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Molecular Imprinting of Luminescent Vesicles

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S Supporting Information

ABSTRACT: Applying molecular imprinting techniques to the surface of functionalized unilammelar fluid vesicles allows the preparation of specific and high-affinity luminescent chemosensors. We have photopolymerized diacetylene containing vesicles in the presence of small peptides as templates yielding imprinted polydiacetylene (PDA) patches in the membrane. They serve as multivalent receptor sites with significantly increased rebinding affinity for the template. All binding sites are surface exposed and accessible for analyte binding. The presence of analytes is signaled with high sensitivity by emission intensity changes of amphiphilic carboxyfluorescein, which is coembedded into the fluid DOPC membrane. The merger of PDA imprinting with dynamic functionalized vesicles overcomes some of the current limitations of molecular imprinting in chemosensor design and may be applied to many different target analytes.

Using a molecule to create its own complementary image is a classic strategy to obtain synthetic receptors in molecular recognition. It is the underlying concept of receptor selection from dynamic libraries[1] and molecular imprinting[2] techniques to produce specific binding sites in synthetic polymers. The techniques have found applications in analytical chemistry, allowing chromatographic resolution of racemates or specific enrichment of analytes,[3] and also in catalysis[4] or in the search for specific enzyme inhibitors.[5] In molecular imprinting, functional monomers are first preorganized in the vicinity of a multivalent analyte, copolymerized with cross-linking monomers, and after removal of the analyte, the polymer contains complementary binding sites rebinding the analyte with high specificity. Although highly developed and widely employed, the technique suffers from some inherent drawbacks. The created binding sites are deeply buried in the imprinted polymer, hampering the removal of the template and the rebinding of an analyte. To overcome this limitation, alternative approaches have been investigated, e.g., placing the molecular imprinted polymer on the surfaces of nanowires or nanotubes, layer-by-layer self-assembled films, or other supports.[6] After grinding the conventionally imprinted particles are still rather large and structurally diverse. The polymerization step connects all monomers covalently, which prevents a dynamic response to the binding of analytes. We have now merged the concept of molecular imprinting with the surface patterning of fluid vesicles using embedded functional amphiphiles, which are partially polymerizable, to obtain imprinted particles with accessible binding sites and a structure that is still dynamic.

Luminescent chemosensors from covalently or noncovalently combined metal complex binding sites with reporter dyes have been developed by us[7] and other groups.[8] A particular useful design principle utilizes analyte-triggered changes in the phase segregation[9] of multicomponent bilayer membranes.[10−12] For this, amphiphilic metal complex receptors and amphiphilic fluorescent dyes are coembedded in synthetic 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) vesicles where they cluster in patches. These assemblies respond to the binding of analytes with altered emission, due to induced changes in the arrangement of the mixed dye-receptor patches.[13]

However, achieving high affinity and selectivity with such chemosensors requires multivalent analyte interaction in the binding process. With the current approaches the defined spatial arrangement of amphiphilic receptor binding sites remains a challenge that can be addressed by molecular imprinting. Transition-metal complex receptor sites that bind analytes through reversible coordination are particularly suitable for imprinting techniques: The analyte coordination is very tight, even in polar solvents, but still reversible for the separation of the bound analyte from the imprinted polymer. For cross-linking, we are using diacetylenes that are known to undergo polymerization in a 1,4 addition-type to generate ene-yne polydiacetylene (PDA) polymers by UV irradiation (254 nm).[14] This photopolymerization in the absence of any external catalyst or initiator is very practical for the imprinting process. The PDA backbone has a characteristic blue color, which changes to red in response to environmental perturbations.[15]

We embedded a polymerizable dinuclear receptor **Zn_2PCDA** (Chart 1) in liquid crystalline **DOPC** (T_m = −20 °C)[16] based vesicles of about 100 nm diameter (see SI for preparation of vesicles and Figures S1 and S2 for their size distribution)[17]in 25 mM HEPES buffer, pH = 7.4. As known from the previous studies, the bis-zinc cyclen complex has affinity to phosphorylated serine and to the imidazole side chain of histidine.[7a] If two metal complex receptor sites (connected by a covalent linkage) are in the right distance, the peptide affinity increased from about 10^4 L/mol for phosphate only binding to about 10^7 L/mol for simultaneous phosphate and imidazole binding. In the presence of peptide **Pep I** (Chart 1), added to the buffer and bearing phoshorylated serine and histidine in its sequence, the metal complex binding sites are organized on the vesicle surface. However, diacetylene monomers with bulky head groups, such as **Zn_2PCDA**, cannot pack close and photopolymerize inefficiently. Therefore **PCDA** (Chart 1) with a smaller carboxylic acid headgroup was added to the membrane allowing the efficient formation of PDA. But the blue to red transition of PDA

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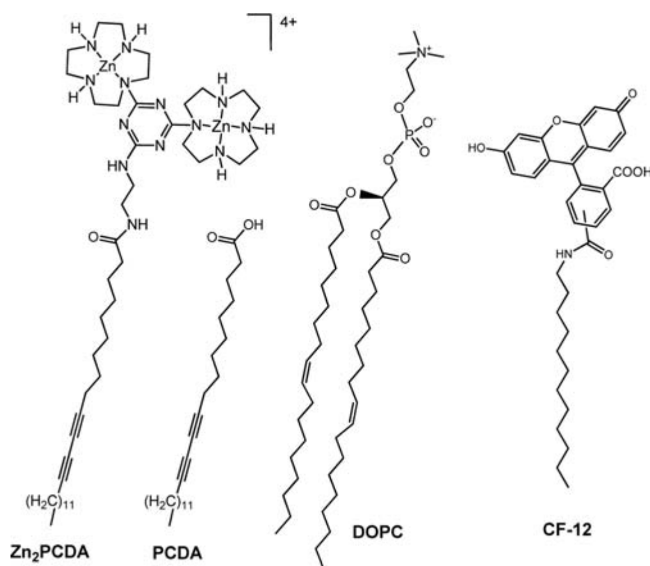
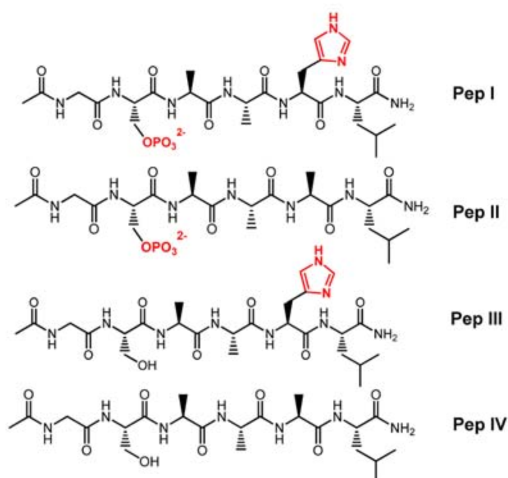
Chart 1. Components for Vesicle Formation (top) and Peptides (bottom) Used for Imprinting[a]





[a]Counter ions are not shown.

absorption did not show sufficient sensitivity for analyte detection at low concentrations, and hence a nonpolymerizable reporter dye **CF-12** (Chart 1) was coembedded in the vesicles. The photopolymerization of the mixed **Zn_2PCDA−PCDA** patches in the bilayer matrix is monitored by the arising blue PDA absorption (λ_{max} = 640 nm) and gives a molecularly imprinted two-dimensional arrangement of the metal complex receptors on the vesicle surface. The template peptide is removed by size exclusion chromatography (see SI). While **Zn_2PCDA** and **PCDA** are now covalently connected in a PDA patch floating in the **DOPC** membrane, the **CF-12** reporter dye is coembedded but not covalently linked to the receptor patch. This allows for a dynamic response of the dye upon analyte binding to the imprinted PDA receptor patch in the **DOPC** membrane. We expect, in the absence of the phosphorylated peptide, the negatively charged dye in close proximity to the positively charged bis-zinc cyclen receptors.[18] The high local concentration of the dye quenches its emission partially. Binding of the phosphorylated peptide to the metal complex receptor leads to local reorganization in the membrane and an increase in the emission of **CF-12**. Figure 1 summarizes schematically the imprinting and detection step.

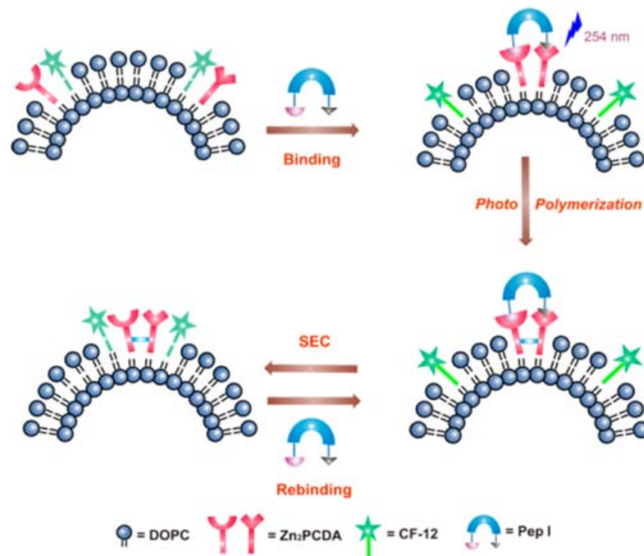


Figure 1. Principle of surface imprinting and luminescent analyte response; SEC is size exclusion chromatography.

The vesicles are prepared from four compounds: **DOPC**, **Zn_2PCDA**, **PCDA**, and **CF-12.** Their relative concentrations determine the stability and sensitivity of the chemosensors and the efficiency of the photopolymerization. Ratios of **Zn_2PCDA** and **CF-12** close to 1:1 gave vesicles with better analyte sensitivity,[19] which is consistent with earlier observations using DSPC-based vesicles.[13] It suggests that a specific receptor/dye composition in the mixed patches is essential to obtain an optimum response. The ratio of **PCDA/Zn_2PCDA** not only affects the photopolymerization[20] but also the sensing capability of the vesicles. Vesicles with an excess of **PCDA** vs **Zn_2PCDA** (ratio **PCDA/Zn_2PCDA** 3:2) led to a high degree of polymerization (Figure S4) but weak sensing ability; an excess of **Zn_2PCDA** vs **PCDA** (ratio **PCDA/Zn_2PCDA** 1:2 or 1:3) gave good sensitivity, but they were difficult to polymerize (Figure S5). A 1:1 ratio of **PCDA** and **Zn_2PCDA** is a suitable balance between the two parameters. The polymerization of the **PCDA/Zn_2PCDA** patches in the vesicles[21] requires only short irradiation of about 1 min to yield the blue PDA backbone (Figure S3), which avoids photobleaching of dye **CF-12**.[22] Unless otherwise mentioned, vesicles with the following composition were used in the experiments: **DOPC** (1×10^{-4} mol/L), **PCDA** (2.6×10^{-5} mol/L), **Zn_2PCDA** (2.6×10^{-5} mol/L), and **CF-12** (2.6×10^{-5} mol/L).[23]

Three other peptides (Chart 1) possessing very similar backbones as **Pep I**, but differing in the number and nature of their binding sites, were used for the imprinting study. Only **Pep I** contains a phosphorylated serine and a histidine unit for simultaneous divalent binding to two bis-zinc cyclen receptors. The other peptides **Pep II−IV** have only one suitable functional group for monovalent coordination to the bis-zinc cyclen complex or contain no coordination site at all. So, only **Pep I** can bind two **Zn_2PCDA** receptors simultaneously on the vesicle surface and produce a molecularly imprinted surface after polymerization.

The binding of the functionalized vesicles to peptides **Pep I−IV** and **pSer** was investigated before and after the described

imprinting procedure by emission titration following the emission intensity increase of **CF-12**. The apparent binding constants (Table 1) were determined by nonlinear fitting of the

Table 1. Apparent Binding Constants of the Zn_2PCDA Receptor Embedded in Vesicles Before and After the Imprinting for Different Analytes

analyte	binding before imprinting [log K][a]	binding after imprinting [log K][a]
pSer	4.0	4.1 (4.0)[b]
Pep I	4.6	7.4 (7.3)[b]
Pep II	3.9	4.0 (3.8)[b]
Pep III	–[c]	–[c]
Pep IV	–[c]	–[c]

[a]Error in determining the binding constant values is ±0.2. [b]Template peptide was added at the step of the vesicle extrusion. [c]No affinity detectable.

experimental data using the Hill equation. The binding of **pSer** and the phosphorylated peptides **Pep I** and **II** to the nonimprinted vesicle is dominated by the reversible coordination of the phosphate moiety to the zinc cyclen binding sites with a typical value of log $K \approx 4$ for **pSer** and **Pep II** (Figures S7 and S8) and a slightly higher log $K = 4.6$ for **Pep I**. A significant increase in affinity is observed only for the rebinding of **Pep I** after the imprinting process. The affinity increases by 3 orders of magnitude.[24]

Figure 2 illustrates the effect of receptor organization by imprinting on the vesicle surface. The rebinding of **Pep I** to the

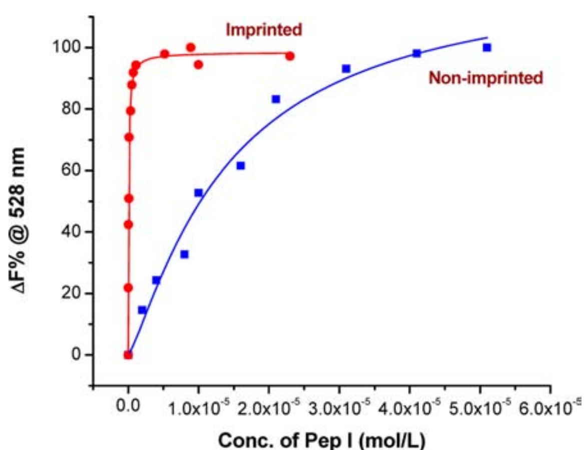


Figure 2. Emission titrations of **Pep I** rebinding to imprinted (red dots) and nonimprinted (blue squares) vesicles, respectively (λ_{ex} = 475 nm).

imprinted vesicle is significantly enhanced in comparison to a nonimprinted sample that has the identical composition but was photopolymerized in the absence of **Pep I** as template (see SI).

Peptides **Pep III** and **IV** show, as expected, no affinity to nonpolymerized or imprinted vesicles (Figure S10 for **Pep III**). The coordination of the imidazole side chain to the zinc cyclen receptors is too weak on its own providing substantial affinity. Only in cooperative action with the phosphate moiety the imidazole contributes to the binding process; already observed in an earlier study[7a] using covalently connected metal complexes. Imprinting in the presence of **pSer** or **Pep II** did not provide any increase in binding affinity for rebinding. Both compounds bind monovalent to the vesicle surface and are therefore not able to affect the distance between bis-zinc cyclen receptor sites. Using any other template than **Pep I** or no template for the imprinting process gave vesicles with an affinity for peptide **Pep I** in the rebinding of about log $K \approx 4$ and no increase in affinity. Furthermore, a vesicle imprinted with **Pep I** did not show any appreciable change in the rebinding affinities for **pSer** or **Pep II–IV**, demonstrating its selectivity for **Pep I**.[25] As a variation of the imprinting process, the template peptide **Pep I** was added already to the membrane–buffer mixture before the extrusion process.[26] The obtained binding and imprinting results (Table 1) are nearly identical to the ones for peptide addition to a solution with extruded vesicles.

In conclusion, we have demonstrated a surface imprinting strategy for **DOPC**-based luminescent vesicular chemosensors in aqueous media (pH = 7.4). Photopolymerizable diacetylene tagged dinuclear zinc cyclen receptors were embedded in the fluid membrane and preorganized in the presence of a peptide as template that is able to bind simultaneously to two receptor sites. Light-induced polydiacetylene formation patterns parts of the vesicle surface with organized arrays of receptor sites. The surface binding sites are easily accessible for rebinding of the target peptide, and the imprinting process increases the binding affinity by 3 orders of magnitude. Our imprinting strategy thus provides a new direction for the selective and sensitive detection of phosphopeptides, whereas the existing strategies are mainly based on employing metal complex fluorophore conjugate,[27] hybrid biosensor,[28] or combinatorial, pattern-recognition-based methods.[29] The extension of the vesicle surface imprinting process to more complex systems with several receptors showing different binding selectivities and to larger peptides, proteins, and other bioanalytical targets can be readily envisaged.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and data. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(17) After extruding through the 100 nm membrane, the vesicles exhibited PDI of 0.2−0.3, shown in Figures S1 and S2.

(18) Spectral features of the embedded **CF-12** were found to be dependent on the relative proportion of **Zn_2PCDA** and the dye, e.g., vesicles with 1:1 ratio of these two components exhibited orange-yellow color and highly quenched emission (Figure S6). At pH 7.4, dye remains mostly in the dianion form, as characterized by the 498−500 nm peak. Different prototropic forms of fluorescein, see: (a) Ali, M.; Dutta, P.; Pandey, S. *J. Phys. Chem. B* **2010**, *114*, 15042. (b) Togashi, D. M.; Szczupak, B.; Ryder, A. G.; Calvet, A.; O'Loughlin, M. *J. Phys. Chem. A* **2009**, *113*, 2757.

(19) **pSer** and **PP_i** were used as analytes for these optimization studies. The apparent binding constant determined for **PP_i** was 4.9 (±0.2).

(20) We compared the intensity of blue PDA peak relative to that of **CF-12** to have a meaningful estimation of the polymer formation.

(21) Polymerized vesicles had comparable diameters as the non-polymerized vesicles; see Figures S1 and S2.

(22) Emission intensity of dye **CF-12** in the absence of analytes increased after photopolymerization depending on the PCDA concentration. We explain this observation with the extrusion of the dye from the polymerized patches. High initial emission intensity lowers the sensitivity of the luminescent vesicle for analytes. Vesicles with a 1:1 ratio of **PCDA/Zn_2PCDA** showed very similar dye emission response before and after the polymerization.

(23) Estimate of the total number of receptors available for binding on the outer vesicle surface, see SI.

(24) Repeating the process for one more cycle did not result in higher affinities. No intensity increase of the blue PDA peak (~640 nm) could be observed when the polymerization was repeated after rebinding of **Pep I**. Presumably, with this specific ratio of **PCDA/Zn_2PCDA** (1:1), the optimum extent of the polymerization was already reached during the first UV irradiation step. Also, the vesicle exhibited reduced analyte response after the second cycle; see Figure S11 for absorption spectra changes.

(25) When **Pep I** was added to a solution of the vesicle samples imprinted with **Pep I** and containing large excess of **pSer** or **Pep II−IV**, the following observations were noted: (a) samples with excess of **Pep III** or **IV** (peptides with no binding affinity) gave very similar rebinding affinities (log K ~6.8) for **Pep I** (Figure S14b); samples containing large excess of **pSer** and **Pep II** showed only nominal changes in the **CF-12** emission in the rebinding titration with **Pep I**. Difference in rebinding affinity of **Pep I** with these analytes is significant but only ~3 orders of magnitude. Binding sites are therefore already saturated with **pSer** or **Pep II**, dyes were expelled, and higher rebinding affinity of **Pep I** cannot induce a change in emission properties any more; see Figure S13.

(26) **Pep I** was added before preparing the vesicle sample by sonication; see SI.

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