Design of a Membrane Fluorescent Sensor Based on Photo-Cross-Linked PEG Hydrogel

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A polymeric hydrogel film was made photochemically for the fluorescent detection of copper ions. An eight-branched PEG macromer having a nitrocinnamate (NC) moiety as the pendant group was synthesized and found to form a gel upon exposure to 365-nm irradiation in the absence of any photoinitiators or catalysts. Peptide **1** (Dns-Gly-His-Lys(NC)) was synthesized with Fmoc solid-phase method and contained three essential parts: a dansyl fluorophore for fluorescent signal transduction, a metal binding site for selective recognition of copper ions from aqueous solution, and a NC group for interaction with PEG-NC macromer. The peptide was found to be efficiently immobilized within the hydrogel network during the photo-cross-linking process. The prepared PEG-NC/**1** gel film is strongly fluorescent and can be quenched by the presence of copper ions. The quenching process was reversed by acid treatment and an excellent reversibility was obtained. The designed membrane sensor showed a superior selectivity toward copper ions as demonstrated by fluorescence spectroscopy and epifluorescence imaging.

1. Introduction

It is of great interest to develop optical sensors for the detection of biologically important metal ions such as copper ions. In the past few years, there appeared a lot of reports on the design of fluorescent chemical sensors for divalent copper ions and some of them exhibited excellent selectivity toward the target ion.[1−9] Our research group is especially interested in developing peptide-derived motifs with a high binding affinity for Cu^{2+}. For example, very recently we reported on highly selective chemosensors based on Gly-His and Gly-Gly-His copper binding motifs.[10,11] Up to now, most of these research studies have been focused on fluorescent probes used in solution phase. Surface-oriented sensors with superior sensing qualities have been very rarely reported.[12] From a practical viewpoint, a sensor on a surface has more preferable properties which allow real-space and real-time measurements. For instance, reversibility is an important parameter in the sensor design. Technically, the reversibility is hard to realize in solution phase. On the other hand, for the surface sensor, after the analysis work is performed, the functionality of the probe/sensor can be restored nondestructively from its complexed form by changing the experimental conditions such as washing out the bound analyte with an acidic solution or putting the sensor in a solution containing competitive ligands.

We report here a surface membrane sensor for the detection of copper ions in which poly(ethylene glycol) (PEG) hydrogel was used as a solid matrix. A small peptide ligand (Dns-Gly-His-Lys(NC), **1**) was immobilized into the gel network for specific interaction with copper ions. Photochemistry has been shown as a very effective and convenient method to construct three-dimensional polymeric networks.[13] Our lab has reported synthesis of a PEG hydrogel via a fast photo-cross-linking approach.[14−16] In our methodogy, photosensitive macromers were first synthesized by modifying branched PEG with chromophores such as nitrocinnamate (NC). A concentrated aqueous solution of the PEG macromer was then applied to long wavelength UV illumination. In this photochemical process, PEG chains are interconnected through the cycloaddition reaction of chromophores thus forming a water-insoluble matrix. This method offers special advantages over conventional physical or chemical methods of network formation: mild reaction conditions, minimum side-product formation, fast gelation, and no need of catalysts, to name a few. In the current study, the nitrocinnamoyl group existed in both the PEG-NC macromer and the peptide ligand, therefore the immobilization of **1** can be realized simultaneously during the photoinduced gelation process (Scheme 1).

2. Experimental Section

Poly(ethylene glycol) (b-PEG, MW = 20 000, eight arms) was purchased from Shearwater Polymers Inc. (Huntsville, AL). All other chemicals and organic solvents were purchased from Sigma-Aldrich (St. Louis, MO) at highest purity available. The deionized water used for physical chemistry experiments was purified by a Modulab 2020 water purification system (Continental Water Systems Corp., San Antonio, TX).

Synthesis of PEG-NC Macromer. PEG-NC was synthesized as earlier reported.[14] In a typical experiment, *trans*-4-nitrocinnamic acid (2.3 g, 12 mmol) was dissolved in 30 mL of warm anhydrous DMF. To this solution, 3.0 g (1.2 mmol OH) of an 8-branched PEG (MW = 20 000) was added, followed by 74 mg (0.606 mmol) of 4-(dimethylamino)pyridine (DMAP) and 1.88 mL (12 mmol) of diisopropylcarbodiimide (DIC). The mixture was reacted for 20 h at 50 °C. Purification was achieved by three times of dissolution/precipitation with toluene/ether.

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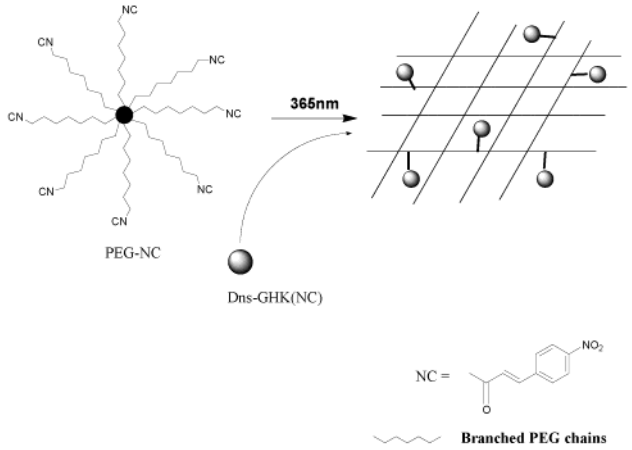
SCHEME 1: Strategy of Photochemical Gelation from PEG-NC Macromer and the Immobilization of Peptide 1 into the Polymer Matrix

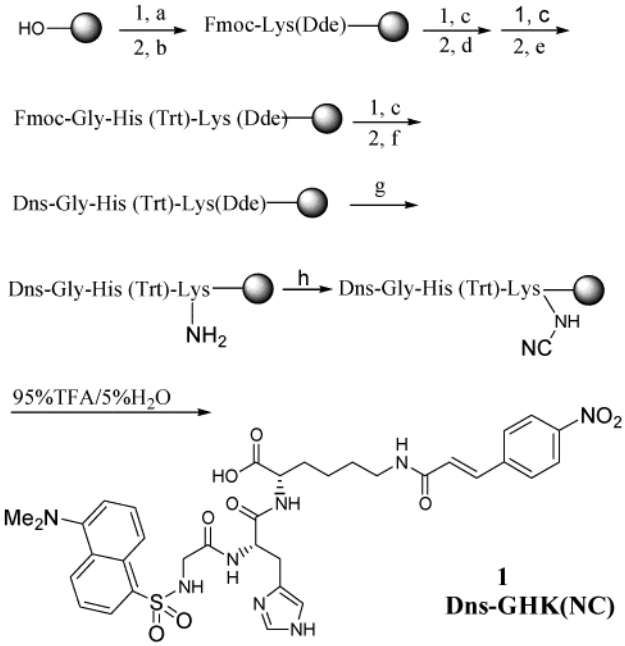


^{1}H NMR showed that the terminal hydroxyl groups were functionalized to a degree of 98%.

Synthesis of Dns-Gly-His-Lys(NC) (1). Peptide **1** (Dns-Gly-His-Lys(NC)) was synthesized via standard solid-phase 9-fluorenylmethoxycarbonyl (Fmoc) chemistry.[17,18] Wang resin was used as the solid phase. Diisopropylcarbodiimide (DIC) and 1-hydroxylbenzotriazole (HOBt) in situ activation method was used for the coupling reactions. The Fmoc groups were de-protected with 20% piperidine solution in DMF. Cleavage of peptide from the resin was completed with CF_3COOH/H_2O (95/5, v/v) for 2.5 h. Following suction under reduced pressure and removal of TFA with N_2 blow-off, crude product was precipitated from cold ether. The solid precipitate was centrifuged and washed with ether and lyophilized under vacuum. Semipreparative RP-HPLC was performed on Waters 2690 separations module. Two solutions were prepared: A: 0.1% TFA in water, B: 0.1% TFA in acetonitrile. A linear gradient (15∼45% B within 30 min, flow rate at 2.5 mL/min) was used for the purification. Column conditions: 7.8 × 300 mm, C18 column. Analytical HPLC was conducted on a small-scale column (4.6 × 75 mm C18 column). Same gradient as the semipreparative method was used. Flow rate = 1 mL/min. ^{1}H NMR data were taken on a Bruker 400 MHz spectrometer. Low-resolution FAB was recorded on a VG-Trio 2000 mass spectrometer. High-resolution FAB was conducted on a 70-4F instrument in the Mass Spectrometry Lab, University of Illinois at Urbana-Champaign. **1**. ^{1}H NMR (400 MHz, methanol-d_4): δ = 8.80 (s, 1H), 8.58 (d, 1H), 8.32 (d, 1H), 8.23 (m, 3H), 7.76 (m, 2H), 7.62 (m, 3H), 7.39 (s, 1H), 7.30 (d, 1H), 6.75 (d, 1H), 4.75 (m, 1H), 4.41 (m, 1H), 3.48 (m, 2H), 3.38−3.28 (m), 3.12 (m, 1H), 2.89 (s, 6H), 1.98 (m, 1H), 1.82 (m, 1H), 1.62 (m, 2H), and 1.50 (m, 2H). FAB-MS: 749.2715(MH^+, calcd 749.2717). UV−Vis (in water): $\epsilon_{214\ nm} = 3.4 \times 10^4$ L mol^{-1} cm^{-1}, $\epsilon_{246\ nm} = 1.5 \times 10^4$ L mol^{-1} cm^{-1}, $\epsilon_{327\ nm} = 1.4 \times 10^4$ L mol^{-1} cm^{-1}.

Photo-Cross-Linking. PEG-NC and peptide **1** were dissolved in ethanol: water (2:1, v/v). Their final concentrations are 1.67 and 3.33 mM, respectively. The dip-coating method was used to prepare thin gel film. Typically, 40 μL of the solution was spread on a quartz slide (Hellma Worldwide, Inc., Plainview, NY). Leave the slide in air 10 min for ethanol to evaporate before illumination. Photo-cross-linking was performed for 10 min using a 4-W 365-nm UV lamp (Spectronics Corp., Westbury, NY) and the lamp was placed above the sample at a distance of 3 cm. After film preparation, the slide was put into pure water to swell for 15 min and then used for physical chemistry experiments.

SCHEME 2: Solid-Phase Synthesis of Peptide 1 (Dns-Gly-His-Lys(NC))[a]



[a] (a) Fmoc-Lys(Dde)-OH, DMAP, DIC, HOBt, 3 h. (b) Ac_2O and pyridine, 0.5 h. (c) 20% Piperidine/DMF. (d) Fmoc-His(Trt)-OH, DIC, HOBt, 0.5 h. (e) Fmoc-Gly-OH, DIC, HOBt. 0.5 h. (f) Dansyl chloride, 0.5 h. (g) 2% Hydrazine, 4 min. (h) 4-*trans*-Nitrocinnamic acid, HOBt, DIC, 0.5 h.

Physical Chemistry Measurements. UV−Vis data were recorded on a Lambda 900 UV/VIS/NIR spectrometer (Perkin-Elmer, Inc., Shelton, CT). The fluorescence spectra of the photo-cross-linked gel films were measured on a Spex Fluorolog 1680 0.22-m double spectrometer (Spex Industries, Inc., Edison, NJ), and the slide was put in a 1-cm fluorescence cuvette with an angle of 45° facing both the incidence and emission light beams to ensure the maximum collection of the fluorescent signals. An epifluorescence microscope (Olympus IX-FLA) was used to obtain the fluorescence images of the cross-linked hydrogel film in different subphases. UV light was used for excitation and an Optronic Magnafire TM CCD camera was used to detect the fluorescence emission. Temperature of measurements was kept at 20 °C. Phosphate buffer solution (pH = 7.0) was used for all the spectroscopic experiments unless indicated otherwise.

3. Results and Discussion

3.1. Synthesis of PEG-NC and Peptide 1. The synthesis of PEG-nitrocinnamate macromer (PEG-NC), prototype of the hydrogel, was achieved through the esterification reaction between a carboxylic acid (*trans*-4-nitrocinnamic acid) and an 8-branched poly(ethylene glycol) with a molecular weight of 20 000. The modification is very efficient (98%) which is confirmed by ^{1}H NMR and absorption spectra. Solid-phase synthesis of **1** is shown in Scheme 2. N-terminal dansylation was done with 1.5 equivalent of dansyl chloride in the presence of 1.5 equivalent of diisopropylethylamine (DIEA) for 30 min. The dansylated peptide may further react with large excess of dansyl chloride to form a dually dansylated peptide thus reducing the synthetic yield. The Dde group was removed with 2% hydrazine in DMF for 4 min.[19] Attachment of the nitrocinnamoyl group to the lysine residue was performed with 3 equivalents

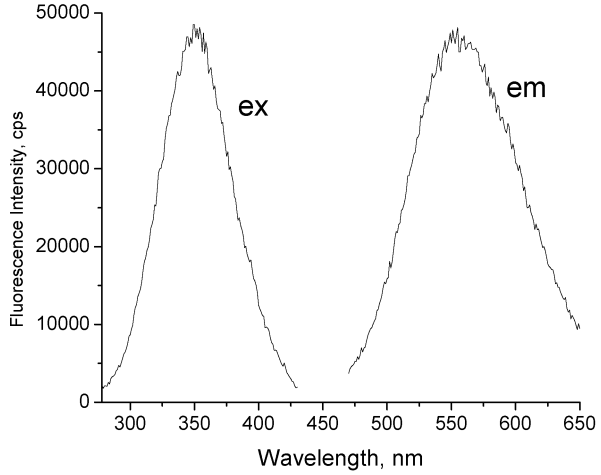


Figure 1. Excitation and emission spectra of peptide **1** (1.0×10^{-5} M) in aqueous solution (pH = 7.0).

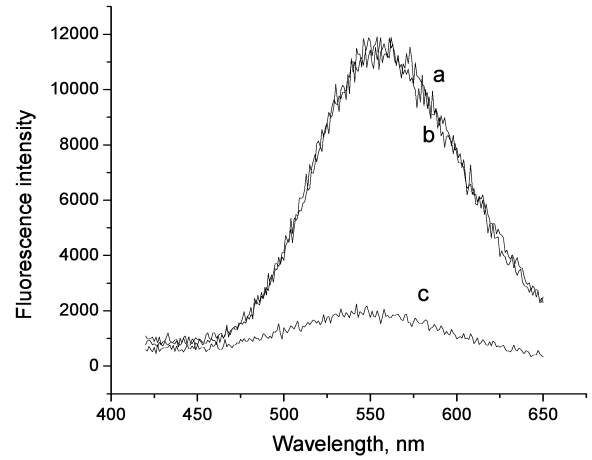


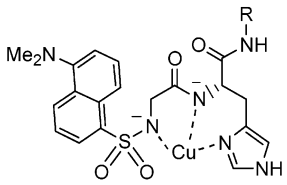
Figure 2. Fluorescent selectivity of peptide **1** toward Cu^{2+}. Concentration of **1** is 5 μM (pH = 7.0). (a) Pure peptide, (b) peptide and miscellaneous ions, (c) peptide, miscellaneous ions, and copper ion (5 μM). The miscellaneous ions contain Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and each has a concentration of 5 μM. Excitation is selected at 350 nm.

of *trans*-4-nitrocinnamic acid activated with DIC. This reaction is somewhat difficult due to the low solubility of nitrocinnamic acid in DMF. Slight heating can help dissolve nitrocinnamic acid completely in DMF. After the attachment reaction, the resin was washed with a sufficient amount of DMF to remove unreacted nitrocinnamic acid.

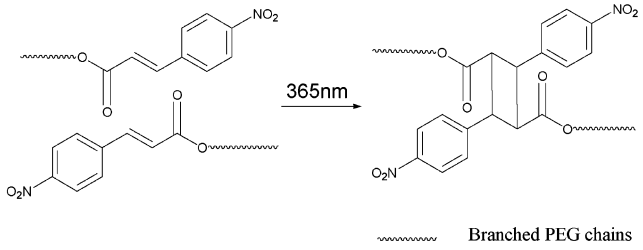
3.2. Fluorescent Properties of Peptide 1 in Aqueous Solution. Peptide **1** has particular structural characteristics. It is composed of three essential parts: a fluorophore (dansyl, i.e., Dns) for fluorescent signal transduction, a metal binding site for selective binding with copper ion, and a photosentive group (NC) for covalent immobilization within the cross-linked PEG-NC matrix. In the fluorescence spectra of **1**, the maximum excitation and emission were observed at 350 and 555 nm, respectively (Figure 1).

As anticipated, the fluorescence of ligand **1** was quenched by Cu^{2+} in aqueous solution and an excellent selectivity toward Cu^{2+} was observed in comparison with several other competitive transition metal ions (Figure 2). It is seen that the fluorescence of an aqueous solution containing **1** and several competitive metal ions (containing Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Ni^{2+}; 5 μM each) is almost overlapped with that of the free ligand (Figure 2, curves a and b). However, when one equivalent of Cu^{2+} was added to the solution, the fluorescence was hugely quenched and only 14% of the fluorescence intensity at 555 nm remained.

SCHEME 3: Binding Model of Dns-Gly-His Motif with Cu^{2+}



SCHEME 4: [2+2] Cycloaddition Reaction of Nitrocinnamate (NC)



The selectivity of **1** toward Cu^{2+} comes from two aspects of factors: one is from the modular binding of the Gly-His motif and the other is from the N-terminal dansylation which thermodynamically enhanced the binding selectivity toward Cu^{2+} (details will be reported elsewhere). The binding model of this ligand with copper is presented in Scheme 3. Three nitrogen atoms of the peptide participated in the binding with copper: the sulfonamide nitrogen, one amide nitrogen, and the unprotonated imidazole nitrogen in the histidine residue.

3.3. Preparation of Gel Film by Photo-Cross-Linking. The synthesized PEG-NC macromer underwent photo-cross-linking to form a hydrogel network due to the [2+2] dimerization of nitrocinnamoyl groups (Scheme 4). An ethanol−water (2:1, v/v) solution containing PEG-NC macromer (1.67 mM) and peptide **1** (3.33 mM) was cast onto a quartz slide. The macromer underwent rapid photo-cross-linking upon irradiation at 365 nm, and formed a transparent water-insoluble gel sheet. Peptide **1** possesses the same cross-linking NC group, thus was immobilized within the gel simultaneously during photo-cross-linking.

3.4. Fluorescent Properties of the Cross-Linked PEG-NC/1 Film and Quenching by Cu^{2+}. In the photo-cross-linking process, peptide **1** reacted with the nitrocinnamoyl groups in PEG-NC macromer and therefore was covalently immobilized. We found out that the fluorescent properties of **1** in the gel were greatly affected by the polymer matrix. On the other hand, the incorporation of **1** endows the gel film special fluorescent properties which can be uniquely quenched by the presence of copper ions in aqueous subphase.

3.4.1. Fluorescence Excitation and Emission. The fluorescence of peptide **1** in the cross-linked gel film presented several different characteristics from that of pure **1** in aqueous solution, which is largely influenced by the PEG network (Figure 3). A red shift (approximately 35 nm) occurred for the excitation wavelength of the dansyl fluorophore and the bandwidth becomes larger; i.e., the full-width at half-maximum (fwhm) is 90 nm (from 336 to 426 nm). In contrast, **1** in aqueous solution presented a fwhm of 67 nm (from 318 to 385 nm). Obviously, the dansyl fluorophore in the gel can be excited at much longer wavelengths.

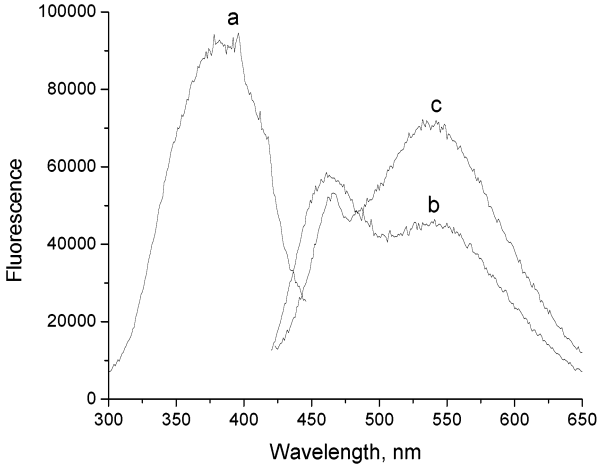


Figure 3. Fluorescence of photo-cross-linked gel film (PEG-NC/**1**). (a) Excitation spectrum, λem = 550 nm, (b) emission spectrum, λex = 350 nm, (c) emission spectrum, λex = 396 nm.

Peptide **1** in the gel film exhibited a fluorescence with maximum at 540 nm. This peak is relatively blue-shifted compared with that in aqueous solution whose maximum is 555 nm. It is known that the dansyl group is highly sensitive to its microenvironment: its emission intensity becomes stronger and exhibits a blue shift in hydrophobic media.[20,21] Therefore it is concluded that dansyl group sensed a more hydrophobic environment in the gel than in aqueous solution. Except the fluorescence from dansyl group, the cross-linked PEG-NC gel itself exhibited a fluorescence emission peak at 465 nm. This peak was partially overlapped with the emission of dansyl fluorophore, thus disturbing the detection of Cu^{2+}-induced fluorescent quenching. Fortunately, the relative ratio of the intensity at 465 nm versus 540 nm can be substantially reduced by using excitation at a longer wavelength (Figure 3, curves b and c). We used 396 nm as the excitation wavelength throughout our experiments to examine the fluorescent properties of the gel film toward binding with metal ions. At this excitation wavelength, the fluorescence of Dns remains a high intensity while the emission of PEG-NC is kept at a relatively low level. Although even longer wavelengths can be used to get the emission spectra, the strong Raman scattering from water gives additional disturbance to the fluorescence of the gel film.[22]

3.4.2. Efficiency of Immobilization. After the solution containing PEG-NC and peptide **1** was cross-linked on a quartz slide, the slide was put in water for swelling and the unconfined peptides were washed out of the gel. Very interestingly, no noticeable fluorescence from the aqueous solution was detected, which means that almost all the ligand molecules were trapped within the gel matrix. To further demonstrate the efficiency of chemical immobilization, we used the same procedure to prepare PEG-NC gel film but a different peptide, i.e., dansyl-Gly-Gly-His-Gly (**2**) was used to be immobilized (synthesis and fluorescent properties of **2** have been reported in ref 11). Obviously this ligand cannot be chemically trapped because it does not possess the NC group for intermolecular cross-linking. The fluorescence of this gel film exhibited just the characteristic emission of pure PEG-NC and no marked peak at 540 nm was observed as that in the PEG-NC/**1** system (Figure 4, curve a). Meanwhile, the aqueous solution in which the gel film was immersed for swelling presented a very strong fluorescence with a maximum at 555 nm, the characteristic emission of dansyl fluorophore in aqueous solution (curve b in Figure 4). These observations showed that ligand **2** was poorly trapped within the gel and got dissolved into the washing aqueous solution.

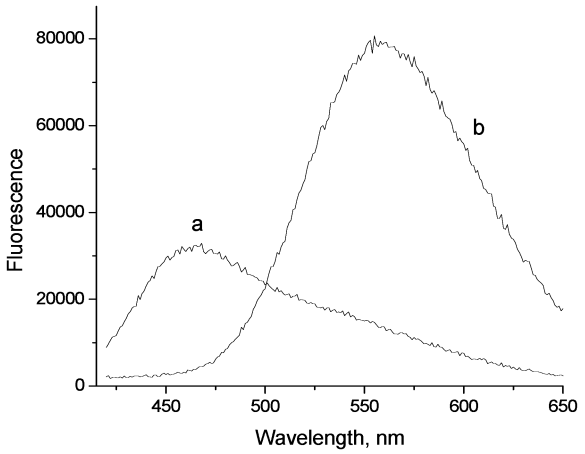


Figure 4. Fluorescence of photo-cross-linked gel film made from PEG-NC and Dns-Gly-Gly-His-Gly (**2**). The time of immersion in water is 30 min. (a) Gel film, (b) the immersing aqueous solution. λ_{ex} = 350 nm.

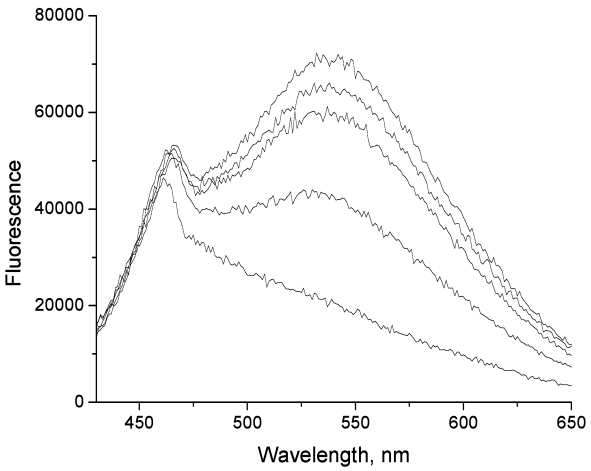


Figure 5. Fluorescence response of the photo-cross-linked gel film (PEG-NC/**1**) with the time of immersion in copper ion solution (10^{-5} M, pH = 7.0). From top to bottom, the immersion time is 0, 5 min, 28 min, 4 h, and 18 h, respectively. Excitation wavelength was selected at 396 nm.

By comparing these results, we conclude that there is an indispensable necessity of chemical bonding for the immobilization of small peptide molecules within a cross-linked polymer matrix.

3.4.3. Fluorescent Quenching by Cu^{2+}. The fluorescence of the PEG-NC/**1** gel film was quenched by copper ions in the aqueous buffer subphase (Figure 5). It is seen that the fluorescence intensity at 540 nm gradually decreased with the immersion time of the slide in Cu^{2+} solution (10^{-5} M). After 18 h of immersion, the fluorescence intensity reached a minimum value. Further immersion in copper solution only caused a marginal change to the emission spectra which indicates that the peptide ligand in the gel was fully complexed with Cu^{2+}. The side peak at 465 nm was composed of two parts: one is the Raman scattering of water and the other is the intrinsic fluorescence of PEG-NC itself as above-noted. From Figure 5, we see that this peak changed very little by the presence of copper ions. Thus copper ions mainly quenched the fluorescence of the dansyl fluorophore due to the specific complexation between the peptide and Cu^{2+}.

3.4.4. Fluorescent Reversibility. Reversibility is an important property of a sensor. After the analysis process is performed, the ligand−analyte complexation should be readily reversed.

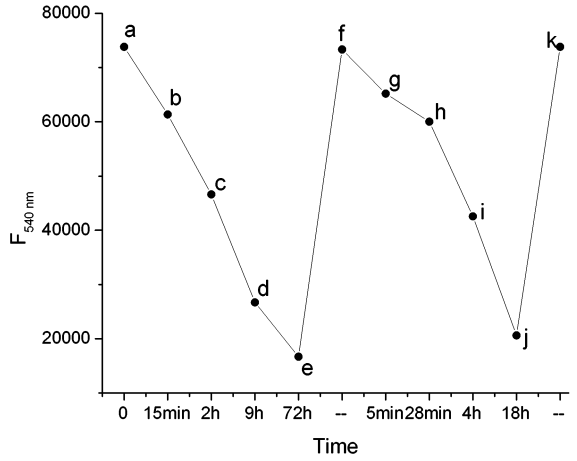


Figure 6. Reversibility of the photo-cross-linked gel film (PEG-NC/**1**). Excitation wavelength was selected at 396 nm. From a to e and f to j, the gel film was put in Cu^{2+} buffer solution (10^{-5} M, pH = 7.0). From e to f and j to k, the film was washed with HCl solution first and then put in aqueous buffer (pH = 7.0).

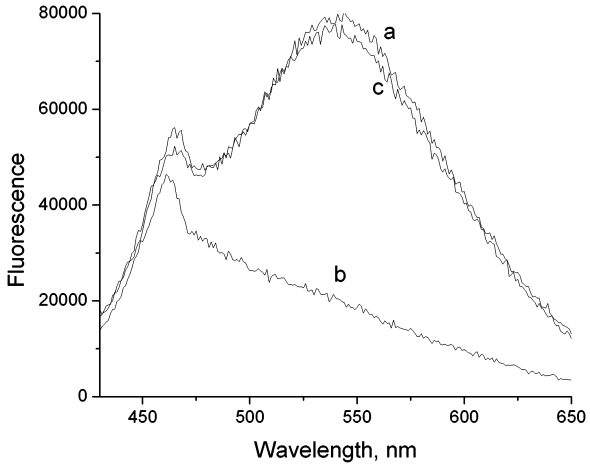


Figure 7. Fluorescent selectivity of photo-cross-linked gel film (PEG-NC/**1**) toward Cu^{2+} (pH = 7.0). (a) In aqueous buffer, (b) in Cu^{2+} solution (10^{-5} M) for 18 h, (c) in miscellaneous ion solution for 24 h. The miscellaneous ions contain Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and each has a concentration of 10^{-5} M. Excitation is selected at 396 nm.

In this process, the analyte (Cu^{2+} herein) was released from the sensor film, so the sensor was restored to its free state and can be used for next-time measurement. We illustrate the reversibility of the photo-cross-linked PEG-NC/**1** gel film in Figure 6. From point a through e, the slide was put in Cu^{2+} solution for 15 min, 2, 9, and 72 h, respectively. The fluorescence decreased with the immersion time. In the next step (from e to f), the slide was put in a HCl solution (pH = 2) first and then in a phosphate buffer (pH = 7.0). The immersion time in each solution can be as short as 1 min. In the acid treatment, the **1**−Cu^{2+} complex dissociated, thus Cu^{2+} was released from the gel film. It is seen here that the fluorescence was efficiently regenerated almost up to the intensity of the original fluorescence. From f to j, again the fluorescence was quenched by Cu^{2+} in aqueous buffer. Similarly from j to k, the fluorescence was restored by washing out Cu^{2+} from the gel with HCl solution. Therefore, an excellent reversibility was observed in this polymer/peptide system.

3.4.5. Selectivity of Cross-Linked Gel Film toward Cu^{2+}. We measured the fluorescent selectivity of the PEG-NC/**1** gel film toward Cu^{2+} (Figure 7). After the gel film was immersed in Cu^{2+} solution for 18 h, the fluorescence intensity of dansyl fluorophore decreased drastically and only 26.4% of the fluorescence intensity left at 540 nm. In contrast, in the miscellaneous ion solution (containing Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Ni^{2+}; 10^{-5} M each) for 24 h, the gel film exhibited a fluorescence which is almost overlapped with the original spectrum (99.6% at 540 nm). Therefore the fluorescence of the gel is highly selective toward Cu^{2+}.

The fluorescent selectivity of the gel film toward copper is also visually evidenced by fluorescent microscopy (Figure 8). Excited by UV light, the gel film in pure water presented a fluorescence of strong green color. However, in the presence of copper solution, the image became dim, indicating that copper ion quenched the fluorescence. Meanwhile, the image is slightly blue which is most probably due to the fluorescence of the PEG-NC gel at 465 nm. In the presence of the miscellaneous ion solution, the image is essentially the same as that in pure water, suggesting that the miscellaneous ions have none or little effect on the fluorescence of the gel film. All of these fluorescent microscopic images are in accordance with the fluorescence spectroscopic measurements shown in Figure 7, and demonstrate the high selectivity of the gel toward Cu^{2+} in aqueous solution.

4. Conclusion

In this study, we have described a method to prepare a membrane sensor for the fluorescent detection of copper ions in aqueous solution. The gel film was prepared from PEG-NC macromer via photo-cross-linking by 365-nm irradiation. Peptide **1** was immobilized efficiently within the gel matrix. The chemical bonding was found to be an important requirement for the immobilization. The gel film exhibited a fluorescence emission which is selectively quenched by copper ions in the aqueous subphase. After the fluorescent quenching, Cu^{2+} can

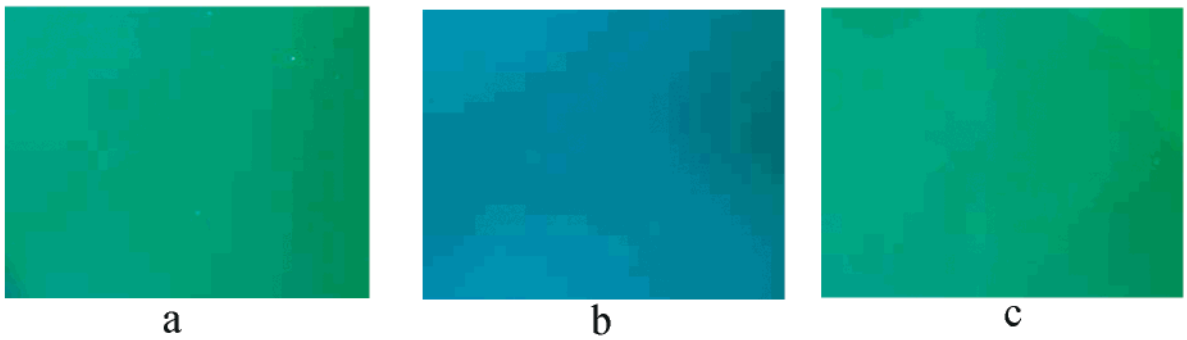


Figure 8. Epifluorescence images of the cross-linked gel film under different subphase. (a) In water, (b) in Cu^{2+} solution (10^{-5} M) for 15 h, (c) in miscellaneous ion solution for 20 h; image size 895 μm × 713 μm. The miscellaneous ions contain Fe^{2+}, Fe^{3+}, Zn^{2+}, Co^{2+}, Ni^{2+}, and each has a concentration of 10^{-5} M. UV light was used for the excitation. Phosphate buffer (pH = 7.0) was used for b and c.

be washed out with HCl solution and thus fluorescence of the gel was restored—a reversibility essential for a practical fluorescent sensor.

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