Artificial Membranes

DOI: 10.1002/anie.201205701

Dynamic Interface Imprinting: High-Affinity Peptide Binding Sites Assembled by Analyte-Induced Recruiting of Membrane Receptors**

*Benjamin Gruber, Stefan Balk, Stefan Stadlbauer, and Burkhard König**

Molecular recognition between membrane-associated receptors and external ligands as stimuli is important for many biological processes.[1] The dynamic formation of domains and the clustering of receptors in fluid membranes play a key role for example, in signal transduction leading to highly specific binding of competing multivalent ligands.[2]

Various model systems for biological membranes have been developed to understand and mimic multivalent interactions at interfaces[3] as well as for applications in delivery, sensing, and catalysis.[4] The principle of template-guided assembly has also been extensively exploited in molecular imprinting,[5] but the noncovalent arrangement of binding sites separated by precisely defined distances still remains a challenge. We have investigated a concept that mimics immunological synapses, where receptors are recruited at a membrane interface and spatially organized by the binding partner, and this orientation triggers a specific response.[6] Our vesicles with membrane-embedded luminescent receptors produce a characteristic optical response in the presence of small peptides as external ligands. Depending on the functional groups present in the peptide, suitable receptors with complementary binding sites are recruited and arranged in the fluid membrane, which triggers a fluorescence resonant energy transfer (FRET) signal.

We have previously reported on the recognition of small biomolecules and phosphorylated proteins by multisite interactions[7] at the lipid–water interface using synthetic vesicle membranes with embedded artificial receptors based on transition-metal complexes of 1,4,7,10-tetraazacyclododecane (cyclen) and nitrilotriacetic acid (NTA). We now expand this concept to the dynamic recruiting of synthetic receptors allowing the multivalent recognition of phosphorylated peptides. The selective recognition of O-phosphoserine (pSer) and histidine (His) moieties in the model peptide **P1** (see Scheme 1) by metal-complex binding sites was investigated in a previous study in homogeneous solution.[8] It was found that in buffered aqueous solution the Zn^{II}–cyclen receptor Zn_2**6** binds peptide **P1** with $\lg K = 4.8$, while dimer Zn_4**6**, allowing a simultaneous two-prong interaction to pSer and His, shows an affinity of $\lg K = 7.5$ (see Table 1 entries 1 and 2, and the Supporting Information). Instead of covalently connecting two receptor sites, as in Zn_4**6**, distinct binding sites are now simply embedded in synthetic lipid bilayers. Recruiting and self-organization by the peptide ligand allows the divalent binding of both pSer and His residues of **P1** at the membrane interface.

Table 1: Summary of the apparent binding constants for peptide **P1** by synthetic receptors in homogeneous solution (entries 1 and 2) and embedded in vesicle membranes (HEPES buffer, pH 7.4, 25 °C).

Entry	Lipid	Receptor	mol%	lg *K*
1	–	Zn_2**6**	–	4.8[a]
2	–	Zn_4**6**	–	7.5[a]
3	DSPC	Zn_2**5**	1	5.9
4	DSPC	Zn_2**5**	10	8.1
5	DOPC	Zn_2**5**	1	8.6
6	DSPC	Zn_2**2**	1	6.2
7	DSPC	Cu**4**	1	5.0
8	DSPC	Zn_2**2** + Cu**4**	1 (each)	6.3
9	DOPC	Zn_2**2**	1	5.5
10	DOPC	Cu**4**	1	5.8
11	DOPC	Zn_2**2** + Cu**4**	1 (each)	8.8

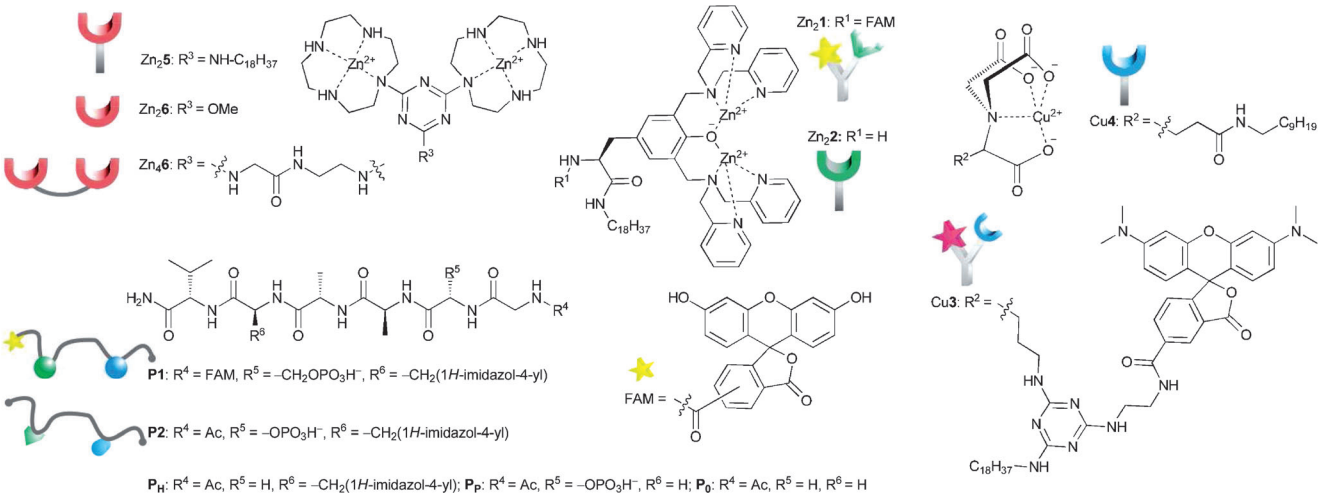
[a] From Ref. [9].

First we embedded Zn_2**5** (1 mol%) in vesicular lipid bilayers made from 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC; Scheme 1) using previously reported procedures (see the Supporting Information). The emission intensity of peptide **P1** increases significantly upon binding to the surface receptors (see the Supporting Information) and a binding constant of $\lg K = 5.9$ (Table 1, entry 3) at ambient temperature was determined. This value is in good agreement with the recognition of pSer by a single Zn_2**5** receptor[7a] and reasonable, because DSPC's high phase-transition temperature (54 °C)[9] restricts diffusion and thus participation of more than a single metal-complex binding site in the peptide recognition (cf. Figure 1 a). Binding affinities could only be enhanced by drastically increasing the vesicle receptor loading to 10 mol% (Table 1, entry 4) resulting in the formation of tightly packed metal-complex patches.[4d] However, replacing the saturated DSPC lipid by unsaturated 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), which has a much lower transition temperature of −20 °C,[9] leads to an increase in the binding affinity to peptide **P1** by more than two orders of magnitude (Table 1, entry 5). The embedded receptor sites can now diffuse in the membrane at room temperature allowing binding of one **P1** by two Zn_2**5** units through a dynamic assembly process (Figure 1 a). The particle

[*] Dipl.-Chem. B. Gruber, M. Sc. S. Balk, Dr. S. Stadlbauer, Prof. Dr. B. König
Institut für Organische Chemie, Universität Regensburg
Universitätsstrasse 31, 93053 Regensburg (Germany)
E-mail: Burkhard.koenig@chemie.uni-regensburg.de
Homepage: http://www-oc.chemie.uni-regensburg.de/koenig/

[**] We thank the Deutsche Forschungsgemeinschaft (DFG) for financial support.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201205701.



Scheme 1. Structures of synthetic receptors and ligands (counterions omitted).

sizes determined by dynamic light scattering were unchanged (Supporting Information, Figure S11), which confirms the binding of the peptide to receptors of one vesicle (intramembrane binding mode) and excludes peptide cross-linking of different vesicles (intermembrane binding).

The experiments show that analyte-induced recruiting of binding sites in a fluid membrane leads to higher binding affinities as a result of multipoint interactions. Next, vesicle membranes with two different embedded receptors, which bind selectively either pSer or His of **P1**, were investigated. The synthesis of the amphiphilic Cu^{II}–NTA complex Cu**4** for the recognition of histidine was reported previously,[7a] the synthesis of the amphiphilic Zn^{II}–DPA complex Zn_2**2** (DPA = 3,5-bis[(bispyridin-2-ylmethylamino)methyl]-4-hydroxyphenyl (DPA) complex Zn_2**2** for the selective recognition of phosphate[10] is described in the Supporting Information. Stable metal-complex-doped DOPC and DSPC vesicle membranes were prepared using Zn_2**2** or Cu**4** and a mixture of both (1 mol% each). For DSPC membranes with one type of receptor embedded, the emission titrations with **P1** revealed monovalent binding through coordination to either Zn–DPA or Cu–NTA,[11] with binding constants of $\lg K = 5.0$ and 6.2, respectively (Table 1, entries 6 and 7). Job's plots with **P1** as the limiting reagent confirm the 1:1 stoichiometry of the binding event (Figure S18). Combining both receptors in gel-phase membranes of DSPC vesicles (Figure 1b,c) does not increase the binding affinity for peptide **P1** ($\lg K = 6.3$; Table 1, entries 6–8). The binding isotherm for the receptor vesicles bearing both complexes resembles the average of the binding properties of the individual receptor vesicles (see the Supporting Information for data). This is explained by the random, but fixed arrangement of the metal-complex receptors embedded in the lipid bilayer, which prevents the formation of a ternary Zn_2**2**–**P1**–Cu**4** complex (see the Supporting Information for Job's plots). DOPC membranes, in contrast, show completely different behavior: Whereas vesicles with either Zn_2**2** or Cu**4** as the embedded receptors give similar binding isotherms and affinity constants ($\lg K =$ 5.5 and 5.8, respectively; Table 1, entries 9 and 10), the liquid-crystalline membrane containing both receptor binding sites shows a significantly higher affinity for peptide **P1** in the nanomolar range ($\lg K = 8.8$, Table 1, entry 11 and Figure 1b,c). We explain this by the divalent binding of **P1** by a heterodimeric Zn_2– and Cu–receptor assembly in the fluid lipid bilayer (cf. Figure 1b,c) resulting from the peptide-induced self-assembly of the membrane receptors. The Job's plot analysis (Figure S19) supports this conclusion.

For bioanalytical applications, the labeling of the peptide analyte must be avoided. Therefore amphiphilic receptor binding sites that signal the presence of the target analyte by induced FRET were developed: the Zn^{II}–DPA complex Zn_2**1** is labeled with carboxyfluorescein (FAM) and the Cu^{II}–NTA complex Cu**3** is labeled with rhodamine (TMR). FRET techniques are widely used in molecular biology to measure distances within biomolecules or to explore membrane structures.[12] We use the FRET signal, which is induced by the close proximity of the membrane-embedded luminescent binding sites to signal the presence of peptide **P2**, which in contrast to **P1**, does not carry a fluorescent label. Divalent binding of **P2** to the membrane-embedded binding sites recruits Zn_2**1** and Cu**3** into close proximity and therefore within the Förster distance of 5.5 nm,[13] resulting in a FRET signal as the optical output (Figure 2a).[14]

The vesicles responded to the presence of the target peptide **P2** with a significant FRET signature. Formation of a Zn_2**1**–**P2**–Cu**3** ternary complex leads to energy transfer from the donor label FAM ($\lambda_{em} = 490$ nm) to the acceptor label TMR ($\lambda_{em} = 580$ nm; Figure 2b and Figure S22 in the Supporting Information). However, an excess of the target peptide is necessary to reach the maximum energy transfer. Control experiments with monovalent ligands ($\mathbf{P_P}$, $\mathbf{P_H}$, and $\mathbf{P_0}$; Scheme 1) did not change the observed emission spectra significantly (Figure S23). We exclude intervesicular FRET through the cross-linking of distinct vesicles as the particle size in solution remains unchanged in the presence of **P2** (Figures S24 and 25). Additionally, vesicles were purified by size-exclusion chromatography before the binding experiments to remove traces of amphiphilic receptors and micellar aggregates from the solution, and the concentrations of the embedded receptors were verified by analysis of the Zn and

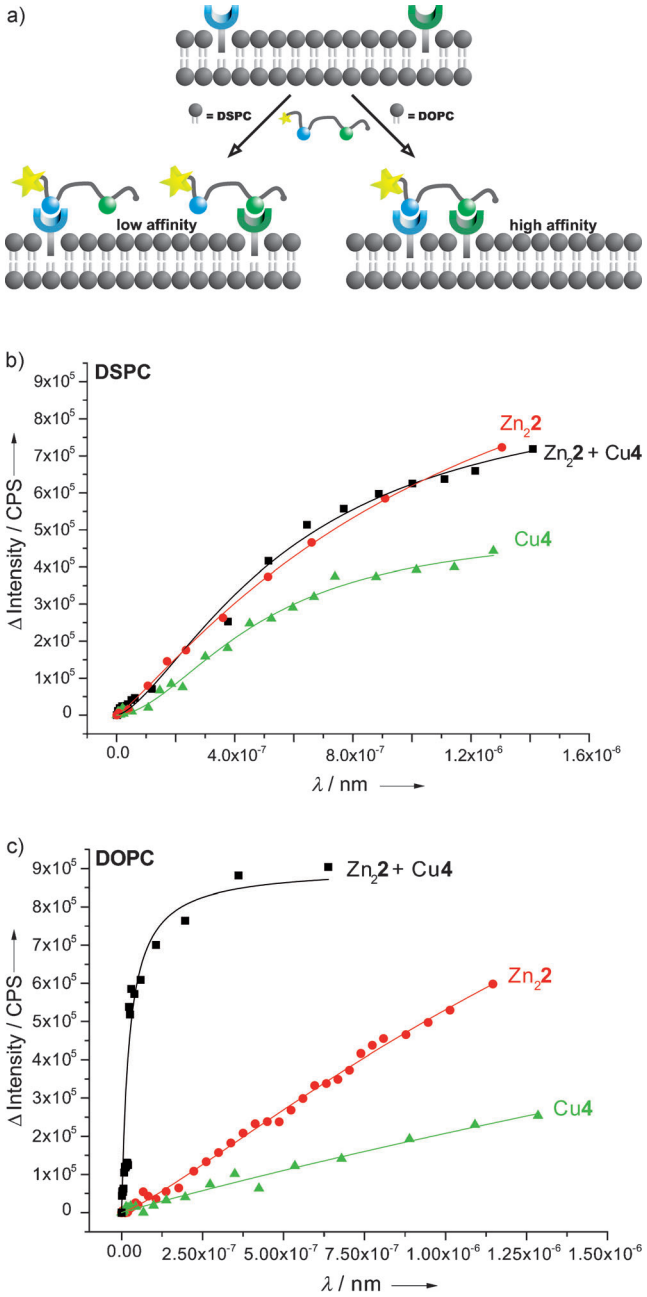


Figure 1. a) Schematic representation of receptor assembly in vesicle membranes: Fixed receptors in gel-phase DSPC membranes versus analyte-induced clustering in fluid DOPC membranes. b,c) Emission titrations of **P1** versus DSPC (b) and DOPC membranes (c) containing Zn_2**1**, Cu**3**, or both receptors.

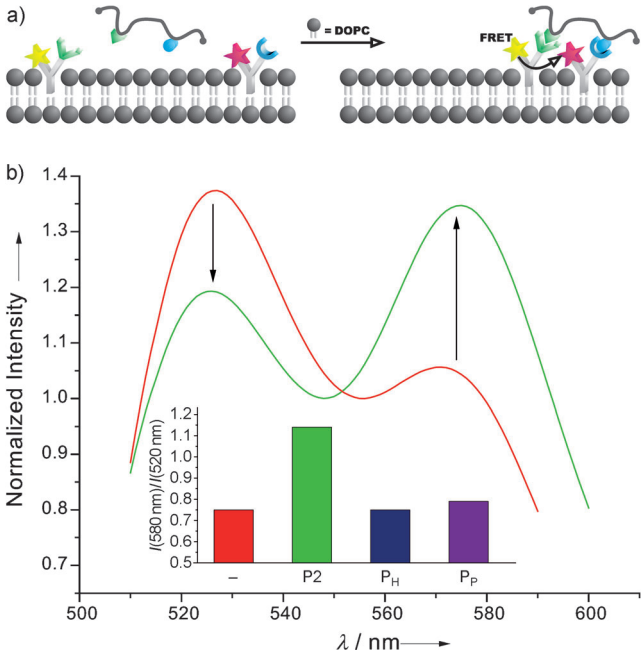


Figure 2. a) Schematic representation of the recruiting of labeled membrane receptors by a peptide ligand resulting in a FRET signal. b) Fluorescence spectra of DOPC vesicles containing labeled receptors Zn_2**1** + Cu**3** (0.5 mol% each) in the absence (red) and presence (green) of peptide **P2**. Relative FRET emission ratio ($I_{580\,nm}/I_{520\,nm}$) of $\mathbf{P_2}$ and monovalent $\mathbf{P_H}$ and $\mathbf{P_P}$ as control (inset).

Cu content using ICP-AES (see the Supporting Information).[15]

In additional control experiments, DOPC and DSPC vesicles with only one of the two binding sites were prepared and used for emission titrations with increasing amounts of corresponding ligands (pSer/$\mathbf{P_P}$ for Zn_2**1** and His/$\mathbf{P_H}$ for Cu**3**). As expected, no significant change in the optical properties of the fluorescent receptor vesicles was observed (Figure S21). DSPC vesicles with both co-embedded receptors Zn_2**1** and Cu**3** (0.1 mol% each) show a small energy transfer between the FAM and TMR labels in the absence of the peptide ligand (Figure S20), which might be explained by the partial formation of Zn_2**1**/Cu**3** heterodimers. Upon addition of any mono- or divalent ligand no change in emission was detected, as the gel-phase state of the DSPC membranes at room temperature limits the diffusion of the embedded metal complexes (Figure S22).

In conclusion, we have demonstrated the specific recognition of peptides by dynamic interface imprinting of synthetic binding sites embedded in vesicle membranes. The amphiphilic metal-complex binding sites are recruited in the membrane by the target peptide, resulting in multivalent interactions and nanomolar binding affinity. The use of amphiphilic binding sites with FRET pair chromophores leads to the self-assembly of a specific analyte epitope at the lipid–water interface and a FRET signal indicating the presence of the target analyte. The experimental data prove that dynamic binding-site recruitment in fluid membranes by analytes (dynamic interface imprinting, DII) leads to the formation of high-affinity epitopes. The process allows the specific detection of functional groups in peptides with nanomolar affinity. To further improve the sensitivity and selectivity of functionalized luminescent vesicles in chemical bioanalysis the number and nature of simultaneously embedded binding sites may be increased, including peptides or nucleotides as recognition moieties. The combined use of several chromophores in the membrane results in a specific spectroscopic output depending on their spatial arrangement. Such functionalized luminescent vesicles may replace antibody-based assays in some applications.

Received: July 17, 2012
Published online: September 11, 2012

Keywords: fluorescence · imprinting · metal complexes · molecular recognition · vesicle membranes

[1] a) B. Alberts, A. Johnson, J. Lewis, *Molecular Biology of the Cell*, *4th ed.*, Garland Science, New York, **2002**; b) M. Luckey, *Membrane Structural Biology*, Cambridge University Press, Cambridge, **2008**; c) S. Singer, *Science* **1992**, *255*, 1671 – 1677.

[2] a) A. Grakoui, S. K. Bromley, C. Sumen, M. M. Davis, A. S. Shaw, P. M. Allen, M. L. Dustin, *Science* **1999**, *285*, 221 – 227; b) M. Renner, C. G. Specht, A. Triller, *Curr. Opin. Neurobiol.* **2008**, *18*, 532 – 540; c) P. Tolar, J. Hanna, P. D. Krueger, S. K. Pierce, *Immunity* **2009**, *30*, 44 – 55.

[3] a) S. Tomas, L. Milanesi, *Nat. Chem.* **2010**, *2*, 1077 – 1083; b) X. Wang, R. J. Mart, S. J. Webb, *Org. Biomol. Chem.* **2007**, *5*, 2498 – 2505; c) T. Schrader, M. Maue, M. Ellermann, *J. Recept. Signal Transduction Res.* **2006**, *26*, 473 – 485; d) K. Bernitzki, T. Schrader, *Angew. Chem.* **2009**, *121*, 8145 – 8149; *Angew. Chem. Int. Ed.* **2009**, *48*, 8001 – 8005; e) A. I. Elegbede, M. K. Haldar, S. Manokaran, J. Kooren, B. C. Roy, S. Mallik, D. K. Srivastava, *Chem. Commun.* **2007**, 3377 – 3379; f) A. Perl, A. Gomez-Casado, D. Thompson, H. H. Dam, P. Jonkheijm, D. N. Reinhoudt, J. Huskens, *Nat. Chem.* **2011**, *3*, 317 – 322.

[4] a) P. Scrimin, P. Tecilla, *Curr. Opin. Chem. Biol.* **1999**, *3*, 730 – 735; b) P. Tecilla, F. Mancin, P. Scrimin, U. Tonellato, *Coord. Chem. Rev.* **2009**, *253*, 2150 – 2165; c) J. Voskuhl, B. J. Ravoo, *Chem. Soc. Rev.* **2009**, *38*, 495 – 505; d) B. Gruber, E. Kataev, J. Aschenbrenner, S. Stadlbauer, B. König, *J. Am. Chem. Soc.* **2011**, *133*, 20704 – 20707; e) D. A. Jose, S. Stadlbauer, B. König, *Chem. Eur. J.* **2009**, *15*, 7404 – 7412; f) E. Mahon, T. Aastrup, M. Barboiu, *Chem. Commun.* **2010**, *46*, 2441 – 2443.

[5] a) L. Chen, S. Xu, J. Li, *Chem. Soc. Rev.* **2011**, *40*, 2922 – 2942; b) M. Komiyama, T. Takeuchi, T. Mukawa, H. Asanuma, *Molecular Imprinting*, Wiley-VCH, Weinheim, **2004**.

[6] a) J. T. Groves, *Angew. Chem.* **2005**, *117*, 3590 – 3605; b) S. Shinkai, M. Takeuchi, *Biosens. Bioelectron.* **2004**, *20*, 1250 – 1259; c) N. W. Turner, B. E. Wright, V. Hlady, D. W. Britt, *J. Colloid Interface Sci.* **2007**, *308*, 71 – 80.

[7] a) B. Gruber, S. Stadlbauer, A. Späth, S. Weiss, M. Kalinina, B. König, *Angew. Chem.* **2010**, *122*, 7280 – 7284; *Angew. Chem. Int. Ed.* **2010**, *49*, 7125 – 7128; b) B. Gruber, S. Stadlbauer, K. Woinaroschy, B. König, *Org. Biomol. Chem.* **2010**, *8*, 3704 – 3714.

[8] A. Grauer, A. Riechers, S. Ritter, B. König, *Chem. Eur. J.* **2008**, *14*, 8922 – 8927.

[9] For phase-transition temperatures and other physical properties of lipids see also LIPIDAT database (http://www.lipidat.tcd.ie/homeLIPIDAT.asp).

[10] S.-i. Tamaru, I. Hamachi in *Recognition of Anions*, *Vol. 129* (Ed.: R. Vilar), Springer, Berlin, **2008**, pp. 95 – 125.

[11] The Zn–DPA complex will coordinate to the serine phosphate moiety, while complex Cu**4** coordinates the imidazole moiety of histidine.

[12] L. M. Loura, M. J. Prieto, *Front. Physiol.* **2011**, *2*, 82.

[13] Integrated DNA Technologies - Technical Bulletins, Fluorescence Resonance Energy Transfer, 2000, http://www.idtdna.com.

[14] In the absence of the target analyte peptide no FRET emission is detected, as the average distance between the two chromophores in the fluid membrane is too large (Figure S20).

[15] As indicated by these measurements, vesicle preparation and purification results in up to 25% loss of embedded amphiphiles. All apparent binding constants reported here, however, were not corrected to reflect this decreased receptor concentration. As a result all lg*K* values are considered "minimum" affinities.

