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Building Fluorescent Sensors by Template Polymerization: The Preparation of a Fluorescent Sensor for L-Tryptophan

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The development of fluorescent sensors for organic molecules is of great practical importance in chemical, biological, and pharmaceutical sciences. Using L-tryptophan as an example, we have studied a new way of making polymeric fluorescent sensors using template polymerization or molecular imprinting techniques. The fluorescent polymers were prepared using functional monomers with a fluorescent probe attached to it. The fluorescence of this polymer could be quenched by 4-nitrobenzaldehyde. Addition of the template molecules, L-tryptophan, increased the fluorescence intensity of the imprinted polymer/quencher mixture in a concentration-dependent fashion, presumably through the displacement of the quencher from the binding sites by L-tryptophan. This fluorescence intensity change upon mixing with L-tryptophan allows the binding event to be detected easily. The sensor also exhibited enantioselectivity for the template molecules. For example, the effect of D-tryptophan on the fluorescence intensity of the polymer is about 70% that of its L-enantiomer. Furthermore, the effect of L-phenylalanine and L-alanine on the fluorescence intensity change is much smaller than that of L-tryptophan. Because the approach used does not require the *de novo* design of the complementary binding site and does not rely on any specific structural features of the template molecule or prior knowledge of its three-dimensional structure, the same principle could potentially be useful for the future construction of practical fluorescent sensors for many other compounds.

INTRODUCTION

The development of fluorescent sensors for organic molecules is of great practical importance in chemical, biological, and pharmaceutical sciences (*1–4*). Recently, many very sensitive fluorescent sensors have been developed for peptides (*5*), metal ions (*6–9*), saccharides (*10–12*), and others (*11,13–19*). Most of the approaches that have been used so far are through *de novo* design of receptors with a built-in fluorescent probe. Such an approach, while has already yielded much progress, is somewhat tedious and requires the prior knowledge of the 3-dimensional structure of the analyte. Furthermore, applying similar strategy for the preparation of fluorescent sensors for other compounds requires almost complete new *de novo* design in each case based on the knowledge of the 3-dimensional structure of each analyte. One approach that does not require the prior knowledge of the 3-dimensional structure of the analyte

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and the complete *de novo* design of the complementary binding site is the construction of polymeric receptors through template polymerization or molecular imprinting.

Molecular imprinting is a technique first demonstrated in the late 1940s by Dickey (*20*). The preparation of imprinted polymers involves (Fig. 1): (1) prearrangement of the print molecule and the monomers at low temperature so that complementary intermolecular interactions among functional groups can develop; (2) polymerization of the monomers under conditions that cause minimal disturbance to the print molecule–monomer interactions; and (3) extraction of the print molecules from the polymers, which leaves behind "receptor sites" that are complementary to the templates or print molecules in terms of size, shape, and functional group orientations. This technique has been used for the preparation of selective recognition sites for a wide variety of molecules (*21–27*). There have also been extensive efforts in developing polymeric receptors that discriminate enantiomers using amino acids or peptides as models (*28–31*). Naturally, such polymeric receptors also have the potential to be developed as molecular sensors (*25,32–36*). One critical component of a practical sensor is the output of a readily detectable signal upon binding and dissociation of the target molecule. In most of the cases, the binding of the imprinted polymeric receptors was studied using chromatographic methods or radioligand binding assays. There have also been efforts in preparing polymeric receptors, for which the binding events could be monitored using other methods such as pH (*37*) and electroconductance measurements (*38,39*). Obviously, fluorescent sensors have the advantages of ready detectability and high sensitivity. Efforts have also been made to prepare fluorescent sensors using molecular imprinting methods with limited success (*15,35,40–43*). Most of these systems rely on the use of fluorescent ligands or fluorotag–ligand conjugates for the fluorescent detections (*35,40–43*). Therefore, such approaches can only be used for the detection of fluorescent molecules, but cannot be used as a way to build fluorescent sensors for nonfluorescent molecules. Developing a way to prepare polymeric receptors with a fluorescent probe built into the receptor could provide a general approach to the preparation of fluorescent sensors using molecular imprinting methods. One difficulty in this approach is that the fluorescent probe built into the polymer may or may not be sensitive enough to the binding event. Gin and coworkers have constructed a polymeric receptor for cholesterol with a fluorescent probe built into it (*15*). However, the fluorescence of the polymeric receptor was not sensitive to the binding event. Therefore, tailor-made fluorescent probes might be necessary for the development of fluorescent sensors for different compounds, which could be technically very difficult (*15*).

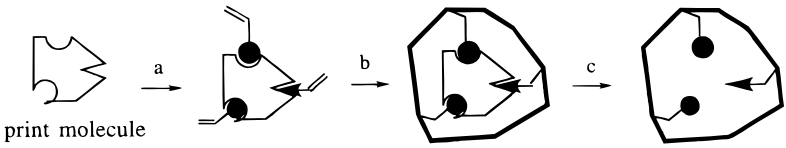


FIG. 1. A cartoon description of the molecular imprinting process.

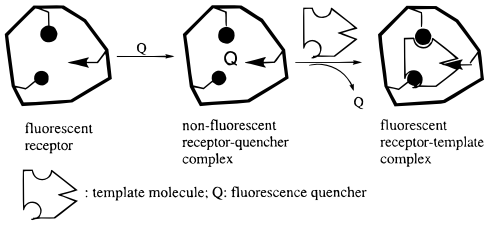


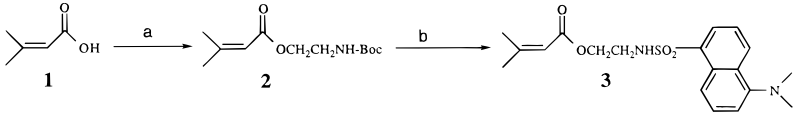
FIG. 2. A cartoon description of the competitive binding of the template molecule and the fluorescence quencher to the imprinted polymeric receptor.

In this report, we describe our efforts in searching for ways to make fluorescent sensors through template polymerization of fluorescent monomers. To develop a method that could be generally applicable to the preparation of fluorescent sensors for nonfluorescent molecules, we used an external fluorescence quencher in studying the binding of the imprinted fluorescent polymer. In the absence of the target molecules, the nonspecific diffusion of the quencher would result in the quenching of the fluorescence of the fluorotags either inside the binding cavities or on the surface of the polymer (Fig. 2). However, in the presence of the target molecules, the displacement of the nonspecific quencher from the binding cavities by the template molecules could result in a change of the fluorescence intensity of the imprinted polymer, therefore, allowing the binding event to be monitored conveniently. With this method, single digit μM concentration detection could be achieved. Further fine-tuning of this method could conceivably lead to the development of practical fluorescent sensors for a wide variety of compounds because the construction of such a fluorescent sensor does not require the print molecule to have certain specific structural features or to be fluorescent itself.

RESULTS AND DISCUSSION

The study is focused on demonstrating the proof of the concept, instead of developing a specific sensor for a particular analyte. We chose L-tryptophan as our initial model template. The imprinted polymers were prepared using 3,3-dimethylacrylic acid-based monomers. The dansyl moiety, attached to 3,3-dimethylacrylic acid (**1**) via an ethanolamine linker, was used as the fluorescent tag (**3**).

Polymer preparation. The synthesis of the fluorescent monomer (**3**) started with the ester formation between *N*-Boc-ethanolamine and 3,3-dimethylacrylic acid (**1**) through activation with DCC (dicyclohexylcarbodiimide) in the presence of DMAP (dimethylaminopyridine) to give **2** in 89% yield (Scheme 1). After the cleavage of



a. Boc-NH-CH_2CH_2-OH, DCC, DMAP,CH_2Cl_2, 89%; b. i) 25% TFA in CH_2Cl_2; ii) Dansyl-Cl,$NaHCO_3$/EtOAc, 80%.

SCHEME 1.

the Boc group using 25% TFA (trifluoroacetic acid) in methylene chloride, the ester (**2**) was reacted with dansyl chloride in the presence of $NaHCO_3$ in ethyl acetate to give the fluorescent monomer **3** in about 80% yield for two steps. The monomer (**3**) was then polymerized using ethyleneglycol dimethacrylate (EGDMA) as the cross linker and AIBN [2,2′-azobis-(2-methylpropionitrile)] as the free radical initiator in the presence of L-tryptophan. Such polymerizations could potentially be carried out either thermally or photochemically. In the photochemical method, the polymerization could be carried out at a lower temperature (0°C) using UV to initiate the generation of free radicals from AIBN. It is known that lower temperature polymerization tends to give polymeric receptors with more specific recognitions (*44,45*). However, when UV initiated polymerization was used at 0°C, no polymerization occurred within the first 48 h presumably because of the absorption of the UV light by the dansyl moiety, which hinders the UV-induced fragmentation of AIBN. Therefore, a thermal polymerization method was used for the preparation of these fluorescent-imprinted polymers. In a typical polymerization reaction, the molar ratio of the template (L-tryptophan), fluorescent monomer (**3**), and the cross linker (EGDMA) was about 1:4:40. Polymerization was carried out at 45°C for 48 h under a continuous flow of nitrogen. The polymer was then dried in a vacuum oven at 35°C overnight, ground and sieved with a 100-mesh sieve, and washed with mixtures of methanol-acetic acid. Then the polymer was dried again in a vacuum oven at 35°C for 12 h before use for the binding studies. Control polymers were prepared using the same method in the absence of the template molecule, L-tryptophan.

Binding studies without the added fluorescence quencher. The binding studies were carried out in two phases because of the low solubility of tryptophan and other amino acids in $CHCl_3$, in which the polymer was suspended. Approximately 10 mg/ml of the polymer was then suspended in chloroform, which was mixed with an equal volume of tryptophan or other amino acids in 0.03 M citric acid aqueous solution. After equilibrating for 4 h, the fluorescence spectrum of the organic phase was recorded (350 nm excitation). The effect of L-tryptophan on the fluorescence emission of the imprinted polymer was first examined by mixing the L-tryptophan solutions at different concentrations with the imprinted polymer suspension. However, only minor changes of the fluorescence emission of the imprinted polymer were observed at L-tryptophan concentration as high as 10 mM (Fig. 3). Specifically, upon mixing with l0 mM of L-tryptophan the emission λ_{max} changed from 481 to 485 nm with very little change in intensity (Fig. 3). This small change was not sufficient for it to be used as a fluorescent sensor. It also indicates that the binding of L-tryptophan probably only caused a minor change in the environment of the dansyl moiety. This is consistent with other literature studies of fluorescent polymers (*15*).

To overcome the problem of low sensitivity of the dansyl moiety to the binding event, we designed the method of using an external quencher to study the binding of the fluorescent polymer. The idea is to use a small molecule quencher, which can freely diffuse in and out of the binding sites, to quench the fluorescence of the dansyl moiety of the polymer (Fig. 2). The occupation of the binding sites of the polymer by the fluorescence quencher would quench the fluorescence of the polymer. However, upon addition of the more specific ligand, the template molecule, the quencher can be displaced, which in turns causes a significant fluorescence intensity change (Fig. 2).

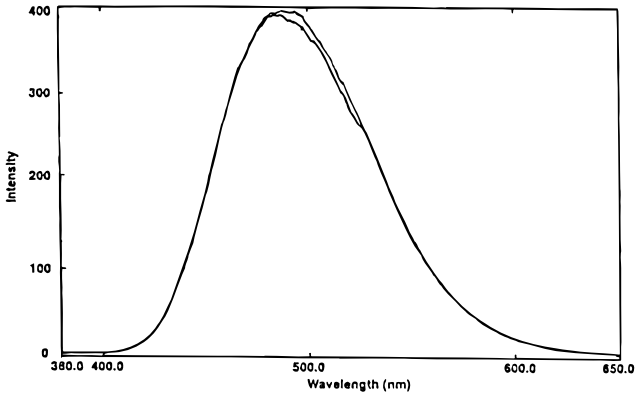


FIG. 3. The effect of L-tryptophan on the fluorescence emission of the L-tryptophan imprinted polymer in the absence of the fluorescence quencher, *p*-nitrobenzaldehyde. Top, imprinted polymer equilibrated with 10 mM L-tryptophan. Bottom, imprinted polymer itself.

The effect of the added fluorescence quencher on the fluorescence of the dansyl moiety in different environments. We selected *p*-nitrobenzaldehyde as the fluorescence quencher because it is smaller than L-tryptophan and is expected to be able to diffuse in and out of the binding cavities for L-tryptophan. We first examined the effect of this quencher, *p*-nitrobenzaldehyde, on the fluorescence of the dansyl moiety in the fluorescent monomer (**3**) in solution, in the control polymer, and in the tryptophan-imprinted polymer (Fig. 4). It was found that *p*-nitrobenzaldehyde was able to quench the fluorescence of the dansyl moiety in a concentration-dependent fashion in all cases. The quenching efficiency was higher for the fluorescent monomer (**3**) in solution than for the polymers (Fig. 4). It is interesting to note that the quenching of the fluorescence of the dansyl moiety of the control polymer was more efficient than that of the imprinted polymer, which in turn was more efficient than the quenching of the fluorescence of the imprinted polymer in the presence of the template molecule, L-tryptophan (Fig. 4). It is easy to understand the lower quenching efficiency of the imprinted polymer in the presence of L-tryptophan because of the protection of the "receptor" sites through the binding of the template molecules, which makes certain part of the imprinted polymer inaccessible by the fluorescence quencher, *p*-nitrobenzaldehyde. It is also easy to understand the difference in the quenching efficiency between the polymers (control or imprinted) and the fluorescent monomer (**3**) in solution because the dansyl moiety in a polymer is not expected to be as readily accessible as it is in solution by the quencher, *p*-nitrobenzaldehyde. However, the different quenching efficiencies for the control and imprinted polymers were somewhat unexpected. This difference helps to further indicate that the "imprinting process" does significantly modify the properties of the polymers even at a macroscopic level so that the overall accessibility of the fluorescent moiety is altered. The effect of the "imprinting process" on the macroscopic overall accessibility of the fluorescent moiety of the polymers can be correlated with the effect of the "imprinting process" on the microscopic structures of the imprinted polymers. For one thing, the structures of the

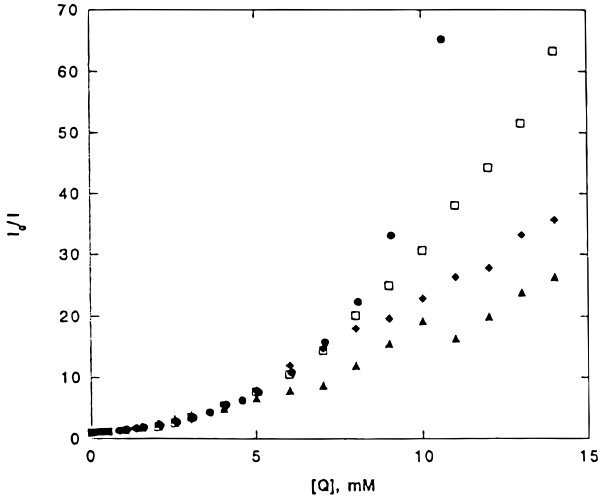


FIG. 4. The concentration-dependent quenching of the fluorescence of fluorescent monomer **3** (●), control polymer (□), L-tryptophan imprinted polymer (◆), L-tryptophan imprinted polymer equilibrated with 10 mM L-tryptophan (▲). *p*-Nitrobenzaldehyde was used as the quencher (*Q*). I_0, fluorescence intensity without the added quencher. *I*, fluorescence intensity at different concentrations of the added quencher. All experiments were run in triplicates.

imprinted polymer are probably more well-organized due to the formation of the "receptor sites" and the structures of the control polymer should be more random. It should also be noted that the emission λ_{max} of the fluorescent monomer **3** (497 nm), the control polymer (485 nm), and the L-tryptophan-imprinted polymer (480 nm) in $CHCl_3$ were also different, further indicating that the environment of the fluorescent moiety in the imprinted polymer was different from that of either its solution form (**3**) or the control polymer. The different structural properties of the imprinted polymer and the control polymer could very well alter the overall accessibility of the polymeric fluorescent moiety and, therefore, the quenching efficiency.

Binding studies with the added fluorescence quencher. Subsequent L-tryptophan binding studies were carried out at fixed concentration of the quencher (3 mM), *p*-nitrobenzaldehyde. At 3 mM concentration of the quencher, the fluorescence intensity of the fluorescent polymer was reduced by more than 75%. Addition of L-tryptophan was able to decrease the quenching efficiency of *p*-nitrobenzaldehyde and, therefore, increase the fluorescence intensity of the imprinted polymer, presumably through the displacement of the quencher from the binding cavities by L-tryptophan (Fig. 2). Figure 5a shows the L-tryptophan concentration effect on the intensity of the fluorescence. The fluorescence intensity of the polymeric fluorescent sensor changed by up to about 40% (Figs. 5a and 6; Table 1) upon mixing with a L-tryptophan aqueous solution in the presence of the fluorescence quencher, *p*-nitrobenzaldehyde, thus allowing the binding event to be detected easily. Addition of L-tryptophan to the mixture of the quencher (3 mM) and the control polymer, however, did not result in such a change (Fig. 5b). To probe the selectivity of the polymeric receptor, we also studied the effect of D-tryptophan, L-phenylalanine, L-alanine, indole, tryptamine, and 3-indolepropionic

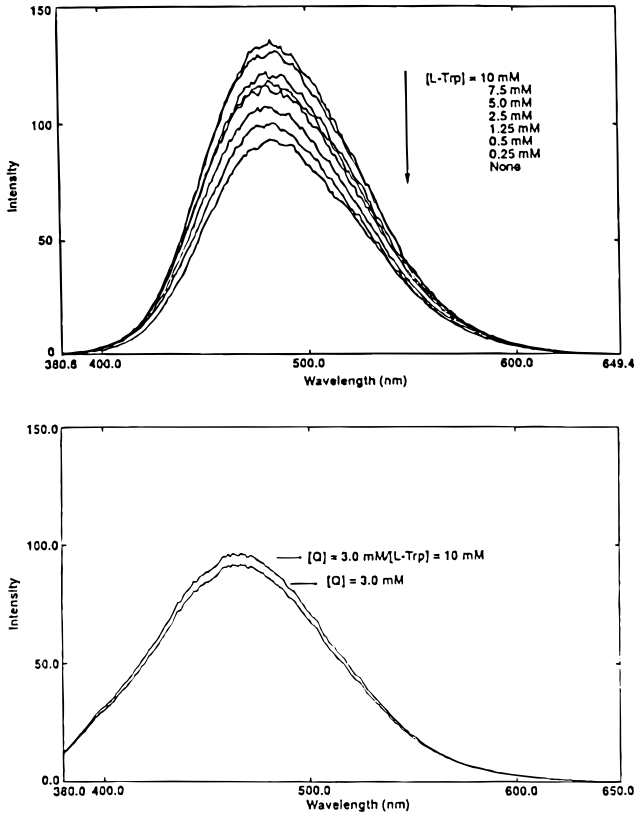


FIG. 5. (a) The effect of L-tryptophan on the fluorescence intensity of the L-tryptophan imprinted polymer in the presence of 3 mM of *p*-nitrobenzaldehyde. (b) The effect of L-tryptophan on the fluorescence intensity of the control polymer in the presence of 3 mM of *p*-nitrobenzaldehyde (*Q*, *p*-nitrobenzaldehyde).

acid using the L-tryptophan-imprinted polymer. As shown in Fig. 6, D-tryptophan, L-phenylalanine, L-alanine, indole, and 3-indolepropionic acid were not able to reduce the quenching effect of *p*-nitrobenzaldehyde as much as L-tryptophan, indicating that their affinities for the polymeric receptors were lower than that of L-tryptophan, the template molecule. Table 1 summarizes the quantitative results from these studies. The use of D-tryptophan in the study was designed to probe the enantioselectivity of the imprinted polymer. As shown in Fig. 6, the effect of D-tryptophan on the fluorescence intensity of the polymer was about 70% that of its L-enantiomer, indicating certain enantioselectivity of the imprinted polymer. The effect of L-phenylalanine was about 60% of that of L-tryptophan, indicating that the imprinted polymer was able to recognize the somewhat minor differences in the side chain structures between the two aromatic amino acids with the same stereochemistry. The effect of L-alanine on the fluorescence intensity of the imprinted polymer was much smaller than that of L-tryptophan ($<$20%) (Fig. 6 and Table 1), indicating that the "imprinting process" was able to afford the recognition of the bulky hydrophobic side chain of tryptophan.