Sensor Particles

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Fluorescent Sensory Microparticles that "Light-up" Consisting of a Silica Core and a Molecularly Imprinted Polymer (MIP) Shell**

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Molecularly imprinted polymers (MIPs) are an established and powerful medium for the selective enrichment and separation of chemical species, in particular for small organic molecules carrying functional groups. They are generally assembled by polymerization of a mixture of functional monomers and cross-linkers in the presence of a template that is analogous to the target compound. After extraction of the template, cavities which are complimentary in shape, size, and electronic or hydrogen-bonding demand remain in the cross-linked polymer matrix, ready to selectively recognize and bind the target molecule.[1] Because their preparation, habitus, and function show parallels to a certain molecular recognition system from nature, MIPs are frequently referred to as "artificial antibodies".[2] Although MIPs can accomplish rather advanced analytical tasks, such as chiral discrimination,[3,4] the key step for future success in a broader range of applications is introducing additional functional features.

One important goal concerns the integration of a signaling element so that the binding events can be directly assessed with a sensitive technique, such as fluorescence.[5] Such signaling MIPs would be a sensor material and would expand the application of MIPs in fluorometric analysis beyond the detection of fluorescent analytes,[6] the fluorescence tagging of analytes prior to detection in the MIP,[7] and displacement assays.[8] In the latter two approaches, the MIP is only used for separation. Detection has to be carried out in a second, discontinuous step, which is not ideal for sensor applications.

MIPs in which fluorescent moieties are directly incorporated in the polymer are scarce. Moreover, the quenching of a covalently embedded dye, lacking designated receptor sites, can only be employed for analytes which are potent quenchers.[9] The perhaps most obvious approach, the covalent integration of a fluorescent probe monomer into a MIP, however has only seldom been accomplished,[10–14] and especially examples showing directional recognition at a designated binding site[12–14] or fluorescence enhancement upon analyte binding are rare.[13] The most appealing type, fluorogenic MIPs that simply "light up" in an analytically useful wavelength range upon binding of the analyte have, to our knowledge, not been reported.[15,16]

To develop MIPs that show an enhancement of fluorescence upon analyte binding and perform well in molecular recognition, we chose *N*-carbobenzyloxy-L-phenylalanine (Cbz-L-Phe or Z-L-Phe, Scheme 1) as our template. It is an established building block in peptide synthesis and a frequent model target for the development of MIPs for enantiopurity control of synthetic peptides.[4] We constructed the fluorescent monomer **1**, from a nitrobenzoxadiazole (NBD) fluorophore carrying a directly fused urea group as the carboxylate recognition site, a short ethylene spacer, and methacrylate polymerizable unit (Scheme 1). When equipped with an electron-donating group in the 4-position, NBD dyes show intense absorption and fluorescence bands at around 450 and 550 nm, respectively, arising from an intramolecular charge transfer (ICT) process,[17] and have thus been used as molecular probes for many years.[18] Accordingly, we reasoned that introduction of the moderate electron-donating 4-urea group and subsequent binding of an electron-rich carboxylate guest at this Y-shaped hydrogen-bonding site should lead to bathochromic shifts and an increase in NBD fluorescence.

Compound **1** was prepared from 4-amino-NBD[19] and 2-isocyanatoethyl methacrylate by using 4-(dimethylamino)-pyridine as a catalyst and butylhydroxytoluene as a stabilizer. Before attempting molecular imprinting, it is important to choose a solvent that is suitable for complex formation between probe monomer and the template as well as for polymerization, in this case for RAFT (reversible addition–fragmentation chain-transfer) polymerization. This technique promised to be the way to obtain the MIP matrix as a thin polymer film on silica microparticles (MPs). RAFT polymerization is not only quasi-living, but also leads to more-homogeneous networks, more-accessible sites, and hence higher binding capacities.[20] MPs were the format of our choice because they can be used as individual sensor units yet can also be integrated in sensor membranes. Investigation of

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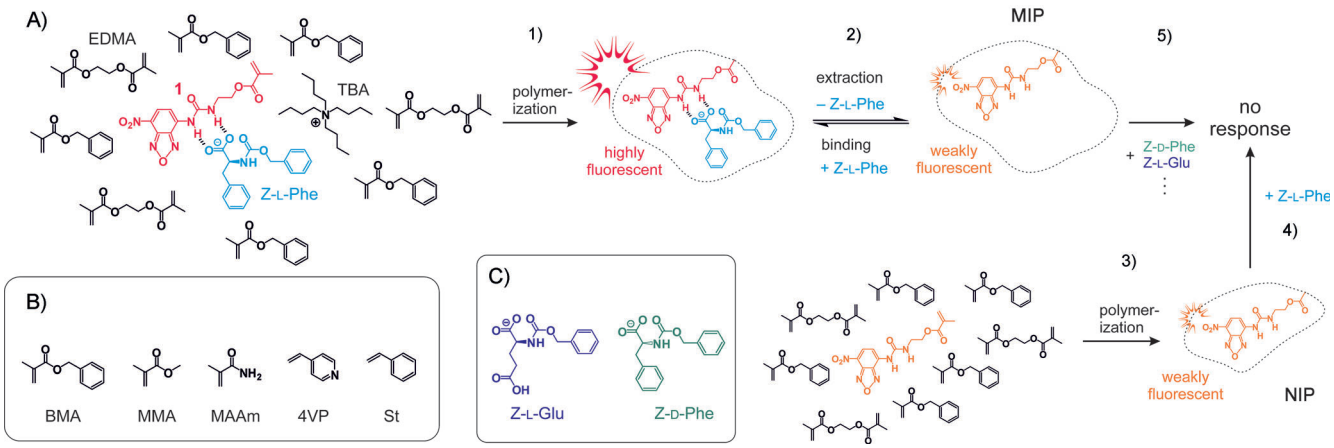
Supporting information for this article (details of syntheses and characterization of compounds and materials; instrumental details; additional NMR, TEM, TGA, FTIR, and optical spectroscopic data) is available on the WWW under http://dx.doi.org/10.1002/anie.201300322.



Scheme 1. A) Preparation of a highly fluorescent MIP (1). Extraction and rebinding of the template switch between high and low fluorescence (2). Preparation of the non-imprinted polymer (NIP, 3) and analytically relevant nonspecific binding (4) and cross-sensitivity (5) reactions complementing the performance parameters. B) Co-monomers and C) other analytes investigated.

1 with the tetrabutylammonium (TBA) salt of Z-L-Phe in various solvents suitable for RAFT polymerization (acetonitrile, chloroform, tetrahydrofuran) revealed that chloroform is the best choice, because both the spectral shift and fluorescence enhancement induced by complex formation are most pronounced in this solvent (Figure 1 and Figure S1 and S2 in the Supporting Information).

One of the crucial points in using urea units for carboxylate recognition through the formation of two directional hydrogen bonds is to adjust the acidity of the urea protons to such an extent that strongest possible complex formation is achieved yet undesired deprotonation is avoided.[21] Deprotonation would give rise to nondirectional electrostatic interactions at the expense of directional H-bonding and would hamper the formation of cavities with desired binding sites. Deprotonation is usually detected in the titration spectra of ureas and carboxylates by a strongly red-shifted (often by about 100 nm) absorption band.[22] In addition, anionic ICT dyes are usually nonfluorescent which is detrimental for fluorescence sensing,[23] and an anionic probe is much less selective in recognizing a hydrogen-bonding guest. Figure 1 A shows that in $CHCl_3$, only after addition of a 7-fold excess of Z-L-Phe, an absorption band at about 520 nm develops, which is tentatively ascribed to deprotonation (for further NMR spectroscopic support, see Section 7 of the Supporting Information).

Figure 1 also reveals that favorable sensing features can be expected for the MIP. As well as bathochromic shifts of about 30 and 10 nm in absorption and fluorescence, respectively, complex formation between **1** and the template leads to pronounced fluorescence enhancement (Figure 1 B). In contrast, in MeCN and THF, both polar solvents with electron lone pairs that can facilitate deprotonation, the bathochromic shifts and fluorescence enhancement upon H-bonding are less pronounced and deprotonation occurs already at equimolar concentrations (Figure S1, S2). Analysis of the titration data of **1** and Z-L-Phe in $CHCl_3$ according to a 1:1 binding model yielded a complex formation constant $\log K = 5.11$ (Figure S3), which is suitable for imprinting.[24]

Being aware of the influence of species concentration on hydrogen bonding and deprotonation equilibria from studies by other groups,[25] a crucial step was to assess the spectroscopic features of the actual pre-polymerization mixture. Figure 1 C shows that at equimolar concentrations (1 mM) of **1** and Z-L-Phe, the amount of $\mathbf{1}^-$ formed in $CHCl_3$ is higher than at 5 μM (Figure 1 A). However, in accordance with the

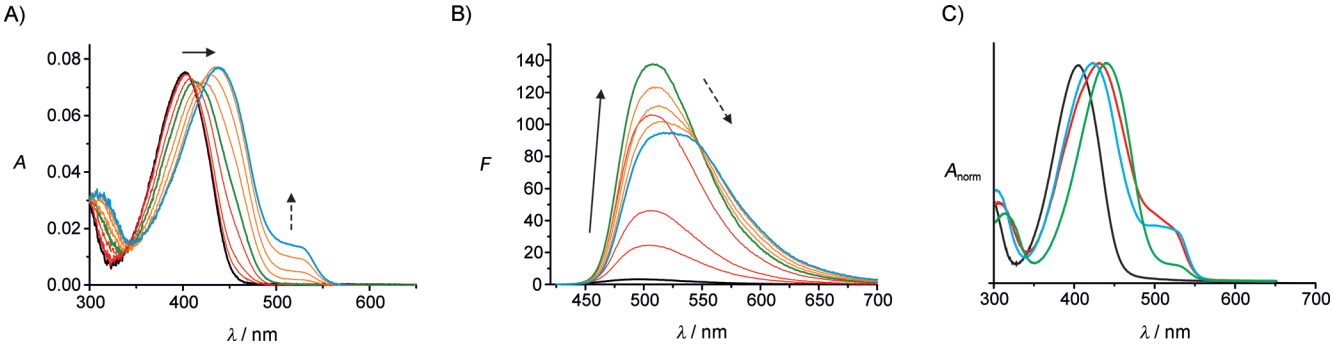


Figure 1. Absorption (A) and fluorescence (B) spectra of **1** ($c = 5$ μM) in $CHCl_3$ in the absence (black) and presence of Z-L-Phe/TBA (red: 0.4, 1, 3 equiv Z-L-Phe; green: 5 equiv; orange: 7, 10, 15 equiv; blue: 20 equiv). Absorption spectra (C) of the pre-polymerization mixtures of **1** ($c = 1$ mM) for the NIP in $CHCl_3$ (black) and the MIP for stoichiometric imprinting of Z-L-Phe/TBA in $CHCl_3$ (green), THF (blue) and MeCN (red).

low-concentration data, stoichiometric imprinting should suffer less from this unwanted side reaction in $CHCl_3$ than in MeCN and THF (Figure 1C). In addition, studies of the temperature-dependent dissociation revealed that the complex is sufficient stable at elevated temperatures (up to 80 °C), which are used in the curing step of the polymerization (here actually 70 °C, Figure S4).

Having established the signaling response, we prepared the sensing material. For this purpose, we chose core–shell MPs because MPs are a very versatile platform and can be employed in many different formats. In addition, thin polymer shells on robust core particles can be reproducibly prepared by surface-initiated polymerization[26] and MIP matrices a few nanometers thick promise to yield fast response times and permit the quantitative extraction of the template in a comparatively short time.[27]

Silica particles of about 300 nm were used as the support. This strategy has the advantages of offering a higher density of the silica core, which facilitates the handling of the beads in various applications, and that silica MPs are well-suited for optical detection techniques. As well as **1** and Z-L-Phe/TBA, EDMA (ethylene glycol dimethacrylate) was used as a moderately polar cross-linker and various (aromatic and aliphatic) monomers of different polarity were employed as additional co-monomers (Scheme 1, Table S1). To achieve a homogeneous polymer layer around the SiO_2 core, a RAFT agent (2-(2-cyanopropyl)dithiobenzoate, CPDB) was coupled onto the aminosilane-activated SiO_2 surface prior to MIP formation. Representative TEM images of the core–shell MIP particles revealing a shell thickness of approximately 10 nm are given in the Supporting Information.

The performance of the sensor particles was assessed in chloroform by titration with the designated analyte Z-L-Phe, the potential competitors Z-D-Phe as the enantiomeric twin, and Z-L-Glu as a conformationally closely related amino acid having two possible H-bonding sites instead of one. As detailed in Table S1, particles CS8 prepared with benzylmethacrylate (BMA) as the co-monomer yielded the highest fluorescence enhancement upon addition of Z-L-Phe and the best discrimination of MIP versus non-imprinted polymer (NIP) as well as the best discrimination against Z-D-Phe and Z-L-Glu. After comparing polymers prepared using the other co-monomers, we ascribe this beneficial effect to the presence of the benzyl groups, which offer additional aromatic moieties for further π–π interactions with the phenyl groups of Phe and Cbz (see Table S1 and description in the Supporting Information).

A typical fluorescence response of CS8 toward Z-L-Phe is shown in Figure 2A,B. The advantage of the thin MIP shell is also clear as full equilibration is obtained in about 15 s (Figure S5). Moreover, Figure 2 also illustrates the different responses of CS8-MIP and -NIP and the discrimination against the two other protected amino acids investigated.[28] Again, a fluorescence enhancement is obtained for CS8 and Z-L-Phe, although not as strong as that of neat **1** in $CHCl_3$ (Figure 1). This difference is because the environment around immobilized **1** in the particle shell is more polar as shown by the red-shifted absorption maximum at 412 nm of **1** in CS8 suspended in $CHCl_3$. The red shift resembles the solvatochromic shifts of **1** in MeCN or MeOH (both 411 nm) and contrasts with **1** in $CHCl_3$ (403 nm) or toluene (404 nm). If we further consider that the fluorescence quantum yield Φ_f of NBD dyes increases with increasing solvent polarity[17] and $\Phi_f(\mathbf{1})$ was determined to be 0.002 in $CHCl_3$, 0.01 in THF, and 0.024 in MeCN, a $\Phi_f = 0.015$ for CS8 supports the conclusion drawn from the absorption measurements.[29] For single-particle assays such an intrinsically higher fluorescence of particles containing **1** is advantageous because sufficient signal intensity can be expected across the entire concentration range. Analytical studies of CS8 finally revealed a quantification limit in a cuvette-based experiment of about 3 μM and a detection limit (3σ) of 60 nM for Z-L-Phe/TBA in $CHCl_3$.

Although the particles would be suitable for the direct assessment of enantiomeric purity during the synthesis of stereochemically pure drugs in the pharmaceutical industry provided organic solvents are employed, binding studies in neat water did not yield a measureable response. Apparently, the thin polymer shell, though prepared from rather nonpolar building blocks, is not hydrophobic enough to shield the hydrogen-bonded complexes efficiently from competing water molecules. To enable the system to detect analytes in

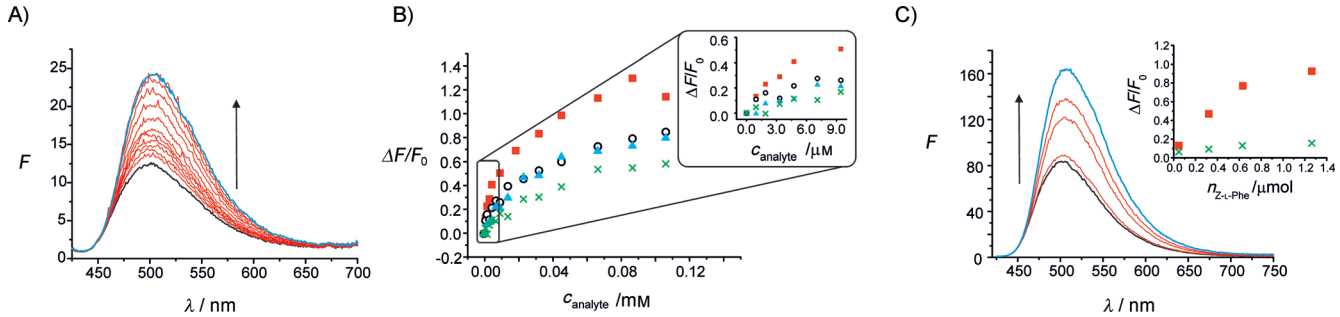


Figure 2. A) Fluorescence spectra of CS8 in $CHCl_3$ in the absence (black) and presence of Z-L-Phe/TBA (red: 0.001–0.085 mM; blue: 0.09 mM). B) Normalized fluorescence changes $\Delta F/F_0$ (with $\Delta F = F - F_0$) versus analyte concentration for CS8 and Z-L-Phe (red squares), CS8 and Z-D-Phe (blue triangles), CS8 and Z-L-Glu (circles), and CSN8 and Z-L-Phe (crosses; CSN8 is the NIP analogue of CS8). Note that the slight decrease at over 0.1 mM is due to the onset of deprotonation. C) Fluorescence spectra of CS8 in the $CHCl_3$ phase in the absence (black) and upon addition of various amounts of Z-L-Phe/TBA to the water phase (red: 0.1–0.7 μmol; blue: 1.4 μmol; after stirring and phase separation). Inset: Corresponding fluorescence titration curves of CS8 (squares) and CSN8 (crosses) with Z-L-Phe (symbols as in B).

aqueous media, we developed a phase-transfer method. In a 10 mm quartz cell, CS8 (10 mg) was suspended in $CHCl_3$ (2 mL) before water (1 mL) was added. 0.1–1.4 μmol of the target Z-L-Phe (as TBA salt) were then added under stirring, and the system was left to settle for 5 min before the fluorescence spectra were measured. Subsequently, the amount of Z-L-Phe that remained in the aqueous phase was measured by HPLC (control experiments of identical regime yet in the absence of sensor particles were conducted to account for the partioning of Z-L-Phe in the neat biphasic system). Figure 2C shows representative titration spectra and curves, verified by the indirect determination with HPLC. It is clear that not only the fluorescence "light-up" of CS8 with Z-L-Phe is preserved in the extraction-based approach, but that nonspecific binding is even reduced (cf. CSN8 data in Figure 2C, inset); the selectivity and response time are also preserved.

In conclusion, we have demonstrated for the first time the integration of a fluorogenic monomer into a MIP matrix that absorbs and fluoresces in an analytically important wavelength range and that "lights up" upon binding to the analyte. The amount of nonspecific binding and the enantiomeric discrimination observed are promising when compared with previous values for MIP sensing systems utilizing fluorescence[11,30] or other detection techniques, such as quartz crystal microbalance (enantiomeric discrimination less than 2).[31] Integration of the MIP as a thin shell on silica microparticles makes handling of the sensor particles straightforward and assay response times fast. We could also demonstrate that such a system cannot only be directly employed in pharmaceutical process-control in organic solvents, but also in a simple phase-transfer assay, which allows the assaying of analytes in aqueous solution. Very recent work by others[16] and the work presented by us herein suggest a bright future for fluorescent MIP sensor particles.

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