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ABSTRACT

From the great variety of BODIPY based-chemosensors able to determine Hg^{2+}, only a small portion has been applied to its determination in environmental and/or biological samples. The lack of studies on the analytical performance of the latter sensors makes interesting the development of investigations oriented to their possible analytical applications. The synthesis of a BODIPY derivative armed with a tetrapod receptor is described. The procedure is based on a previous publication, and the modifications performed to improve the synthesis include alternative procedures with different objectives, as the consecution of a multigram synthesis, improving the low yields of some of the previously proposed procedure steps, simplifying the experimental steps, achieving the desired purity requirements for use with analytical purposes, and enriching the characterization of the implied structures. The characteristics of its selectivity towards Hg^{2+} have been investigated, and the OFF–ON fluorometric response, based on a photo-electron transfer (PET) mechanism, served as the base for the development of a method able to determine Hg^{2+} in environmental waters at ng mL^{-1} levels.

The intrinsic fluorescence of the BODIPY core is inhibited and the probe exhibits a weak fluorescence (i.e. "OFF" state due to the deactivating PET effect). Upon complexation, Hg^{2+} interacts with the lone-pair electrons on the nitrogen atoms of the receptor moiety so that the electronic transfer from the receptor to the photo-excited fluorophore is slowed down or switched off (i.e. "ON" state due to the suppression of the deactivating PET effect by coordination of the analyte to the probe).

Regarding the complex photostability in aqueous solution, it is mandatory to conduct the experiments at darkness due to its photodegradation. The stoichiometry studies indicated a 1:2 relationship for the BODIPY-Hg^{2+} complex. The high selectivity towards mercuric ions is considerably influenced by pH, being necessary to conduct the experiments in a pH value higher than 6. Calibration samples were prepared by adding appropriate amounts of Hg^{2+} between 20.0–120.0 ng mL^{-1}, at a constant BODIPY concentration of 1 μmol L^{-1}. After agitating for 5 min at darkness, phosphate buffer (pH=7.50) was added, and it was diluted to the mark with water. Fluorescence measurements were carried out at 18 °C, exciting at 515 nm, and obtaining fluorescence emission at 538 nm. The method has been satisfactory applied to Hg^{2+} determination in environmental water samples.

1. Introduction

Mercury is a widespread pollutant with distinct toxicological effects. Despite a reduction of its industrial use as a result of stricter regulations, high concentrations of mercury are still present in many environmental compartments [1,2].

It is usually found in many products of daily life such as paints, electronic equipment and batteries, and it exists in a variety of different forms (metallic ion, and as part of organic salts and complexes). Once released into the environment (especially aquatic systems), inorganic Hg can be transformed via biotic and/or abiotic methylation to monomethylmercury, a highly toxic and bioaccumulative Hg form in organisms and humans. These environmental and health problems have prompted the development of methods for the detection and quantitation of mercury, especially in situations where conventional techniques are not appropriate.

Among the use of mercury-indicating methodologies, which are developed to provide critical information for mercury hazard assessment and mercury pollution management, the use of luminescent chemosensors for the determination of Hg^{2+} traces in

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environmental and biochemical samples is of current interest [3–8], as it is evident in several critical reviews about heavy metal ions chemosensing, and in a recent report on the analytical application of two of the most used families of chemosensor molecules for mercury detection, rhodamines and BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene, difluoroborondipyrromethene) derivatives [9].

It is interesting to note that, as a heavy metal ion, Hg^{2+} coordination with a fluorophore usually induces quenching of fluorescence because of the spin orbit coupling effect. However, in order to avoid Hg^{2+} fluorescence quenching, specific Hg^{2+}-induced reactions have been developed for the design of turn-on Hg^{2+} chemodosimeters. For example, a number of Hg^{2+} chemodosimeters have been designed via desulfuration followed by cyclization to afford the corresponding derivative, with the drawback of being irreversible [9].

Turn-on probes based on the photoinduced electron transfer (PET) mechanism are more attractive, since they normally display instant and reversible Hg^{2+} sensing ability, and examples of this kind of probe have been reported. Classical PET probes for metal cations contain three parts: fluorophore, spacer and receptor. Usually, the spacer between the receptor part and the fluorophore part of the molecule is helpful to remove the emission-quenching effect of Hg^{2+} and, therefore, the Hg^{2+} coordination of the receptor only displays the PET blocking effect, and results in the turn-on response, as it is the case in BODIPY derivatives based chemosensors [9].

The possibilities of the BODIPY core-based fluorophores for the study of biological systems “in vitro” as well as “in vivo”, in technologies that need energy transfer processes, and in the chemical analysis fields, have given rise to structure/properties studies which, at the same time, have motivated the development of new synthetic methodologies [10,11].

In this way, since they were first discovered in 1968 by Treibs and Kreuzer [12]. BODIPY dyes have become very popular in the fluorescent chemosensor field due to their notable properties, starting from the relatively moderate redox potential of the BODIPY core, which is a requisite at the construction of fluorescent probes based on electron transfer processes [13].

The excellent luminescent properties of the BODIPY dyes included their photochemical stability, the relatively high molar absorption coefficients and fluorescence quantum yields, insignificant triplet-state formation, excitation and emission wavelengths in the visible spectral region with high intensity narrow emission peaks, good solubility and resistance toward self-aggregation in solution and fluorescence lifetimes in the nanosecond range [14–16].

Chemosensors based on BODIPY system follow a mechanism in which the fluorophore, i.e. difluoroborondipyrromethene, is connected to the aromatic ring that acts as spacer from the receptor moiety. The aromatic ring includes a substituent in the *para* position that is a strong electron-donor (amino, hydroxyl or ether group, usually) and in the absence of the analyte inhibits fluorescent emission. The receptor system can have other groups with lone pair electrons, and/or suitable coordination geometry that increase selectivity to the target of interest. The benzene ring is not only a spacer, allowing delocalization between receptor donor groups and the fluorophore. When complexation with the analyte is produced, electron transmission to the fluorophore is disconnected, and then it shows its fluorescent properties [9].

Several BODIPY derivatives have been described and applied to the determination of Hg^{2+} in different environmental and biochemical samples. A Hg^{2+} chemosensor containing a thiacalix [4] (*N*-phenylazacrown-5)ether ligand linked by a spacer to a BODIPY core was described by Bitter et al. [17]. A BODIPY derivative with an *o*-aminophenol chelator at the *meso*-position, which acts as an specific fluorescence turn-on chemosensor to Hg^{2+} has been reported [18]. Yuan et al. have reported the synthesis and sensing characteristics of a new class of colorimetric and fluorimetric dual-channel assay to the specific detection of Hg^{2+} in the presence of other cations, that comprises both PET and ICT processes in a single molecule [19]. Du and coworkers have synthesized and characterized a highly selective fluorescent chemosensor with an open-chain azadioxadithia (NO_2S_2) chelator for Hg^{2+} determination [20]. A fluorescent chemosensor which exhibits selective fluorescence towards Hg^{2+} was presented by Fan et al. [21], which is unaffected by the presence of anions existing in environment and organisms. Lu et al. reported a fluorescent turn-on chemosensor able to selectively determine Hg^{2+} not only in aqueous media, but also within living cells [22], and a BODIPY-based fluorescent probe highly selective to Hg^{2+} in methanol [23]. Three fluorescent chemosensor molecules with polyamide receptors were designed and synthesized by Wang et al. two of them having high sensitivity towards Hg^{2+} [24]. The very weak basal fluorescence of the BODIPY-based chemosensors was largely enhanced after the addition of Hg^{2+}, because the reductive PET quenching process is impeded due to the positively cooperative metal-chemosensor complexation. A BODIPY appended thiacrown molecule able to detect Hg^{2+} and Ag^{+} in a H_2O:CH_3CN medium has been also reported. The involved PET mechanism is blocked in presence of Hg^{2+}, which interacts with the sulfur atoms of the thiacrown part [25]. Fan et al. have developed a BODIPY derivative turn-on chemosensor able to sensitively and selectively detect Hg^{2+} even in the presence of cysteine or in sulfur-rich environments, which are known to form stable complexes with Hg^{2+} [26]. A chemosensor capable of selectively detecting Hg^{2+} over competing metal ions was designed by Vedamalai and Wu [27]. It is interesting to note that fluorescent probes for in vivo imaging are highly demanded; therefore, complexes with aqueous solubility are especially attractive.

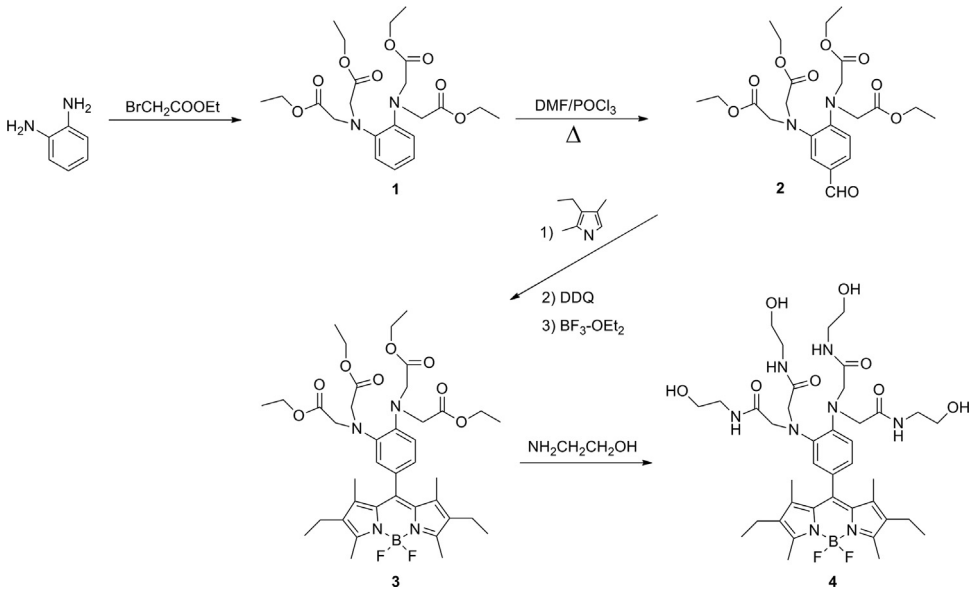
In the present paper, the synthesis of a BODIPY derivative armed with a tetrapod receptor is described. The procedure is based on a previous publication, and the modifications performed to improve the synthesis have been indicated [24]. The characteristics of its selectivity towards Hg^{2+} have been investigated, and the OFF–ON fluorimetric response, based on the PET mechanism, served as the base for the development and application of a method able to determine Hg^{2+} in environmental waters.

2. Experimental

2.1. Synthesis of diaminophenyl BODIPY derivative

A modification of the synthesis of Wang et al. [24] for the preparation of the BODIPY derivative was employed (Scheme 1). The synthesis was performed including alternative procedures with different objectives, as the consecution of a multigram synthesis, improving the low yields of some of the previously proposed procedure steps, simplifying the experimental steps, achieving the desired purity requirements for use with analytical purposes, and enriching the characterization of the implied structures.

Preparation of 1. A suspension of o-phenylenediamine (4.4 g, 40.7 mmol), ethyl bromoacetate (26.8 mL, 241 mmol), sodium iodide (5.1 g, 34 mmol), and diisopropylethylamine (33.3 mL, 200 mmol) in 40 ml of acetonitrile was refluxed in nitrogen atmosphere for 7 h, cooled to room temperature, poured into water (200 mL) and extracted with dichloromethane (3 × 150 mL). The organic layer was dried over sodium sulfate, filtered and concentrated at vacuum until a brown oil that was crystallized and recrystallized from ethanol gives 1 (10.79 g, 58.5%). ^{1}H-RMN ($CDCl_3$): δ (ppm) 7.04 (m, 2-H), 6.94 (m, 2H), 4.30 (s, 8H), 4.10



Scheme 1. Synthesis procedure of chemosensor 4.

(q, 8H, J=7.2 Hz), 1.19 (*t*, 12H, J=7.2 Hz); ^{13}C-RMN ($CDCl_3$): δ (ppm) 171.16, 141.60, 123.29, 121.60, 60.68, 52.59, 14.21 (Additional information on the assignations in Tables S1 and S2).

Preparation of 2. A stirred solution of 1 (1.5 g, 3.3 mmol) in DMF (33 mL) and dry pyridine (6.6 mL) was cooled in an ice bath and phosphorus oxychloride (25 mL, 260 mmol) was gradually added with a compensated pressure funnel, drop by drop during 10 min and then stirred for an hour. After that, the reaction mixture was heated during 40 min at 75 °C, cooled to room temperature, diluted to 150 mL with dichloromethane and poured onto ice-water (200 mL) with sodium carbonate. After careful agitation of the mixture, additional amounts of sodium carbonate were added to complete neutralization of the generated HCl. The aqueous phase was extracted with dichloromethane (3 × 200 mL), the combined organic layers were dried over sodium sulfate, filtered, concentrated at vacuum and crystallized from ethanol to give **2** as a yellow solid (1.11 g, 70%)0.^{1}H-RMN ($CDCl_3$): δ (ppm) 9.80 (s, 1-H), 7.53 (d, 1-H, J_m=2 Hz), 7.45 (dd, 1-H, J_m=2 Hz, J_o=8 Hz), 7.06 (d, 1-H, J_o=8 Hz), 4.38 (s, 4-H), 4.24 (s, 4-H), 4.11 (q, 4-H, J=7.2 Hz), 4.06 (q, 4-H, J=7.2 Hz), 1.17 (t, 12-H, J=7.2 Hz); ^{13}C-RMN ($CDCl_3$): δ (ppm) 191.05, 170.44-170.37, 147.46, 141.15, 131.13, 126.05, 122.50, 120.81, 60.77-60.89, 52.17-52.27, 14.1 (Additional information on the assignations in Tables S3 and S4).

Preparation of 3. To a solution of **2** (1.00 g, 2.0 mmol) and 2,4-dimethyl-3-ethyl-pyrrol (0,60 mL, 4.2 mmol) in 400 mL of absolute dichloromethane under nitrogen atmosphere, four drops of trifluoroacetic acid were gradually added and it was stirred at room temperature during 5 h. Then, a solution of dichlorodicyanobenzoquinone (DDQ, 520 mg) in dichloromethane (40 mL) was added and stirred for 15 min. After that, diisopropylethylamine (12.5 mL, 71 mmol) and 12.5 mL of BF_3-OEt_2 (12,5 mL, 100 mmol) were added and stirring for another 30 min. The mixture was washed with water (300 mL), extracted with dichloromethane (3 × 100 mL), the organic phase was dried over sodium sulfate, filtered and concentrated under vacuum. The crude was purified by flash chromatography (hexane-ethyl acetate 7:3), and 3 crystallized from ethanol (0.897 g, 57%). ^{1}H-RMN ($CDCl_3$): δ (ppm) 7.10 (d, 1-H, J_o= 6.8 Hz), 6.90 (d, 1-H, J_m=1.6 Hz), 6.81 (dd, 1-H, J_o=6.8 Hz, J_m=1.8 Hz), 4.34 (s, 4-H), 4.29 (s, 4-H), 4.10 (q, 4-H, J=5.6 Hz), 4.04 (q, 4-H, J=5.6 Hz), 2.50 (s, 6-H, J=6 Hz), 2.27 (q, 4-H, J=6 Hz), 1.28 (s, 6-H), 1.19 (t, 6-H, J=5.6 Hz), 1.16 (t, 6-H, J=5.6 Hz), 0.95 (t, 6-H, J=6 Hz); ^{13}C-RMN ($CDCl_3$): δ (ppm) 170.35, 153.21, 141.35, 139.77, 138.21, 132.070, 130.55, 129.66, 122.18, 121.34, 120.43, 60.24, 54.93, 52.26, 42.95, 16.65, 14.23, 13.76, 13.73, 12.10, 10.93 (Additional information on the assignations in Tables S5 and S6).

Preparation of 4. 2-aminoethanol (15 mL) was added to a solution of 3 (0.350 mg, 0.45 mmol) in acetonitrile (15 mL), and the reaction mixture was refluxed under nitrogen atmosphere during 3.5 h. After that, it was cooled and concentrated under vacuum to eliminate the acetonitrile. Then, it was added to a NaCl saturated solution (150 mL), the resulting mixture was neutralized with sodium dihydrogen phosphate and extracted with dichloromethane (3 × 150 mL). The organic phase was dried over sodium sulfate, filtered, concentrated under vacuum and crystallized from ethanol/ethyl ether to give 4 (220 mg, 60%). ^{1}H-RMN (DMSO-d_6): δ (ppm) 8.10 (t, 2-H, J=5.2 Hz), 8.04 (t, 2-H, J=5.2 Hz), 6.93 (d, 1-H, J_o=8.4 Hz), 6.73 (d, 1-H, J_m=1.6 Hz), 6.68 (dd, 1-H, J_o=8.4 Hz, J_m=1.6 Hz), 4.60 (t, 2-H, J=5.6 Hz), 4.57 (t, 2-H, J=5.6 Hz), 4.04 (s, 4-H), 4.00 (s, 4-H), 3.20-3.30 (m, 8-H, J=6 Hz), 2.99 (q, 4-H, J=6 Hz), 2.94 (q, 4-H, J=6 Hz), 2.37 (s, 6-H), 2.23 (q, 4-H, J=7.2 Hz), 1.20 (s, 6-H), 0.89 (t, 6-H, J=7.2 Hz). ^{13}C-RMN (DMSO-d6): δ (ppm) 169.26, 152.06, 140.74, 137.98, 131.58, 127.52, 59.13, 54.17, 40.77, 15.85, 14.07, 11.67, 10.36 (Additional information on the assignations in Tables S7 and S8).

2.2. Chemicals and reagents

The stock solution of Hg (II) was prepared by dissolving 0.0514 g of $Hg(NO_3)_2$ in 1.00 L (1.5×10^{-4} mol L^{-1}) of Milli-Q water containing a few drops of concentrated HNO_3, and was further diluted whenever necessary. Ion metal stock solutions were prepared by diluting Li(I), Na(I), K(I), Mg(II), Ca(II), Ba(II), Fe(II), Cu(II), Zn(II), Cd(II), Co(II), Pb(II), Fe(III) and Al(III) standard solutions AAS grade (Merck). Stock solution of BODIPY 2.5×10^{-5} mol L^{-1} was prepared by dissolving 50.0 μg of BODIPY in 250.0 mL of ultrapure water. A 0.5 mol L^{-1} phosphate buffer of pH=7.50 was also used. All other used chemical reagents were of analytical reagent grade. All stock solutions were stored in amber glass bottles at 4 °C. Working standard solutions were prepared daily by proper

dilution with ultrapure water. Ultrapure water was provided by a Milli-Q system (Millipore, Bedford, MA, USA).

Real samples were tap and waste water collected in Faculty of Biochemistry and Biological Sciences, Universidad Nacional del Litoral, Santa Fe city, Argentina.

Special care was taken in the preparation and handling of solutions and containers to minimize any possible risk of Hg(II) contamination. Before using, calibrated flaks were left overnight in 10% (v/v) HNO_3 (analytical reagent grade) and rinsed with ultrapure water to eliminate contamination.

2.3. Reaction characterization

With the purpose of characterizing the reaction between 4 and Hg^{2+}, appropriate aliquots of their stock solutions were added into a 3 mL quartz in order to have concentrations of 2 μmol L^{-1} and 5 μmol L^{-1} of 4 and Hg^{2+}, respectively, after completing to the final volume (3 mL) with phosphate buffer. After homogenization, emission spectra were registered in the spectrofluorimeter at several excitation wavelengths between 500 nm and 525 nm. The same procedure was conducted to record the absorption spectrum in the spectrophotometer. Blank solutions without Hg^{2+} addition were also measured. With the aim of evaluating possible variations associated with the order of addition of reagents, the latter solutions were also prepared assaying several orders of addition of aliquots of 4, Hg^{2+} and phosphate buffer.

Later, stability of both 4 and 4-Hg^{2+} complex was assessed at darkness by registering emission spectra every 15 min during 90 min of three solutions containing 2 μmol L^{-1} of 4, without and with Hg^{2+} (2 μmol L^{-1} and 5 μmol L^{-1}), respectively. Eventually, more spectra were recorded after exposing the solutions at light for short periods of time, in order to gather information about the complex photostability.

The influence of organic solvent in the studied reaction was evaluated by substituting different volumes of phosphate buffer for ethanol in a solution having Hg^{2+} (5 μmol L^{-1}) and 4 (2 μmol L^{-1}) in order to reach percentages of ethanol ranging between 0% and 40%. All solutions were maintained under agitation, and emission spectra were recorded at the previously established optimal conditions every 5 min during 25 min.

With the purpose of analyzing the temperature effect on the reaction, three solutions containing 0, 2 and 5 μmol L^{-1} Hg^{2+} and 2 μmol L^{-1} 4 were prepared. Emission spectra of several portions of each solution were registered in the spectrofluorimeter after reaching temperatures between 10 and 35 °C every 5 °C.

Aqueous solutions of 2 μmol L^{-1} 4 and 5 μmol L^{-1} Hg^{2+} were prepared in order to study the influence of the pH on the analyte recognition. Appropriate aliquots of $HClO_4$ or NaOH solutions were added to the 4 in its free and complex states in order to gather emission spectra at different pH values.

Besides, the influence of Hg^{2+} concentration was evaluated on several solutions containing fixed concentrations of 4 (1.75 μmol L^{-1}), and variable concentrations of Hg^{2+} ranging from 0 to 1.5 mg L^{-1}. Emission spectra were recorded and emission fluorescence was measured at 538 nm (λ_{EX}=515 nm). In order to establish the stoichiometry of the reaction, both Job [28] and Yoe–Jones [29] methods were applied. Regarding the first, emission spectra of several solutions containing variable concentrations between 0 and 4 μmol L^{-1} of both Hg^{2+} and 4 were registered, taking into account that, in all cases, the sum of the contribution had to be equal to 4 μmol L^{-1}. Concerning the Yoe–Jones method, the analyzed solutions were prepared maintaining constant the Hg^{2+} concentration (4 μmol L^{-1}) while varying BODIPY concentrations between 0 and 3 μmol L^{-1}.

An interference study was performed to gather information regarding the reaction between 4 and the following metallic ions: Li^+, Na^+, K^+, Ag^+, Mg^{2+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Pb^{2+}, Fe^{3+} and Al^{3+}. Emission spectra were registered for solutions having 0.3 μmol L^{-1} of each metallic ion and 1 μmol L^{-1} of 4.

In all experiments, after the addition of both Hg^{2+} and 4 aliquots, solutions were maintained under agitation for 5 min at darkness. Then, they were diluted to final volume with phosphate buffer (pH=7.50, 0.5 mol L^{-1}).

2.4. Analytical parameters

Calibration samples were prepared by adding appropriate amounts of Hg^{2+} stock solution, and 120.0 μL of 1.65×10^{-5} μmol L^{-1} 4 stock solution into 2.00 mL volumetric flasks. Seven standards were performed (three replicates) at the following Hg^{2+} concentrations: 0, 20.0, 40.0, 60.0, 80.0, 100.0 and 120.0 ng mL^{-1}, at a constant 4 concentration of 1 μmol L^{-1}. After agitating for 5 min at darkness, 320.0 μL of phosphate buffer (pH=7.50, 0.5 mol L^{-1}) were added to each solution, and they were diluted to the mark with milli-Q water. After homogenization, fluorescence measurements were carried out in the optimal established conditions, using 5 nm of excitation and emission slit widths, exciting at 515 nm, and obtaining fluorescence emission at 538 nm. The photomultiplier tube (PMT) voltage was set at 680 V, and the temperature was fixed at18 °C. Analytical figures of merit were calculated using the ACOC software [30,31].

2.5. Analytical applications

Real samples were prepared by spiking tap and waste water with stock solution of Hg^{2+} to have three different concentration levels: 30, 60 and 90 ng mL^{-1}. Then, the solutions were passed through Whatman filter papers (London, England). Before recording the emission spectra, 1.40 mL of each sample solution and 120.0 μL of 1.65×10^{-5} μmol L^{-1} 4 stock solution were placed into 2.00 mL volumetric flasks. After agitating for 5 min at darkness, 320.0 μL of phosphate buffer (pH=7.50, 0.5 mol L^{-1}) were added to each solution, and they were diluted to the mark with milli-Q water. This procedure was performed in duplicate.

2.6. Instrumentation

UV–vis measurements were performed using a Spectrophotometer Varian Model Cary 50 Bio.

The proposed method was developed in a spectrofluorimeter Varian Cary Eclipse equipped with a xenon lamp, emission and excitation Czerny-Turner monocromators. The spectrofluorimeter was connected by means of a GPIB IEE – 488 card to a PC computer.

^1H and ^{13}C NMR spectra were recorded on a Bruker 400 AC/PC instrument at 400 and 100 MHz, respectively in $CDCl_3$ or DMSO-d_6. Assignments were confirmed by homo- and hetero-nuclear double-resonance, DEPT (distortionless enhancement by polarization transfer). TMS was used as the internal standard (δ=0.00 ppm) and all *J* values are given in Hz.

A Crison MicropH 501 m was used for pH measurements. Microwave digestions were carried out using a CEM MARSx microwave synthesizer.

3. Results and discussion

3.1. Integral characterization of the chemosensor and its complex with Hg^{2+}

3.1.1. Absorbance and fluorescence spectral features

In order to evaluate the spectral behavior of the chemosensor, both the absorption and fluorescence characteristics of 4 in

absence and presence of Hg^{2+} were inspected. Phosphate buffer (pH=7.50) was added to the reaction mixture to guarantee that the lone pair electrons on the nitrogen were available to interact with the metal ion. Besides, the 1:2 stoichiometry was taking into account to conduct the reaction. As expected, a selective response toward Hg^{2+} was produced after complexation with the receptor subunit, with a 40-fold fluorescence increment due to the blocking effect on the PET mechanism (Fig. 1A). The latter response is coincident with that of BODIPY-type fluorophores, i.e. intense emission spectra, and excitation and emission fluorescence bands in the VIS spectral region as well.

Upon photo-excitation of the BODIPY fluorophore, one electron is promoted from the HOMO to its LUMO. Subsequently, one electron from the HOMO of the electron-rich receptor moiety is transferred to the single occupied HOMO of the attached BODIPY fluorophore. As a result, the intrinsic fluorescence of the BODIPY core is inhibited and the probe exhibits a weak fluorescence (i.e. "OFF" state due to the deactivating PET effect) [11].

Upon complexation, Hg^{2+} interacts with the lone-pair electrons on the nitrogen atoms of the receptor moiety preventing the electron delocalization (i.e. the electron-donor character of the receptor subunit is blocked) (Scheme 2). Consequently, the energy of the HOMO of the receptor is diminished so that the electronic transfer from the receptor to the photo-excited fluorophore is slowed down or switched off (i.e. "ON" state due to the suppression of the deactivating PET effect by coordination of the analyte to the probe).

Furthermore, the latter interaction can be clearly visualized inspecting both the absorption spectra of 4 and 4-Hg^{2+} mixtures (Fig. 1B). In the presence of Hg^{2+}, a slightly absorbance decrease is observed, as well as a shift of the maximum absorbance wavelength from 515 nm to 519 nm. The latter effects can be attributed to changes in the energetic levels of the compound due to complexation to Hg^{2+}.

Regarding the experiments related to the order of addition of reagents, the results showed that the signal is highly modified according to the order in which each compound is added to the solution. The optimum signals are achieved when 4 and Hg^{2+} firstly react in absence of phosphate buffer, due to the fact that the reaction is initiated at higher concentrations of both components, and at a slightly more acidic pH, which contributes to stabilize Hg^{2+}. According to that, the order of addition was established as 4, Hg^{2+}, and finally phosphate buffer.

3.1.2. *Stability study*

With the aim of evaluating the stability of the chemosensor in solution, several absorbance spectra of the 4 stock solution were recorded at different times. The latter study indicated that this solution remains stable for at least seven days.

Regarding the stability of the 4-Hg^{2+} complex, the small signal increment during the first 15 min of reaction appreciated in Fig. 2, indicates that the kinetic of the complex formation is relatively slow. The signal remained constant until 50 min were reached, and then started to show a small intensity decrease. As can be seen in Fig. 3, it is mandatory to perform the experiments at darkness because the complex is highly affected by light exposure.

3.1.3. *Influence of organic solvent, and reaction time*

Considering that the presence of organic solvent in the reaction medium may improve the stability of the BODIPY-type complexes,

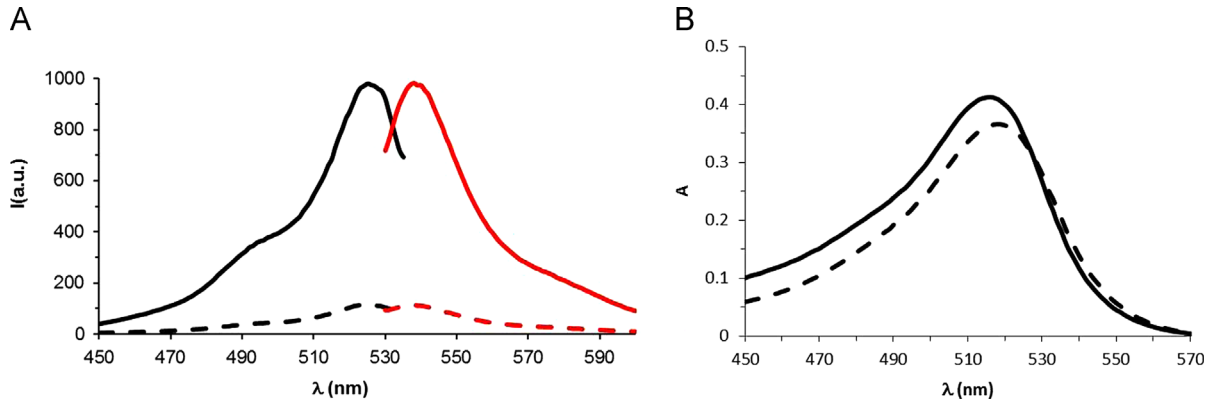
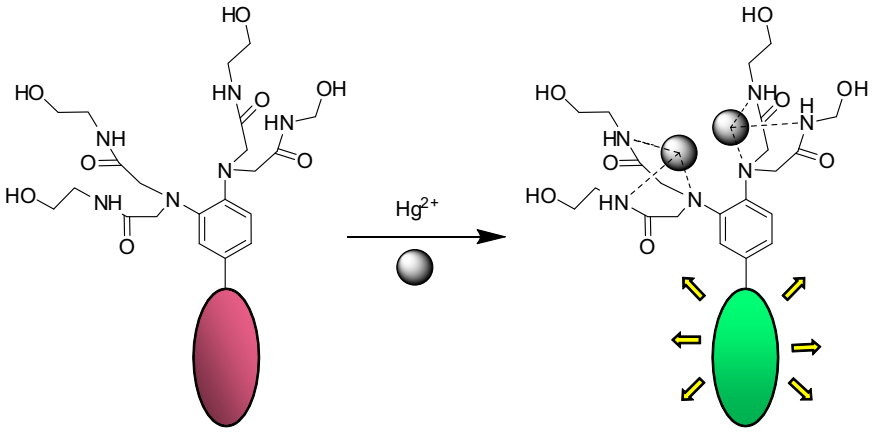


Fig. 1. (A) Excitation (black lines, λ_{EX}=525 nm) and emission (red lines, λ_{EM}=538 nm) fluorescence spectra of 4 (2 μmol L^{-1}) in absence (dashed lines) and presence (solid lines) of Hg^{2+} (5 μmol L^{-1}). (B) Absorbance spectra of 4 (2 μmol L^{-1}) in absence (solid line) and presence (dashed line) of Hg^{2+} (5 μmol L^{-1}).



Scheme 2. OFF–ON fluorescence mechanism of the sensor in the presence of Hg^{2+}.

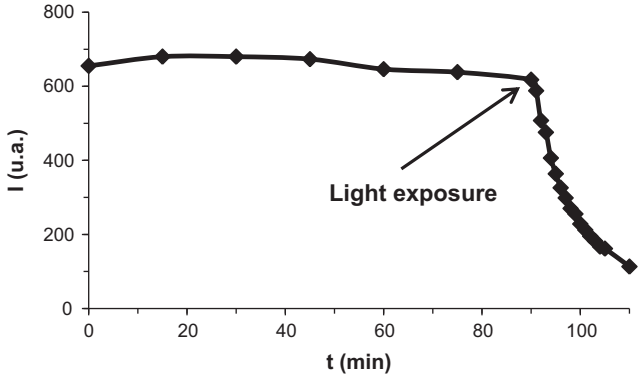


Fig. 2. Stability as a function of time, and light exposure of a solution containing 4 (2 μmol L^{-1}) and Hg^{2+} (5 μmol L^{-1}), λ_{EM}==538 nm, λ_{EX}==515 nm.

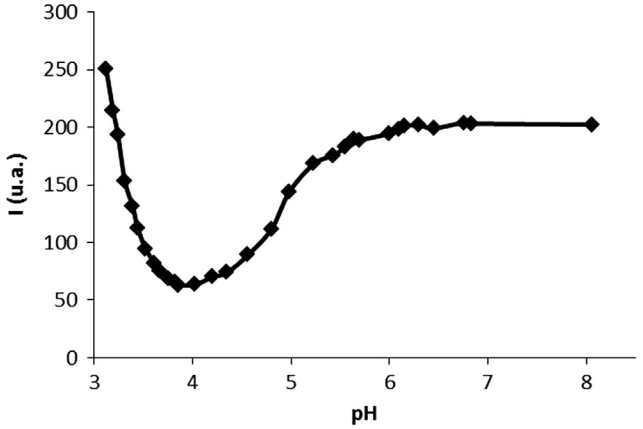


Fig. 3. *Variation* of the fluorescence *intensity* (λ_{EX}=515 nm, λ_{EM}=538 nm) of the 4-Hg^{2+} complex as a function of pH for a solution of 2 μmol L^{-1} 4 and 5 μmol L^{-1} Hg^{2+} in buffer pH=7.50.

the influence of the ethanol concentration on the fluorescent signal was evaluated. Besides, this study was conducted through time with the aim of comparing the results with the experiments performed in the absence of organic solvent (see Section 3.1.2), and to establish the optimum reaction time in which the fluorescent signal should be measured.

Although the addition of ethanol to the reaction mixture remarkably increases the fluorescent signal, i.e. 45% of signal increment in the presence of 40% of ethanol, its inclusion was avoided in order to simplify the analytical procedure, due to the aqueous nature of the samples under analysis. Besides, the lower concentration levels of ethanol produced small signal increments. This decision was also supported by the results gathered in the kinetic study, which showed similar kinetics of the complexation reaction at different percentages of organic solvents, i.e. a minor signal increment during the first 15 min, followed by a constant signal trend for further times (Fig. S9). The reaction time to record the analytical signal was established as 15 min.

3.1.4. Influence of the temperature

The influence of the temperature on the analytical response is an important parameter that should be analyzed in order to gather information related to its influence on the reaction kinetic, as well as likely viscosity variations that can be generated in the reaction media. In our case, the experiments conducted between 10 °C and 35 °C revealed that in the presence of Hg^{2+} the highest signal intensity is obtained at the lowest temperature. Then, the signal suddenly decreased until reaching 30 °C, to remain constant at higher temperatures. This latter phenomenon can be ascribed to the higher number of collisions between molecules associated to the temperature increment, which promotes the deactivation of the complex excited state through mechanisms different than luminescent emission. In absence of Hg^{2+}, the signal stayed invariant as changes in temperature occurred for the whole experiment. We performed an experiment in which the complex was heated to 35 °C and then cooled to 18 °C, to investigate if the decreasing of the signal is due to quenching or photodegradation of the optical probe. When the solution was heated to 35 °C the fluorescence diminished respect to the same solution at 18 °C and, after cooling the solution to 18 °C, the fluorescence is increasing again, which is indicating a temperature quenching effect. Taking into account the significant incidence of this parameter on the reaction, the temperature was set to 18 °C, due to its closeness to ambient temperature, and the satisfactory signal that is obtained at this temperature value (Fig. S10).

3.1.5. Influence of pH

The lone-pair electrons on the nitrogen atoms of the 4 sensor play a crucial role in the coordination with Hg^{2+}. Therefore, pH is an important factor that can significantly affect the recognition of the metallic cation by the sensor.

The pH-dependent fluorescence of the 4-Hg^{2+} complex is shown in Fig. 3. It can be seen that under acidic conditions (i.e. pH=3) the complex shows noticeable fluorescence which diminishes as the pH of the solution approaches 4. When the pH is shifted to more alkaline conditions, the fluorescence intensity increases until a plateau is reached (i.e. I∼200 for pH=6–8).

The pH-dependent fluorescence of the free 4 chemosensor (i.e. without the addition of mercuric ions) is shown in Fig. S11. In this case the maximum fluorescence intensity is reached at very acidic pH values (i.e. I∼30 for pH=0.8). When the solution pH is shifted to more alkaline conditions, the fluorescence gradually diminishes until a plateau is reached at∼2.5.

In order to calculate the pK_a value of the free chemosensor in the excited state, the algebraic method proposed by Wilson and Lester was used [32]. Selected points from the fluorescence-pH curve (Fig. S11) were introduced in Eq. (1):

$$\log R = \mathrm{pH} + \log \frac{I_a - I_m}{I_m - I_b} \quad (1)$$

where: I_a and I_b correspond to the fluorescence intensity at acidic and basic pH, respectively; I_m is the total fluorescence intensity of the mixture of the basic and acidic forms and it lies between I_a and I_b, and R is the fluorescence ratio. The selected points from the fluorescence-pH curves are shown in Fig. S11, and the logarithms of the R values versus the corresponding pH values are depicted in Fig. S12. A pKa value of 1.24 is obtained from pH intercept at log R=0 (Fig. S12).

Taking into account the pH-dependent fluorescence of the chemosensor 4, the use of buffer pH=7.50 was selected in order to register a reasonable fluorescence response of the sensor to Hg^{2+}. It is expected that at this pH value the lone-pair electrons on the nitrogen atoms of the chelator are not protonated and therefore, able to interact with mercuric ions. Upon metal ion complexation (by active participation of the lone-pair electrons) the PET effect is diminished and an enhancement in the emission intensity of the fluorophore is produced.

3.1.6. Influence of Hg^{2+} concentration and stoichiometry study

A preliminary study regarding the influence of Hg^{2+} concentration on the fluorescence signal of the complex showed a typical behavior which corresponds to the formation of complexes (data not shown). At low concentrations of Hg^{2+} the receptor's binding sites are available to interact with the analyte and a linear trend

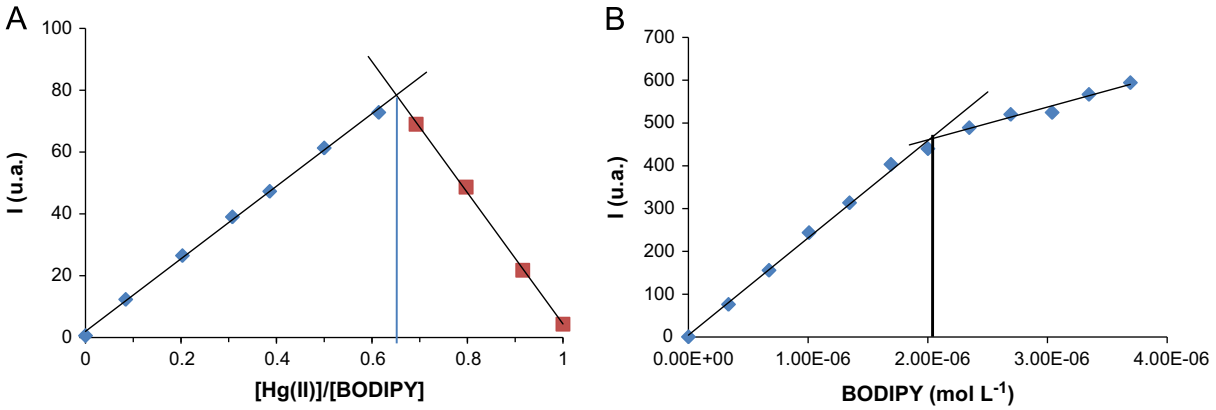


Fig. 4. (A) Job's plot for 4 and Hg^{2+}. It consists of a graphical representation of the fluorescence intensity (λ_{EX}=515 nm, λ_{EM}=538 nm) as a function of the molar fraction. The total concentration was kept constant at a value of 4 μmol L^{-1} (buffer pH==7.50). (B) Yoe–Jones's plot for the chemosensor and Hg^{2+}. It consists of a graphical representation of the fluorescence intensity (λ_{EX}=515 nm, λ_{EM}=538 nm) as a function of the concentration of BODIPY. The concentration of the metal ion was fixed at 4 μmol L^{-1} (buffer pH==7.50).

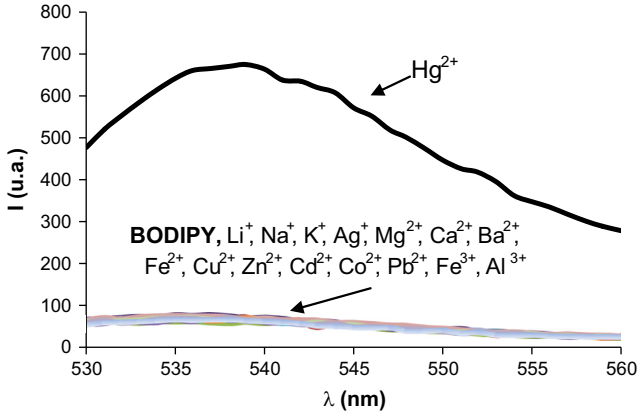


Fig. 5. Study of the BODIPY signal in absence and presence of different metallic cations (λ_{EX}=515 nm).

Table 1
Statistical results for the liner regression of the Hg^{2+} calibration curve.

Statistical parameters[a]	Values
Slope	4.35
Standard deviation on slope	0.06
Intercept	21
Standard deviation on intercept	4
R^b	0.9969
γ^{-1} (ng mL^{-1})	2.3
$LOD_{Long\text{-}Winefordner}$ (ng mL^{-1})	2.7
$LOD_{Clayton}$ (ng mL^{-1})	5.5

[a] Parameters obtained with the analytical software ACOC [30,31].
[b] Correlation coefficient of the linear regression of predicted vs. nominal concentration.

is observed. At high concentrations of Hg^{2+} the recognition process is affected due to the fact that most of the binding sites on the receptor are already interacting with the analyte and therefore, the intensity of the signal is slowed down.

The stoichiometry of the complex formed between 4 and Hg^{2+} was analyzed using the method of Job's plot (Fig. 4A). A maximum fluorescence is observed when the molar fraction of Hg^{2+} is 0.66, which is indicative of a 1:2 stoichiometry for the complex between 4 and Hg^{2+}. Therefore, two mercuric cations that interact with the 4 tetraamide fragments are required to produce a maximum fluorescence signal. The good agreement between the straight lines and the experimental points is indicative of the high stability of the complex.

In addition, the Yoe–Jones analysis was performed (Fig. 4B). In this case, a satisfactory fitting of the experimental points with the straight lines is also observed. The fluorescence increases linearly until a point in which the slope changes. This point allows finding a stoichiometry of 1:2 for the complex 4:Hg^{2+}. According to that, the indicative of a 1:2 coordination pointed in the previous analysis was confirmed.

3.1.7. *Interference study*

The selectivity of the 4 chemosensor towards mercuric ions was assessed by testing the fluorescence response in the presence of various metallic cations under identical experimental conditions (Fig. 5). It can be clearly seen that the interaction between Hg^{2+} and the tetraamide chelator is highly selective, and it yields a noticeable enhancement of the fluorescence intensity ($I \sim 80$ for the free state and $I \sim 700$ for the complex). The other metal ions do not affect significantly the fluorescence signal of the free 4 (i.e. $I < 80$). The two arms to which each electron-donor amine of the receptor is tailored, comprise functional groups with lone-pair electrons and with a particular spatial geometric configuration that is proper to interact selectively with Hg^{2+} upon analyte recognition. The chemical structure of the chemosensor 4 is adequate to allow the determination of Hg^{2+} in aqueous samples (in which a variety of metallic cations are present) in a simple way (i.e. without performing any laborious separation and/or purification steps). In addition, taking into account that inorganic Hg can be transformed via biotic and/or abiotic methylation to monomethylmercury, we evaluated monomethylmercury (II) chloride as an interferent, and we found that the fluorescence of the probe is not affected by the presence of this ion.

3.2. *Figures of merit and analytical applications*

The statistical parameters obtained by least-squares fitting of the calibration curve of Hg^{2+} can be seen in Table 1.

Although the calculated LOD is close to the limit stated by the EPA (2 ng mL^{-1}), it is necessary to point out that in order to apply the chemosensor reported in this work to evaluate Hg^{2+}

contamination in drinking water, a preconcentration step is necessary.

In addition, as one application for Hg^{2+} sensitive probes is the control of contamination in seawater, we evaluated how the ionic strength of the media, at values similar to those found in seawater, affect the analytical signal. For that, a solution containing 1.60 μg ml^{-1} of Hg^{2+} was measured in absence and presence of 16.000 μg ml^{-1} of NaCl and, in the presence of that NaCl concentration, the fluorescence of the complex decreased by about 95% as compared to the probe alone. Similar results were obtained when a higher concentration of NaCl was used. On the other hand, according with the Environmental Protection Agency, the limit of inorganic Hg^{2+} in industrial waste water is more than 50 ng ml^{-1} [33], and the proposed method can be applied for distinguishing the safe levels in this case.

With the aim of testing the applicability of the analytical method under development, water samples found to be free from Hg^{2+} were spiked with this analyte, and a recovery study was performed. Table 2 summarizes the results obtained from duplicate analyses of three different Hg^{2+} levels in two real matrices. The satisfactory analytical results suggest that the proposed method is appropriate for the environmental determination of Hg^{2+} in aqueous samples.

In Table 3, the most important analytical characteristics of some published BODIPY chemosensors for Hg^{2+} detection are summarized and compared with the method developped in the present work. As can be seen, LOD values are only given for four chemosensors (21, 22, 26, 27). The calculated LOD of 5.5 ng ml^{-1}, according with the Clayton criterium, given for the chemosensor used in this work, is higher than the 2 ng ml^{-1} values given for chemosensors named BDP6 and BDP10. With respect to interferences, the selectivity of the developed chemosensor is very high, being similar or higher than the reported selectivities of the chemosensors summarized in Table 3. It is important to note that only in few cases (21, 22, 26, 27), analytical applications have been developed for evaluating Hg^{2+}, in living cells (21, 22, 27) and natural waters (26). For natural waters, the chemosensor reported in this work shows a LOD higher than the reported for the chemosensor BDP10 (26), but in that case, interferences from Ag^{+} and Cu^{2+} are reported.

Table 2
Predicted values for the determination of Hg^{2+} in water samples.

	Hg^{2+}	
	Nominal (ng mL^{-1})	Found (ng mL^{-1})[a]
Tap water[b]	30.0	32.9 (9) [110]
	60.0	59.9 (9) [99]
	90.0	94.8 (3) [105]
Waste water[b]	30.0	25.4 (9) [85]
	60.0	68.3 (4) [114]
	90.0	75.8 (2) [84]

[a] Experimental standard deviation of duplicates, in the last significant figure, in parenthesis. The recoveries are indicated in square brackets.
[b] From Santa Fe City (Santa Fe, Argentina).

4. Conclusions

From the great variety of BODIPY based-chemosensors and rhodamine based-chemodosimeters able to determine Hg^{2+}, only a small portion have been applied to determine this toxic cation in environmental and/or biological samples. The lack of studies on the analytical performance of the latter sensors makes interesting the development of investigations oriented to their possible implementation in new analytical challenges.

The application of BODIPY-type chemosensors to determine Hg^{2+} is based on an OFF–ON fluorimetric response, based on the PET mechanism. The proposed improvements in the synthesis procedure of 4 have given rise to both significant yield enhancement and experimental work simplification. In the presence of Hg^{2+}, 4 experiments a noticeable fluorescence intensity increase, due to the efficient blocking of the deactivating PET mechanism. The formation kinetic of the 4-Hg^{2+} complex is fast, and is highly influenced by the order of addition of reagents. Regarding the complex photostability in aqueous solution, it is mandatory to conduct the experiments at darkness due to its photodegradation. The stoichiometry studies indicated a 1:2 relationship for the 4-Hg^{2+} complex. The high selectivity of 4 towards mercuric ions is considerably influenced by pH, being necessary to conduct the experiments in a pH value higher than 6.

The satisfactory statistical parameters obtained for the calibration curve, i.e. sensitivity toward Hg^{2+} below the environmental limit stated by EPA, allows the application of the proposed methodology to the detection and quantitation of Hg^{2+} in environmental waters.

Table 3
Analytical characteristics of BODIPY chemosensors for Hg^{2+} detection.

Chemosensor*	Reaction medium	λ_{ex} (nm)	λ_{em} (nm)	LOD (ng mL^{-1})	Interferences	Analytical applications	References
BDP1	CH_3CN	490	529	–	Cu^{2+}, Fe^{3+}, Ag^{+}	–	[17]
BDP2	DMSO:HEPES buffer 1:99 (v/v) pH=7.2	483	510	–	–	–	[18]
BDP3	THF:HEPES buffer 30:70 (v/v) pH=7.2	540	578	–	–	–	[19]
BDP4	H_2O:CH_3CH_2OH 7:3 (v/v) pH=7.0	490	512	–	Ag^{+}, Cl^{-}, Br^{-}, CO_3^{2-}, SCN^{-}, $CH_3CO_2^{-}$	–	[20]
BDP5	HEPES buffer pH=7.2	480	507	15	Ag^{+}, Pb^{2+}, Cu^{2+}	Living cells	[21]
BDP6	DMSO:HEPES buffer 1:99 (v/v) pH=7.2	470	509	2	Ag^{+}, Pb^{2+}	Living cells	[22]
BDP7	CH_3OH	483	513	–	–	–	[23]
BDP8a		527	538			–	[24]
BDP8b	Phosphate (0.1 M) solution, pH=7.5	527	541	5.5	–	–	[This work]
BDP9	H_2O:CH_3CN 60:40 (v/v)	498	507	–	Ag^{+}	–	[25]
BDP10	CH_3CH_2OH:HEPES buffer (20 mM HEPES, 100 mM $NaNO_3$, 1:1(v/v), pH=7.0)	495	513	≤ 2	Ag^{+}, Cu^{2+}	Natural waters	[26]
BDP11	CH_3OH	492	520	5.6 x 10^2	Co^{2+}, Fe^{3+}	Living cells	[27]

* The structures of the summarized chemosensors are reported in Ref. [9].

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Appendix A. Supplementary mateial

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.09.009.

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