

NJC

Accepted Manuscript



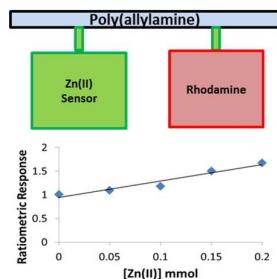
This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

A Ratiometric Fluorescent Hydrogel Sensor for Zinc(II) Based on a Two Fluorophore Approach



A hydrogel sensor has been developed to measure the concentration of Zn(II) upon swelling in aqueous buffer.

A Ratiometric Fluorescent Hydrogel Sensor for Zinc(II) Based on a Two Fluorophore Approach

Graham R.C. Hamilton¹, Lewis Fullerton¹, Bridgeen McCaughan¹, Ryan F. Donnelly² and
John F. Callan^{1*}

1. School of Pharmacy and Pharmaceutical Sciences, The University of Ulster, Northern Ireland, BT52 1SA.; 2. School of Pharmacy, Queens University of Belfast, Northern Ireland.

* To whom correspondence should be addressed: email: j.callan@ulster.ac.uk

Abstract: A polymeric ratiometric fluorescent sensor for Zn(II) has been developed based on a Zn(II) responsive naphthalimide fluorophore (λ_{EM} 535 nm) and a Zn(II) insensitive Rhodamine calibration fluorophore (λ_{EM} 579 nm) both coupled to a common poly(allyl amine) backbone. A concentration dependent increase in the ratiometric response ($I_{535 \text{ nm}} / I_{579 \text{ nm}}$) was observed in the 0 – 25 mM Zn(II) ion range for the sensor in aqueous buffer. The effect of dilution on the ratiometric intensity of the polymeric sensor was also studied and no change in ratiometric response was observed upon dilution to 50% of its original concentration. In contrast, when the polymeric sensor was incorporated within a Gantrez AN-139 hydrogel matrix, a linear ratiometric response was observed upon addition of increasing amounts of Zn(II) to the gel. Therefore, this approach offers the opportunity to determine Zn(II) concentration in environments where sensor concentration may vary dramatically.

Introduction: The development of “smart materials” that respond to changes in their local environment (i.e. pH, ion concentration etc.) or upon exposure to external stimuli (i.e. light or ultrasound) are of considerable interest in biomedical / pharmaceutical research.¹⁻³ Polymeric hydrogels have been widely studied in such materials due to their low cost, relative ease of preparation and biocompatibility.⁴ One such area where polymeric hydrogels are beginning to attract attention is in the development of Microneedles (MNs).⁵ MNs are minimally-invasive devices that, upon skin insertion painlessly by-pass the stratum corneum barrier.⁶ While MNs have mainly been studied as drug delivery vehicles⁷ they have also been investigated for their potential in blood diagnostics. The novelty of this concept lies in the fact that if drugs can diffuse from MN swollen by moisture from the viable skin, then drugs and diagnostic markers in skin interstitial fluid could also be extracted for analysis.

Our research is focussed on developing polymeric hydrogel MNs with fluorescence sensors incorporated in the back-plate as a minimally invasive method to determine the concentration of important blood analytes such as sodium, potassium and glucose in interstitial fluid. These sensors, designed according to the Photoinduced Electron Transfer (PET) mechanism, switch their fluorescence from “Off” to “On” upon binding a target analyte.⁸ However, two main challenges must be overcome to permit the use of such sensors in MNs or other hydrogel materials. The first is to ensure the sensor remains within the MNs during the swelling process. The second is to compensate for the sensors dilution during the swelling process as the fluorescence intensity observed not only depends on the level of analyte bound but also the sensor concentration. Our approach to overcoming these obstacles are: (i) chemically attaching the sensor to a high molecular weight polymer that due to its size will be unable to escape through the pores of the swollen hydrogel and (ii) utilise ratiometry where the intensity of the sensor will be compared to that of an analyte insensitive calibration

fluorophore. The resulting ratiometric polymeric sensor would then be incorporated within the hydrogel matrix.

In this manuscript, we describe the synthesis and photophysical evaluation of a ratiometric polymeric sensor for zinc. Zinc is an essential mineral that plays a key role in proper immune system function.⁹⁻¹¹ It also plays a critical role in the wound healing process¹² and supports normal growth and development during pregnancy, childhood, and adolescence.^{12,13} Therefore, the ability to measure Zn(II) concentration accurately is of extreme importance. To this end, we have prepared the Zn(II) specific fluorescent sensor **4** and grafted it onto a poly(allyl amine) backbone along with a Rhodamine calibration fluorophore resulting in ter-polymer **6**. We determine the ability of **6** to accurately measure Zn(II) concentrations in aqueous buffer and illustrate its potential to overcome the problem of sensor dilution. Finally, we incorporate **4** within a Gantrez AN-139 hydrogel matrix and determine its ability to respond to different levels of Zn(II) ion through changes in fluorescence intensity.

Results and Discussion: Sensor **4** was prepared in four steps from commercially available precursors following a convergent synthesis (Scheme 1). Briefly, intermediate **1** was first prepared by a condensation reaction between 4-bromo-1, 8-naphthalic anhydride and 6-aminohexanoic acid.¹⁴ In parallel, intermediate **3** was formed by first reacting 2-pyridine carboxaldehyde with N-(2-aminoethyl) acetamide in a reductive amination reaction followed by hydrolysis of the acetyl protecting group under acidic conditions.¹⁵ Intermediates **1** and **3** were then combined together in an *N*-substitution reaction to produce sensor **4** which was obtained as a pure yellow solid after chromatographic purification.

The fluorescence spectrum of **4**, recorded in HEPES buffer solution ($\text{pH} = 7.4 \pm 0.1$) displayed a broad emission band centred at 530 nm when excited at 440 nm, consistent with

emission from the naphthalimide fluorophore.¹⁶⁻¹⁸ **4** possesses a Zn (II) selective dipicolylamine receptor coupled to a naphthalimide fluorophore via an ethyl spacer in a PET format. The dipicolylamine receptor co-ordinates Zn(II) between its two pyridine nitrogen atoms and the tertiary amino nitrogen atom.¹⁹ This chelation of the tertiary amine lone pair increases its oxidation potential cancelling the thermodynamic driving force for PET that otherwise occurs in the unbound state.²⁰ To ensure this amine lone pair remains unprotonated and free to bind Zn(II) ions at physiological pH, a pH-fluorescence titration was performed. The results are shown Figure 1 and reveal the fluorescence intensity of **4** increasing with decreasing pH due to the lone pair becoming protonated and cancelling the PET process. When relative fluorescence intensity was plotted as a function of pH a sigmoidal curve was observed with the fluorescence remaining low until pH 7.0 and then increasing until pH 5 where it leveled out (Figure 1b). Using a plot of $-\log (F_{\text{MAX}} - F) / (F - F_{\text{MIN}})$ against pH (where F_{MAX} is the maximum fluorescence intensity, F_{MIN} the minimum fluorescence intensity and F the measured fluorescence intensity) the pKa of the tertiary amine was calculated as 6.24.²¹ This pKa value ensures the tertiary amine of the receptor will be less than 10% bound with protons at physiological pH and free to bind Zn(II) ions.

To determine the effectiveness of **4** to bind Zn(II) ions at physiological pH, we prepared solutions of **4** (5 μM) with varying amounts of ZnCl_2 up to a concentration of 1.8 mM in a 9:1 HEPES (0.1 M, pH 7.4 ± 0.1):MeOH solvent system, and recorded the fluorescence spectrum at each concentration. As shown in Figure 2a, a concentration dependant increase in intensity was observed upon increasing Zn(II) concentration. Indeed, good linearity was observed in the 0.30 mM – 1.50 mM range (Figure 2b). Given the binding stoichiometry between dipicolylamine and Zn(II) is known to be 1:1,²² the binding constant $\log\beta$ was calculated from the equation $\log(F_{\text{MAX}} - F) / (F - F_{\text{MIN}}) = \log[\text{Zn(II)}] + \log\beta$ and found to be 3.031 ± 0.0029 .²¹ The quantum yield (Φ_F) of **4**, calculated with reference to Rhodamine B,

was 0.068 in the absence of Zn(II) increasing to 0.24 in the presence of 1.80 mM Zn(II) with the limit of detection (LOD) determined as 18.8 μM .²³

To generate a ratiometric polymeric sensor for Zn(II) based on the fluorescence properties of **4**, rhodamine isothiocyanate was first grafted onto a 15 kDa poly(allyl amine) through a facile thiourea bond to form co-polymer **5** (Scheme 2). Subsequently, **4** was attached to co-polymer **5** using standard carbodiimide chemistry to form ter-polymer **6** which was purified by extensive dialysis to remove unreacted monomers. The loading ratio for rhodamine and **4** was purposely kept low (<10%) to avoid any possible energy transfer occurring between the two fluorophores. The stacked ¹H NMR spectra of poly(allyl amine), **4** and **6** are shown in Figure S1. The resonance's in the upfield region of **6** (0.75 – 1.80 ppm) are broader than those observed in the same region for **4**, and most likely reflect both the protons in or near the poly(allyl amine) backbone and several methylene protons from the aliphatic chain of **4**. Peak broadening is a common feature associated with the ¹H NMR chemical shift of protons in or near the backbone of polymers and results from an ineffective averaging of their chemical shift anisotropies.²⁴ The ¹H NMR spectrum of **6** also displays peaks in the downfield region (6.60 – 8.80 ppm) representing the aromatic protons of the naphthalimide and rhodamine units that appear much sharper due to their distance from the polymer backbone.

The fluorescence spectrum of **6**, recorded in HEPES buffer solution (pH = 7.4 \pm 0.1), displayed characteristic naphthalimide emission centred at 535 nm when excited at 440 nm and characteristic rhodamine emission centred at 579 nm when excited at 555 nm (Figure 3a). While the dipicolylamine moiety has proven selectivity for Zn(II),²² it has been shown that immobilisation of sensor molecules on a polymeric backbone can alter selectivity through a co-operative binding effect.²⁵ Therefore, the selectivity of **6** was tested against a range of physiological and environmentally important cations and the ratiometric response (I_{535}/I_{579})

plotted for each metal ion. As shown in figure S2, a significant enhancement was observed for Zn(II) with only minor effects for the other metal ions illustrating excellent selectivity for Zn(II). To establish the range over which this ratiometric response for Zn(II) was linear, solutions containing a fixed concentration of **6** and increasing amounts of zinc chloride were prepared. While a Zn (II) concentration dependent increase in the naphthalimide intensity was observed at 535 nm, the intensity of the Rhodamine emission at 579 nm remained relatively constant (Figure 3a). A plot of ratiometric intensity (I_{535}/I_{579}) against Zn(II) concentration is shown in Figure 3b and shows good linearity in the 0-25 mM range with the LOD determined as 0.64 mM²³. The ability of **6** to measure Zn(II) over a significantly wider range than observed for **4**, is attributed to the ratiometric detection adopted for **6** compared to single wavelength detection for **4** which helps to compensate for factors such as photobleaching etc.

For fluorescent sensors to operate effectively in environments where their concentration may vary significantly, it is important that the sensing construct can account for such variations and still accurately determine analyte concentration. In this context, we are interested in incorporating such sensors in swellable polymeric hydrogels that can swell up to twice their own weight upon absorption of water.²⁶ To determine if **6** is suitable to operate in such environments we prepared a solution of **6** (containing the equivalent of 23.6 μ M **4** and 2.24 μ M rhodamine) and performed serial dilutions to 50% of the original concentration using distilled water. According to the equation, $I_F = 2.3I_0 \epsilon c l \Phi$, where I_F is emission intensity, I_0 is excitation intensity, ϵ is the extinction co-efficient, c is the concentration, l is the path length and Φ is the quantum yield of fluorescence, the intensity of fluorescence is directly proportional to the concentration of the fluorophore, and, consequently, one would expect the intensity of both the naphthalimide and rhodamine components to decrease upon dilution as observed in Figure 4a. However, when the ratiometric change was plotted as a

function of dilution the ratiometric intensity remained relatively constant (Figure 4b) at a value of 1. Therefore, in the absence of Zn(II) the intensity of both fluorophores reduce in relative terms by the same amount leading to a constant ratiometric value over this dilution range.

To determine if polymeric hydrogel films containing **6** could respond to varying Zn(II) concentration, **6** (containing the equivalent of 23.6 μM of **4** and 2.24 μM rhodamine) was incorporated in a Gantrez AN-139 hydrogel matrix. 1 cm^2 films approximately 1 mm thick in diameter were bathed in solutions containing varying amounts of Zn(II). Fluorescence spectra of the films were recorded using a fibre optic probe. Upon incorporation of the polymeric sensor into the Gantrez films, a slight change in the λ_{MAX} of both fluorophores was observed: 510 nm for naphthalimide and 578 nm for rhodamine. As shown in figure 5a, the intensity from both the naphthalimide and rhodamine units show an overall reduction with increasing Zn(II) addition. However, the ratiometric intensity (I_{510}/I_{578}) increased with increasing Zn(II) addition. This can be explained as follows: while both fluorophores experience an “intensity reduction effect” due to dilution, the naphthalimide component also experiences a “PET intensity enhancement effect” due to more Zn(II) binding switching fluorescence “On”. Therefore, while it is observed that the absolute intensity of both fluorophores reduces, the naphthalimide intensity does not decrease as much as the rhodamine. The net result is an increase in the ratiometric intensity upon increasing Zn(II) addition as shown in Figure 5b. This is in contrast to the behaviour upon dilution in the absence of Zn(II) where the ratiometric response remained constant (Figure 4b). The LOD for **6** in this gel matrix was determined as 0.016 mM.²³

In conclusion, we have developed a ratiometric polymeric sensor for Zn(II) with good selectivity and capable of measuring the concentration of this ion in the 0-25 mM range in aqueous buffer (9:1 H₂O:MeOH). Furthermore, the ratiometric change between the intensity

of the calibration fluorophore and that of the Zn(II) sensitive naphthalimide fluorophore was shown to remain constant when diluted to 50 % of its original concentration. However, when incorporated within a swellable hydrogel matrix, the sensor displayed an increasing ratiometric response upon Zn(II) addition. To the best of our knowledge, this is the first example of a ratiometric fluorescence sensor capable of operating in conditions of high dilution.

Experimental

Materials and methods:

All chemicals were purchased from Sigma-Aldrich (Gillingham, UK) except for 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide and triethylamine which were purchased from Fischer Scientific (Leicestershire, England) and used without further purification. All solvents were also purchased from Sigma-Aldrich (Gillingham, UK), while deuterated solvents were obtained from Cambridge Isotope Laboratories Inc. (Andover, USA). In pH studies hydrochloric acid and sodium hydroxide were used to lower and increase pH respectively. Dialysis cassettes were purchased from Sigma-Aldrich (Gillingham, UK) and used as received. NMR spectra were obtained on Varian 500 MHz instrument at $25.0 \pm 1^\circ\text{C}$ and processed using Bruker software. Mass spectra were obtained on Finnigan LCQ-MS apparatus. Absorption spectra were obtained using a Varian Cary UV-Vis spectrophotometer and fluorescence spectra using a Varian Cary Eclipse fluorescence spectrophotometer and the Varian Cary Fibre Optic Coupler. pH was measured by a pH meter (Hanna Instruments).

Synthesis of Compounds 1-6

6-(6-bromo-1,3-dioxo-benzo[de]isoquinolin-2-yl)hexanoic acid (1):

4-bromo-1, 8-naphthalic anhydride (1.5 g, 5.4 mmol) and 6-aminohexanoic acid (0.72 g, 5.45 mmol) were refluxed in ethanol (80 mL) for 18 hours. Upon cooling crystals formed, which were filtered under vacuum, giving 6-(6-bromo-1,3-dioxo-benzo[de]isoquinolin-2-yl)hexanoic acid (**1**) as a yellow solid (1.58 g, 72 % yield). mp: 161 °C. ¹H NMR: (500 MHz, CDCl₃) δ (ppm): 8.68 (1H, Ar-H), 8.60 (1H, Ar-H), 8.42 (1H, Ar-H), 8.05 (1H, Ar-H), 7.87 (1H, Ar-H), 4.20 (2H, N-CH₂), 2.41 (2H, -CH₂-), 1.78 (2x 2H, -CH₂-), 1.05 (2H, -CH₂-). ¹³C NMR (125 MHz, CDCl₃): 179.29, 163.55, 133.22, 132.04, 131.22, 131.07, 130.59, 130.22, 128.96, 128.05, 123.05, 122.19, 40.23, 37.77, 27.63, 26.47, 24.32. -ve ESI MS: Expected: 389 Da, Obtained: 388 Da. IR: V_{max} (cm⁻¹): 3090, 2960, 2870, 1700, 1660.

N-isopropenyl-N',N'-bis(2-pyridylmethyl)ethane-1,2-diamine (2):

2-pyridine carboxaldehyde (0.95 mL, 10 mmol), N-(2-aminoethyl) acetamide (0.31 mL, 5 mmol) and sodium triacetoxyborohydride (3.03 g, 143 mmol) were stirred in 1,2-dichloroethane (20 mL) for 3 hours under N₂. 2 M sodium hydroxide (40 mL) was added to quench the reaction. The organic layer was retained and the aqueous layer was separated and extracted with dichloromethane (2 x 80 mL). The organic layers were combined, washed with brine, separated and dried with anhydrous sodium sulphate. The mixture was filtered and the organic layer was evaporated to dryness, giving N-isopropenyl-N',N'-bis(2-pyridylmethyl)ethane-1,2-diamine (**2**). ¹H NMR: (500 MHz, CDCl₃) δ (ppm): 8.59 (2H, Ar-H), 7.64 (2H, Ar-H), 7.62 (1H, C-NH-C=O), 7.37 (2H, Ar-H), 7.20 (2H, Ar-H), 3.91 (4H, N-CH₂-Ar), 3.38 (2H, N-CH₂), 2.77 (2H, N-CH₂), 2.04 (3H, O-CH₃). ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 170.02, 159.12, 149.07, 136.57, 123.19, 122.15, 59.89, 52.50, 37.59, 23.19. -ve ESI MS: expected: 389 Da, obtained: 388 Da. IR: V_{max} (cm⁻¹): 3280, 3060, 2930, 2850, 1655, 1590, 1570.

N',N'-bis(2-pyridylmethyl)ethane-1,2-diamine (3):

2 was dissolved in 5 M hydrochloric acid (100 mL) and refluxed for 24 hours. The solution was cooled and the pH of this was adjusted to 10 using NaOH. The solution was then extracted with DCM (3 x 150 mL), the organic layers combined and dried with sodium sulphate. This solution was filtered and evaporated to dryness, forming N',N'-bis(2-pyridylmethyl)ethane-1,2-diamine (**3**) (0.8 g, 67 % yield). ¹H NMR: (500 MHz, CDCl₃) δ (ppm): 8.46 (2H, Ar-H), 7.58 (2H, Ar-H), 7.40 (2H, Ar-H), 7.05 (2H, Ar-H), 3.80 (2x 2H, -N-CH₂-Ar), 3.05 (2H, NH₂), 2.80 (2H, N-CH₂). ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 159.59 149.01, 136.31, 122.90, 121.92, 60.73, 57.30, 39.60. +ve ESI MS: expected: 242 Da, obtained: 243 Da. IR: V_{max} (cm⁻¹): 3280, 3050, 3010, 2930, 2820, 1590, 1570.

6-[6-[2-[bis(2-pyridylmethyl)amino]ethylamino]-1,3-dioxo-benzo[de]isoquinolin-2-yl]hexanoic acid (4):

1 (0.11 g, 0.28 mmol), **3** (0.25 g, 1 mmol) and triethylamine (1 mL) were refluxed in 2-methoxyethanol (15 mL) for 30 hours under N₂. The solution was evaporated to dryness and the resulting oil purified using column chromatography with silica gel (DCM:MeOH 5:1 increasing to 3:1), giving 6-[6-[2-[bis(2-pyridylmethyl)amino]ethylamino]-1,3-dioxo-benzo[de]isoquinolin-2-yl]hexanoic acid (**4**), a yellow solid (80 mg, 15 % yield). mp: 157-158 °C. ¹H NMR: (500 MHz, CDCl₃) δ (ppm): 8.68 (1H, Ar-H), 8.49 (2H, Ar-H), 8.43 (1H, Ar-H), 8.18 (1H, Ar-H), 7.82 (1H, Ar-H), 7.71 (1H, Ar-H), 7.62 (2H, Ar-H), 7.50 (2H, Ar-H), 7.20 (2H, Ar-H), 6.65 (1H, Ar-NH-C), 4.00 (2H, -N-CH₂), 3.89 (4H, -NH₂-N-), 3.52 (2H, N-CH₂), 2.81 (2H, -N-CH₂), 2.20 (2H, -CH₂-), 1.54 (2H, -CH₂-), 1.30 (2H, -CH₂-), 1.22 (2H, -CH₂-). ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 176.94, 165.00, 164.44 158.68, 150.55, 149.09, 136.76, 135.00, 131.11, 130.28, 127.78, 124.45, 123.38, 123.06, 122.40, 120.48, 109.45, 104.17, 59.63, 51.12, 41.12, 40.01, 33.90, 27.821, 26.47, 24.59. (%): HRMS (+ve

m/z) Expected: 551.2533 Da, Obtained: 552.2255 Da. IR: V_{\max} (cm^{-1}): 3300, 2950, 2850, 1680, 1640, 1390.

Free-basing poly(allylamine) hydrochloride:

Poly(allylamine) hydrochloride (1 g, 17.5 mmol) was dissolved in distilled water (40 mL) and the pH adjusted to 12. The solution was dialysed against water using dialysis cassettes (exclusion weight: 700 Da) for 24 hours, with 3 water changes. Remaining solution in the cassettes was evaporated to dryness, forming 600 mg of poly(allylamine).

Grafting of Rhodamine B isothiocyanate to poly(allylamine) to form 5:

Poly(allylamine) (238 mg, 4.7 mmol) was dissolved in hot ethanol (50 mL) and rhodamine B isothiocyanate (100 mg, 0.19 mmol) was added. The solution was stirred for 18 hours. The solution was purified by dialysis against 50:50 MeOH: H₂O for 36 hours, with 3 solvent changes. The solution was then freeze-dried, with co-polymer **5**, formed as a dark-red solid.

Grafting of 4 onto to co-polymer 5 to form ter-polymer 6:

4 (50 mg, 0.088 mmol), N-Hydroxysuccinimide (NHS) (50 mg, 0.44 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide (EDC) (84 mg, 0.44 mmol) were dissolved in DMF:PBS:MeOH (35:10:5) (50 mL) and stirred for 15 minutes. To this **5** (50 mg, 0.88 mmol) was added in MeOH:PBS (1:1) (30 mL), and the contents stirred for 18 hours under N₂. The solution was purified extensively by dialysis against 80:20 H₂O: MeOH solution (24 hours, 3 solution changes). The solution was evaporated to dryness, giving ter-polymer **6**, a clear, red solid.

Preparation of the 6 loaded Gantrez Hydrogel.

5.5 mL of a saturated solution (MeOH:H₂O) of **6** was stirred with 5.5 mL of a pre-formed 7.5 % PEG (mw:10,000 Da), 15 % Gantrez AN-139, 3 % sodium carbonate, 74.5 % H₂O solution until completely homogenous. The contents were then centrifuged for 10 minutes at 3500 RPM and subsequently cast into moulds. The moulds were then allowed to dry at 25 °C for 48 hours and cured at 80 °C for 24 hours. The films were then removed from their moulds and used in the sensing experiments.

Photophysical Experiments

Quantum Yields

Quantum yield values of **4** were determined in ethanol using a Rhodamine B standard at low [Zn(II)] = 0 mM and high [Zn(II)] = 1.80 mM.

Fluorescence-pH titration for 4.

A 5 µM solution of **4** was made prepared in 200 mL of H₂O:MeOH (9:1) solution. The solutions pH was adjusted from acidic (pH 3.0) to basic (pH 12.0) using 5 M HCl and 5 M NaOH ensuring volume addition was as low as possible to avoid dilution. The fluorescence spectra were recorded approximately every 0.5 pH units.

Fluorescence – Zn(II) titration for 4 and Terpolymer 6.

A 5 μM solution of **4** was prepared in 200 mL of a 0.1 M HEPES and 0.05 M KCl buffered (pH 7.4):MeOH (9:1) solution. The Zn(II) concentration was adjusted from 0 to 1.8 mM by the addition of 20 μL aliquots of 1 M of zinc chloride and the fluorescence spectrum recorded after each addition using an excitation wavelength of 450 nm. A similar titration was performed for **6** (comprising 23.6 μM sensor **4** and 2.24 μM rhodamine B) where the concentration of Zn(II) was adjusted from 0 to 25 mM using 100 μL aliquots. The fluorescence spectra were recorded after each aliquot using excitation wavelengths of 450 nm and 555 nm.

*Ratiometric response of **6** upon dilution.*

A saturated solution of **6** was made up in a H_2O :MeOH (9:1) buffered solution (0.1 M HEPES, 0.05 M KCl, pH 7.4). Fluorescence spectra of the solution were recorded upon excitation at 450 nm and 555 nm. The solution was then diluted with the buffer solution until 50 % of its original concentration was reached with the fluorescence spectra recorded at specific intervals.

*Ratiometric response of Hydrogel containing **6** upon increasing Zn(II) addition.*

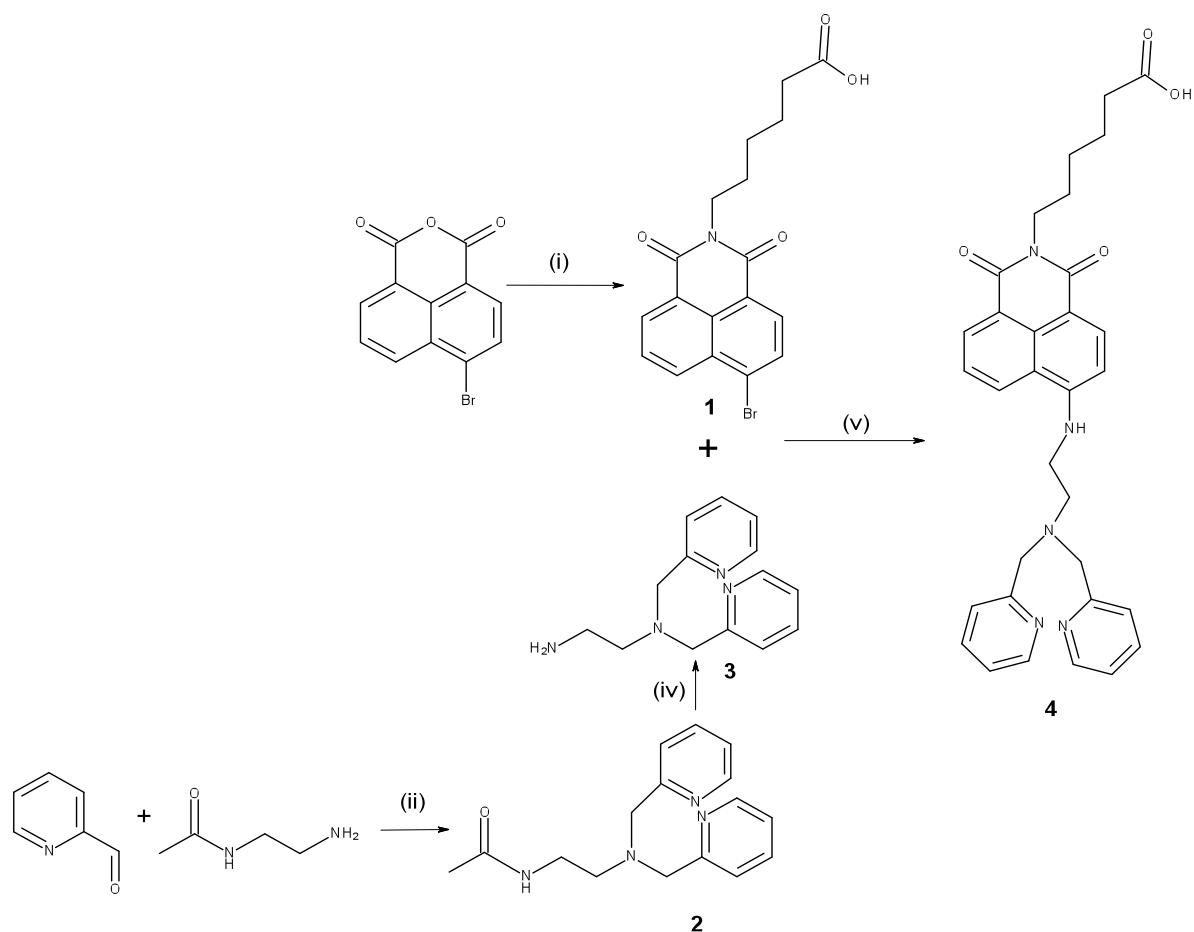
The fluorescence spectra of a 1 cm x 1 cm x 0.1 cm disc of hydrogel were first recorded upon excitation at 450 nm and 555 nm. 10 μL of 0.5 M zinc chloride solution was then added to the gel and allowed to fully absorb. The fluorescence spectra were again recorded upon excitation at 450 nm and 555 nm. This process was repeated until a total of 40 μL of Zn(II) was added in 10 μL aliquots with the fluorescence spectra recorded after each aliquot was added.

References

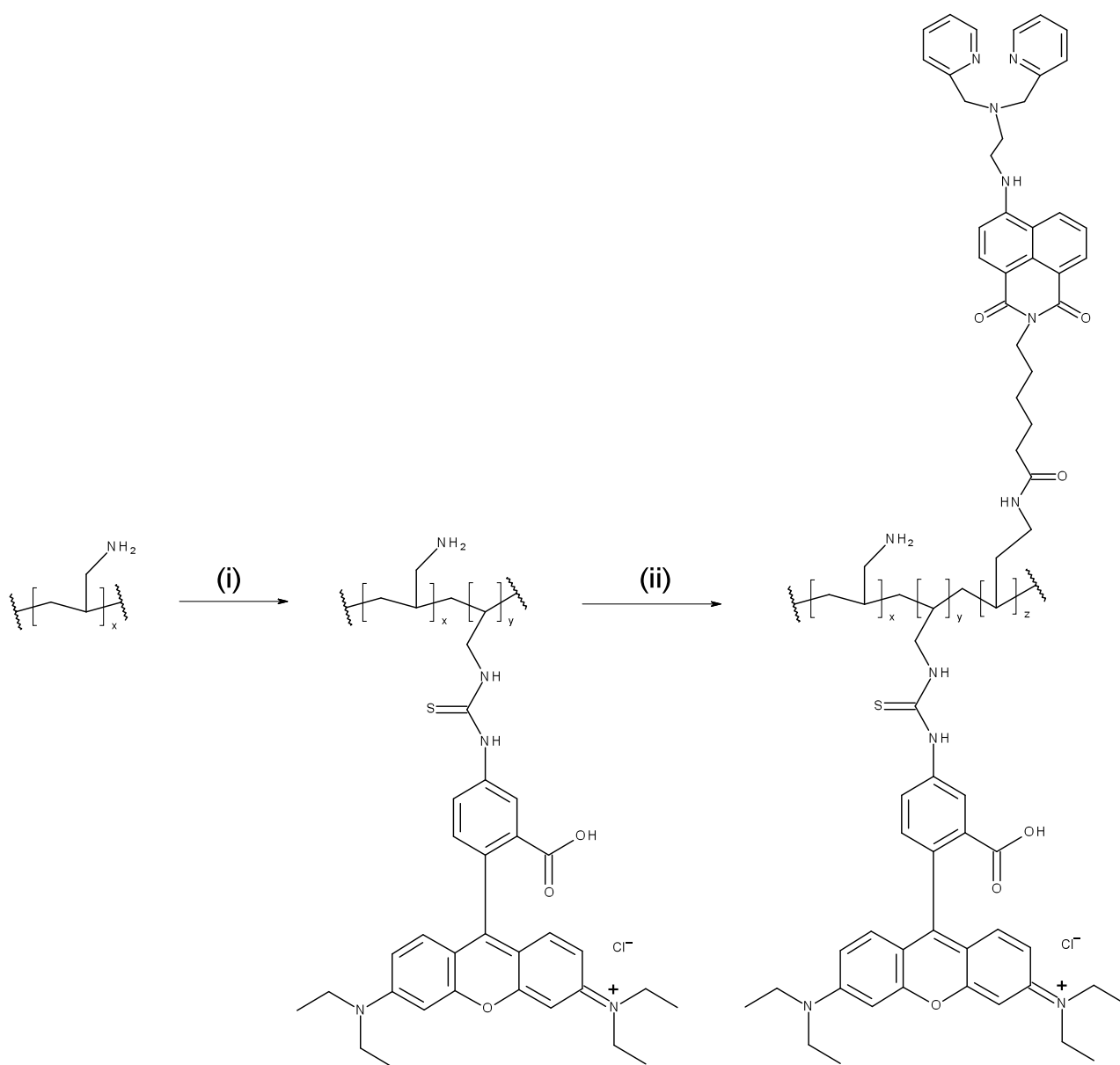
1. K. Iseda, K. Kokado, K. Suda, *React. Funct. Polym.*, 2013, **73**, 961.
2. E. M. White, J. Yatvin, I. Grubbs, J. A. Bilbrey, J. Locklin, *J. Polym. S. Pol. Phys.*, 2013, **51**, 1084.
3. Z. Zhu, C. Wu, H. Liu, Y. Zou, X. Zhang, H. Kang, C. J. Yang, W. Tan, *Angew. Chem. Int. Ed.*, 2010, **122**, 1070.
4. S. Aoyagi, H. Izumi, Y. Isono, M. Fukuda, H. Ogawa, *Sensor. Actuat. A-Phys*, 2007, **139**, 293.
5. D. V. McAllister, P. M. Wang, S. P. Davis, J-H. Park, P. J. Cantella, M. G. Allen, M. R. Prausnitz, *P. Natl. Acad. Sci. USA.*, 2003, **24**, 13755.
6. S. Kaushik, A. Hord, D. Denson, D. McAllister, S. Smitra, M. Allen, M. R. Prausnitz, *Anesth. Analg.*, 2001, **92**, 502.
7. R. F. Donnelly, T. R. R. Singh, A. D. Woolfson, *Drug Deliv*, 2010, **17**, 187.
8. J. F. Callan, A. P. de Silva, D. C. Magri, *Tetrahedron*, 2005, **61**, 8551.
9. P. J. Fraker, L. E. King, *Annu. Rev. Nutr.*, 2004, **24**, 277.
10. K. M. Dean, Y. Qin, A. E. Palmer, *Biochim. Biophys. Acta*, 2012, 1406.
11. C. J. Frederickson, J-Y Koh, A. I. Bush, *Nat. Rev. Neurosci.*, 2005, 1.
12. B. L. Vallee, K. H. Falchuk, *Physiol. Rev.*, 1993, **73**, 79.
13. J. E. Coleman, *Annu. Rev. Biochem.*, 1992, **61**, 987.
14. S. Trupp, P. Hoffmann, T. Henkel, G. J. Mohr. *Org. Biomol. Chem.*, 2008, **6**, 4319-4322.
15. M. Schatz, M. Leibold, S. P. Foxon, M. Weitzer, F. W. Heinemann, F. Hampel, O. Walter, S. Schindler. *Dalton Trans.*, 2003, **8**, 1480-1487.

16. N. I. Georgiev, I. S. Yaneva, A. R. Surleva, A. M. Asiri, V. B. Bojinov, 2013. *Sens. Actuator B-Chem.*, 2013, **184**, 54.
17. N. I. Georgiev, V. B. Bojinov, P. S. Nikolov, *Dyes Pigments*, 2011, **88**, 350.
18. L. Y. Zhao, Q. L. Mi, G. K. Wang, J. H. Chen, J. F. Zhang, Q. H. Zhao, Y. Zhou, *Tetrahedron Lett.*, 2013, **54**, 3353.
19. H. G. Lee, J. H. Lee, P. S. Jang, H. M. Park, S-J. Kim, Y. Kim, C. Kim, R. G. Harrison, *Tetrahedron*, 2011, **67**, 8073.
20. A. de Silva, H. Q. N. Gunaratne T. Gunnlaugsson, A. J. M. Huxley, C. P. McCoy, J. T. Rademacher, T. E. Rice, *Chem. Rev.*, 1997, **97**, 1515.
21. S. Kamila, J. F. Callan, R. C. Mulrooney, M. Middleton, *Tetrahedron Lett.*, 2007, **48**, 7756.
22. T. Gunnlaugsson, T. C. Lee, R. Parkesh, *Org. Biomol. Chem.*, 2003, **1**, 3265.
23. A. Shrivastava, V. B. Gupta, *Chron. Young. Sci.*, 2011, **1**, 2, 21-25.
24. N. Singh, N. Kaur, J. Dunn, R. Behan, R.C. Mulrooney, J. F. Callan, *Eur. Polym. J.*, 2009, **45**, 272.
25. N. Singh, N. Kaur, C. Ni Choitir, J. F. Callan, *Tetrahedron Lett.*, 2009, **50**, 4201.
26. T. R. R. Singh, P. A. McCarron, D. Woolfson, R. F. Donnelly, *Eur. Polym. J.*, 2009, **45**, 1239.

Figures and Diagrams



Scheme 1 Synthesis of Zn(II) sensor 4. (i) 6-aminohexanoic acid, EtOH, reflux, 8 hours (ii) sodium triacetoxyborohydride, 1,2-dichloroethane, N₂, stirring, 3 hours. (iii) hydrochloric acid (5 M), reflux, 24 hours. (iv) 2-methoxyethanol, triethylamine, N₂, reflux, 30 hours.



Scheme 2 Synthesis of ter-polymer **6**. (i) EtOH, rhodamine B isothiocyanate, stirring, 18 hours (ii) **4**, NHS, EDC, compound, N_2 , stirring, 12 hours.

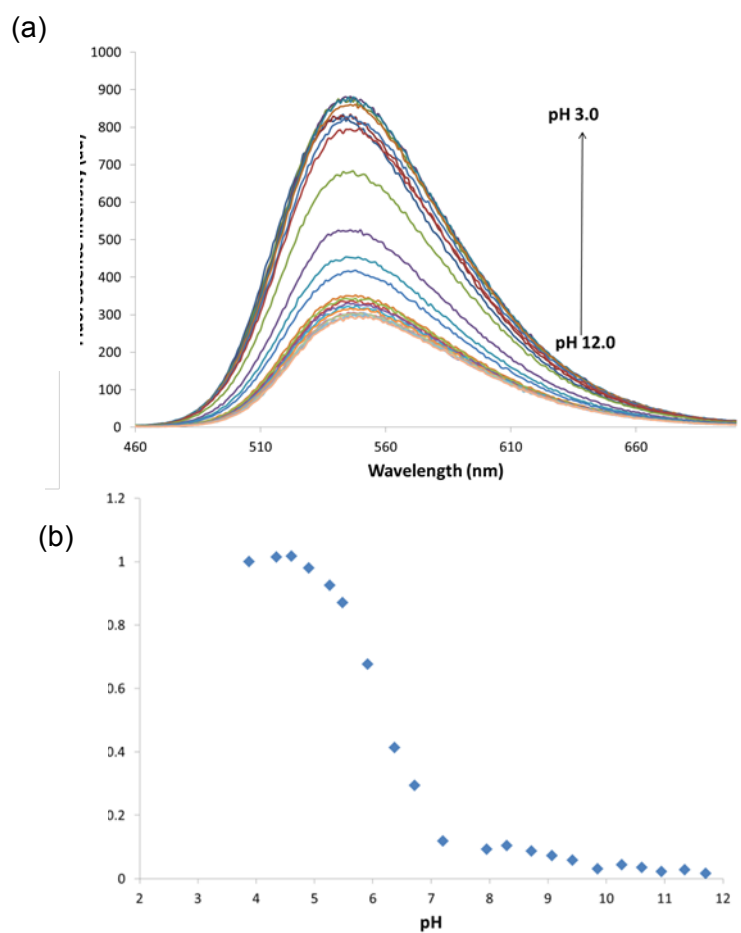


Figure 1 (a) Fluorescence spectra of **4** recorded as different pH values (b) plot of relative intensity against pH for **4**. [**4**] = 5 μ M. Solvent= 9:1 (H₂O:MeOH).

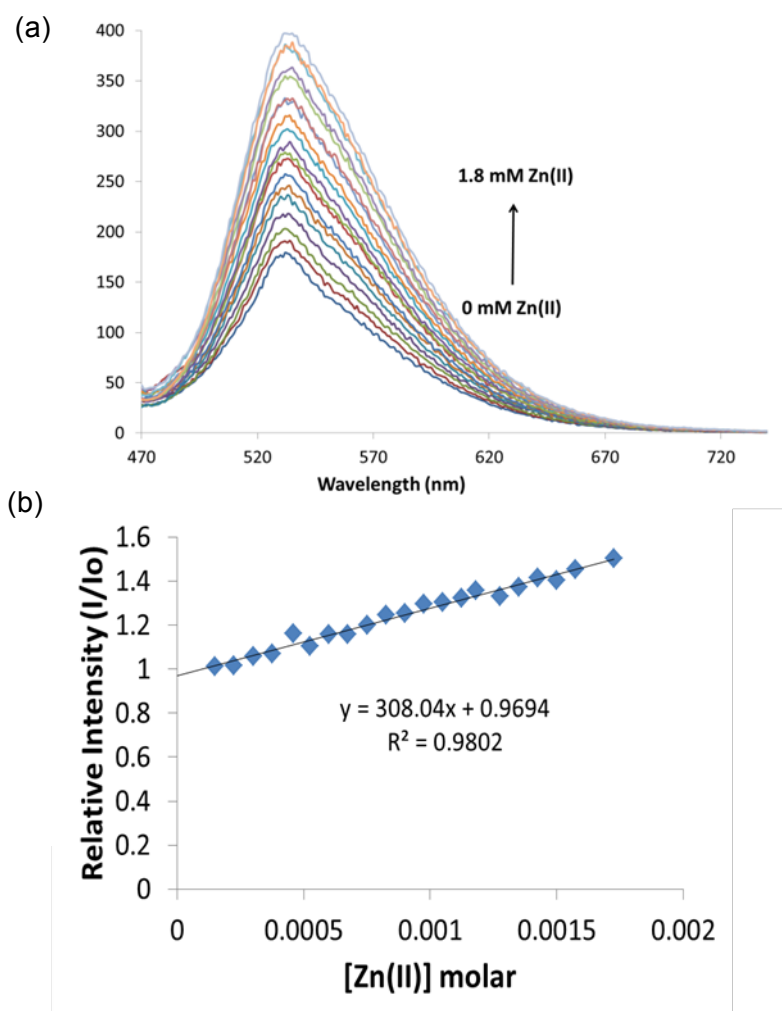


Figure 2 (a) Fluorescence spectra of **4** recorded at different Zn(II) concentrations and (b) plot of relative fluorescence intensity against zinc concentration. [**4**] = 5 μ M. Solvent= 9:1 (HEPES (0.1 M, pH 7.4 \pm 0.1):MeOH).

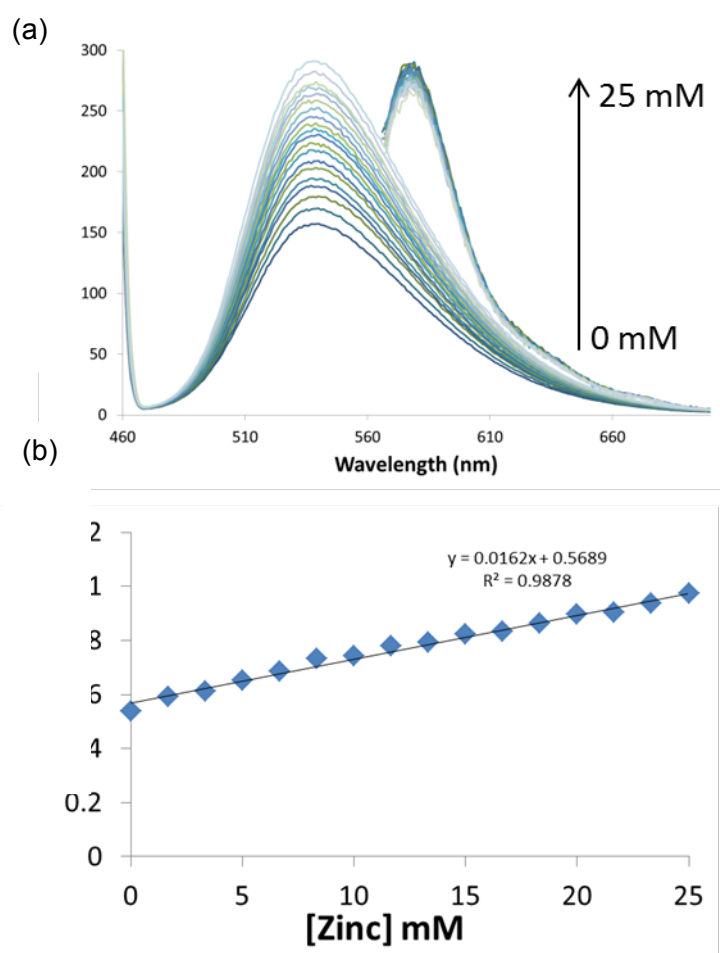


Figure 3 (a) Fluorescence spectra of **6** recorded at different Zn(II) concentrations and (b) plot of ratiometric intensity (I_{535}/I_{585}) against zinc concentration. Solvent= 9:1 (HEPES (0.1 M, pH 7.4 ± 0.1):MeOH).

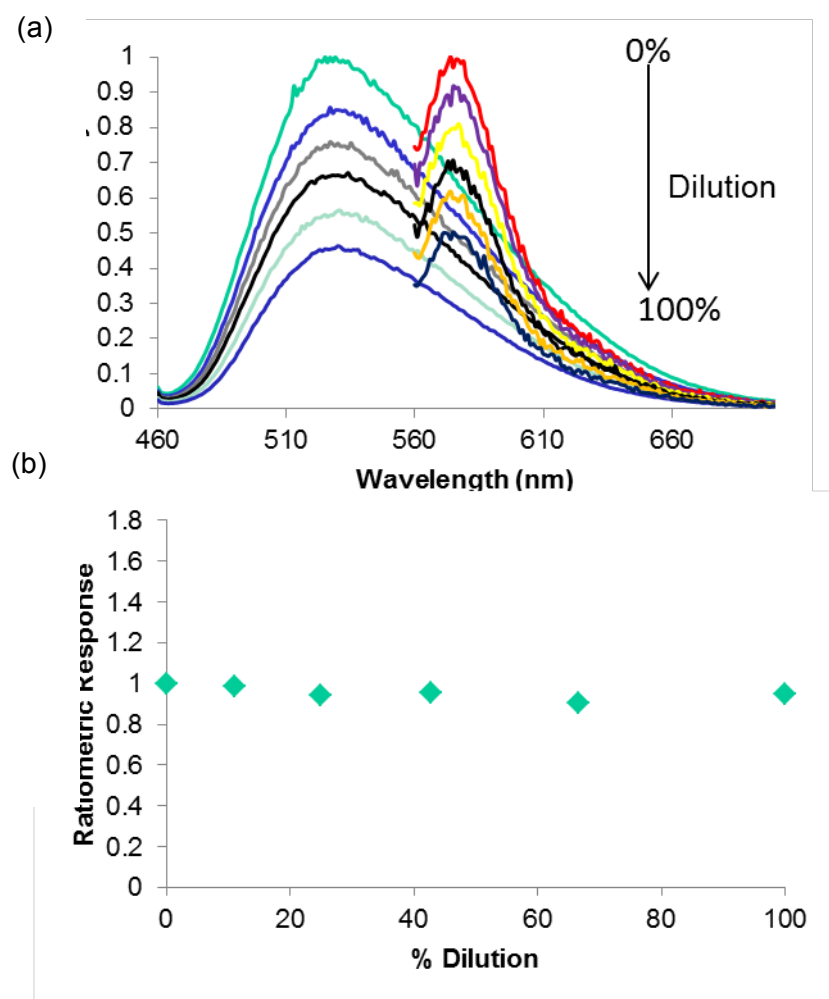


Figure 4 (a) Fluorescence spectra of **6** recorded at different levels of dilution and (b) plot of ratiometric intensity (I_{535}/I_{585}) for **6** against percentage dilution.

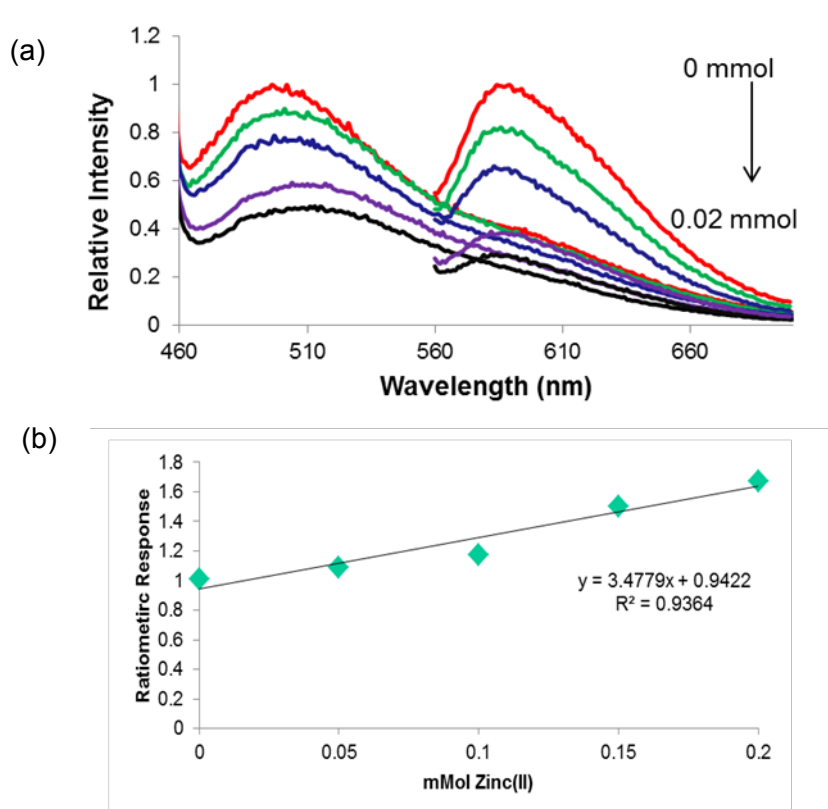


Figure 5 (a) Fluorescence spectra of **6** after incorporation within a hydrogel matrix, recorded after addition of Zn(II) and (b) plot of ratiometric intensity (I_{510}/I_{578}) as a function of Zn(II) addition.