Luminescence Chemosensors

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Modular Chemosensors from Self-Assembled Vesicle Membranes with Amphiphilic Binding Sites and Reporter Dyes

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Transition metal complexes with vacant coordination sites have found wide use as binding sites in the development of molecular probes and chemosensors.[1] Such probes typically consist of the guest binding site and a luminescent reporter group that enables convenient monitoring of ligand-binding events. The luminescent moiety can either be a part of the actual molecular chemosensor, usually in close proximity to the analyte binding site,[2] or a separate dye that is first non-covalently attached to the binding site and later displaced by the analyte (indicator displacement assay).[3] Both strategies have drawbacks however, as the rational design of luminescent chemosensors is still difficult and their synthesis laborious, while the response of indicator displacement assays is typically not reversible. Herein we describe a novel approach to signal analyte binding by noncovalent co-embedding of amphiphilic non-fluorescent binding sites[4] and reporter dyes into the membrane of small unilamellar vesicles. The receptor binding is communicated through the membrane to the co-embedded dyes,[5] which change their emission properties. We demonstrate a simple and versatile approach to combine several different recognition sites with fluorescent reporter groups in a lipid bilayer.[6]

Three amphiphilic artificial binding sites, 1,4,7,10-tetraazacyclododecane (cyclen)–zinc(II) complex **1** for phosphate anion binding,[7] a nitrilotriacetic acid (NTA)–copper(II) complex **2** for the recognition of imidazole derivatives,[1a] and a benzoaza crown ether (BACE) **3** as host for ammonium ions,[8] and two amphiphilic fluorophores based on carboxyfluorescein (**4**) and coumarin (**5**) were incorporated into vesicle membranes (Figure 1). Compounds **1** and **5** were reported previously by us;[4c,9] syntheses of compounds **2, 3**, and **4** are described in the Supporting Information.

The widely used carboxyfluorescein (CF) dye was selected owing to its sensitivity to local electrostatic potential[10] and easy derivatization with alkyl amines. The sensitivity of coumarin derivative **5** to changes in the local environment had been observed in an earlier study.[11]

Luminescent vesicular receptors (LVRs; Figure 2) with phospholipid bilayer-embedded receptors and fluorescent dyes were prepared following our previously reported procedure.[4c] Vesicles **LVR-1,5**, containing co-embedded cyclen–zinc(II) complex **1** and coumarin derivative **5**, exhibit a strong fluorescence emission with a maximum intensity at 408 nm. In the presence of increasing amounts of phosphate anions, such as pyrophosphate (PP_i) or phosphoserine (pSer), this emission intensity is significantly decreased (Figure 3 top). The embedded zinc(II)–cyclen complexes retain their known selectivity for phosphate anions, and the addition of other anions gave no emission response (see Supporting Information). We explain this change in emission intensity by a rearrangement of the membrane structure induced by the phosphate anion coordination towards the embedded receptor molecules: Dye **5** and artificial receptor **1**, if embedded together in the lipid bilayer, are not expected to be homoge-

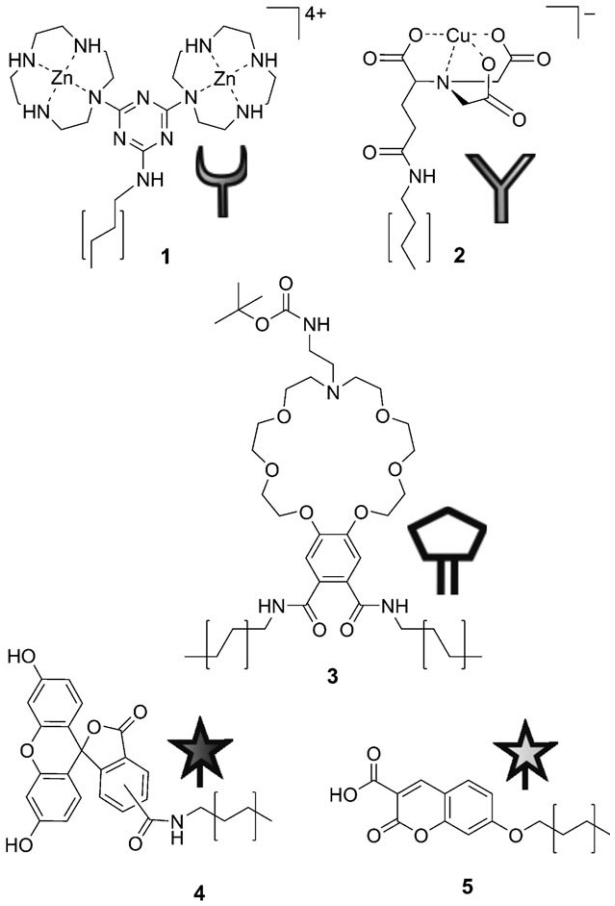

Figure 1. Amphiphilic artificial binding sites based on Zn^{II}–cyclen (**1**), Cu^{II}–NTA (**2**), and BACE (**3**), and amphiphilic fluorescent reporter dyes derived from carboxyfluorescein (**4**) and coumarin (**5**).

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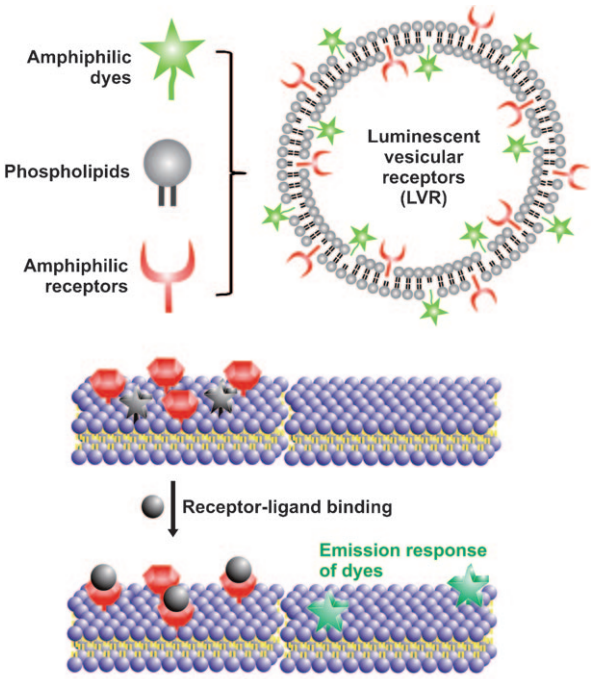

Figure 2. Preparation of luminescent vesicular receptors (LVRs) and their analyte response: Self-assembly of lipids, amphiphilic artificial receptors, and amphiphilic fluorescent dyes in buffered aqueous solution yields surface-modified vesicles that respond to receptor–ligand binding by a change in their emission intensity.

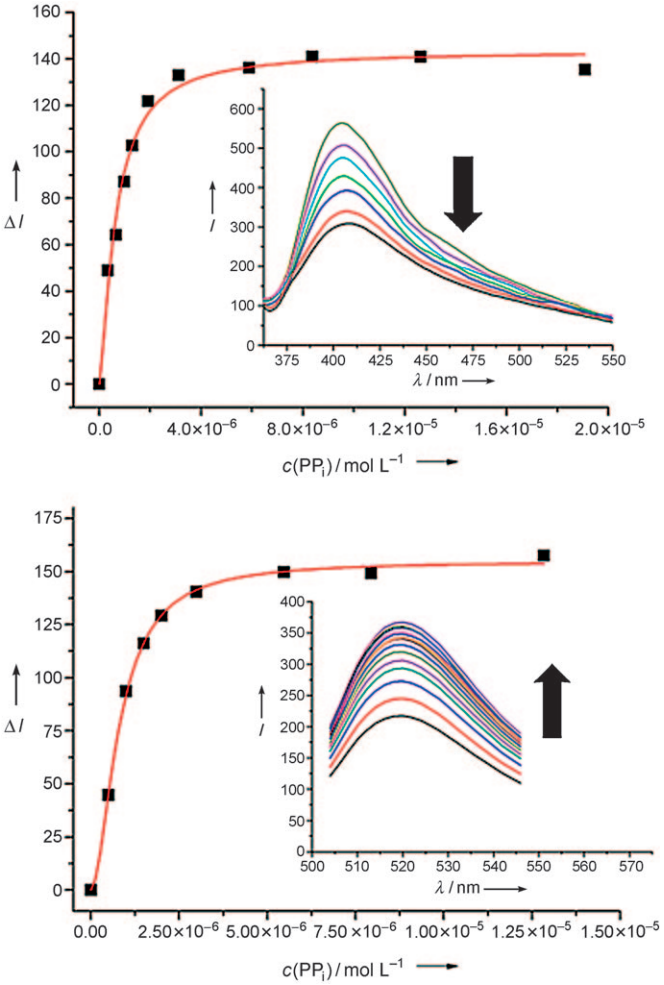

Figure 3. Top: Binding isotherm obtained from emission titration of **LVR-1,5** versus PP_i ($\lambda_{ex}=349$ nm, $\lambda_{em}=405$ nm). Bottom: Binding isotherm obtained from emission titration of **LVR-1,4** versus PP_i ($\lambda_{ex}=495$ nm, $\lambda_{em}=520$ nm).

Table 1: Binding affinities of luminescent vesicular receptors (LVRs) to several analytes derived from emission titrations.

	Receptor(s)	Reporter	Analyte	log *K* (±0.1)
LVR-1,4	**1**	**4**	PP_i	6.2
LVR-1,4	**1**	**4**	pSer	5.9
LVR-1,5	**1**	**5**	PP_i	6.0
LVR-1,5	**1**	**5**	pSer	5.9
LVR-2,4	**2**	**4**	4-Me-Im	7.1
LVR-2,4	**2**	**4**	His-OMe	7.1
LVR-1,2,4	**1**+**2**	**4**	PP_i	6.0
LVR-1,2,4	**1**+**2**	**4**	pSer	5.8
LVR-1,2,4	**1**+**2**	**4**	4-Me-Im	6.2
LVR-1,2,4	**1**+**2**	**4**	His-OMe	6.1
LVR-1,2,3,4	**1**+**2**+**3**	**4**	PP_i	6.1
LVR-1,2,3,4	**1**+**2**+**3**	**4**	4-Me-Im	6.0
LVR-1,2,3,4	**1**+**2**+**3**	**4**	Gly-OMe	5.8

neously distributed but to form relatively closely arranged mixed patches in the membrane, as its structure is disturbed by the rather bulky embedded molecules. Such non-uniform distribution translates into a different environment for dye molecules within the membrane. While a certain portion of dye molecules remains free enough to provide initial emission, those molecules, which are in a close proximity to the ligand, are "switched-off" owing to the interactions of receptor and dye. These interactions are imposed by the tightly packed membrane structure of the surrounding lipid phase and do not occur in homogeneous aqueous solution.[12] Binding of bulky phosphate anions to the receptors changes solvation and charge of the binding sites and thereby changes the miscibility of the receptor and dyes. These phase-state alterations, in turn, cause a segregation of the patches and the surrounding zone and thus the lateral expelling of dye molecules from the mixed phase. Similar phenomena, though more complex in nature, underlie the response of cell membranes, which are essentially composed of lipids with various embedded active sites, to external stimuli and substrates.[13] Environment-sensitive dyes, such as coumarin derivatives, are known to respond to such altered conditions resulting from the binding event,[14] in this case by a decrease of their emission intensity.

Apparent binding constants were determined from emission titration binding isotherms and are summarized in Table 1. The obtained affinities for PP_i and pSer are in the micromolar range and in good agreement with affinities previously determined by other methods.[4c] To confirm that the specific anion–metal-complex interaction is responsible for the emission response and to exclude effects of unspecific adsorption, the measurements were repeated with vesicles that do not contain zinc(II)–cyclen binding sites. These vesicles also exhibit blue fluorescence, but indeed give no

emission response on excess addition (50–100 equiv) of PP_i or pSer.

Different reporter dyes can be used to adjust the optical properties of the vesicular receptors: **LVR-1,4** obtained from the co-embedding of zinc(II)–cyclen complexes **1** and amphiphilic carboxyfluorescein **4** showed a green fluorescence emission with a maximum intensity at 520 nm if excited at 495 nm. As the optical response of environment-sensitive dyes depends on their structure and properties and also on their miscibilities with the substrate-free and occupied receptor, it can vary substantially, and upon addition of increasing amounts of phosphate anions the emission at 520 nm shows a strong increase (Figure 3, bottom). The apparent binding constants derived from the binding isotherms of **LVR-1,4** and of **LVR-1,5** are however identical for pSer and for PP_i (Table 1) within the error of the experiment. This fact fits our above hypothesis and gives rise to the assumption that a variety of dyes can be employed as long as they change their emission properties upon an alteration of their environment. As vesicles functionalized with **4** show an increase in emission upon guest binding ("switch-on" fluorescence), which is more desirable for most analytic applications than an emission decrease ("switch-off"), compound **4** was used as reporter dye for all subsequent binding studies.[15]

To provide more experimental support for our mechanistic hypothesis, we also prepared **LVR-1,4** with different concentrations of receptors and dyes. As a result, the relative emission response to a certain amount of analyte increases (or decreases) with an increasing (decreasing) amount of embedded receptors and dyes (see Supporting Information), which fits well with our suggested model of receptor/dye patches.

The amphiphilic copper(II)–nitrilotriacetic acid (NTA) complex **2** was then investigated as binding site. Cu^{II}–NTA complexes coordinate imidazole ligands and are widely used in immobilized metal-affinity chromatography (IMAC),[16] for example for the separation of histidine(His)-tagged biomolecules.[17] Vesicles **LVR-2,4** containing the Cu^{II}–NTA complexes **2** and the CF dye **4** were prepared; the particles showed emission at 520 nm, analogous to **LVR-1,4**. Binding studies revealed that the emission intensity increased considerably in the presence of imidazole derivatives, whereas other additives—cationic or anionic—induced no response (see the Supporting Information). The analyte selectivity of the functionalized vesicle is therefore determined by the selectivity of the embedded Cu^{II}–NTA complexes.

To increase the complexity, two different binding sites were incorporated simultaneously in one vesicle: **LVR-1,2,4** contains cationic[18] Zn^{II}–cyclen complexes **1** and anionic[14] Cu^{II}–NTA complexes **2** as binding sites and CF reporter groups **4**. The particles exhibit green fluorescence at 520 nm, which increases in the presence of either phosphate anions or imidazoles (Supporting Information, Figure S5). Binding affinities for PP_i and pSer were similar to those determined for **LVR-1,4**, whereas the derived $\log K$ values for 4-methyl imidazole (4-MeIm) and His were found to be up to one order of magnitude lower than for **LVR-2,4**, but still in the micromolar range and thus higher than for Cu^{II}–NTA complexes in homogeneous aqueous solution.[19] The selectivities of the individual binding sites for their corresponding ligands were retained. Vesicles containing both metal complexes did not respond to other analytes by a change of their emission, such as ammonium or sulfate ions (Supporting Information, Figure S5). We therefore assume that our suggested model for a binary system (with one receptor and one dye) also works with ternary (two receptors and one dye) or even more complex systems.[20] Furthermore, the addition of phosphate and imidazole derivatives to **LVR-1,2,4** can be sequential, with each guest producing an incremental increase in fluorescence intensity. This again supports our suggested model of (multi-)receptor and dye patches in the membrane that are rearranged to some extent by an additional binding of analytes to the receptor moieties.

To increase the complexity of the vesicular receptors even further, a third binding site was added to the cationic and anionic transition metal complex receptors **1** and **2**. Therefore we prepared the amphiphilic benzoaza crown ether derivative **3**[8] and incorporated it together with **1**, **2** and **4** into the luminescent vesicles **LVR-1,2,3,4** that now bears three fundamentally different molecular recognition sites on the bilayer surface. The benzoaza crown ether moiety is a well-established low-affinity binding site for the molecular recognition of ammonium ions. Incorporated in the luminescent vesicles, the binding selectivity of the crown ether is combined with the optical properties of the bilayer-embedded fluorescent dye. The emission properties of **LVR-1,2,3,4** and binding affinities towards phosphate and imidazole are again in good agreement with **LVR-1,2,4** (Table 1). However, **LVR-1,2,3,4**, in contrast to **LVR-1,2,4**, also responds to the presence of ammonium ions, such as the C-terminal protected amino acid glycine (Gly-OMe), by increase in emission intensity. The binding affinity for Gly-OMe was determined to be $\log K = 5.8$, which exceeds ammonium ion affinities for the benzoaza crown ether moiety in aqueous solution by three to four orders of magnitude. We explain the significant binding affinity increase by the special properties affecting intermolecular interactions at the interface of hydrophobic membrane and water. Such effects are reported for related examples.[9,21] The improved signaling mechanism that uses the embedded dye furthermore allows a more sensitive detection.

To finally demonstrate the reversibility of the receptor–ligand binding on the vesicular surface, the vesicles were saturated with analyte and then separated from these bound analytes by size exclusion chromatography and reused for emission titrations, with nearly identical results (Supporting Information, Figure S7).

In summary, a novel kind of luminescent vesicular chemosensors for the recognition of biologically important ions and molecules was obtained by the self-assembly of lipids, amphiphilic binding sites, and reporter dyes that are sensitive to their environment. As a proof of principle, three different amphiphilic binding sites **1**–**3**, based on Zn^{II}–cyclen (**1**) or Cu^{II}–NTA complexes (**2**) and benzoaza crown ether (**3**), and two fluorescent dyes, based on coumarin and carboxyfluorescein, were co-embedded into membranes of unilamellar vesicles. Although not covalently linked or directly coordinated to a single receptor site, as in classical indicator displacement systems, the reporter dyes signal analyte bind-

ing to the receptor sites by changes of their emission properties. We explain this response by an analyte binding-induced rearrangement of the doped bilayer membrane. Embedding of bulky binding sites and dyes leads to phase separation and the initial formation of patches with gathered receptors and dyes. Subsequent receptor binding events change conditions such as solvation and charge in the local receptor environment and release dye molecules for signaling. This redistribution of dye molecules finally explains the emission response of the environment-sensitive dyes. This "substrate-catch–dye-release" signaling mechanism based on the delicate interplay between binding events and phase properties of local environment of the receptor site also provides an opportunity for fine-tuning of the system response by simple altering the ligand-to-dye ratio and vesicle composition. Vesicles prepared from mixtures of binding sites show an additive response corresponding to the selectivity of all incorporated receptors. Analytes, such as pSer, His-OMe, and Gly-OMe, bind reversibly and are detected at (sub)-micromolar concentrations. The key advantage of the self-assembled functionalized nanoparticles is the non-covalent pooling of multiple binding sites and fluorescent reporter groups that decrease the synthetic effort of chemosensor preparation. It is likely that many other binding sites and luminescent probes can be combined in this way, which paves an easy way to analytical nanoparticles that are adjustable at will in their binding and response properties.

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