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# Combinatorial approach to the development of fluorescent sensors for nanomolar aqueous copper†

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**Abstract**

New fluorescent sensors for aqueous copper ions were developed via screening of metal ligand libraries. Identified ionophores were linked to a dansyl fluorophore reporter, affording copper ion sensors based on fluorescence quenching. Simple chemical modification of identified ionophores generated sensors responsive to a broad range of copper ion concentrations (10 nM–35 μM). © 2000 Elsevier Science Ltd. All rights reserved.

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The development of molecular sensors is an important goal of broad consequence, ranging from environmental monitoring to the study of intracellular processes.[1] For instance, fluorescent probes for measuring intracellular concentration of calcium ions have afforded conceptually new tools for cellular biology.[2] Fluorescence has frequently been used as a readout output due to its high sensitivity. In the case of metal ions, the physical mechanism of fluorescence sensing depends on the spin state of the metal ion.[3] Many promising fluorescent sensors, based on both protein and synthetic scaffolds, have been developed for a variety of metals, including Ca(II),[4] Zn(II),[5] Pb(II)[6] and Cu(II).[7] New trends in molecular sensing suggest that the integration of multiple signals, originating from a number of sensors having varying characteristics, provide more powerful sensing abilities than single sensor-based systems.[8] This, perhaps, shifts the ultimate goals of the chemical and biochemical community in this area, traditionally focused on the development of receptors of highest selectivity and affinity, to the generation of sets of receptors (ionophores, organic molecule receptors) covering a desirable range of properties.

We wish to report on the development of fluorescent chemosensors for copper ions in water. Our design was based on new copper ionophores generated via screening of combinatorial ligand libraries. The dansyl fluorophore was covalently linked to the ionophore,[9] followed by the

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† This paper is dedicated to Professor Harry H. Wasserman on the occasion of his 80th birtday.


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attachment of the hybrid structure to polymeric microspheres. The resultant chemosensors, compatible with an optical fiber detection system developed by Walt,[10] detected nanomolar aqueous copper ions in minutes as determined by fluorescent microscopy.

The first phase of our study centered on the generation of combinatorial libraries of metal ligands on solid support.[11] We synthesized a library based on three related scaffolds **I–III** (1470 members) in a split-remix format on Tentagel polymer resin (Fig. 1). The three different side chains were introduced via orthogonal Boc/Fmoc protection protocol. Incubation of the resultant library with a solution of $CuCl_2$ in HEPES buffer was followed by treating the beads with copper selective staining reagent (thiourea, $NH_2C(S)NH_2$) as schematically shown in Fig. 2. The positive beads were separated and decoded in a standard fashion.[12] Screening the library with a 2 μM solution of $CuCl_2$ identified multiple positive beads within hours. Employing the same library, 19 beads were positive at 10 nM concentration of Cu(II) in aqueous buffer. However, 5 days of incubation were required to identify the positive structures at this concentration, which we ascribed in part to slow diffusion of ions into the polymeric matrix. Two representative ligands **1** and **2** are shown in Fig. 2. All identified ionophores contained a carboxylic group as well as an aromatic nitrogen heterocycle. Pyrazine **1** was selected for subsequent detailed studies.

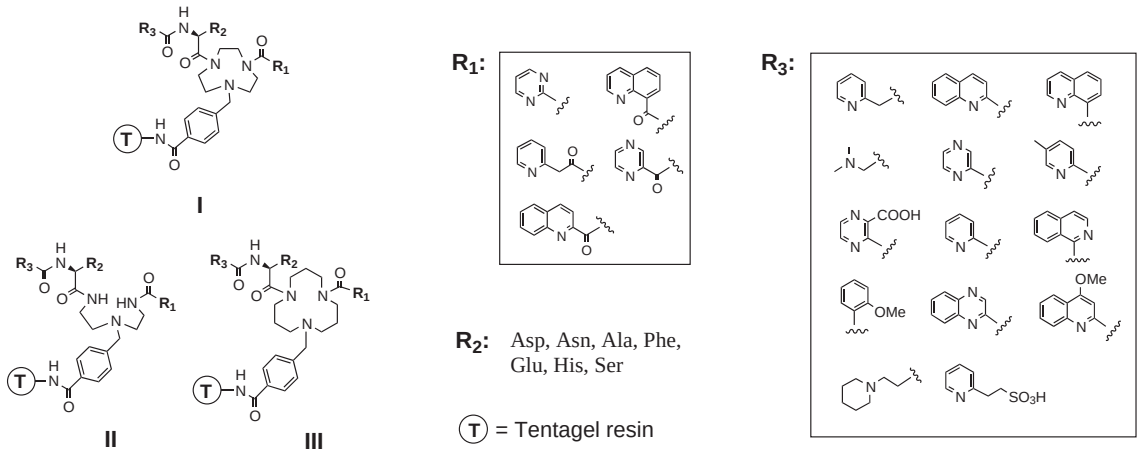



Figure 1. A library of ionophores was generated based on three scaffolds **I–III**

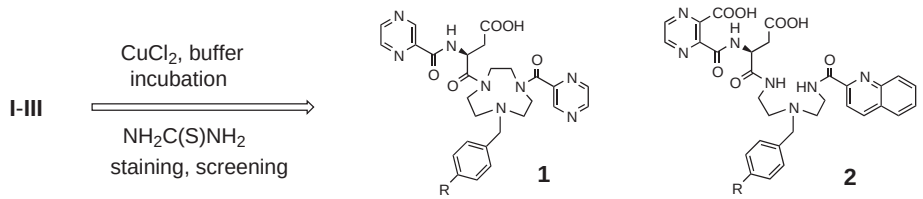
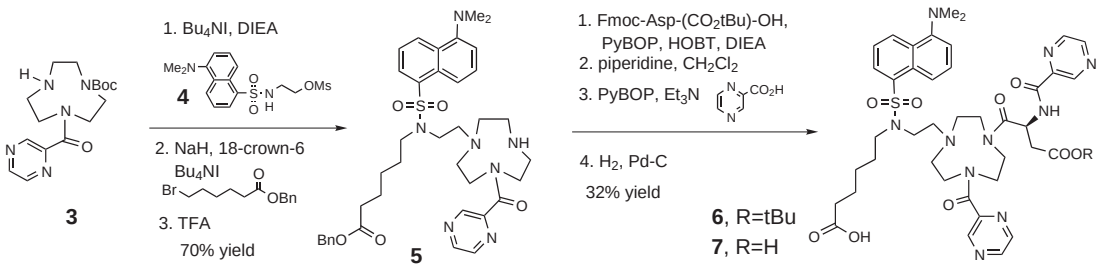



Figure 2. Screening for copper binding

The benzyl group in **1** was replaced by a new linker containing the dansyl fluorophore (Scheme 1). Starting material **3**, readily available from 1,4,7-triaza-cyclononane, was alkylated with mesylate **4**, followed by the subsequent alkylation of the sulphonamide with benzyl 6-bromohexanoate to yield compound **5**. The buildup of the third arm of the cyclen-based

structure was achieved via standard peptide coupling protocol, affording sensors **6** and **7**.[13] As was hoped, 0.1 equiv. of $CuCl_2$ per ionophore led to reproducibly measurable quenching of fluorescence in aqueous methanol ($H_2O$:MeOH 4:1). Increasing the amount of $CuCl_2$ to 1 equiv. resulted in a 50% decrease in fluorescence intensity. In addition, the quantitative fluorescence studies have been performed in the presence of other metal ions to determine relative selectivity of **6**, which demonstrated high relative selectivity values for Cu(II) ions ($S_{Cu/Co}>500$, $S_{Cu/Ni}=180$, $S_{Cu/Fe}=40$).[14]




Scheme 1.

The free carboxylic acid residue in **7** was subsequently used to attach the compound to polymeric microspheres (3.1 μm). The choice of microspheres was determined by the size of the microwells at the tip of optical fibers, developed by the Walt laboratory.[15] The resultant microsphere sensors were examined via fluorescent optical microscopy in the presence of aqueous copper ion solution. The results are summarized in Fig. 3.[16] Evidently, reproducible

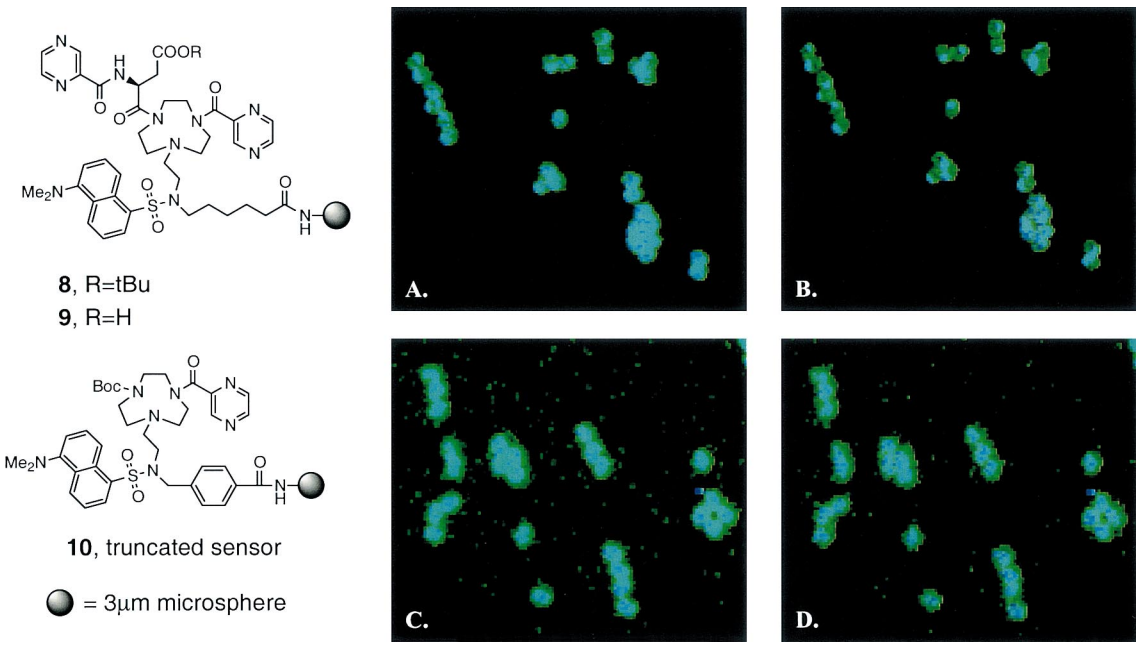



Figure 3. (A) Sensor **9** in HEPES buffer. (B) Sensor **9** in 50 nM $CuCl_2$ in HEPES, 20 min. (C) Sensor **10** in HEPES buffer, 0 min. (D) Sensor **10** in 5 μM $CuCl_2$ in HEPES, 60 min. 50× magnification

quenching of **9** in the presence of 50 nM $CuCl_2$ solution in HEPES buffer was observed within minutes (maximum quenching was reached within 20 minutes; **9**, $K_d = 10$ nM).[17] *tert*-Butyl ester **8** showed weaker affinity for copper ion by a factor of 39 ($K_d = 390$ nM). Apparently, the free carboxyl moiety contributes significantly to copper complexation. Furthermore, the presence of both pyrazine rings is crucial for copper ion recognition as demonstrated by dramatically reduced sensitivity of truncated sensor **10** ($K_d = 35$ μM). Reproducible quenching of **10** was detected in the presence of 5 μM solution of $CuCl_2$, three orders of magnitude lower sensitivity in comparison to **9**. Thus, simple chemical modifications of the highest affinity ionophore provided three unique sensors responsive to a range of copper ion concentrations spanning three orders of magnitude (nM–μM).[18]

In summary, we have developed sensitive fluorescent copper sensors via combinatorial approach. Due to the availability of a variety of staining reagents for transition metals, the power of the combinatorial approach in the search for selective ligands is evident. We have demonstrated that a combinatorial method provided high affinity receptors, which may, in turn, be desensitized via simple chemical disassembly or chemical modifications. The chemical simplicity, and robust nature of synthetic chemosensors is a very attractive characteristic of these systems.

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13. Product **7** was purified by column chromatography, and the purity determined by RP-HPLC and HRMS [calcd for $C_{44}H_{59}O_9N_{10}S$: 902.4109; found: 903.4207 ($M+H^+$)]. The receptor **7** was coupled to 3.1 μm resin using PYBOP, HOBt and DIEA in DMF shaken for 1 h. Following coupling, the resin was washed three times each with DMF, EtOAc and MeOH. The *t*-butyl ester was deprotected with TFA/$H_2O$ (95:5) (4 h) and washed three times with DMF, 2×DMF/DIEA (10%) (5 min each), 3×DMF, 3×MeOH. Following each wash, beads were centrifuged to separate solvent. Resin (3.1 μm) was purchased from Bangs Laboratories (Fishers, IN).
14. *Fluorescence assay in solution:* 10 μM of the complete receptor **1** in 10 mM HEPES was added separately to equivalent amounts of 10, 8, 6, 4, 2 μM $Cu^{2+}$ in 10 mM HEPES. The mixed solutions were analyzed by a spectrofluorometer. A control experiment was performed to assess the binding of Cu ions to the fluorophore itself, where 0.5 μM dansyl–ethanolamine was combined in MeOH with 0, 0.1, 0.2, 0.3, 0.4 and 0.5 μM Cu solutions. No quenching was observed in the control experiment.
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16. *Fluorescence assay with microspheres:* A small Petri dish was coated with a very thin layer of silicon adhesive and left to dry 0.5 h prior to bead application. GE silicon adhesive (GE Silicone II, 100% silicone) is commercially available. Dried beads were diapersed in $CH_2Cl_2$ and allowed to dry overnight. A Cu(II) solution was subsequently added to the Petri dish, and the images were focused and enhanced digitally. Petri dishes were covered to prevent evaporation. Following capture, images were further enhanced in Adobe PhotoShop. Imaging was done with a Nikon Fluor-50X on a Nikon Eclipse E800 upright microscope equipped with a mercury lamp and excitation (255–270 nm) and emission filters (470–540 nm). IPLab Spectrum version 2.1 software was used to both capture and digitally enhance images. Enhancement was also done in Adobe PhotoShop Version 5.0.
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