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A Hydrogel Based Zinc(II) Sensor for use in Fluorescent Multi-Well Plate Analysis.

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Abstract: A polymeric hydrogel containing a photoinduced electron transfer (PET) based probe for Zn(II) has been formulated into the wells of a 96-well plate. Upon addition of Zn(II) ions to selected wells, the fluorescence of the gel was observed to increase in a concentration dependent manner in the 0.25 – 1.75 mM range. The millimolar binding constant observed for this probe is higher than that reported for other Zn(II) probes in the literature and offers the possibility to determine the concentration of this ion in environments where the Zn(II) concentration is high. The combination of the multi-well plate set-up with fluorescence detection offers the possibility of high-throughput screening using low sample volumes in a timely manner. To the best of our knowledge, this is the first reported example of a polymeric hydrogel sensor for zinc with capability for use in fluorescence multi-well plate assay.

1.0 Introduction: Fluorescent multi-well plate analysis has emerged as a popular technique for the detection of many different types of analyte ranging from metal ions and small molecules to proteins and nucleotides.[1,2] The high throughput capability of the multi-well set-up, coupled with the sensitivity of fluorescent detection, enables a large number of samples to be processed in a relatively short time period using small sample volumes. Most protocols require the sensing (or binding) component to be fixed to the surface of the well to facilitate removal of un-bound substrates in one or more wash steps.[3] A convenient and inexpensive method for such attachment is through the in-situ formation of hydrogels within the wells of the multi-well plate.[4,5] Such a strategy usually requires the sensor to by covalently bound within the hydrogel matrix to prevent leaching during the swelling or wash steps.

In this manuscript, we report a hydrogel based fluorescent sensor for zinc based on the physical properties of an acrylamide hydrogel in combination with a photoinduced electron transfer (PET) operated Zn(II) sensor. Sensors based on the PET mechanism switch their fluorescence from “Off” to “On” upon binding a target analyte due to a cancellation of the PET process that otherwise de-activates the excited state.[6,7] Our interest in Zn(II) as a target analyte stems from its important biological roles such as maintaining proper immune system function,[8] involvement in the wound healing process[9] and supporting normal growth and development during pregnancy, childhood, and adolescence.[10] Zinc is also an essential trace element, with an average of 2-3 grams being present in adult humans[11] making it the second most abundant trace element in the body.[12] Abnormal levels of zinc have been associated with certain diseases such as Alzheimers,[13] ischaemic stroke,[14] and epilepsy.[15] In developing sensors for the detection of analytes such as Zn(II), care must be taken to ensure the probe is selective for the target over other relevant analytes and be sensitive in the appropriate range. The majority of Zn(II) probes reported in the literature have binding constants in the nanomolar range[16] meaning they become saturated and ineffective at measuring Zn(II) in environments where its concentration reaches micromolar or millimolar levels.[17] Indeed, Zn(II) concentrations in synaptic vesicles of forebrain neurons have been reported to be in the μM to mM range,[18, 19] illustrating the

need for lower affinity sensors that can operate at these levels. In this manuscript, we present a low affinity hydrogel based Zn(II) responsive sensing system capable of detecting Zn(II) in the millimolar concentration range. The hydrogel was fixed to the surface of a 96-well plate making it amenable for high throughput Zn(II) measurements.

2.0 Results and Discussion:

2.1 Synthesis of monomer 5: Methacrylate functionalised monomer **5** was prepared in five steps following a convergent synthesis as outlined in Scheme 1. The synthesis of dipicolylamine (DPA) containing receptor component **2** has already been described by our group in a previous communication.[20] In parallel to the preparation of **2**, 4-bromo-1,8-napthalic anhydride was reacted with 2-(2-aminoethoxy)ethanolto form alcohol substituted **3**, which was then combined with **2** in an N-substitution reaction forming compound **4**. After esterification of **4** with methacryloyl chloride, methacrylate containing monomer **5** was obtained after purification using flash chromatography.

*2.2 Photophysical Characterisation of **5***: Before **5** was deemed suitable for inclusion within a polymeric hydrogel matrix, it was first necessary to determine its sensing properties in solution, to ensure it was capable of selectively binding Zn(II) in aqueous buffer at physiological pH. The tertiary amino nitrogen of **5** is the principal atom involved in the PET process to the napthalimide fluorophore. Due to the basicity of this amine group, the lone pair of electrons present on the N-atom can become bound to adventitious protons that cancel the PET process even in the absence of Zn(II). Therefore, it is imperative that this lone pair remains unbound at physiological pH so the receptor is free to bind Zn(II). To ensure this was the case, a pH titration was carried out where the pH of an aqueous solution of **5** was adjusted from 2.5 to 11 and the fluorescence spectrum recorded at each pH interval. The results are shown in Figure 1a and reveal an increase in fluorescence intensity upon decreasing pH due to a cancellation of the PET process as described above. When the relative fluorescence intensity at 535 nm was plotted as a function of pH a sigmoidal curve was obtained (Fig 1b) which clearly revealed that the fluorescence of **5** was quenched at pH

7.4 and free to bind Zn(II). Indeed, from a plot of -log(F_{MAX}- F)/(F- F_{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 5.77 meaning it was only 2.3% bound with protons at this pH.[21]

Having established the suitability of **5** to operate at physiological pH, the next step was to determine its fluorescence response to Zn(II). Solutions containing **5** (5 μM) in a 19:1 HEPES (0.1 M, pH 7.4 ± 0.1) : DMSO solvent system were prepared that contained a range of Zn(II) concentrations up to a final concentration of 6 mM. The fluorescence spectrum of each solution was recorded and is shown in Figure 2a. The results reveal a concentration dependent increase in the fluorescence intensity of **5** attributed to cancellation of the PET process as a result of Zn(II) chelation by the tertiary amine and two pyridine nitrogen atoms of the DPA receptor. This chelation event, increases the oxidation potential of the tertiary amine removing the thermodynamic driving force for PET and switching fluorescence "On".[22] When the relative fluorescence intensity at 535 nm was plotted as a function of Zn(II) concentration good linearity was observed in the 0.5 – 6.0 mM range (Figure 2b). The LOD was determined as 0.236 mM following a literature precedent.[23] As, the binding stoichiometry between Zn(II) and the dipicolylamine receptor is known to be 1:1[24], the binding constant was determined by plotting –log(F_{MAX}-F)/(F-F_{MIN}) against –log[Zn(II)] and found to be 2.51 M^{-1}.[25] This binding constant is in a similar range to that reported by us in a previous communication where the structure of the fluorophore-spacer-receptor components of the probe were identical, the only difference being the tethering group attached to the imide nitrogen.[20] The reason for the high binding constants observed in these single DPA receptor, napthalimide fluorophore based probes when compared to the QZ or Zinspy family of sensors is not fully understood. It has been shown by Lippard's group that reducing the number of DPA binding units from two to one in the QZ and Zinspy probes raised the binding constant by four orders of magnitude to ≈10 μM.[26] In addition, placing a methyl group *ortho* to the pyridine nitrogen atom of the DPA unit also had an increasing effect on the binding constant by sterically restricting access to the receptor pocket.[26] Therefore, the use of single

DPA receptor based Zn(II) probes in combination with steric effects can significantly reduce the propensity of Zn(II) to bind to this type of receptor. Perhaps, the conformational geometry of the napthalimide fluorophore in **5** has a more pronounced steric effect than the fluorescein unit used in QZ and Zinspy, that, in combination with the single DPA receptor increases the binding constant of **5** to the millimolar range. Furthermore, the long flexible tethering unit connected to the imide nitrogen of **5** could adopt a conformation that positions it in the proximity of the DPA receptor and hinder Zn(II) binding. Whatever the reason, it is clear these napthalimide-DPA Zn(II) probes have significantly higher binding constants than others reported in the literature.

To determine the selectivity of **5** for Zn(II) against other physiological and environmentally relevant ion, solutions were prepared containing **5** (5μM) and the metal ions of interest (5 mM) in 19:1 HEPES (0.1 M, pH 7.4 ± 0.1) : DMSO solution. The fluorescence spectrum of each was recorded and the results, expressed as the % fluorescence enhancement relative to the blank, are shown in figure 3a. The results reveal that while Ni(II), Co(II) and Cu(II) quenched the fluorescence of **5**, the remaining ions had practically no effect, even at the high concentrations used in this study. The quenching effect of Ni(II), Co(II) and Cu(II) was not unexpected as these ions are known to quench the exited state of fluorophores by electron or energy transfer mechanisms.[27]However, cellular concentrations of Ni(II) and Co(II) are very low and are not likely to interfere with **5**'s ability to measure Zn(II), while Cu(II) has been shown to reach levels as high as 0.35 mM in neuronal cells.[19] To demonstrate the ability of **5** to measure Zn(II) in a competitive environment containing Cu(II), a solution was prepared containing **5** (5 μM) in the presence 5 mM Zn(II) of 0.35 mM Cu(II). The results are shown in Fig 3b and reveal only a minor quench in the intensity of **5** in the presence of Zn(II) and Cu(II) relative to Zn(II) alone illustrating its ability to measure Zn(II) in an environment where the Cu(II) concentration in relatively high.

2.3 Multi-Well plate Analysis: To prepare a polymeric hydrogel with **5** covalently linked in its structure, the monomer was co-polymerized with N'N dimethylacrylamide (DMAA) in the

presence of the cross-linker N,N' methylenebisacrylamide (MBA) under the assistance of a sodium metabisulfate : potassium persulfate : iron (II) sulfate (3.15 mM: 0.45 mM: 0.81 mM) initiator system. This particular hydrogel was chosen as it can be prepared at room temperature, is rapidly formed and has been deemed effective for use in fluorescent multi-well plate sensing.[2] The process of hydrogel formation involved dissolving all monomers, cross-linker and initiators in ice-cold water, then aliquoting into the wells of a 96-multiwell plate, and allowing the gel to set at room temperature for two hours. When the hydrogels had cured successfully they were subsequently washed with buffer (HEPES 0.1M pH 7.4 ±t 0.1) to remove unreacted reagents, ensure full swelling of the gel and equilibrating the pH of the gel to 7.4. Photographs of the resulting gel, shown alongside a control gel with no **5** present and prepared in a scintillation vial for ease of illustration, are shown in Figure 4. It is evident from these photographs that Sol-Gel transition was effective and that **5** was homogenously distributed throughout the gel (figure 4). This was confirmed when the fluorescence spectra of a range of wells (n = 5) containing gels were recorded using a Varian Cary Eclipse fluorimeter with multi-well plate capability. A consistent spectral profile was observed from each well with typical napthalimide emission observed and an average intensity of 440.5 nm ± 6.5 across all wells (figure 5). However, when a range of Zn(II) concentrations were added to these wells in HEPES buffer (0.1M pH 7.4 ± 0.1), a concentration dependant increase in fluorescence intensity was observed that was linear in the 0.25 – 1.75 mM range (figure 6) with a limit of detection (LOD) of 0.174 mM.[23] Therefore, this suggests that the gels equilibrate with the Zn(II) solution effectively and the covalently immobilised **5** is capable of chelating Zn(II) which manifests itself in an increased fluorescence intensity

3.0 Conclusion: In conclusion, a small molecule Zn(II) probe, comprising a napthalimide fluorophore connected to a single DPA receptor in a PET format, has been prepared and effectively incorporated within a polymeric hydrogel sensing system. When solutions of Zn(II)

were added to the gel, a concentration dependent increase in fluorescence intensity was observed with good linearity in the 0.25 – 1.75 mM range and a LOD of 0.174 mM. The observed binding constant, is, to the best of our knowledge, higher than any other Zn(II) probe reported in the literature. This may enable the detection of Zn (II) where its concentration is high, such as in the synaptic vesicles of forebrain neurons.[28] The ability to formulate the sensor into a hydrogel compatible with fluorescence multi-well plate analysis is an added advantage, and enabled the rapid screening of 21 samples in under ten minutes. This demonstrates the potential of this system as a method to screen large numbers of samples in a relatively short time scale with minimal operator input.

4.0 Materials & Methods.

4.1 Reagents and instruments:

All chemicals were purchased from Sigma-Aldrich (Gillingham, UK) 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. NMR spectra were obtained on Varian 500 MHz instrument at 25.0 ± 1 ℃ 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 fitted with plate reader accessory. pH was measured by a pH meter (Hanna Instruments).

*4.2 Synthesis of compounds **1-5**.*

The synthesis of compound **1**[29] and **2-3**[20] have been reported before.

*4.3 Synthesis of 6-[2-[bis(2-pyridylmethyl)amino]ethylamino]-2-[2-(2-hydroxyethoxy) ethyl]benzo [de]isoquinoline-1,3-dione (**4**):* **1** (1.00 g, 2.75 mmol), **3** (2.37 g, 9.81 mmol) and triethylamine (9.80 mL) were refluxed in 2-methoxyethanol under N_2 for 17 hours. The solution was evaporated to dryness and the resulting oil purified using column chromatography with silica gel (5:1 CH_3Cl:MeOH) giving **4** as a yellow powder (0.30 g, 20.64% yield). Mp: 143-147 ℃. ^{1}H NMR: (500 MHz, CDCl) δ (ppm): 8.84 (1H, Ar-H,), 8.64 (1H, Ar-H), 8.59 (2H, Ar-H), 8.43(1H, Ar-H), 7.94 (1H, Ar-H), 7.71 (1H, Ar-H), 7.56 (2H, Ar-H), 7.38 (2H, Ar-H), 7.16 (2H, Ar-H), 6.56 (1H, -NH), 4.45 (2H, N-CH_2), 4.02(4H, N-CH_2-Ar), 3.87(2H, O-CH_2-CH_2), 3.70 (2X 2H, O-CH_2-CH_2), 3.41(2H, NH-CH_2-CH_2), 3.06(2H, N-CH_2-CH_2), 2.69(1H, -OH). ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm): 164.25, 163.47, 157.74, 149.64, 149.59, 135.66, 134.10, 130.38, 129.16, 126.85, 123.30, 122.32, 121.61, 121.37, 119.72, 107.80, 103.00, 71.25, 67.72, 60.84, 58.76, 50.06, 39.95, 39.86, 38.21. +ve ESI MS: Expected: 525.2, Obtained: 526.3. V_{max} (cm^{-1}): 3300, 2960, 2850, 1680, 1640, 1590, 1390

*4.4 Synthesis of 2-[2-[6-[2-[bis(2-pyridylmethyl)amino]ethylamino]-1,3-dioxo-benzo[de] isoquinolin-2-yl]ethoxy]ethyl 2-methylprop-2-enoate (**5**):* **4** (0.30 g, 0.51 mmol), methacryloyl chloride (83.7 µL, 0.86 mmol) and triethylamine (199.4 µL) were refluxed in DCM under N_2 for 8 hours. The solution was evaporated to dryness and the resulting oil purified using column chromatography with silica gel (100% DCM to 9:1 DCM:MeOH) giving **5** as a yellow oil (0.136 g, 40.03% yield). Mp: 112-115 ℃. ^{1}H NMR (500 MHz, D_6MSO) δ (ppm): 8.53 (1H, Ar-H), 8.44 (1H, Ar-H), 8.32 (2H, Ar-H), 8.11 (1H, Ar-H), 7.61 (1H, Ar-H), 7.40 (4H, Ar-H), 7.05 (2H, Ar-H), 6.46 (1H, Ar-H), 5.81 (1H, -CH_2), 5.30 (1H, -CH_2), 4.27 (2H, N-CH_2-CH_2), 4.12 (2H, O-CH_2-CH_2), 3.79 (4H, -N-CH_2-Ar), 3.72 (2H, CH_2-CH_2-O), 3.67 (2H, -O-CH_2-CH_2), 3.45 (2H, CH_2-NH), 2.84 (2H, N-CH_2), 1.65 (3H, -CH). ^{13}C NMR: (125 MHz, D_6MSO) δ (ppm): 165.00, 164.44, 158.76, 151.11, 148.16, 137.08, 134.72, 131.11, 130.00, 128.04, 124.72, 123.77, 122.36, 120.56, 107.78, 103.89, 68.27, 67.79, 63.62, 58.67, 51.95, 40.57, 38.35, 30.29, 25.01, 23.35, 16.68. +ve ESI MS: Expected 593.3, Obtained: 594.4. V_{max} (cm^{-1}): 2960, 2920, 2855, 1720, 1690, 1645, 1380.

4.5 Hydrogel formation: To prepare gels, aliquots of **5** (0.609 mL, 148 µM), monomers (6% w/v MBA in DMAA) (0.350 mL, 10% v/v), distilled water (1.182 mL) and sulphuric acid (0.172 mL, 6.15 mM) were added to an iced vial. This solution was allowed to cool to 0℃ for 5 minutes. Potassium persulfate (0.630 mL, 0.45 mM) sodium metabisulphate (0.368 mL, 3.15 mM) and iron (II) sulphate (0.189 mL, 0.81 mM) were added. The solution was immediately aliquoted (160 µL) into 21 wells of multiwell plates and gels were allowed to cure for 2 hours. Gels were washed three times with distilled water and equilibrated overnight with 0.1M HEPES/ 0.05M KCl (pH 7.40 ± 0.10) buffer.

*4.6 Fluorescence pH titration of **5**:* A 5 µM solution of **5** was prepared in 100 mL of H_2O:DMSO (19:1) solution. The solution pH was adjusted from acidic (pH 3.0) to basic (pH 11.0) 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.

*4.7 Fluorescence- Zn(II) titration of **5**:* A 5 µM solution of **5** was prepared in 200 mL of a 0.1 M HEPEs and 0.05 M KCl buffered (pH 7.4):DMSO (19:1) solution. The Zn(II) concentration was adjusted from 0 to 6 mM by the addition of 100 µL aliquots of 1 M of zinc chloride and the fluorescence spectrum recorded after each addition using an excitation wavelength of 450 nm.

*4.8 Fluorescence Zn(II) on sensor **5** loaded-hydrogel:* Gels were prepared as described above in a 7 x 3 format in a 96-well plate. To each row, solutions containing 0.25, 0.50, 0.75, 1.00, 1.25, 1.50 or 1.75 mM Zn(II) (3 x 160 µL) (0.1 HEPES and 0.05 mM KCl, pH 7.40 ± 0.10) were added. These were allowed to incubate overnight. Solutions were aspirated off, and the fluorescence spectrum using an excitation wavelength of 450 nm.

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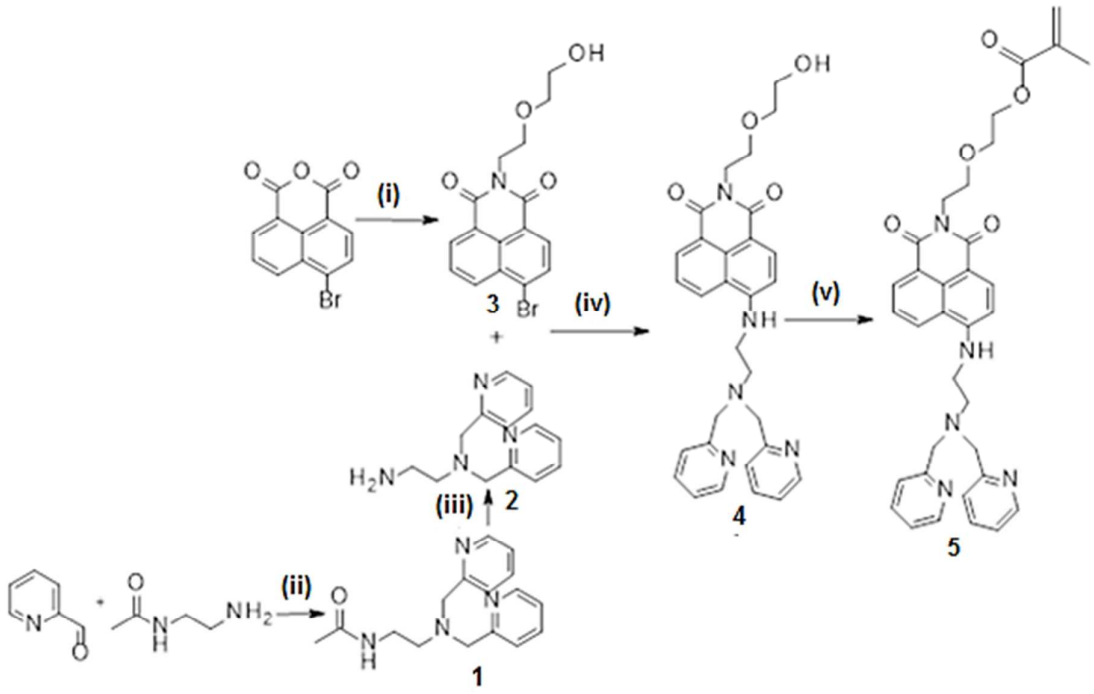
Figures & Diagrams



Scheme 1 Synthetic scheme for the formation of sensor **5**. (i) 2-aminoethoxy ethanol, EtOH, reflux, 12 hours. (ii) Sodium triacetoxyborohydride, N_2, 2-dichloroethane, stirring, 3 hours. (iii) Hydrochloric acid (5M), reflux, 24 hours. (iv) 2-methoxyethanol, triethylamine, N_2, reflux, 30 hours. (v) DCM, methacryloyl chloride, N_2, triethylamine, 6 hours.

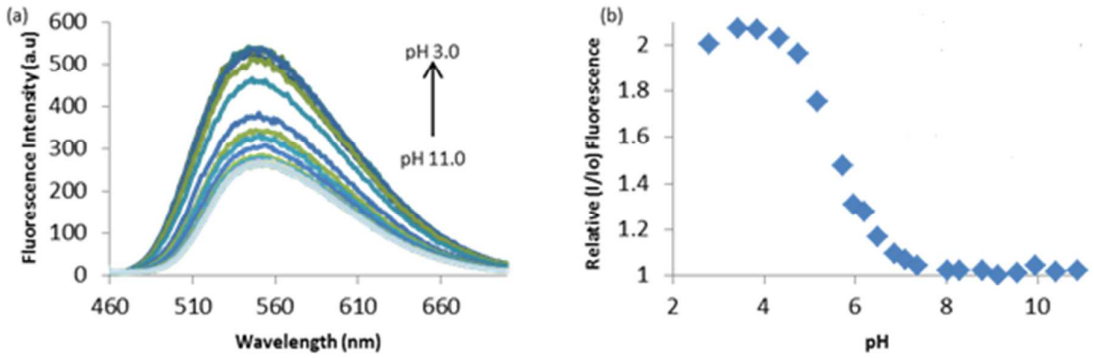


Figure 1 (a) Fluorescence spectra of **5** recorded in H_2O at different pH values ranging from 3.0 to 11.0. (b) Plot of relative fluorescence intensity (I/Io) at 535 as a function of pH. [**5**] = 5 μM. λ_{EX} = 450 nm.

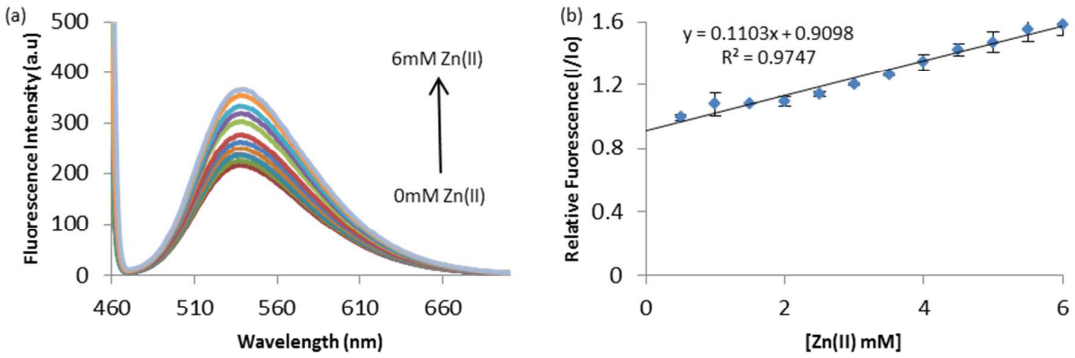


Figure 2: (a) Fluorescence spectra of **5** recorded in the presence of varying Zn(II) concentration (b) plot of relative fluorescence intensity (I/Io) at 535 nm for **5** as a function of Zn(II) concentration. [**5**] = 5 μM, λ_{EX} = 450 nm, solvent = 19:1 HEPES (0.1 M, pH 7.4 ± 0.1) : DMSO.

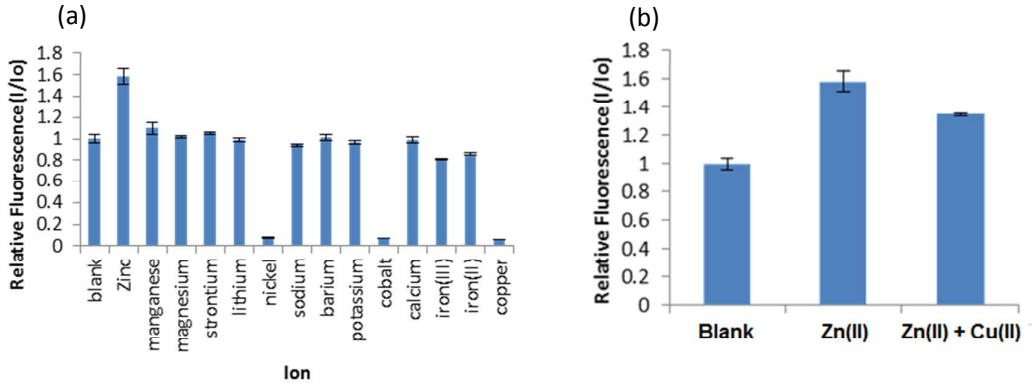


Figure 3 (a) Bar chart showing the fluorescence response of **5** in the presence of a range of range of different ions. [**5**] = 5 μM, [ion] = 5 mM, λ_{EX} = 450 nm, solvent = 19:1 HEPES (0.1 M, pH 7.4 ± 0.1) : DMSO. (b) Plot showing relative fluorescence enhancement for a solution containing **5** + 5 mM Zn(II) alone and **5** + 5 mM Zn(II) in the presence of 0.35 μM Cu(II).

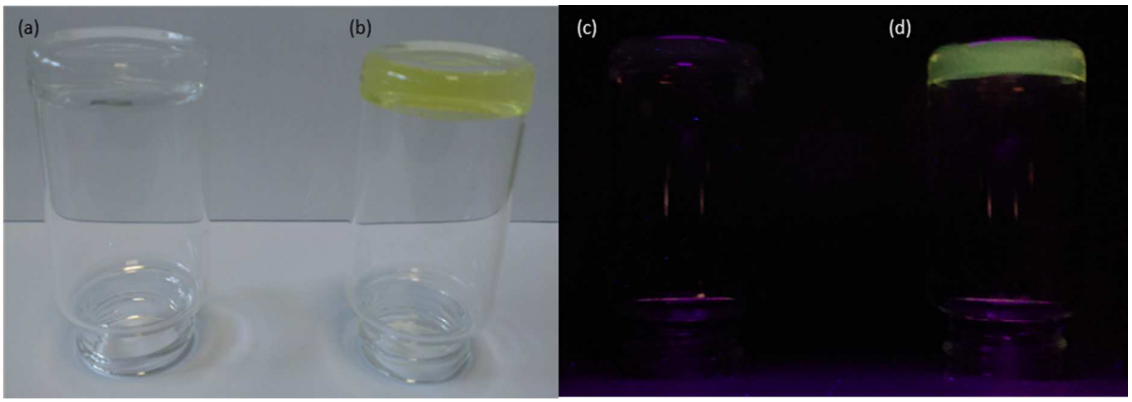


Figure 4 Photographs of hydrogels (a) and (c) with no **5** present during polymerisation and (b) and (d) with 148 µM **5** present during polymerisation recorded in ambient light (a+b) and upon excitation at 365 nm (c+d).

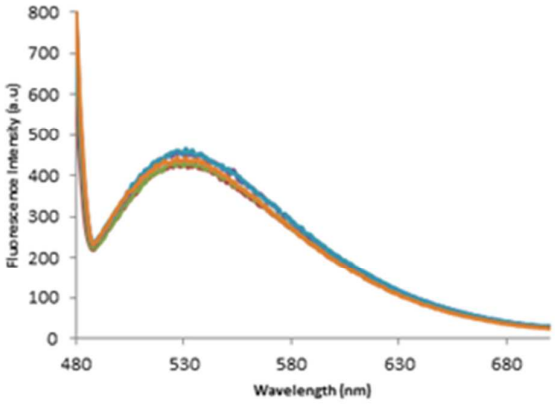


Figure 5 Fluorescence spectra from 5-wells of a multi-well plate coated with a hydrogel containing **5**. Inset shows the mean fluorescence intensity of the 5 wells (± SEM) at 530 nm (λ_{EX} = 450 nm).

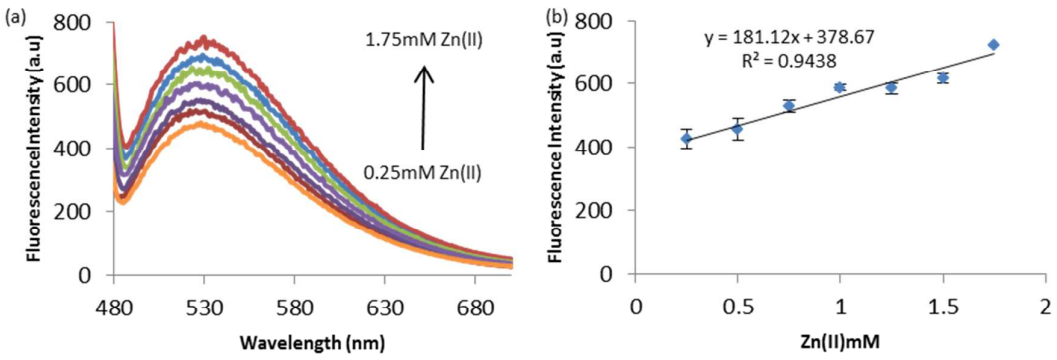
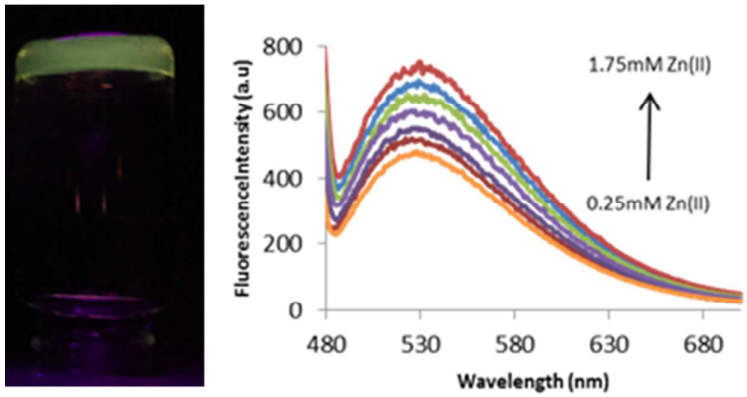


Figure 6 (a) Fluorescence spectra from wells of a multi-well plate coated with a hydrogel containing 5 with increasing amounts of Zn(II) added (b) plot of fluorescence intensity at 530 nm against Zn(II) concentration for wells treated with increasing amounts of zinc in (a). For clarity, only one spectrum is shown for each concentration in (a) but data plotted in (b) represents the mean (± SEM) result where n = 3.

A Hydrogel Based Zinc(II) Sensor for use in Fluorescent Multi-Well Plate Analysis.



A polymeric hydrogel sensor for zinc has been developed for use in fluorescence multi-well plate assay.