in acidic solution (pH ∼2) during 60 min at 60 °C [33]. Formation of 4,5-benzopiazselenol was monitored from the emission spectra, recorded upon excitation at 380 nm to avoid the interference of DAN (Fig. 3). For the sample in absence of HP-β-CD fluorescence emission was practically negligible in the aqueous solution and the product formed was extracted with cyclohexane before recording the spectrum (curve 1). This last spectrum was similar to that obtained for 4,5-benzopiazselenol by other authors in the same organic solvent [23], confirming

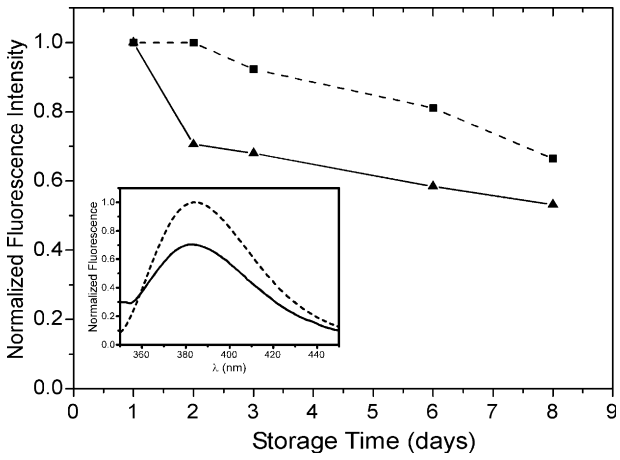

Fig. 2. Fluorescence intensity of DAN (▲) and DAN/HP-β-CD complex (■) immobilized in sol–gel matrix at the same DAN concentration (37 μM), recorded at 382 nm as a function of storage time. Inset: fluorescence emission spectra of the immobilized DAN (continuous line) and DAN/HP-β-CD complex (dotted line) recorded the second day of storage.

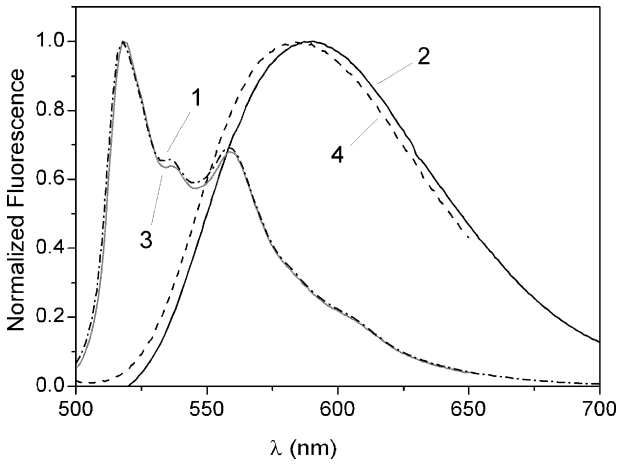

Fig. 3. Fluorescence emission spectra of 4,5-benzopiazselenol (λ_{exc} = 380 nm) obtained from reaction of selenite 0.5 μM with: (a) DAN in aqueous solvent, after extraction with cyclohexane (curve 1); (b) DAN/HP-β-CD complex in aqueous solution (curve 2); (c) DAN/HP-β-CD complex in aqueous solvent after extraction with cyclohexane (curve 3) and (d) DAN/HP-β-CD complex immobilized in a sol–gel matrix (curve 4).

the reactivity of free DAN against selenite. In contrast, for the sample in presence of HP-β-CD, a fluorescence spectrum was directly recorded in the aqueous solution, which was clearly different of that obtained in cyclohexane, showing a single width band around 586 nm (Fig. 3, curve 2). Changes in the fluorescence emission spectrum of 4,5-benzopiazselenol as a function of solvent were previously reported by other authors, which observed a hypsochromic shift as decreasing the polarity of the solvent [33]. Therefore, to confirm that this band effectively corresponds to the benzopiazselenol formed within the cyclodextrin, solution was extracted with cyclohexane, and its spectrum was recorded and compared to that obtained, in the same solvent in absence of HP-β-CD. Both spectra were similar (curves 3 and 1, respectively, in Fig. 3), indicating that the incorporation of DAN into the cyclodextrin cavity not only does not restrict the interaction of selenite with the two amino groups of DAN but also provides a suitable hydrophobic environment for fluorescence sensitization of 4,5-benzopiazselenol.

Once confirmed that incorporation of DAN into the cyclodextrins does not modify its reactivity against selenite, the next step was to check the ability of the sol–gel immobilized DAN/HP-β-CD complex to generate 4,5-benzopiazselenol. Monoliths containing DAN/HP-β-CD complex were immersed in a solution containing selenite (0.5 μM) and incubated for 60 min at 60 °C. After this treatment, monoliths were placed in fluorescence cuvettes and their emission spectrum was recorded upon excitation at 380 nm (curve 4 in Fig. 3). The fact that the spectra were practically the same both with and without sol–gel, confirms that DAN keeps being active against selenite and therefore it can be used as a selenite sensor. Since the diffusion of selenite through the matrix could slow down the sensor response, we proceeded to determine if the time selected in the previous experiment (60 min) was enough to complete the reaction between selenite and DAN/HP-β-CD complex within the sol–gel matrix. Monoliths containing the inclusion complex were incubated at 60 °C at a constant concentration of selenite (5 μM) and the fluorescence of 4,5-benzopiazselenol was monitored as a function of the incubation time. A continuous increase in the fluorescence signal was observed at 586 nm, to reach practically a constant value around 150 min (data not shown).

The performance of the developed sensor was examined by a calibration curve in which the sensitivity of the sensor to selenite was measured. Samples containing the immobilized DAN/HP-β-CD complex were incubated for 150 min at 60 °C with increasing concentrations of selenite, up to 0.6 μM. For each selenite concentration the fluorescence emission spectrum of 4,5-benzopiazselenol was recorded. Appropriate blanks were subtracted from each sample. A plot of the fluorescence signal observed at 586 nm as a function of selenite concentration is shown in Fig. 4a. Its linear range spanned very low concentrations of selenite, up to 0.6 μM, with a good correlation coefficient (r^2 = 0.9907). The same linear response (r^2 = 0.9969) was found up to 5 μM of analyte (see inset in Fig. 4a). A lower detection limit of 13 nM was calculated from the slope of the plot and the standard deviation of the blank measurements, as recommended by IUPAC [45]. This detection limit was slightly higher than that observed with previous extraction of piazselenol in cyclohexane [46], probably because the compound shows a higher quantum yield in this solvent than in HP-β-CD. However, the sensor shows important advantages with respect to the conventional assay, since it allows working *in situ* directly on the sample without the need of further manipulation, making only use of environment-friendly reagents. In addition, the sensor could be used in turbid and coloured samples since the reaction occurs within the sol–gel matrix and not in the sample, so all the information regarding selenite concentration is contained inside this matrix.

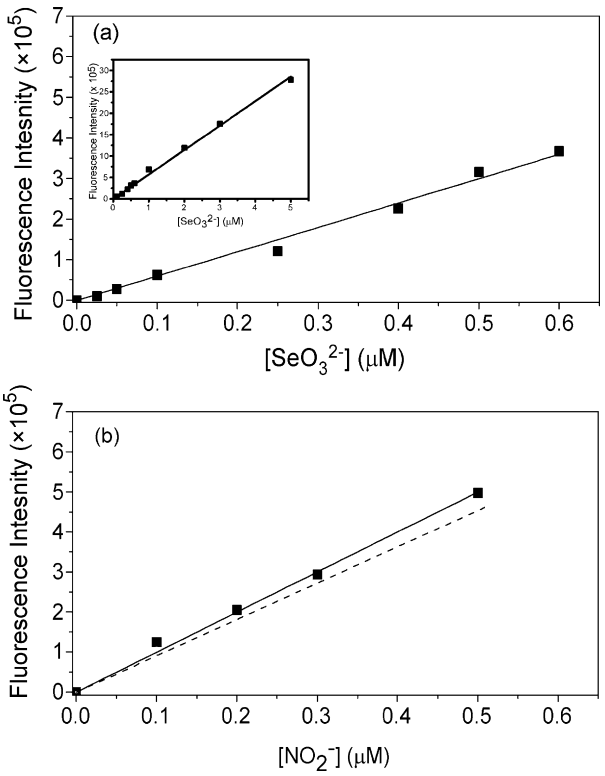

Fig. 4. (a) Calibration curve of the selenite sensor based on immobilization of DAN/HP-β-CD complex in a sol–gel matrix. Inset: calibration curve for the immobilized system with the *x*-axis expanded in the range of 0–5 μM. (b) Calibration curve of the nitrite sensor determined using the operational conditions of the selenite sensor (150 min of incubation time at 60 °C) (—) and using the experimental conditions described in Ref. [43] (60 min at 37 °C) (- - -).

3.3. Simultaneous detection of selenite and nitrite

Once verified the suitability of the sensor to quantify selenite and given that previously we had shown that it is suitable to determine nitrite [43], we explored the possibility of use the sensor to simultaneously quantify both selenite and nitrite. With this goal we performed a first trial to check if the operating conditions selected for selenite (incubation during 150 min at 60 °C) were also appropriate for the nitrite detection. Fig. 4b shows the calibration curve obtained under these experimental conditions from different concentrations of nitrite in the range 0–0.5 μM. The plot was linear in all the concentration range, showing a slope which was very close to that achieved at the experimental conditions previously selected for the nitrite sensor (60 min at 37 °C) [43].

Taking into account this result, we proceeded to study the simultaneous determination of both substances in the same aqueous solution. A sample was prepared containing nitrite and selenite at the same concentration (0.5 μM) and a monolith with the immobilized DAN/HP-β-CD complex was transferred to the sample and incubated during 150 min at 60 °C. After that time, monolith was placed in a quartz cuvette and the fluorescence spectrum of 4,5-benzopiazselenol was recorded. Then, the monolith was dissolved in 2 mL of 2.8 M NaOH in quartz cuvettes and the fluorescence spectrum of NAT was taken. Both spectra were compared with those obtained when only 0.5 μM selenite or 0.5 μM nitrite were present in the sample (Fig. 5). Results show that the fluorescence signal of 4,5-benzopiazselenol and NAT obtained for the mixture of analytes was similar to that recorded separately for each one of them, indicating that the developed sensor is able to quantify, with high sensitivity, both substances in the same aqueous sample, with no further sample pre-treatment.

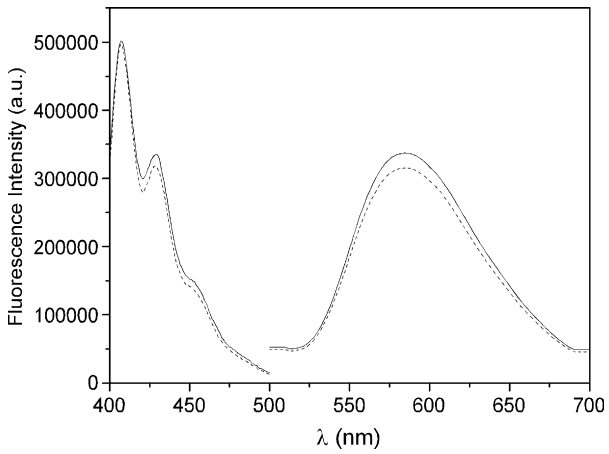

Fig. 5. Simultaneous detection of 0.5 μM nitrite and Se (IV) in the same aqueous solvent using the dual fluorescent sensor developed in this work. The figure compares the fluorescence emission spectra of NAT (left) and 4,5 piazselenol (right), recorded upon incorporation of the immobilized DAN/HP-β-CD complex in a sample containing the mixture of analytes (continuous line), with those obtained when only nitrite or selenite were present in the sample (dotted line).

3.4. Applications of the sensor

In order to demonstrate the utility of the developed sensor for the analysis of environmental samples research work was conduced to the simultaneous determination of nitrite and selenite in water samples from different natural sources. Spring water, well water, as well as tap water and double distilled water, were analysed for comparisons by the proposed procedure. Analysis was carried out within 24 h of sample collection to avoid the interconversions. Results are given in Table 1. Nitrite was found in all samples (between 0.19 and 0.36 μM), while selenite concentrations were below the detection limit. Hence, spiking experiment was carried out for both species. Recoveries were satisfactory which indicates the capability of the present method to simultaneously determine selenite and nitrite in environmental water samples with good reproducibility and high sensitivity.

In conclusion, in this work we have developed a dual fluorescent sensor based on the immobilization of the DAN/HP-β-CD complex in a sol–gel matrix. The inclusion of DAN into the cyclodextrin before the immobilization step clearly reduced the leaching of the dye from the matrix, though it was not completely prevented. The sensor was able to quantify selenite concentrations at submicromolar level with a minimum detection limit of 13 nM. Furthermore, sample pre- or post-treatment are not necessary. In addition, the immobilized complex serves to prepare a highly sensitive reagentless dual-analyte chemical sensor for simultaneous quantification of both nitrite and selenite in the same aqueous sample. The dual sensor allows working *in situ* directly on environmental (natural water) samples without the need of further manipulation and could have wide applications not only in the environmental field but also in clinical diagnosis since both analytes can bring serious risks to human health and environment if they exceed certain concentration levels which are above the minimum detection limit of the sensor. Applications of the sensors in body fluids such as urine, blood or plasma, together with the search for new strategies to reduce the leaching of the dye from the sol–gel matrix are now under investigation and will be the scope of a future work.

Table 1
Selenite and nitrite concentration (mean ± standard deviation) of water samples.

Samples	SeO_3^{2-} (μM)		NO_2^- (μM)	
	Standard addition	Amount found (±0.02)	Standard addition	Amount found (±0.03)
Spring water	–	<0.013	–	0.32
	0.10	0.12	0.10	0.39
	0.50	0.50	0.50	0.84
Tap water	–	<0.013	–	0.36
	0.10	0.12	0.10	0.49
	0.50	0.47	0.50	0.89
Well water	–	<0.013	–	0.32
	0.10	0.10	0.10	0.45
	0.50	0.50	0.50	0.87
Distilled water	–	<0.013	–	0.19
	0.10	0.11	0.10	0.28
	0.50	0.52	0.50	0.69

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