J Fluoresc (2012) 22:381–389
DOI 10.1007/s10895-011-0970-8

ORIGINAL PAPER

Fluorescence Detection by Intensity Change Based Sensors: A Theoretical Model

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Received: 30 July 2011 / Accepted: 6 September 2011 / Published online: 13 September 2011
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Abstract According to Fluorescence Detection by Intensity Changes (FDIC) the fluorescence intensity of many fluorophores depends on the non-covalent (specific and/or non-specific) interactions these fluorophores would be able to establish with the solvent and, more interestingly, with other surrounding molecules. This latter effect is the basis of FDIC for analytical purposes. In this paper, a preliminary study of FDIC applications using a fluorophore supported in a solid medium (sensor film) is presented. First, a mathematical model relating the analyte concentration with the immobilized fluorophore fluorescence is deduced. The model includes all the different mechanisms explaining this relationship: index of refraction or dielectric constant modification, scattering coefficient alteration and sensor film volume increase. Then, the very first experimental results are presented, using different fluorophores and solid supports. The best results were obtained using polyacrylamide (PAA) polymers and coralyne as the fluorophore. This sensor film is applied for albumin and polyethylenglycol determination and the results are compared with those obtained using coralyne in solution. Albumin quenches the coralyne fluorescence in both cases (solution and film), while PEG quenches coralyne fluorescence in films but increases it in solution. These results suggest that the outstanding fluorescence change mechanism is sensor films is the film volume increases, which is different than those observed in solution.

Keywords FDIC · Non-covalent interactions · Sensors · Polyethylenglycol · Albumin

Electronic supplementary material The online version of this article (doi:10.1007/s10895-011-0970-8) contains supplementary material, which is available to authorized users.

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Introduction

Control of molecular environmental effects on emission is crucial for the design and development of original detection systems and sensors based on fluorescence. This depends on the understanding of the molecular interactions established between the corresponding fluorescent probe, analyte and medium; non-covalent interactions play an important role in probe emission [1, 2]. Let us consider that the fluorescence quantum yield is given by:

$$\phi_F = \frac{k_F^r}{k_F^r + k_F^{nr}} \quad (1)$$

k_F^r and k_F^{nr} being the radiative and non-radiative excited state decay constants respectively (throughout the paper the subindex F will be used for indicating a parameter depending on the fluorophore). It has been well documented in the literature regarding liquid media that quenching phenomena are produced by specific interactions

between the corresponding quencher molecule and fluorescent probe, via an increase in k^{nr} that produces a decrease in fluorescence quantum yield.

Previous works by our group have shown that the addition of numerous chemical compounds induces changes in the fluorescence signals of certain probes (e.g. coralyne and berberine cations), which affect emission intensity without producing significant changes in emission wavelength [3, 4]. These changes not only involve quenching but also enhancements in emission with regard to intrinsic fluorescence of the corresponding probe in a given medium. A large number of non-fluorescent molecules produce this phenomenon [5–8], which seems to be general. We have found that electrostatic, non-specific probe-analyte interactions are responsible for emission increases.

In order to provide a theoretical support for these effects, a first model was developed to account for experimentally-obtained emission increases for the above-mentioned probes [9] when used in a silica gel surface for thin layer chromatography detection. According to this model, non-specific, dipolar interactions between probe and analyte contribute to the efficiency of the fluorescence emission, through analyte polarizability (α), creating a microenvironment that isolates the fluorescence probe and prevents non-fluorescent decay mechanisms. Chromatographic applications have been developed for quantitatively detecting a variety of non-fluorescent analytes under HPLC and TLC conditions [3, 4]. In these cases, chromatographic detection is referred to as FDIC (Fluorescence Detection by Intensity Changes). FDIC response in these systems has been explained as a balance between radiative and non-radiative processes due, respectively, to the non-specific and specific interactions established between probe and analyte, in a given medium.

Recently, we have reported a more general model for fluorescence in solution which tries to explain how the solvating ability of the medium (given by its refraction index n_m and its dielectric constant ε_m) affects, via non-specific interactions, the fluorescence intensity (F) of a given fluorophore dissolved in that medium [10]. This model starts with a general equation for the fluorescence intensity read in a spectrofluorometer in specific instrumental conditions:

$$F = K_a^{ins}\phi_{F,m}a_{F,m}c_F \tag{2}$$

K_a^{ins} being a constant depending on the instrumental conditions (including I_0 , the intensity of the light source at the excitation wavelength), $a_{F,m}$ and $\phi_{F,m}$ being the molar absorptivity and the fluorescence quantum yield of the fluorophore in that medium (the subindex m refers to a parameter depending on the medium) at the excitation and fluorescence wavelengths respectively and c_F being the fluorophore concentration. Then, the effect of the medium on $a_{F,m}$ and $\phi_{F,m}$ was deduced as:

$$a_{F,m} = a_F^v\left(\frac{f_{n,m}^2}{n_m}\right) \tag{3}$$

$$\phi_{F,m} = \frac{n_m f_{n,m}^2}{n_m f_{n,m}^2 + \Upsilon_F^{ISC} e^{P_F^{S-T} f_{\varepsilon-n,m}}} \tag{4}$$

a_F^v being the fluorophore molar absorptivity in the absence of the medium, Υ_F^{ISC} being the quotient k_v^{nr}/k_v^r ($k_v^r$and $k_v^{nr}$are the radiative decay constant and non-radiative decay constant in the absence of the medium, i.e. in vapor phase), P_F^{S-T}being a parameter depending on the fluorophore properties and $f_{n,m}$ and $f_{\varepsilon\text{-}n,m}$ being parameters depending on n_m and/or ε_m (n_F being the index of refraction of the fluorphore)

$$f_{n,m} = \frac{3n_m^2}{2n_m^2+1} \tag{5}$$

$$f_{\varepsilon-n,m} = \frac{\left(\frac{\varepsilon_m-1}{2\varepsilon_m+1}\right)}{1-2\left(\frac{\varepsilon_m-1}{2\varepsilon_m+1}\right)\left(\frac{n_F^2-1}{n_F^2+2}\right)} \tag{6}$$

Combining (2)–(6) gives the general fluorescence equation considering the effect of the medium. When an analyte is dissolved in a solvent containing a fluorophore, the n_m and/or the ε_m and thus the fluorescence intensity will change. From a macroscopic point of view, the analyte effect on both parameters can be represented by its volume molar fractions (X_S and X_A for the solvent and the analyte respectively; throughout the subindexes S and A refer to solvent and analyte respectively), according to the classical treatment by Onsager (for dielectric constant) and Lorentz-Lorenz for index of refraction:

$$\frac{n_m^2-1}{n_m^2+2} = \frac{n_S^2-1}{n_s^2+2} + \left(\frac{n_A^2-1}{n_A^2+2} - \frac{n_S^2-1}{n_s^2+2}\right)X_A \tag{7}$$

$$\varepsilon_m = \varepsilon_S + (\varepsilon_A - \varepsilon_S)\,X_A \tag{8}$$

When (3–8) are substituted in (2), an equation is obtained relating the fluorescence intensity variations of a medium with the analyte concentration. This can be considered the mathematical model describing FDIC.

In this paper we propose a mathematical model which can be used as the theoretical support for FDIC based optical sensors. In addition, a flow cell designed for this

kind of experiments will be presented, the ability of different immobilization procedures as a basis for FDIC will be explained, different fluorophores will be tested and finally the very first application of the method for albumin and polyethylenglycol determination will be shown.

Experimental Methods

Apparatus

Measurements were carried out with a Perkin Elmer LS-50B luminometer. The flow cell containing the sensor film was placed on an optical fiber plate reader accessory. A FIA system was used, consisting of a Wilson (Minipuls 3) peristaltic pump and a six-way manual injection valve, together with 0.5 mm inner diameter PTFE tubes . A UV Atom 75-Fotomatic lamp was also used for sensor film preparation.

Reagents

Acrylamide (AA), N,N-bis-acrylamide (BAA), ammonium persulphate (APS), and bovine serum albumin (BSA) were used for sensor film preparation (Sigma). Berberine, Auramine-O, Stains all, Tiazol Orange, Fluorescein, Ethidium bromide and, mainly, Coralyne chloride (Across Chimica, Geel, Belgium) were used as fluorescent probes. Bovine serum albumin (BSA) and polyethylenglycol (PEGs) (Sigma), were used as analytes. Assays were also performed using fluorescein linked BSA (BSA-fluorescein, Sigma).

Bidistilled Milli-Q water and methanol (Panreac, Spain) were used as solvents. For preparation of methanol/water mixtures, the solvents were degassed by means of an ultrasound bath for 30 min before mixing.

Polyacrylamide Sensor Film Preparation Procedure

Different procedures were applied for the preparation of polyacrylamide-coralyne (PAA-Cor) based sensor films, depending on the solvent used and on the way that probe incorporation was performed. In general, all procedures used were based on probe trapping into the solid structure of polyacrylamide (PAA), which is in-situ formed from its monomer.

Sensor Films for Aqueous Solutions

Basic Procedure The basic procedure was based on a method reported elsewhere [11] designed to prepare sensor films based on immobilized glucose oxidase-fluorescein. 20 mg of AA and 4 mg of BAA were mixed with 85 μl of a coralyne solution (1 mg/ml) in bidistilled water. 15 μl of an APS solution (0.4 mg/ml) was then added. APS catalyzes acrylamide polymerization. Dissolved oxygen was removed by bubbling nitrogen through the solution. The cocktail was spread on a hollow (20×9×0.1 cm) cut into a glass plate, covered with a glass film and irradiated with the UV-lamp (254 nm) for 60 min. The obtained film was stored in water at 4 °C.

Dispersion Based Procedure A modification was introduced into the basic procedure. After completing solubilization of AA, BAA and coralyne, 100 μl of an appropriate concentration of BSA solution was added to improve the dispersion of the fluorescent probe before APS addition. In this case, the mixture was carefully stirred to prevent bubble formation.

Water/Methanol Mixtures

Where water/methanol mixtures were used, the procedure was different to those previously described. A polyacrylamide film with AA, BAA and APS in water was prepared. A coralyne solution (1 mg/ml) in methanol was also prepared, and the film dipped into this solution for 1 h. After removing the excess coralyne, the film was stored at 4 °C in the appropriate water/methanol mixture until its use. No BSA was used in the film preparation in this case.

Other Fluorophores and Immobilization Procedures for Sensor Film Fabrication

Other sensor films were prepared using different fluorophores in combination with two general immobilization methods (see Table 1): a) Entrapment using silicone, polyamide, PVC, polyacrylamide (PAA). In the first three cases, the polymer and the probe were dissolved in the same solvent and then evaporated; PAA was obtained as described in 2.3.1; b) Covalent attachment using cellulose and Ultrabind™ (the probe was attached to the reactive groups of the film with a covalent bond). In all cases, recommended preparation procedures were used (most of them compiled by Cass [12], and reviewed by Cooper and Cass [13]).

Flow Cell Description

The flow cell was designed in our laboratory (Fig. 1). The main part of the cell is a stainless steel piece (a) (2×2.5×2 cm) with a hollow (b) (0.5×1.5×0.3 cm). The cell is closed with a holed methacrylate cover (c) in which the film is fixed using a perforated Mylar film (d). The stainless steel component has two stainless steel tubes (e) (2 mm

Table 1 Dipole moments and energy gap values calculated for the different fluorophores considered

Fluorophore	$\mu_F^{S(v)}$ (D)	$\mu_F^{T(v)}$ (D)	ΔE_F^{S-T} (Kcal mol^{-1})	$\Delta E_F^{S-T}\Delta\mu_F^2$ (D^{2}Kcal mol^{-1})
Auramine	4,12	2,59	35,7	367
Berberine	1,93	2,92	21,4	−103
Coralyne	4,38	1,44	20,9	357
Ethidium bromide	1,97	3,32	65,1	−462
Fluorescein	3,48	6,56	12,7	−392
Stains all	1,33	1,83	22,6	−36
Tiazol Orange	3,30	1,74	24,2	191

outer diameter) to allow the fluid to circulate inside the cell. The two main pieces of the cell are joined by four screws (f) and a silicon o-ring (g) to avoid fluid losses. The sensor cell has a volume of 225 μl.

Measurements

Experiments were carried out either in water or in water/methanol mixtures. Solvent flowed across the cell at 0.4 ml/min and the fluorescence was monitored at the corresponding Coralyne maxima (water: λ_{exc}=425 nm and λ_{em}=460 ; water/methanol: λ_{exc}=427 nm and λ_{em}=478). 1 ml analyte was injected and the corresponding transient signal obtained, the maximum/minimum of this signal being the measured fluorescence. Signals obtained for PEGs and BSA were negative with regard to the fluorescent probe baseline, and in these cases the intensity in the minimum (F_{min}) of the peak were used for signal measurements:

$$analytical\ parameter = \Delta F = \frac{F_0 - F_{min}}{F_0} \tag{9}$$

For measurements in solution the same analytical parameter will be used, replacing F_{min} by fluorescence intensity observed after the analyte addition (F).

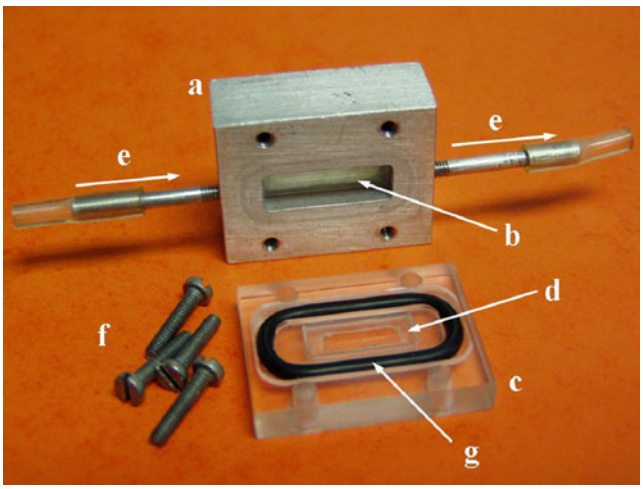

Fig. 1 Flow cell used for fluorescence measurements (for description, see "Flow Cell Description")

Computational Methods

All calculations were carried out using the Gaussian03 package. The B3LYP hybrid DFT functional [14–16] has used throughout this paper in combination with the 6–31G (d,p) basis set [17]. Time dependent-density functional theory using the B3LYP functional (TD-B3LYP) was employed for excitation energies using B3LYP optimized ground state geometries.

Model for FDIC Sensor Films

General Model

Consider a fluorophore immobilized in a film. When another compound (the analyte) comes into contact with this film, the fluorescence of the fluorophore can be changed by two main mechanisms:

A. The analyte can modify the index of refraction (n_m) and/or the dielectric constant (ε_m) of this film.

Equation 2 gives the fluorescence intensity generally used for explaining measurements in solution, but it is not valid when working in solids or films. Based on the Yang revision of the Kubelka-Munk theory, we have developed an optical model for describing the fluorescence signal in thick films [18]. According to this model, the fluorescence emitted from the film has two main components: the forward and the backward components. The backward is the more frequently used and is given by the simplified equation [19]:

$$F = K_b^{ins} L S_{ex,m} \phi_{F,m} a_{F,m}^{3/4} c_F^{3/4} \tag{10}$$

K_b^{ins}being a constant having the same nature as K_a^{ins}, L and $S_{ex,m}$ being the thickness and the scattering coefficient at the excitation wavelength of the film respectively. For the sake of clarity we have retained the sub-index "m" referring to the thick film. In addition to L, the most important differences between Eqs. 2 and 10 are the exponent of both, the fluorophore concentration and the fluorophore molar absorptivity (it has been consid-

ered that neither the solvent nor the solid support absorbs at the fluorophore excitation wavelength), and the appearance of the film scattering coefficient. Working with fluorescence in solution, the $a_{F,m}$ and $\phi_{F,m}$ dependences on the ε_m and n_m can be deduced from Eqs. 3 to 6 , and the dependence of these parameters on the analyte concentration from Eqs. 7 and 8. However, for applications of these equations to this case, two aspects should first be dealt with:

1) The effect of the thick film solid support on ε_m and n_m . In the absence of analyte, the ε_m (ε_0) and n_m (n_0) depends on the solid and the solvent; these dependences can be obtained applying a similar approach to that used for obtaining (7) and (8). The presence of analyte in the film causes an alteration in the volume occupied by the solvent. The mathematical treatment gives rise to similar expressions:

$$\frac{n_m^2-1}{n_m^2+2}=\frac{n_0^2-1}{n_0^2+2}+\left(\frac{n_A^2-1}{n_A^2+2}-\frac{n_0^2-1}{n_0^2+2}\right)X_A \qquad (11)$$

$$==>\ n_{c,m}^2=n_{c,0}^2 \quad + \quad \left(n_{c,A}^2-n_{c,0}^2\right)X_A$$

$$\varepsilon_m=\varepsilon_0+(\varepsilon_A-\varepsilon_0)\,X_A \qquad (12a)$$

the notation n_c^2being used for the sake of clarity. It is easy to demonstrate that as n^2 increase n_c^2 also increase.

2) The a_F and ϕ_F dependence on the ε_m and n_m obtained from Eqs. 3–6 is extremely complicated and very difficult to handle. To overcome this problem, a mathematical study was carried out in order to obtain a simplified expression for the $\left(\phi_F a_F^{3/4}\right)$dependence on ε_m and n_m (see [19]).

$$\phi_{F,m}\left(a_{F,m}\right)^{3/4}=\left(\phi_{F,m}\left(a_{F,m}\right)^{3/4}\right)_0 \left(1+\left(N_{A,0}-3P_F^{S-T}E_{A,0}\right)X_A\right) \qquad (12b)$$

$\left(\phi_{F,m}\left(a_{F,m}\right)^{3/4}\right)_0$ being the $\phi_{F,m}(a_{F,m})^{3/4}$ value in the absence of analyte and $N_{A,0}$ and $E_{A,0}$ being:

$$E_{A,S}=\frac{\varepsilon_A-\varepsilon_0}{(\varepsilon_0+2)^2} \qquad (13A)$$

$$\frac{n_{c,A}^2-n_{c,0}^2}{0.18+n_{c,0}^2}=N_{A,0} \qquad (13B)$$

and represent the analyte availability of changing the index of refraction and the dielectric constant of the film. According to this equation, analytes having a higher index of refraction and/or lower dielectric constant than the sensor film will produce an increase in the fluorophore fluorescence intensity; this is the same effect observed for analytes interacting with fluorophores in solution.

When Eqs. 12a and 12b is substituted in (10) the general fluorescence model is obtained:

$$F=K_b^{ins}LS_{ex,m}\left(\phi_{F,m}\left(a_{F,m}\right)^{3/4}\right)_0 \left(1+\left(N_{A,0}-3P_F^{S-T}E_{A,0}\right)X_A\right)c_F^{3/4} \qquad (14)$$

In the analyte absence (X_A=0) Eq. 14 gives:

$$F_0=K_b^{ins}LS_{ex,m}\left(\phi_{F,m}\left(a_{F,m}\right)^{3/4}\right)_0 c_F^{3/4} \qquad (15)$$

and the parameter ΔF (see 2.9) can be finally obtained:

$$\Delta F=\frac{F-F_0}{F_0}=\left(N_{A,0}-3P_F^{S-T}E_{A,0}\right)\ X_A \qquad (16)$$

As can be seen, the parameter ΔF does not depend on the instrumental conditions (as F does) and only depends on the analyte ability of changing the index of refraction and the dielectric constant of the film ($N_{A,0}$ and $E_{A,0}$), on the fluorophore (P_F^{S-T}) and the analyte concentration, becoming this parameter more robust than F.

B. The analyte can modify the physical dimensions of the film.

This effect will be observed when high molecular weight analyte molecules (i.e. higher than about 1000) are being monitored and will be especially important when working with hydrogels (i.e., polyacrylamide) as solid supports. This give rise to two additional effects: 1) Since the film structure changes, the scattering coefficient also changes proportionally to the analyte concentration. When working with hydrogels and in the conditions used in this paper these variations can be disregarded [11]; 2) As the fluorophore amount in the film does not change but the volume increases, its final concentration diminishes proportionally to the analyte concentration according to:

$$C_F=C_F^0(1-X_A) \qquad (17)$$

C_F^0 being the original fluorophore concentration and the ΔF is given by [19]:

$$\Delta F=\left(N_{A,0}-3P_F^{S-T}E_{A,0}-\frac{3}{4}\right)X_A-\frac{3}{4}\left(N_{A,0}-3P_F^{S-T}E_{A,0}\right)X_A^2 \qquad (18)$$

As can be seen due to the sensor volume increase a new term depending on the analyte concentration

appears which always decreases as the analyte concentration increases. This term is very important because even in the non-specific interactions absence (index of refraction or dielectric constant changes), the analyte will produce a generic decrease in the fluorescence intensity.

Additional Considerations

The analyte will not be in the film originally but in the sample solution, for which the concentration (C_A) is usually required to be measured. The relationship between X_A and the analyte concentration in the film ($C_{A,m}$, in M) is well known

$$X_A = C_{A,m}\frac{M_A}{\rho_A} \tag{19}$$

M_A and ρ_A being the analyte molecular weight and density (g L^{-1}), respectively. Different models can be used for relating C_A and $C_{A,m}$ depending on the predominant mechanism between the film solid and the sample, and the sample hydrodynamics (continuous flow, flow injection, etc). At this point we are not interested in obtaining a different model for each different case, but rather a general expression. In our opinion, the Langmuir isotherm could be the appropriate solid film/sample interaction:

$$c_{A,m} = K_h\frac{K_A^m c_{A,m}^{\max} c_A}{1 + K_A^m c_A} \tag{20}$$

$c_{A,m}^{\max}$ being the maximum analyte concentration in film (saturation condition) and K_A^m depending on the analyte/film distribution constant. A constant K_h will also be added containing those parameters related with the hydrodynamics [19].

Results and Discussion

Preliminary Studies

A) Fluorophore study

The fluorophore to be immobilized is of crucial importance, because its fluorescence should be prone to modification by the analyte. According to the initially proposed model, this can be partially evaluated from the product $P_F^{S-T}\Upsilon_F^{ISC}$ (see [10] for a full explanation). The higher the $P_F^{S-T}\Upsilon_F^{ISC}$, the higher the environmental effect on its quantum yield and the better the fluorophore for sensor film application. It is very difficult to give an exact evaluation of this parameter, but it can be very crudely be approximated to:

$$P_B^{S-T}\Upsilon_F^{ISC} \propto \Delta\mu_F^2\Delta E_F^{S-T}$$
$$\Delta\mu_F^2 = \left(\mu_F^{S(v)}\right)^2 - \left(\mu_F^{T(v)}\right)^2 \tag{21}$$

$\mu_F^{S(v)}$ and $\mu_F^{T(v)}$ being the fluorophore dipole moment in the singlet and triplet states respectively in the absence of medium (in vapour phase), ΔE_F^{S-T} being the fluorophore excited to triplet state energy gap in the absence of the medium and α being a fitting parameter. The fluorophore dipole moments and the energy gap can be theoretically estimated by computational methods. For these first assays, different fluorophores frequently used as fluorescent labels for biological applications or thin layer chromatography were chosen. Table 1 shows the dipole moment, the energy gap and the $\Delta\mu_F^2, \Delta E_F^{S-T}$ calculated. The results indicate that Auramine and Coralyne seem to be the best fluorophores. As will be shown later, assays have been performed with Coralyne and all the other fluorophores, and the results obtained tend to confirm this hypothesis (Table 2).

B) Sensor Film Material

In order to produce an appropriate sensor film, the supporting material has to be carefully chosen and the following aspects should be taken into account: the fluorophore must be efficiently retained and in such a quantity that it can be easily detected and should be stable enough in the sensor film medium, the fluorophore leaching should be minimised and the analyte should be detected.

When preparing durable sensor films, the fluorophore can be immobilized in the sensor film in two different ways: attached to the surface (covalently or adsorbed) or entrapped (free or cross-linked) in the film structure. Tests were performed using both types of method. First, ultrabind(TM) and functionalized cellulose commercial films were used in which the fluorophore can easily be covalently attached. Secondly, polyacrylamide (PAA), PVC, silicone and polyamide were used in which the fluorophore is entrapped. Table 2 summarizes the results obtained with coralyne and berberine (used as a reference fluorophore). As can be seen, entrapment gives better results in general. Polyacrylamide was finally chosen and this support was also tested with the rest of the fluorophores, but no improvements over Coralyne were observed (Table 2).

C) Analyte selection.

These first assays were performed using three different analytes: glucose, polyethylenglycol and

Table 2 Probes, analytes and immobilization methods tested. **a** Entrapment, **b** Covalent attachment, **c** Adsorption. (*) Phosphate buffer pH=7.5

Probe	Method	Solvent	Film external appearance	Results
Coralyne	Polyacrylamide[a]	Water	Non-homogeneous film: problems with APS	Low signals with analytes. Coralyne leaching
		MeOH	Homogeneous film	Coralyne leaching but signal of the analytes observed
	Polyamide[a]	HCOOH	Homogeneous film	Fast coralyne leaching
	Silicone[a]	Chloroform	Non-homogeneous film	Needs a support which gives a high background signal
	PVC[a]	THF	Homogeneous film	No signal with analytes
	Cellulose[c]	Aqueous media*	Homogeneous film	Coralyne leaching
	Ultrabind™ [c]	Aqueous media	Homogeneous film	Coralyne leaching
Berberine	Polyacrylamide[a]	Water	Non-homogeneous film: problems with APS	No spectra observed
	PVC[a]	THF	Homogeneous film	No signal with analytes
	Cellulose[c]	Aqueous media	Homogeneous film	Badly resolved observed spectra
	Ultrabind™ [c]	Aqueous media	Homogeneous film	Bad observed spectra
Auramine 0	Polyacrylamide[a]	Water	Homogeneous film	No observed spectra
Stains All	Polyacrylamide[a]	Water	Non-homogeneous film	No observed spectra
Tiazol Orange	Polyacrylamide[a]	Water	Non-homogeneous film	No observed spectra
BSA-Fluorescein	Polyacrylamide[a]	Water	Homogeneous film	No signal with analytes
	Cellulose[b]	Aqueous media	Homogeneous film	No observed spectra
	Ultrabind™ [b]	Aqueous media	Homogeneous film	No signals with analytes
Ethidium Bromide	Polyacrylamide[a]	Water	Bad formed film: problems with APS	
	Glass[b]	Aqueous media	Non-homogeneous film	Support gives high background signal

albumine. Coralyne entrapped in PAA was the only system giving good results, using albumine and PEG as analytes. In the following section these results are described in more detail.

Coralyne-PAA Sensor Film for BSA Determination in Water

A) Sensor film preparation

Coralyne-PAA-films prepared using the basic procedure (2.3.1) presented two problems. First, no significant variations in sensor film fluorescence were observed when BSA was injected as an analyte. Second, coralyne escaped from the cross-linked structure of polyacrylamide in the sensor-film and was dissolved into the water flow. This produced a substantial drift in the baseline. It is known that an adjustment of AA and BAA ratios allows the pore structure of the resulting polyacrylamide to be tailored [20]. Therefore, different proportions of these reagents were used in the polymerization reaction to obtain a pore structure suitable for coralyne retention. However, no results were obtained. Likewise, a closer inspection of these films revealed that coralyne precipitates during preparation, given its insolubility with APS. This precipitation was avoided when dimethyl sulfoxide was used for probe solubilization. However, this was not useful for improving the analytical signal.

These problems were partly overcome with sensor films prepared using the dispersion based procedure (2.3.1). This involves BSA being added after solubilization of AA, BAA and coralyne, but before APS addition and prior to film formation. This prevents coralyne precipitation caused by APS. The protein seems to protect the coralyne probe in some way, favouring its dispersion in the film. In addition, while the reaction in the basic procedure takes 60 min, it is completed in only 15 min in the case of the dispersion based procedure. This is probably due to the cross-linking effect of BSA in PAA formation which helps the polymer and probably a BSA-Molecularly Imprinted Polymer like structure is actually formed.

B) BSA determination

To evaluate the effect of the BSA concentration on the sensor-film performance, three sensor films were prepared with different initial concentrations of BSA: 5, 10 and 20 mg/ml. The higher the starting BSA concentration, the more homogeneous the film; the film prepared from 5 mg/ml of BSA showed some aggregates of precipitated coralyne. Once the films were prepared and

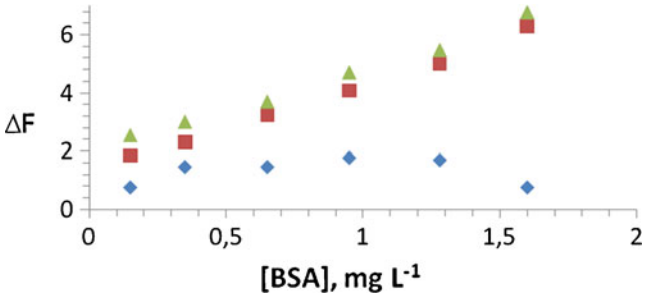

Fig. 2 ΔF variation with BSA concentration obtained for different BSA amount in the sensor film formation: ■ 20 mg/ml BSA, ♦ 10 mg/ml BSA and ▲5 mg/ml BSA

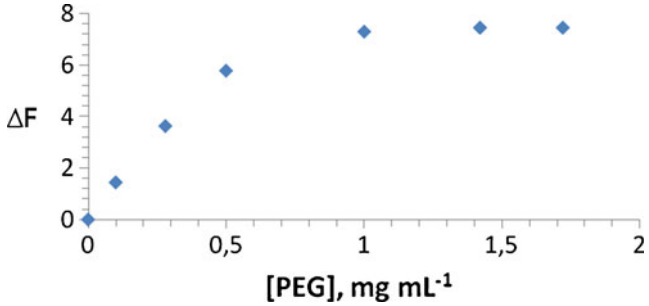

Fig. 4 ΔF observed in coralyne films with different concentrations of PEG 4250 in methanol-water of 50% (v/v) mixture

their spectra recorded, they were tested for BSA detection. In all cases a quenching effect of fluorescence by albumin was observed.

Figure 2 shows the ΔF observed using the three sensor films. As can be seen, films containing 10 and 20 mg/l BSA show linearity, the slope of the calibration lines being similar. The film giving homogeneity problems (5 mg/ml of starting BSA) did not show a regression between the fluorescent signal and the analyte concentration. The coralyne-PAA film have a one week lifetime (intensity reduction of about 50%).

In order to obtain some information about the fluorescence change mechanism, we made an experimental study of the BSA effect on coralyne fluorescence in water solution. As in sensor films, the BSA produces quenching in fluorescence; Fig. 3 shows the ΔF versus BSA concentration. According to the model in solution, BSA acts modifying the dielectric constant of the medium surrounding the fluorophore. These results could suggest that the fluorescence change mechanism is similar in films and in solution; however since the slope of the ΔF versus [BSA] is lower in sensor film than in solution and the linearity is worse , it could be deduced that the fluorescence change also comes from the volume increase; the original albumin present in the film models the PAA structure in such a way that the albumin analyte does not produce additional changes.

Sensor Film for PEG Measurement

Development of sensor films for analyte detection in organic media should also be possible. We explored the preparation (by means of the dipping procedure, see 2.3.2) and performance of sensor-films in methanol-based media for detecting PEGs.

Although PAA-coralyne sensor-films were prepared with the dipping procedure in pure methanol, it was not possible to perform measurements when using pure methanol as flow solvent. There is a shrinking in the polyacrylamide pore structure causing the sensor to collapse. This effect has been experimentally noticed in our laboratory with methanol and other organic solvents, such as acetone and acetonitrile, for PAA films. PAA-based sensor films prepared by the dipping procedure are solid gels that must be kept in a liquid medium. If dried, they become rigid and can no longer be used.

In general terms, the pore size of a PAA gel depends on the amount of water molecules trapped in its reticulate structure. When the film is additionally brought into contact with another solvent, shrinking/swelling phenomena affect the pore structure. This process is quasi-reversible: when the film initially prepared and tested in methanol is used again in an aqueous medium, it experiences swelling and partially recovers its initial aspect.

It was possible to work with water/methanol mixtures. With this solvent, no baseline drift problems were observed,

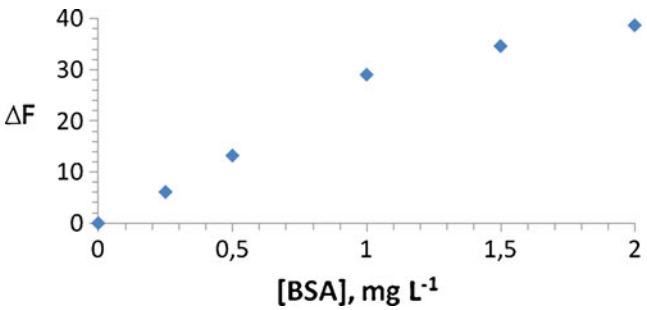

Fig. 3 BSA additions to a solution of coralyne (0.02 mg/ml) in water. ΔF variation (λ_{exc}=410 nm and λ_{em}=470 nm) *vs*. BSA concentration

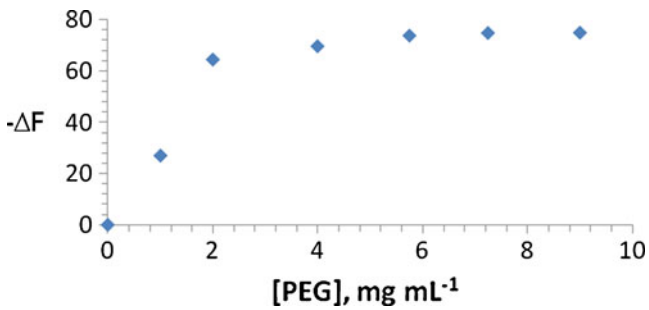

Fig. 5 PEG 4250 additions to a solution of coralyne (0.02 mg/ml) in water. −ΔF (λ_{exc}=410 nm and λ_{em}=470 nm) *vs*. PEG concentration

for either the basic or the dispersed procedure, and the films can be used during at least two weeks. In the case of water/methanol mixtures, pore shrinking/swelling greatly mitigates the coralyne loss from the PAA structure.

Figure 4 shows the fluorescence intensity variation with PEG concentration (a PEG with molecular weight of 4250) obtained using a dipping-based PAA-coralyne sensor film and working in a 50% (v/v) water/methanol mixture. As can be seen, the PEG produces a decrease in the sensor film fluorescence. As with BSA, a parallel study of the PEG effect on coralyne fluorescence in solution was also carried out (Fig. 5). As can be seen, in contrast with the films, PEG produces an increase in coralyne fluorescence, indicating that the fluorescence change mechanism in solution is due to index of refraction modification. These results also suggest that, as with BSA, the increase in the sensor film volume is the dominant mechanism of the fluorescence change; this effect is able to compensate the coralyne fluorescence increase by the PEG observed in solution.

Conclusions

The application of the FDIC phenomenon as a fluorescence detection method in optical sensor systems has been presented and mathematically modelled. Preliminary experiments in which the analyte produces a similar or a different fluorescence change mechanism compared to solution have been described. All these results suggest that the outstanding fluorescence change mechanism is the increase in the sensor volume due to the analyte being able to overcome all other mechanisms.

Further experiments with other types of analyte, sensor films and fluorophores will be performed in the near future in order to obtain better validation of the model and for a better understanding of the differences between film and solution. In addition to chemical detection and its use as a detector in liquid chromatography or capillary electrophoresis, the methodology can also be tested for sensing physical properties.

Acknowledgements Authors thank the Spanish Ministerio de Ciencia e Innovacion (MICIN) of Spain (projects CTQ 2008-06751-C02-01/BQU and CTQ 2008–00959). A.D-C. thanks to the MICIN for a grant.

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