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ORIGINAL PAPER

Immobilization and Characterization of 2,3-diaminonaphthalene/cyclodextrin Complexes in a Sol–Gel Matrix: A New Fluorimetric Sensor for Nitrite

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Abstract The aromatic diamino compound 2,3-diaminonaphthalene (DAN) has been extensively used to detect and quantify nitrite ions in biological and environmental samples. We have immobilized the DAN reagent in a porous silicate glass matrix, via previous incorporation of the dye in HP-β-CD. Changes in fluorescence intensity were used to characterize the inclusion complexes and determine the association constant and stoichiometry of the process. Fluorescence spectrum of these complexes was also used to monitor their immobilization within the sol–gel matrix. Reactivity of the immobilized complexes was evaluated with increasing concentrations of nitrite up to 10 μM (with a detection limit around 20 nM). Results show that sol–gel immobilization does not modify the reactivity of the dye against nitrite and serves to prepare a highly sensitive ready to use fluorescence-based sensor for the specific measurement of nitrite at submicromolar concentrations with no further sample pretreatment.

Keywords Sol-gel encapsulation · Cyclodextrin · DAN · Nitrite · Fluorescent sensor

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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Introduction

Determination of anion nitrite is of general importance in various fields such as environment, food and life sciences, because of its harmful impact on human health. The toxicity of nitrite is primarily due to its interaction with blood pigment to produce the condition known as meth-aemoglobinaemia. Nitrite can also react in the human body with secondary or tertiary amines to form *N*-nitroso compounds, some of which are known to be carcinogenic and mutagenic (see [1] and references herein). Due to these toxic effects, numerous analysis methods have been developed for determination of nitrite in food products, natural waters and biological fluids, such as blood, plasma and urine [2–10]. However, despite considerable advance in last years, accurate quantitative determination of nitrite, specially in complex biological fluids, is still a challenging analytical task. The greatly differing basal concentrations reported for this anion in plasma and urine of humans are a clear evidence for the existence of unresolved analytical problems, many of which arise from preanalytical factors including generation and treatment of the biological sample to be analyzed and from contamination by extraneous nitrite from water and glassware [11].

The most famous and most frequently used method of analysis of nitrite in biological fluids is the Griess colorimetric assay [4, 11, 12]. Although this analytical assay is simple and non expensive, it requires previous

treatment of the sample before analysis because the recognition of numerous interferences (free reduced thiols, proteins, and other plasma constituents) in the assay and does not allow quantitative determination of the anion below 200 nM [11]. An alternative analytic method for nitrite determination is based on the nitrosation of the aromatic diamino compound 2,3-diaminonaphthalene (DAN). This fluorimetric method has been also extensively used to detect and quantify the anion in biological and environmental samples with high sensitivity [2, 13–16]. As shown in Fig. 1, the assay is based on the reaction, under acidic conditions, of nitrite with DAN (slightly fluorescent) 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 much higher quantum yield than NATH itself. Despite this method offers great sensitivity and versatility, allowing to quantify nitrite concentrations as little as 10 nM [17], there is a great difficulty in employing the assay to detect traces of nitrite in certain biological samples because of high blank values, as well as the fluorescence quenching, and interference by inherent biological substances and colorimetric chemicals [5, 15, 18, 19], which often complicate the accurate detection of the anions. In addition, DAN is possibly a carcinogenic reagent [20] and shows poor solubility in water. Most of these problems could be avoided by using an efficient procedure for immobilization of the indicator on an appropriate polymer matrix. Encapsulation of DAN in a solid support, such as sol–gel matrix, should largely reduce its toxicity, providing additional advantages including easy probe handling and enhanced stability of the encapsulated dye [21–27]. Moreover, due to the tunable porosity of the sol–gel glasses, the matrix could act as a filter, avoiding the interferences by biological components during the sample collection.

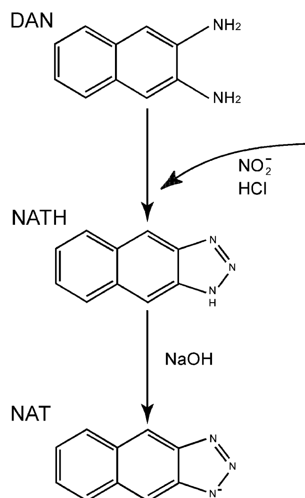

Fig. 1 Generation of NAT from the nitrosation of DAN via nitrite

Encapsulation of molecules within sol–gel materials is currently obtained from the hydrolysis of alkoxide precursors, resulting in a colloidal sol solution. Then, a buffered aqueous solution containing the molecule of interest is added to the sol producing a polycondensation reaction, which leads to the formation of a transparent highly porous gel, which encloses the species within their pores. However, due to the size of these pores (20–50 Å), small molecules can diffuse between the interconnected pores, and finally escape into the surrounding buffer [28–30]. Consequently, direct immobilization of DAN within the sol–gel matrix would not be effective in terms of protection against DAN toxicity and interference or quenching effects from biologicals. Previous incorporation of the dye within molecular assemblies (e.g., micelles, liposomes, cyclodextrins, dendrimers, etc.) and the subsequent encapsulation of the system into the matrix, could overcome this problem [30–32]. Recently, our group has incorpored DAN in β-cyclodextrins (β-CD), cyclic oligosaccharides consisting of seven glucopyranose units with hydrophobic interiors [33]. Results showed that an important part of the DAN molecule is spontaneously inserted into the β-CD cavity through hydrophobic interactions, forming stable 1:1 complexes which continue to be reactive against nitrite. 2-hydroxypropyl-β-cyclodextrin (HP-β-CD) is a derivative of β-CD also formed by 7-glucopyranose unit compounds, but it has an average degree of substitution of 0.67 hydroxypropyl group at the 2, 3 and 6 hydroxyl sites, which greatly increase water solubility as well as its average size [34, 35]. Because of these properties, an easier complex formation would be obtained with even more effective matrix entrapment. Therefore, HP-β-CD has been selected in this work to complex DAN molecules which were subsequently encapsulated in sol–gel matrixes. Changes in fluorescence intensity were used to characterize the new inclusion complexes and determine the association constant and stoichiometry of the process. Fluorescence spectrum of these complexes was also used to monitor their immobilization within the sol–gel matrix and the ability of the immobilized DAN to yield NAT was evaluated at different nitrite concentrations.

Materials and methods

Reagents

2,3-Diaminonaphthalene was provided by from Molecular Probes Inc. (Eugene, OR, USA). A stock solution of DAN in *N*,*N*′-dimethylformamide was prepared at 7.4 mM and stored in the dark at −20 °C before use. Tetraethyl

orthosilicate (TEOS) and 2-hydroxypropyl-β-cyclodextrin were obtained from Sigma-Aldrich (Spain). All other reagents used were of analytical or spectroscopic grade. A standard sodium nitrite stock solution (1 mM) and sodium phosphate buffers (50 mM, pH 7.3) were prepared with deionised doubly distilled water.

Fluorescence spectra

Fluorescence emission spectra of DAN and of naphthotriazole anion (NAT) were performed on a PTI-QuantaMaster spectrofluorometer. Excitation wavelengths at 340 and 382 nm for DAN and NAT, respectively, were utilized. The experimental samples (sol–gel monoliths and aqueous solution) were placed in 10×10 mm path length quartz cuvettes. Background intensities due to the sol–gel matrix were always taken into account and subtracted from the sample.

Experimental procedure for preparing the DAN/HP-β−CD inclusion complex

DAN/HP-β-CD inclusion complexes were prepared in phosphate buffer (pH 7.3) at room temperature. The DAN concentration in the buffer was maintained constant at 74 μM in all experiments while the cyclodextrin concentration was varied. In brief, 0.5 mL aliquots of a solution of DAN in buffer (370 μM) were added to 2 mL of HP-β-CD with concentrations between 0–20 mM (final concentration) in different flasks, which were covered with aluminium foil. All solutions were magnetically stirred for 48 h at room temperature in the dark before used.

Immobilization in sol–gel matrix

Silica stock solution was prepared using TEOS as precursor. Briefly, 4.46 mL of this product were mixed under vigorous stirring with 1.44 mL of H_2O and 0.04 mL HCl (0.62 M) at 22°C in a closed vessel. After 1 hour, 1 mL of the resulting sol was mixed with 1 mL of deionised doubly distilled water, and submitted to rotaevaporation for a weight loss of 0.6 g (i.e., 0.6 g are approximately the alcohol mass resulting from alkoxyde hydrolysis). Afterwards, 0.7 mL of the aqueous sol were mixed with 0.7 mL of the DAN/HP-β-CD complex (74 μM/20 mM) in a disposable polymethylmethacrylate cuvette. Gelation occurs readily after mixing. Following gelation, transparent monoliths, having a size of ~9×9×12 mm, were washed three times with phosphate buffer solution and were wet aged in 0.5 mL of the same buffer at 4 °C during 24 h. For nitrosation experiments, this buffer was replaced by a 0.62 M HCl solution. Cuvettes were covered with parafilm and stored in the dark at that temperature before use.

Nitrosation and alkalinization with nitrite

Monoliths containing the DAN/HP-β-CD complex were removed from the disposable cuvettes and incubated during 60 min at 37 °C in 2.5 mL of sodium nitrite aqueous solution at different concentrations. Afterwards, monoliths were placed in quartz cuvettes containing 2 mL of 2.8 M NaOH solution and let stand at 37 °C until the monolith was totally dissolved (approximately 30 min). Fluorescence measurements were then performed.

Results and discussion

Incorporation of DAN in HP-β-CD

The fluorescence spectra of DAN in phosphate buffer and DAN incorporated in HP-β-CD in the same buffer are shown in Fig. 2. It can be observed an enhancement in the fluorescence intensity in presence of increasing concentrations of HP-β-CD from 0 to 20 mM. There is only a slight shift to lower wavelengths of the emission spectrum in going from water to HP-β-CD, which indicates that the NH_2 groups of DAN in the CD complex are probably exposed to the water phase as in the case of the DAN/β-CD inclusion complex [33].

Stoichiometry and association constant of the inclusion complex

Fluorescence enhancement observed in the emission spectra of DAN upon addition of DAN/HP-β-CD was then used to calculate the association constant (K) and the stoichiometry of the DAN/HP-β-CD inclusion complex. Determination of

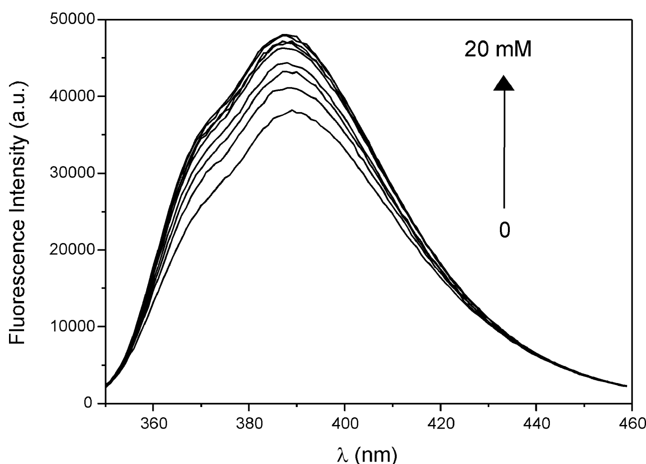

Fig. 2 Fluorescence emission spectra of DAN (74 μM) in phosphate buffer containing increasing concentrations of HP-β-CD, from 0 to 20 mM

K is most accurately done by using non-linear least-squares fitting to the following equation:

$$\frac{I}{I_0} = 1 + \left(\frac{I_{max}}{I_0} - 1\right)\frac{[CD]_0 K}{1 + [CD]_0 K} \quad (1)$$

where I_0 and I represent the fluorescence intensities in the absence and in presence of CD, respectively, $[CD]_0$ denotes the analytical concentration of cyclodextrin and I_{max} is the limiting intensity of fluorescence obtained when all DAN molecules are complexed. This equation assumes that stoichiometry of the inclusion complex is 1:1, an assumption that can be tested using the Benesi–Hildebrand double-reciprocal plot [36], which represents $1/(I–I_0)$ versus $1/[CD]_0$. The plot will be linear if only 1:1 complexes are formed.

Figure 3 shows the fluorescence intensity increasing of DAN at the emission maximum as a function of HP-β-CD concentration. The solid line shows the fit of the curve to Eq. (1), which yields a value for the association constant of $K=426$, and $I_{max}/I_0=1.3$. When the Benesi–Hildebrand plot is constructed a straight line is obtained ($r=0.997$) which is indicative of a 1:1 stoichiometry for complexes (inset in Fig. 3). As was expected, these values are in the same range than those obtained for DAN and β-CD [33].

Immobilization of DAN/HP-β-CD complex in sol–gel matrix

The excellent optical properties of the sol–gel matrix did allow for the spectroscopic characterization of the immobilized DAN/HP-β-CD inclusion complex using the fluorescence emission spectrum of the dye. Fluorescence spectrum of the immobilized complex was directly recorded from the sol–gel monolith placed in a 10×10 mm quartz cuvette and excited at 340 nm. Figure 4 shows that this spectrum is

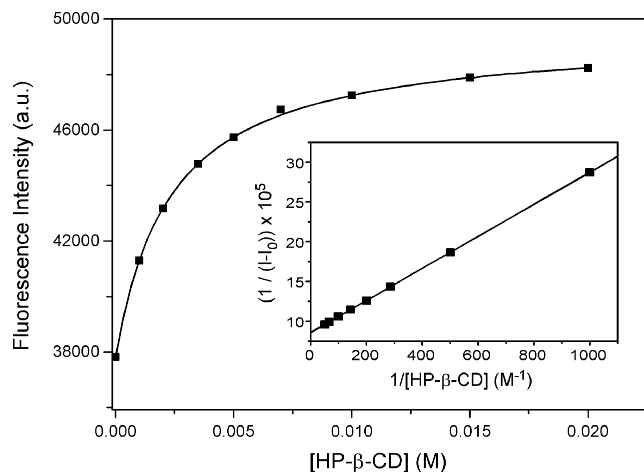

Fig. 3 Influence of HP-β-CD concentration on the fluorescence signal of DAN (λ_{exc}=340 m; λ_{em}=388 m). Inset: Benesi–Hildebrand plot for 1:1 DAN/HP-β-CD complexes

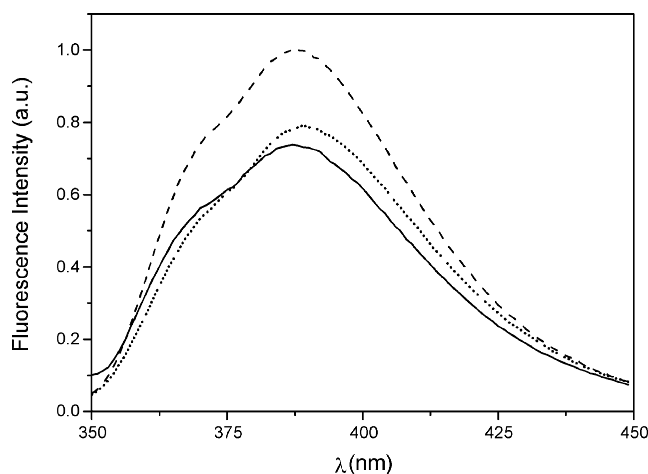

Fig. 4 Fluorescence emission spectra of DAN in phosphate buffer (…), DAN/HP-β-CD complex in phosphate buffer (- - -) and DAN/HP-β-CD complex immobilized in sol–gel matrix (—) upon excitation at 340 m

similar to that recorded for the inclusion complex in phosphate buffer, and slightly shifted to lower wavelengths with respect to that obtained for DAN in absence of cyclodextrin. This confirms that during the immobilization process DAN remains inserted into the cyclodextrins cavities. The lost of fluorescence intensity observed in the emission spectrum of the immobilized DAN/HP-β-CD inclusion complex could be due to the release of DAN from the monolith surface during the washing process.

The stability of the immobilized complex with aging time was investigated from the fluorescence spectra of DAN recorded at different days. Results showed that, unlike DAN in absence of HP-β-CD, which once immobilized easily leaks out through the pores of the sol–gel matrix, the complex is retained inside of the monolith. Once prepared the monolith and maintained hydrated during 10 days only a loss of 20% in the fluorescence signal was observed (data not shown).

Ability of the sol–gel immobilized DAN/HP-β-CD complex to yield NAT

Immobilization of DAN/HP-β-CD into the sol–gel matrix could modify the reactivity of DAN against nitrite preventing its analytical use as nitrite sensor. To rule out this possibility, the ability of the immobilized complex to yield NAT was investigated. Monoliths containing DAN/HP-β-CD were immersed in a solution containing an excess of nitrite ion (50 μM) and incubated for 40 min at 37°C. To ensure the formation of NAT, which needs an alkaline environment, the monolith was taken out of the nitrite solution and placed in a quartz cuvette in presence of NaOH. 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 before measurements. It should be noted that the monolith dissolution at this point would not affect to the above mentioned advantages of non sample pre-treatment with the proposed assay, i.e. non pre-treated sample solutions would be incubated with the matrix and then, with the already acquired nitrite information, the matrix would be replaced into the basic solution. Figure 5 shows the emission spectrum, recorded upon excitation at 382 nm, after this treatment. The spectrum shows two major emission bands at 408 and 430 nm and a shoulder with low emission at ~450 nm. This spectrum coincides with the fluorescence emission of NAT recorded upon nitrosation and subsequent alkalinization of DAN/HP-β-CD complex in solution (Fig. 5), thus confirming that upon sol–gel immobilization DAN maintains its reactivity against nitrite. Both spectra slightly shift to the red when compared to the emission spectrum of NAT in solution (Fig. 5) indicating that after alkalinization NAT remains inserted into the cyclodextrin cavity.

Effect of nitrite concentration on the DAN/HP-β-CD complex fluorescence in sol–gel matrix

The aforementioned experiment suggests the potential use of the immobilized DAN/HP-β-CD complex as nitrite sensor. In our previous work with DAN/β-CD complex in solution, an incubation time of 40 min was obtained for complete DAN nitrosation [33]. Since the diffusion of nitrite through the matrix could slow down this process, we proceeded to determine the time required to complete the

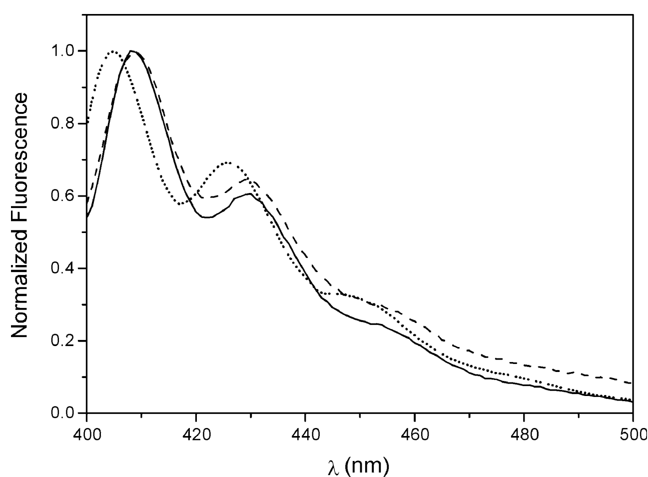

Fig. 5 Normalized fluorescence emission spectra of NAT (λ_{exc}= 382 nm) after incubation of DAN in phosphate buffer (…), DAN/HP-β-CD complex in phosphate buffer (- - -) and DAN/HP-β-CD complex immobilized in sol–gel matrix (—) with an excess of nitrite concentration

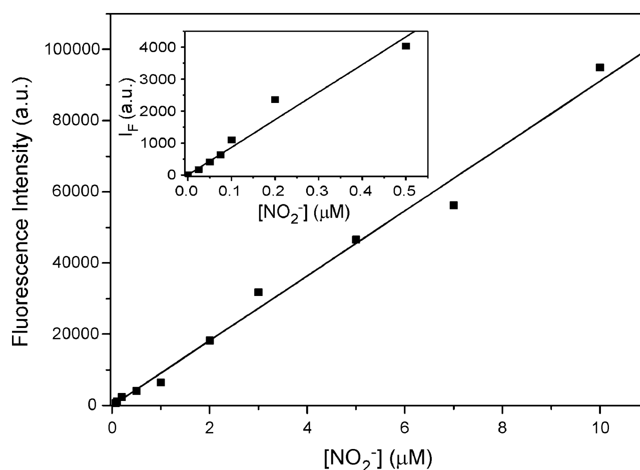

Fig. 6 Dependence of the fluorescence intensity of NAT (λ_{exc}= 382 nm; λ_{em}=408 nm) as a function of nitrite concentration. Inset: Dependence for nitrite concentrations smaller than 0.5 μM

nitrosation of the DAN/HP-β-CD complex in the sol–gel matrix by using a kinetic study. Monoliths containing the inclusion complex were incubated in a nitrite solution during different periods of time and the emission maximum of NAT was monitored after previous dissolution of the sol–gel matrix in the NaOH solution. A steady increase of the fluorescence was observed until completion of nitrosation reaction was achieved, which occurred after approximately 60 min (data not shown).

To check if the increase in the fluorescence intensity of NAT is proportional to the nitrite concentration, samples containing DAN/HP-β-CD in sol–gel matrix were incubated for 60 min at 37°C with increasing concentrations of nitrite up to 10 μM. For each nitrite concentration the fluorescence emission spectrum of NAT was recorded. Appropriate blanks (i.e., identical samples without nitrite) were subtracted from the sample emission to eliminate the background fluorescence. A plot of the fluorescence signal observed at 408 nm as a function of nitrite concentration is shown in Fig. 6. It can be observed that the response was linear in the range studied (up to 10 μM) although the system can also detect much lower concentrations with a detection limit close to 20 nM (see graphic inset for concentrations smaller than 0.5 μM). Since the nitrite concentrations in body fluids such as plasma and urine range between 80 and 2,000 nM [11, 37], they should be directly determined from our sensor. In addition the high sensitivity of the prepared sensor allows for handling diluted samples that further reduce the presence of interference substances [28].

The present assay is, therefore, much more sensitive than the traditional Griess colorimetric assay [4], and shows a sensitivity only slightly lower than that reported for the current procedure (when DAN is used in solution) [17]. However, our method shows important advantages since it

allows to work *in situ* directly on the sample without the need of further manipulation, and it also permits the measurement on turbid and coloured samples. Nitrosation reaction occurs within the sol–gel matrix and not in the sample, so all the information regarding nitrite concentration is contained inside this matrix after the incubation period. In addition, during the incubation time, the matrix can act as a filter, in biological samples, avoiding the interaction with DAN of certain proteins (e.g. serum albumin), as was previously reported by Fernández-Cancio et al. [10]. In the same way, the immobilized DAN could be also used to determine nitric oxide in biological fluids, only by maintaining the monolith hydrated in neutral buffer instead of acid media. All these possible applications are now under investigation and will be the scope of a next work.

In conclusion, we have immobilized the DAN reagent in a porous silicate glass matrix, via previous incorporation of the dye in HP-β-CD. An important part of the DAN molecule is spontaneously inserted into the HP-β-CD cavity through hydrophobic interactions, forming stable 1:1 complexes. Immobilization of the complexes in the sol–gel matrix does not modify the reactivity of the dye against nitrite and serves to prepare a highly sensitive reagentless fluorescence-based sensor for the specific measurement of nitrite ions at submicromolar concentrations with no sample pre-treatment.

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