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# Fluorometric detection of nitric oxide using 2,3-diaminonaphthalene incorporated in β-cyclodextrin

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**Abstract**

The aromatic diamino compound 2,3-diaminonaphthalene (DAN) has been used in numerous occasion to detect nitrite and nitric oxide in biological fluids; however, the sensitivity of this method relies on a strong alkalinisation of the sample which, in many cases, limits the general applicability of the method. In this work, we propose a new fluorescent method to quantify nitric oxide at physiological pH, based on the reaction of this radical with DAN, previously incorporated in β-cyclodextrin (β-CD). Changes in the fluorescence intensity of DAN were used to characterize the DAN/β-CD inclusion complexes and determine the association constant and stoichiometry of the process. Reactivity of the DAN/β-CD complex was then evaluated in presence of different concentrations of NO. Results show that the inclusion of DAN into the β-cyclodextrin does not prevent the formation of the fluorescent product 2,3-naphthotriazole (NATH), which remains into the cyclodextrin increasing considerably its fluorescence quantum yield, even at neutral pH. A plot between NO concentration and fluorescence intensity of NATH was built to determine the sensitivity of the DAN/β-CD complex to the radical. A linear relationship was found with a lower detection limit ∼20 nM.


*Keywords:* Nitric oxide; 2,3-Diaminonaphthalene; β-Cyclodextrin; Fluorescence

## 1. Introduction

The paramagnetic radical nitric oxide, NO, one of the smallest and simplest biologically actives molecules in nature, is produced by various cell types in picomolar to nanomolar range and plays an important role in various physiological phenomena. It has been shown to be an important mediator in vasodilation, neurotransmission and cellular cytotoxicity [1–3] and alterations of its concentration, as compared to an optimal level, are related to several diseases such as atherosclerosis, cardiac failure, hyperthension, hepatitis B and alzheimer [1,4,5]. Although NO is expected to be related to several other physiological processes, its instability, low concentration and short half-life in biological systems make it difficult to be detected. In this respect, during the past decade, extensive research efforts motivated by the requirement of improved methods for NO detection were initiated. Although numerous spectroscopic and electrochemical methods have been recently developed (reviews in [6–8]), the fact is that, to date, the measurement of the concentration of NO under physiological conditions in terms of selectivity, sensitivity and experimentally feasibility continues to be a challenging analytical problem. Fluorescence spectroscopy is one of the techniques which can satisfy these requirements. Among the fluorometric methods developed to quantify NO one of the most used due to its low cost and high sensitivity is based on the reaction of NO with the fluorescent compound 2,3-diaminonaphthalene (DAN) [9]. As shown in Fig. 1, DAN reacts rapidly with NO-derived *N*-nitrosating agents ($N_2O_3$), generated from the interaction of NO with $O_2$, to yield the fluorescent product 2,3-naphthotriazole (NATH). Fluorescence of NATH is rather low and usually interferes with that from DAN [10]; 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.

*Abbreviations:* CD, cyclodextrin; DAN, 2,3-diaminonaphthalene; NAT, 2,3-naphthotriazole anion; NATH, 2,3-naphthotriazole; NO, nitric oxide; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine

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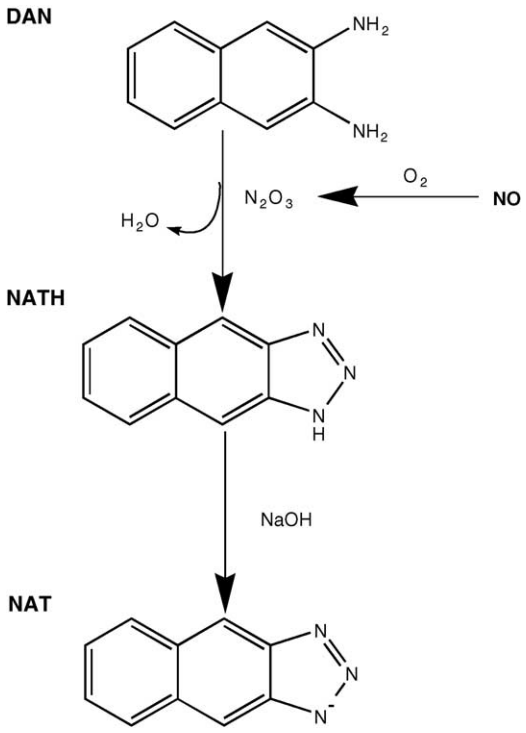


Fig. 1. Common reaction pathway for NO quantification with 2,3-diaminonaphthalene (DAN).

Therefore, in the usual NO detection procedure, a strong alkaline solution is added to the NATH solution in order to obtain higher sensitivity. Addition of the strong alkaline solution allows quantify NO concentrations as little as 10 nM [11] but results in many cases a limitation of the method. Alkalinisation impedes the direct determination (in vivo) of the analyte, increases the manipulation of the samples and the time of the analysis and prevents the immobilization of the indicator in certain polymeric supports which are unstable in alkaline medium [12]. Kungl and co-workers [10] introduced a modification of the method using time-resolved fluorescence spectroscopy, which allows the determination of NO at neutral pH. The authors observed that changes in the fluorescence lifetimes of DAN and NATH could be used to detect the release of micromolar amounts of NO at physiological conditions. The method is applicable for samples in vivo, but its sensitivity is not very high and its use is limited by the cost and complexity of the instrumentation.

We present here a sensitive and easy to use fluorometric method based on the nitrosation of DAN, in which NO can be quantified at physiological pH, without the need to add an alkaline solution to the sample. With this goal and taking into account that certain fluorescent compounds increase their fluorescence upon incorporation into cyclodextrins (CDs) [13–15], we have investigated the interaction of DAN with α- and β-CD at physiological pH. Changes in fluorescence intensity were used to characterize the inclusion complexes and determine the association constant and stoichiometry of the process. The ability of the DAN/β-CD complex to yield NATH in presence of NO was evaluated at physiological conditions and the possibility of using this methodology for NO determination was investigated.

## 2. Experimental

### 2.1. Reagents

2,3-Diaminonaphthalene and the NO donor *S*-nitroso-*N*-acetyl-DL-pencillamine (SNAP) were obtained 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. α-Cyclodextrin (α-CD) and β-cyclodextrin (β-CD) were obtained from Fluka (Switzerland). All other chemicals were of analytical or spectroscopic reagent grade. Sodium phosphate buffers (50 mM, pH 7) were prepared with deionised doubly distilled water.

### 2.2. Spectroscopic measurements

Absorption spectra and absorbance measurements were carried out using a Shimadzu spectrophotometer (UV-1603, Tokyo, Japan). Fluorescence spectra and intensity measurements were performed on a SLM-8000C (SLM Instruments Urbana, IL) with excitation wavelength at 340 and 383 nm for DAN and NATH, respectively. All measurements were carried out at 37 °C by use of a thermostated cell holder and thermostatically controlled water bath.

### 2.3. Experimental procedure for preparing the inclusion complex DAN/cyclodextrin

The incorporation of DAN in cyclodextrin was studied at neutral pH and 37 °C. The DAN concentration in the buffer was held constant in 14.8 μM, while the α-CD or β-CD concentration was varied from 0 to 12 mM. Aqueous solution of cyclodextrins were prepared by weight in their own quartz cuvettes. A 500 μL aliquot of 74 μM DAN solution (prepared by dilution of stock solution in phosphate buffer solution) was directly transferred into the quartz cuvettes, which were perfectly sealed with Teflon stoppers. All solutions were magnetically stirred for 48 h, in the darkness and at room temperature, before used.

### 2.4. NO generation

Nitric oxide was generated from SNAP, which spontaneously releases NO in aqueous solution. The solutions of SNAP in buffer, ranging from 0.1 to 2 μM, were prepared immediately before each experiment. In order to reduce the long half-life of SNAP, which is approximately 10 h at neutral pH [16], $Cu^{2+}$ (0.1 mM final concentration) was added to the solution. At these conditions, the half time of the SNAP was reduced to approximately 8 min at 37 °C. Generation kinet-

ics of the radical was spectrophotometrically monitored from decomposition of SNAP at 342 nm ($\varepsilon = 700\,M^{-1}cm^{-1}$).

## 3. Results and discussion

### 3.1. Incorporation of DAN in cyclodextrins

Absorption and steady-state fluorescence studies were made with the purpose of investigating the possible formation of inclusion complex between DAN and α- or β-CD. These compounds are cyclic oligosaccharides of six and seven D-glucopyranose units, respectively, which form inclusion complex in aqueous solution with various molecules (guests) with suitable characteristics of polarity and dimension. The cavity within CDs is hydrophobic and less polar than the surrounding water molecules, thus the chemical and spectral properties of the guest are usually modified upon inclusion into the CD cavity [13–15]. Absorption spectra of DAN were recorded at different cyclodextrin concentrations, ranging from 0 to 10 mM. In the presence of α-CD, the absorption spectra were practically identical to that observed for DAN in solution (data not shown). However, in presence of β-CD, a small shift to higher wavelengths was observed in the spectrum (Fig. 2). The fluorescence spectra of DAN in absence and in presence of increasing concentrations of both cyclodextrins are shown in Fig. 3. The results show that the fluorescence of DAN does not vary in presence of α-CD, whereas a substantial fluorescence enhancement is observed upon addition of β-CD. The enhancement of the intensity suggests formation of inclusion complex between DAN and β-CD, stabilized through hydrophobic interactions and/or hydrogen bonds. Within the cavity the motion freedom degree of DAN should be limited due to these interactions, thus the probability of radiationless transition decreases, increasing fluorescence intensity. We should note that there is only a slight shift in the position of the emission maxima of DAN in going from water to β-CD, which indicates that the $NH_2$ groups of DAN in the CD complex are probably exposed to the water phase [17].

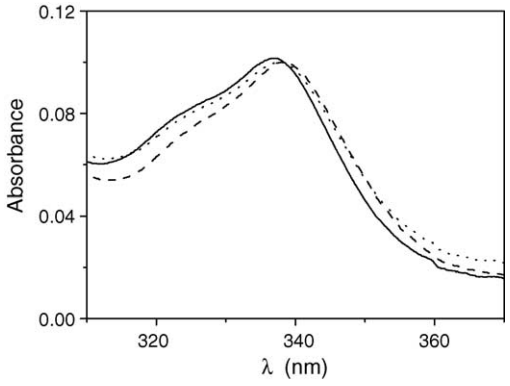


Fig. 2. Absorption spectra of DAN (14.8 μM) in absence (—) and in presence of β-CD, 5 mM (· · ·) and 10mM (- - - -).

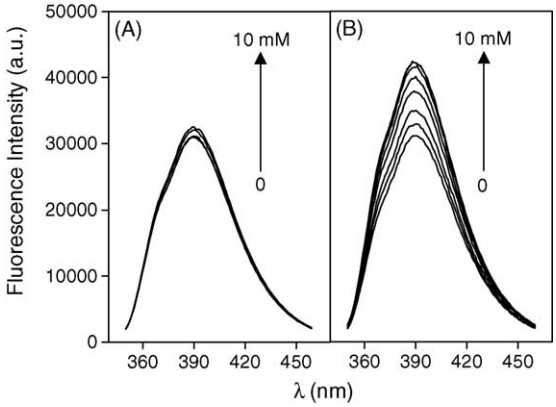


Fig. 3. Fluorescence emission spectra of DAN (14.8 μM) in phosphate buffer containing increasing concentrations of (A) α-CD and (B) β-CD, from 0 to 10 mM.

### 3.2. Stoichiometry and association constant of the inclusion complex

The change in the absorption spectra of DAN in presence of the different β-CD concentrations is too small to allow estimation of the association constant ($K$). Fluorescence enhancement observed in the emission spectra of DAN upon addition of β-CD was then used to calculate $K$ and the stoichiometry of the DAN/β-CD inclusion complex. Determination of $K$ is most accurately done by using non-linear least-squares fitting to the following equation [18]:

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

where $I_0$ and $I$ represent the fluorescence intensities in the absence and in presence of CD, respectively, $[CD]_0$ 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 [19], which represents $1/(I - I_0)$ versus $1/[CD]_0$. The plot will be linear if only 1:1 complexes are formed.

Fig. 4 shows the fluorescence intensity increasing of the DAN at 390 nm, measured at 37 °C as a function of β-CD concentration. The solid line shows the fit of the curve to Eq. (1), which yields a value for the association constant of $K = 380$, and $I_{max}/I_0 = 1.4$. When the Benesi–Hildebrand plot is constructed (inset in Fig. 4) straight line is obtained ($r = 0.999$) which is indicative of a 1:1 stoichiometry for complexes. The association constant is in the same range that the value obtained for other naphthalene derivatives such as 1- and 2-naphtol [20], but lower than that obtained for the association of DAN to *p*-sulfonic sodium calixarene [21], a caged molecule like cyclodextrin. The strong interaction between DAN and calixarenes is attributed to the hydrogen bonding established between the sulphonyl groups of calixarene and

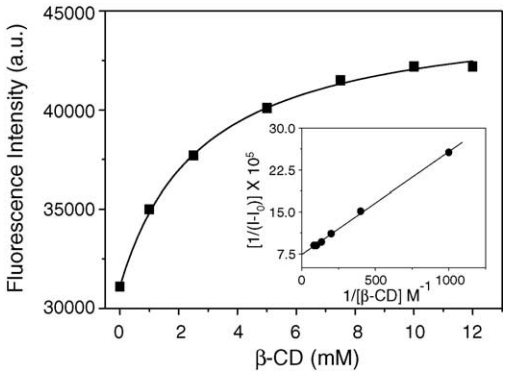


Fig. 4. Influence of β-CD concentration on the fluorescence signal of DAN ($\lambda_{exc}$ = 340 nm; $\lambda_{em}$ =390 nm). *Inset:* Benesi–Hildebrand plot for 1:1 DAN/β-CD complexes.

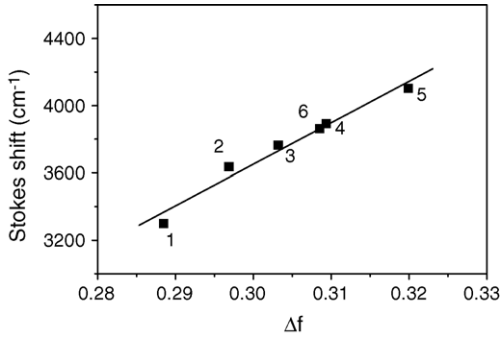


Fig. 5. Stokes shift ($\Delta\bar{\nu}$) as a function of orientation polarizability ($\Delta f$), in different media. The numbers indicate the solvents in Table 1.

the amino groups of DAN. Taking into account the shape and dimensions of β-CD and DAN, the relatively low association constant obtained for the inclusion complex supports the hypothesis that the naphthalene group of DAN is inserted into the non-polar cavity but, at difference of calixarenes, the two $NH_2$ groups remain exposed to the water phase.

### 3.3. Polaririty surrounding DAN guest

In order to gain further insight about the inclusion process it is important to determine the dielectric constant $\varepsilon$ of the medium surrounding DAN. Values of $\varepsilon$ close to 48 (the dielectric constant of the inner β-CD cavity) indicate that an important part of the guest molecule is inserted into the cavity, while larger values suggest most of the molecule exposed to the water polar solvent [22]. The dielectric constant was estimated by application of the Lippert equation [23], which relates the Stokes shifts, in $cm^{-1}$ ($\Delta\bar{\nu} = \bar{\nu}_{abs}^{max} - \bar{\nu}_{fluo}^{max}$), of the fluorophore in different solvents to the orientation polarizability, $\Delta f$, defined as:

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (2)$$

where $n$ represents the refractive index of the solvent. Absorption and emission spectra of DAN in different water–ethanol mixtures were recorded and compared to that obtained for the DAN/β-CD complex, and the Stokes shift was calculated from the absorption and emission maxima (Table 1). Fig. 5 shows the Lippert plot ($\Delta\bar{\nu}$ against $\Delta f$) for the mixtures listed in Table 1. $\Delta\bar{\nu}$ increases as the medium polarizability increases and the dependence with $\Delta f$ is almost linear, indicating that the same specific effects due to hydrogen bonding were present in all mixtures [23]. The equation that best fits the dependence at 298 K is $\Delta\bar{\nu} = 25031\Delta f - 3856$ ($r = 0.989$). Using this equation with $n = 1.3459$ (this value was estimated by measuring in our laboratory the refractive index of the 10 mM cyclodextrin solution) and assuming that the DAN environment is relaxed when is complexed with β-CD, we have determined a dielectric constant of the medium surrounding DAN of 48. This value is similar to that one of the inner β-CD cavity, and indicates the insertion of an important part of the molecule into the β-CD cavity. The good correlation between the stokes shift of DAN/β-CD complex and that obtained for DAN in the ethanol–water mixtures suggests that the same specific interactions occurring in the polar mixtures (hydrogen bonding between the water molecules and the amino groups of DAN) are operating in the DAN/β-CD complex. These results together with the substantial fluorescence enhancement of DAN upon addition of β-CD and the relatively low association constant of the inclusion complex confirm, as was previously suggested, the total insertion of the naphthalene group into the β-CD, with the amino groups extending outside of cavity. The proposed structure should leave free the amino groups to react with other reactive agents such as NO derivatives.

Table 1
Spectral characteristics of DAN in different solvents, together with the refractive index ($n$) and the dielectric constants ($\varepsilon$) of the solvents

| | Solvent | $\bar{\nu}_{abs}$ ($cm^{-1}$) | $\bar{\nu}_{em}$ ($cm^{-1}$) | Stokes shift ($cm^{-1}$) | $n$ | $\varepsilon$ |
|---|---|---|---|---|---|---|
| 1 | EtOH | 29283 | 25974 | 3309 | 1.3614 | 24.3 |
| 2 | EtOH:$H_2O$ (79:21) | 29542 | 25907 | 3635 | 1.3655 | 36.4 |
| 3 | EtOH:$H_2O$ (58:42) | 29586 | 25840 | 3746 | 1.3621 | 48.7 |
| 4 | EtOH:$H_2O$ (36:64) | 29674 | 25773 | 3901 | 1.3539 | 61.4 |
| 5 | $H_2O$ | 29762 | 25641 | 4121 | 1.3330 | 78.5 |
| 6 | β-CD 10 Mm | 29586 | 25707 | 3879 | 1.3459 | 48.0[a] |

$\bar{\nu}_{abs}$ and $\bar{\nu}_{em}$ are, respectively, the wavenumbers of the absorption and emission maxima.
[a] Determined from the fit of the Lippert plot (see Fig. 5).

### 3.4. Effect of NO concentration on the DAN/β-CD complex fluorescence

Incorporation of DAN into the cyclodextrin cavities could modify the reactivity of the molecules against NO derivatives, preventing its analytical use as NO sensor. To rule out this possibility, the ability of the DAN/β-CD complex to yield NATH (see Fig. 1) was evaluated by addition of SNAP (a spontaneously NO-releasing compound) to samples containing DAN (14.8 μM), in absence and in presence of β-CD (10 mM). Samples were maintained at 37 °C during 60 min and the emission spectra were recorded upon excitation at 383 nm, to avoid the interference of DAN. Curve A in Fig. 6 shows the emission spectrum of NATH generated from the nitrosation of DAN in the aqueous solution, while curve B corresponds to the spectrum of NATH recorded in the cyclodextrin solution. Fluorescence intensity was at least 2-fold higher in presence of β-CD and the spectrum was clearly different, showing two major emission bands centered at 408 and 430 nm and a shoulder with low emission around 450 nm. This spectrum is quite similar to that obtained in solution for the anionic species NAT, under alkaline conditions (see Fig. 2 in [24]). As was discussed in Section 1, the fluorescence of NATH is not very high and increases considerably at alkaline pH due to the deprotonation of the amino group (see Fig. 1). The higher fluorescence and the similarity between the spectrum recorded in cyclodextrin and that of NAT suggests that the p*K* of NATH decreases considerably when the molecule is inserted into the β-CD cavity.

To determine the time required to complete the nitrosation of DAN/β-CD, the increasing in the fluorescence intensity at the emission maximum of NATH (410 nm) was monitored for different periods of time upon addition of SNAP (inset in Fig. 6). Fluorescence intensity progressively increased up to a maximum value which was reached after ∼20–30 min of reaction, showing that an incubation time of 30 min was enough to complete the nitrosation of DAN/β-CD. The time for reaction completion was similar to the reported at the same temperature for DAN in solution [24], suggesting that the

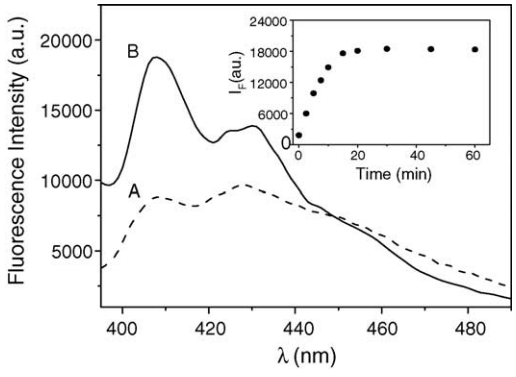


Fig. 6. Fluorescence emission spectra of NATH after incubation of DAN (curve A) and DAN/β-CD (curve B) with SNAP (2.3 mM). *Inset:* Kinetics of DAN/β-CD nitrosation followed by NATH formation at $\lambda_{exc}$ = 383 nm and $\lambda_{em}$ = 410 nm ($T$ = 37 °C).

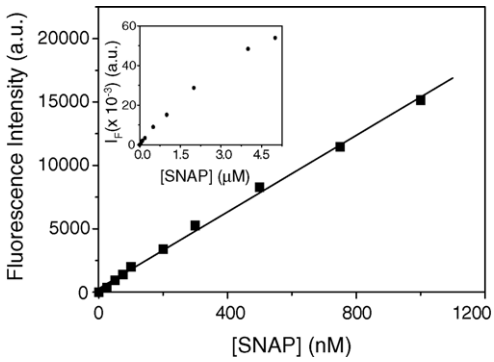


Fig. 7. Dependence of the fluorescence intensity of NATH as a function of SNAP concentration. *Inset:* Expanded scale of the correlation curve of fluorescence intensity with the amount of NO. [DAN] = 14.8 μM; [β-CD] = 10 mM.

incorporation of DAN into the cyclodextrin does not restrict the interaction of the nitrosating agents with the two amino groups of DAN, as was expected from the model previously proposed for the inclusion complex.

Dependence of fluorescence intensity of NATH as a function of SNAP was analysed to determine the sensitivity of the DAN/β-CD complex to the radical nitric oxide. Samples containing DAN/β-CD were incubated for 30 min with increasing concentrations of SNAP, up to 1 μM and fluorescence intensity was recorded at 430 nm. For every SNAP concentration, three sets of assays each containing duplicate samples were performed to assess the reproducibility and reliability of the method. The fluorescence response was linear in the range studied (Fig. 7). Examination of the expanded scale between 0 and 5 μM revealed that the linearity of the assay was retained practically up to 4 μM (inset in Fig. 7). A lower detection limit of 20 nM was determined from the slope of the plot and the standard deviation of the blank measurements, as recommended by IUPAC [25]. This assay is, therefore, much more sensitive than the traditional Griess colorimetric assay, which lower limit of detection is 1–2 μM, and than the method proposed for Kungl and co-workers from the fluorescence lifetimes of DAN [10]. Even thought the sensitivity of our assay is slightly lower than that reported for the current procedure (when the NATH solution is alkalinised [11]), the method shows important advantages as compared to this one, since allows to work directly at physiological conditions, reducing the sample manipulation and the time of analysis. In addition, due to the low cost of β-CD and DAN, the method is much less expensive than some other more sensitive NO fluorescent or electrochemical assays currently in use [26,27]. On the other hand, this new assay opens the possibility of immobilizing the DAN/CD complex in polymeric supports which are unstable in alkaline medium. Immobilization of the inclusion complex in solid supports, such as sol–gel matrix, should allow to build a fluorimetric sensor for nitric oxide having additional advantages including easy handling and enhanced stability of the encapsulated probe. Moreover, due to the tunable porosity of the sol–gel glasses [28], the matrix

could 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. [24]. The possible immobilization of the inclusion complex in a sol–gel matrix is now under investigation in our laboratory and will be the scope of a new work.

In conclusion, we have developed a sensitive fluorimetric assay for quantification of nitric oxide based upon the incorporation of DAN in β-CD and the subsequent reaction of NO with the DAN/β-CD complex. An important part of the DAN molecule is spontaneously inserted into the β-CD cavity through hydrophobic interactions, forming stable 1:1 complexes. Inclusion of DAN into the cyclodextrin does not modify the reactivity of the probe against NO, but decreases the p*K* of the nitrosation product NATH, increasing noticeably its fluorescence quantum yield. The radical can be quantified at physiological pH, even at concentration as lower as 20 nM, without the need to add an alkaline solution to the sample.

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