Luminescent vesicular receptors for the recognition of biologically important phosphate species†

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The anion binding ability of bis-zinc cyclen complexes in buffered aqueous solution was investigated using indicator displacement assays (IDA) as well as luminescent labelled complexes. A high affinity to phosphate anions, such as UTP or pyrophosphate was observed in IDA while there was no observable binding of other anions. The binding affinity, and as a result the selectivity, between different phosphate anions correlates with their overall negative charge and steric demand. Complexes bearing luminescent labels did not respond to the presence of phosphate anions in homogeneous solution, but did if embedded as amphiphiles in small unilamellar vesicle (SUV) membranes. The scope of possible anionic analytes was extended to phosphorylated protein surfaces by using such metal complex-functionalized vesicles bearing oligoethylene glycol residues in an optimized amount on their surface to suppress non-specific interactions. Under physiological conditions these surface-modified vesicles show a selective response and nanomolar affinity for α-S1-Casein, which is multiple phosphorylated, while not responding to the corresponding dephosphorylated Casein or BSA. The vesicular luminescent metal complexes do not currently reach the sensitivity and selectivity of reported enzymatic assays or some chemosensors for phosphate anions, but they present a novel type of artificial receptor for molecular recognition. Membrane-embedding of multiple, different receptors and their possible structuring on the vesicular surface is expected to improve affinities and selectivities and may allow the design of artificial antibodies.

Introduction

Molecular recognition of phosphate anions under physiological conditions is of interest as they are ubiquitously present in nature:[1] in RNA and DNA, in phosphorylated saccharides and phosphorylated proteins.[2] The nucleotide adenosine triphosphate (ATP) is the molecular currency of intracellular energy transfer,[3] and pyrophosphate ($P_2O_7^{4-}$, PP_i), the product of ATP hydrolysis, plays an important role in intracellular signalling.[4] Therefore, the development of artificial phosphate anion receptors for use under physiological conditions is of continuous interest. Such sensors are useful tools for the detection of biologically important phosphates,[5] with applications in molecular biology, life and environmental sciences.

Recent reports reveal that transition metal complexes with vacant coordination sites are well suited to serve as phosphate ion binding sites.[6] A widely used binding unit in phosphate chemosensors is the zinc(II)-dipicolylamine (Dpa) complex as demonstrated by Hamachi,[7] Hong[8] and Smith.[9] Macrocyclic 1,4,7,10-tetraazacyclododecane (cyclen) transition metal complexes were reported as phosphate binding sites by Kikuchi[10] and Kimura.[11] We have recently reported the use of zinc(II)-cyclen as promoters in ester hydrolysis,[12] detection of phosphorylated peptides[13] and proteins,[14] and for a sterically guided molecular recognition of nucleotides, nucleobases and phosphates in supramolecular self-assembled systems.[15,16]

There are two typical ways to signal the binding of an analyte to a synthetic receptor: a luminescent group is located closely to the binding site and responds to the binding event by a change in its emission properties.[17] Alternatively, an indicator-displacement assay (IDA) based on the competitive binding of a pH indicator and the analyte to the non-labelled binding site is used to signal the interaction of the analyte and the receptor.[18]

We describe here the preparation and binding properties of several 1,4,7,10-tetraazacyclododecane (cyclen) Zn(II) complexes and small unilamellar vesicles, which contain amphiphilic luminescent cyclen Zn(II) complexes as phosphate anion binding sites embedded in the vesicle membrane.

Results and discussion

Syntheses of Zn(II)-cyclen complexes

The previously reported triazine-bis-zinc cyclen complex **1** was modified by the introduction of fluorescent groups (**2**, **3**), substitution with an alkyl chain (**4**) or both (**5**, **6**). Fig. 1 summarizes all prepared compounds. Complexes **1**,[12b] **2**[14] and **4**[15] were synthesized as previously reported and the preparation of compound **3** is described in the ESI.†

The synthesis of complexes **5** and **6** is shown in Scheme 1. Amphiphilic, fluorescent compounds **10** and **11** were obtained by Williamson ether synthesis with alkyl bromide on 8-hydroxycoumarin-3-carboxylic acid ethyl ester **7**[19] and subsequent saponification. Binuclear Zn(II)-cyclen complexes **5** and **6** were

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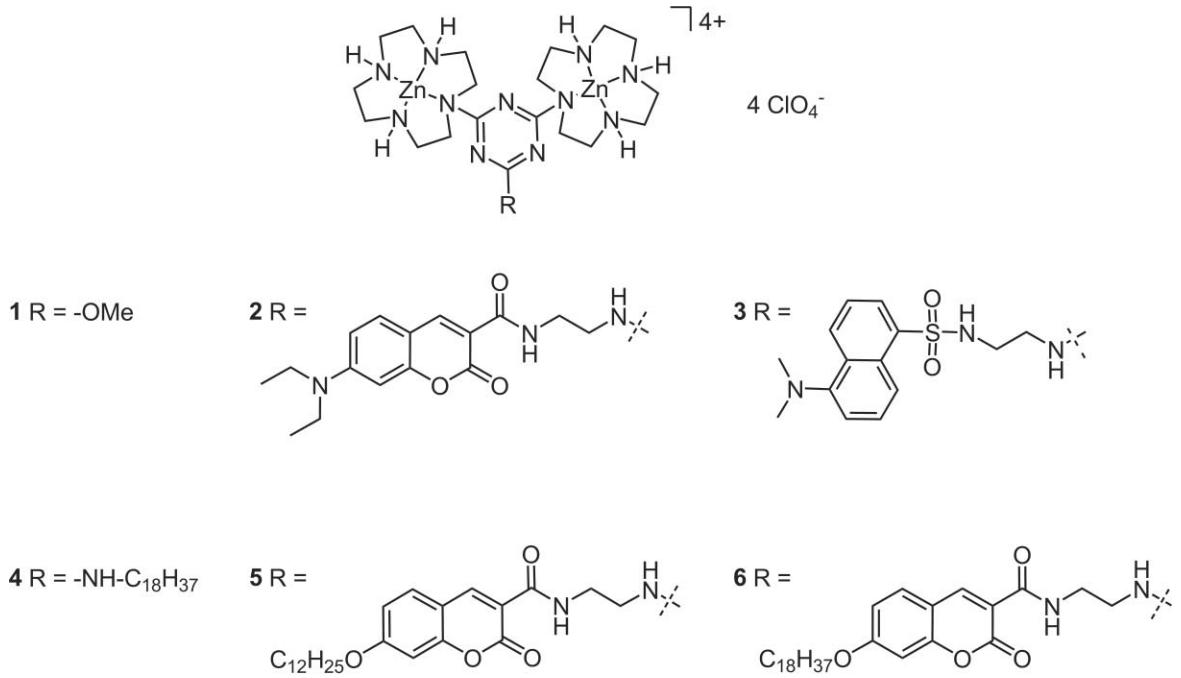
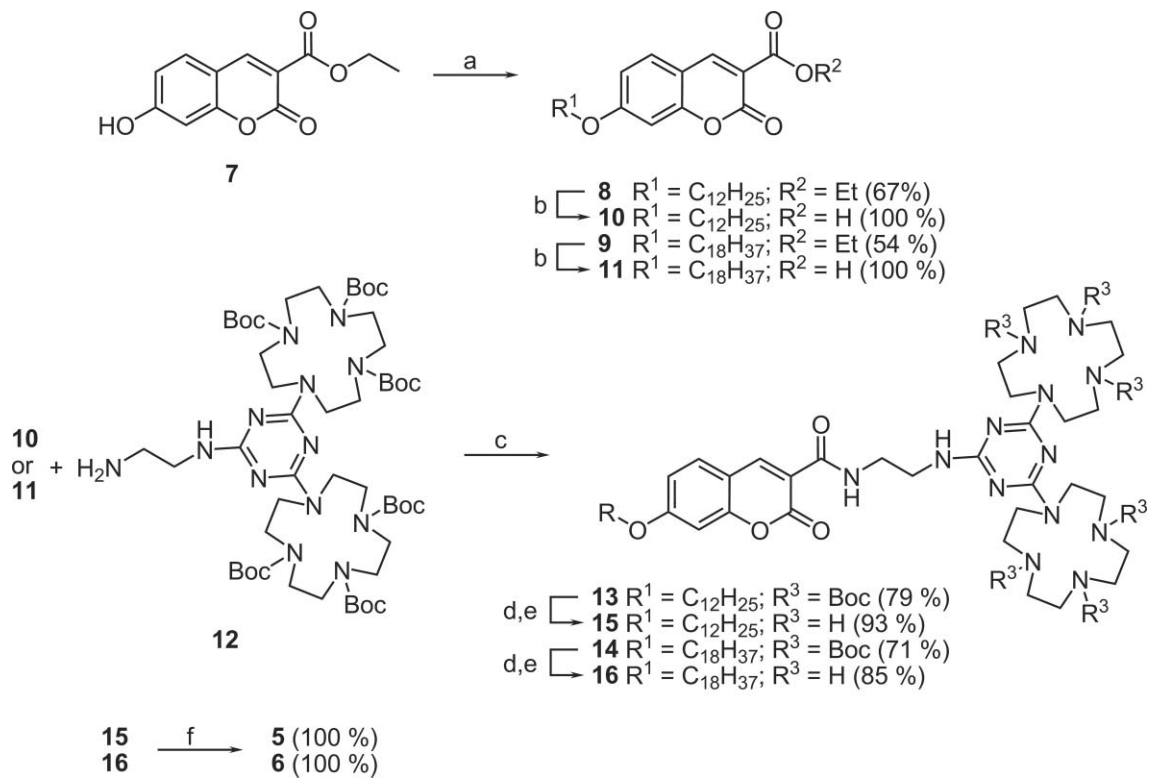


Fig. 1 Fluorescent and amphiphilic binuclear Zn(II)-cyclen complexes **1–6** for phosphate anion binding in aqueous media.



Scheme 1 Synthesis of fluorescent amphiphilic binuclear Zn(II)-cyclen complexes **5** and **6**. (a) Br-$(CH_2)_n$-CH_3 (n = 11 or 17), K_2CO_3, DMF, 80 °C, 20 h; (b) NaOH, THF, reflux, 5 h; (c) TBTU, HOBt, DIPEA, DMF, 40 °C, 2.5 h; (d) HCl/ether, RT, o/n; (e) basic ion exchanger resin H_2O, MeOH; (f) $Zn(ClO_4)_2$, MeOH, 65 °C, 20–24 h.

then prepared by amide formation using standard peptide coupling conditions in solution.

Removal of the Boc protecting groups and subsequent basic ion exchanger resin gave the free amine ligands, which finally were treated with two equivalents of a methanolic solution of $Zn(ClO)_4$. Detailed experimental procedures and analytical data of the prepared compounds are provided in the Experimental Part and in the ESI.†

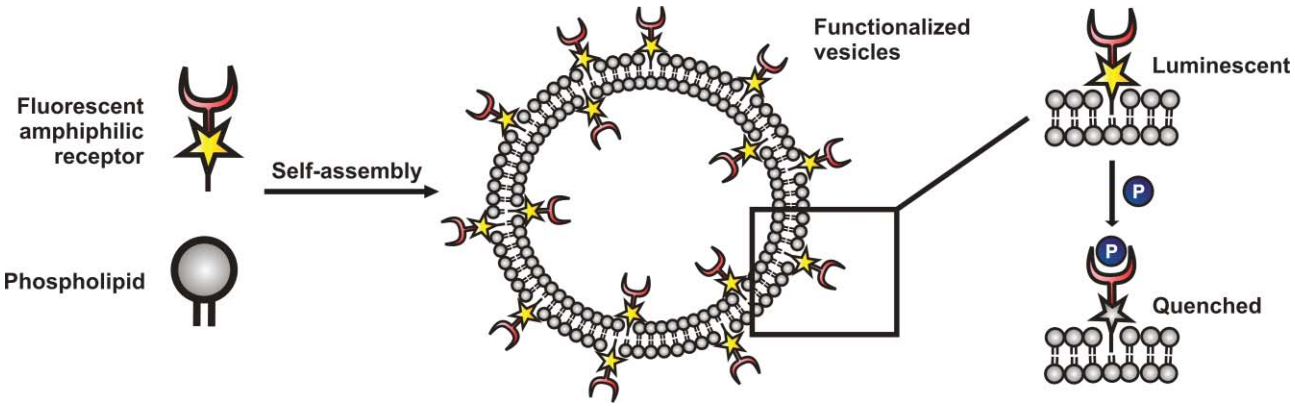


Fig. 2 Scheme of functionalized vesicles with surface exposed receptors that respond to phosphate anions by decreasing fluorescence emission.

Preparation of Zn(II)-cyclen modified vesicles

Membrane-functionalized vesicles (vesicular receptors) were prepared from a mixture of commercially available synthetic phospholipid 1,2-distearoyl-sn-*glycero*-3-phosphocholine (DSPC) and amphiphilic Zn(II)-cyclen complexes **4**, **5** and **6** (10 mol% in respect to used DSPC) by the well-established film-hydration method.[19] The resulting multilamellar vesicles (MLVs) were homogenized by extrusion to yield small unilamellar vesicles (SUVs) of a defined size of 80–100 nm.

The individual receptor units of the obtained vesicles are assumed to be equally distributed in both layers of the liposomal membrane as reported for similar surface modified vesicles.[20] Thus, we established a correction factor f describing the outer surface exposed receptors as a fraction of its entire quantity of matter. This factor enables the determination of the effective concentration of available binding sites on the outer layer of the vesicle (for details see Experimental Part). As the main phase transition temperature (T_m) of DSPC vesicles is reported to be 54 °C,[21] no transverse (flip-flop) diffusion is assumed to occur at room temperature.

Characterization of vesicle dispersions

The particle size, particle number and sample dispersity of the prepared vesicle dispersions were determined by dynamic light scattering (DLS)[22] and the average hydrodynamic diameter of the functionalized vesicles was found to range from 80 to 100 (± 5) nm (Table 2). Generally, homogenized SUV dispersions are assumed to be free of impurities and thus no further purification is required. Nevertheless vesicles can be passed through size exclusion chromatography (SEC) columns to ensure complete exclusion of unimolecular amphiphiles or aggregates of lower molecular weight.[23] All prepared vesicle dispersions were stored as buffered aqueous solutions at 6 °C and used within 2 weeks.

Phosphate anion binding studies

Zn(II) complexes of cyclen possess very high stability constants (log $K_{Zn\text{-}cyclen} = 16.2$),[24] and as a result no decomplexation of the artificial receptors is assumed to occur under the conditions of the following binding studies. Initially, the binding properties of Zn(II)-cyclen **1** to various phosphate species were investigated in homogeneous aqueous solution by an indicator displacement assay utilizing pyrocatechol violet (PV) in HEPES buffered solution (10 mM, pH 7.4) by UV-VIS spectroscopy. Upon coordination to zinc cyclen complexes, PV shows a colour change resulting from a decreasing absorption at $\lambda_{max} = 443$ nm and an increasing absorption at $\lambda_{max} = 636$ nm (Fig. 3, left).[25] By addition of aliquots of aqueous solutions (HEPES buffer 10 mM, pH 7.4) of the sodium or potassium salts of ATP, ADP, cAMP, GTP, PPi, hydrogen phosphate and phenyl phosphate to a 1 : 1 mixture of **1** and PV (35 μM each) the indicator is partially or fully displaced (Fig. 3, right). The binding constants (log K) of **1** to the different phosphate anions (Table 1) were derived from the concentrations of PV and the respective phosphate anion at 50% release of the indicator. The addition of other anions, such as SO_4^{2-}, NO_3^{2-}, N_3^-, CO_3^{2-}, Br^-, Cl^-, ClO_4^-, tartrate, ascorbate or acetate, did not displace the PV indicator from the metal complex: the absorption at $\lambda_{max} = 443$ nm remains unchanged and only a slight decrease at $\lambda_{max} = 636$ nm is observed.[26] Under the experimental conditions, no hydrolysis of pyrophosphate or ATP was induced by the bis-zinc cyclen complexes over several hours as confirmed by HPLC-MS and NMR (see ESI†).

The binding affinity is clearly influenced by the number of negative charges on the phosphate, as was previously reported for other phosphate anion receptors,[27] and the steric demand of

Table 1 Summary of binding constants to various phosphate species obtained by indicator displacement assays in solution for the binuclear Zn(II)-cyclen motif. Error limits for the determined binding constants are ± 0.2

		log K			
Entry	Phosphate species	**1**	**VR-4**	**VR-5**	**VR-6**
1	Pyrophosphate	5.9[a]	7.1[c]	6.6	6.6
2	UTP	5.4[c]	7.2[c]	—	—
3	ATP	5.9[a]	6.5[c]	6.6	6.6
4	GTP	5.8[a]	6.5[c]	—	—
5	GDP	–[b]	5.2[c]	—	—
6	UDP	4.9[c]	5.2[c]	—	—
7	Fructose-1,6-bisphosphate	–[b]	6.4[c]	—	6.1
8	Inorganic phosphate	4.9[a]	5.2[c]	—	5.5
9	p-Ser	–[b]	4.3[c]	—	—
10	Ph-O-PO_3	4.0[a]	4.2[c]	—	—

[a] Binding affinities were obtained by IDA methods (indicator dye: PV) with UV-VIS absorption titration. [b] No binding experiments were performed. [c] Binding affinities were obtained by IDA methods (indicator dye: CMS) by emission titration ($\lambda_{ex} = 396$ nm, $\lambda_{em} = 480$ nm).

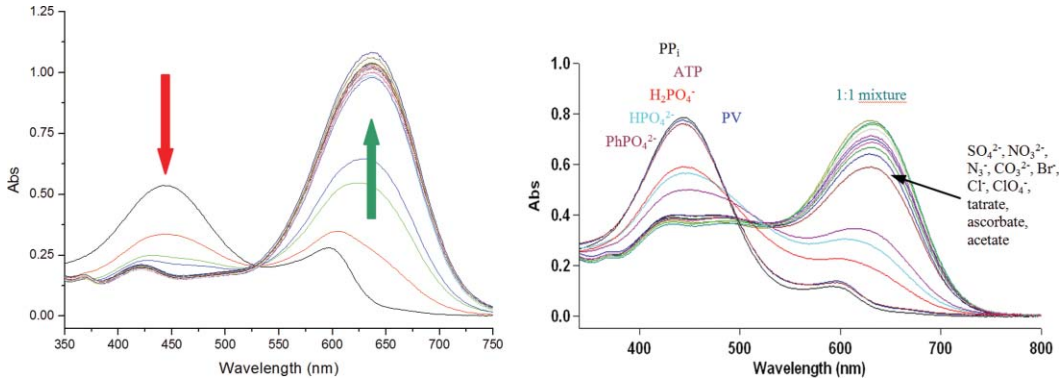


Fig. 3 (Left) Addition of **1** (0–105 μM) to a constant concentration of **PV** (35 μM). Titrations were performed at 25 °C in 10 mM HEPES buffer, pH 7.4. (Right) UV/Vis spectra of a 1 : 1 mixture **1** and **PV** (50 μM, $\lambda_{max} = 636$ nm) in the presence of various anions (250 μM). Only phosphate anions are able to displace the indicator with $\lambda_{max} = 443$ nm. The displacement, and therefore the binding ability of **1**, is proportional to the number of negative charges on the phosphate.

Table 2 Characterization of vesicular receptors

Vesicle	Molar composition	Size	λ_{ex}	λ_{ex}
VR-4	DSPC/**4** 10 : 1	80 ± 5 nm	—	—
VR-5	DSPC/**5** 10 : 1	95 ± 5 nm	349 nm	406 nm
VR-6	DSPC/**6** 10 : 1	95 ± 5 nm	349 nm	406 nm
VR-6P	DSPC/DSPC-PEG350/ **6** 5.6 : 4.4 : 1	100 ± 5 nm	349 nm	406 nm

the molecule. Nucleotides like ATP have four negative charges at the given pH and show the highest binding constants (log $K \sim 6$) together with PP_i, which has only three negative charges but is sterically much less demanding, and as a result also exhibits a very high charge density. Nucleoside diphosphates exhibit log K values which are a half up to one order of magnitude lower (~5), similar to inorganic phosphate whereas phenyl phosphate again shows a decreased affinity with a log K of 4.2. For cAMP (log $K = 3.8$), for example, partial displacement of the indicator was observed only upon addition of an excess of analyte (> 6 eq). As the IDA method represents an indirect method for the determination of the binding event, we used the Zn(II)-cyclen complexes **2** and **3**, which bear a fluorescence label and investigated their response in homogeneous aqueous buffered solution to phosphate anions (*e.g.* PP_i, ATP, GTP, ADP, Na_2HPO_4, GDP and other nucleotides). However, none of the added anions induced a significant change in the absorption or emission properties of **2** or **3** (see ESI†). The coordination of a phosphate anion obviously does not influence the photophysical properties of the covalently attached fluorophores.

Having acquired this information on phosphate anion binding of complexes **1**, **2** and **3** in homogeneous solution we turned our attention to self-assembled surface-modified vesicles for anion sensing[16] and molecular recognition.[20,28] Thus, a set of vesicular receptors modified by the amphiphilic phosphate binding moieties **4**–**6** were prepared.

A vesicular receptor (**VR-4**) with the hydrophobic binuclear Zn(II)-cyclen complex **4** was prepared and its binding affinity (Fig. 6) to various phosphate anions was investigated by IDA methods employing coumarin methyl sulfonate (CMS) as an indicator dye (Fig. 4, left). The highest binding constant was found for UTP (7.2), which exceeded the affinities of the other tested nucleotides ATP and GTP (both 6.5). This may be explained by the binding of both phosphate and imide moieties of UTP to the Zn(II)-macrocycles (see ESI†).[15] However, no difference in

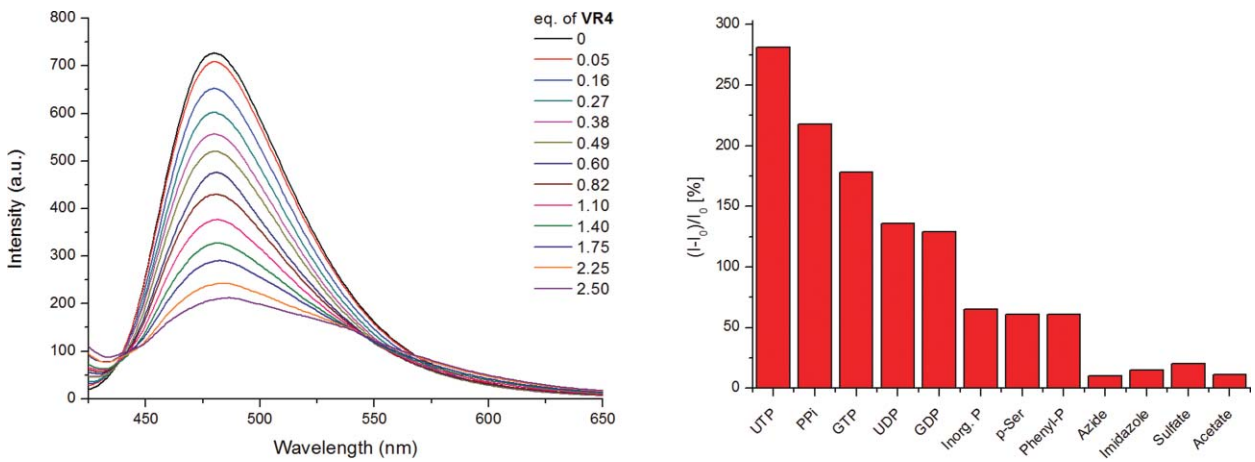


Fig. 4 (Left) Fluorescence quenching of CMS in the presence of **VR-4**. (Right) Relative changes in emission intensity obtained by displacement with various analytes.

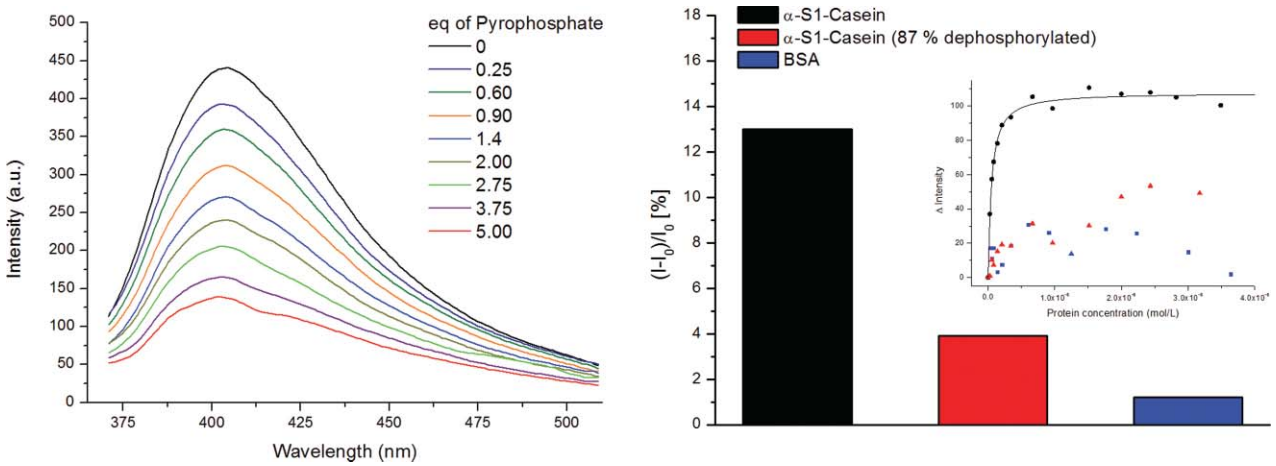


Fig. 5 (Left) Fluorescence quenching of **VR-6** in the presence of pyrophosphate anions. (Right) Relative emission response of **VR-6P** in the presence of different proteins (0.05 equivalents). Inset plot shows corresponding binding isotherms of α-S1-Casein, dephosphorylated α-S1-Casein and BSA to VR-6P [5×10^{-6} M Zn(II)].

binding affinities of the nucleoside diphosphates UDP and GDP was observed. GDP and UDP both show a binding constant of 5.2 as, due to the weaker coordination capabilities of diphosphates, the affinity is one to two orders of magnitude lower compared to the respective triphosphates. Pyrophosphate, due to its small size and high charge density, exhibited a remarkable binding affinity with a log K value of 7.1. Fructose-1,6-bisphosphate also binds tightly (log $K = 6.4$), which might be explained by the favourable interaction with two bis-Zn(II)-cyclen moieties revealing additive or even cooperative action: following the binding of the first phosphate group, the binding of the second phosphate group is facilitated by the preformed substrate–receptor complex. Inorganic phosphate showed the same binding constant as the diphosphates GDP and UDP. At the given pH value inorganic phosphate exists predominantly as a dianion, having one negative charge less than GDP and UDP. The similar binding affinities might be explained by the lack of steric hindrance of inorganic phosphate as well as by an increase of its acidity upon complexation by the bis-Zn(II)-cyclen moieties, resulting in an additional negative charge. The phosphate monoesters phenylphosphate and phosphoserine, in contrast, bind with an affinity one order of magnitude lower than inorganic phosphate, obviously due to their larger steric demands and only two acidic protons present at the phosphate moiety. For all tested and compared compounds the respective binding constants to the vesicular receptor were found to be higher than those to complex **1**. Only a minor increase was found for the monophosphates phenylphosphate and inorganic phosphate, whereas the difference in the binding constants for pyrophosphate amounted to an entire order of magnitude. The ion selectivity of vesicular receptor **VR-4** was investigated by the addition of other anionic compounds like sulfate, azide and acetate (see ESI† and Fig. 4, right). Furthermore, imidazole was tested as a potential ligand, as the bis-Zn(II)-cyclen moieties are known to have a weak affinity for histidine residues.[29] None of these compounds showed a considerable affinity towards the vesicular receptor **VR-4**. Thus, binding constants for these compounds could not be determined, but were estimated to be smaller than log $K = 2$.

Using the amphiphilic fluorescent binuclear Zn(II)-cyclen **6** and its corresponding vesicular receptor **VR-6**, a direct signalling of the phosphate ion binding event was possible (Fig. 2): upon addition of phosphate anions, such as PP_i, ATP, fructose-1,6-bisphosphate and inorganic phosphate, the emission intensity at 405 nm of the coumarin label decreased (Fig. 5 left). To the best of our knowledge this technique of anion sensing with membrane-embedded fluorescence-labeled metal complexes has not been reported so far. The determined binding affinities exceed the micromolar range and are consistent with the corresponding values obtained by the indicator displacement assay for the vesicular receptor **VR-4** and compound **1**. Modifying the tethered hydrophobic alkyl chain of the binuclear Zn(II)-cyclen complex from C_{18} to C_{12} (compound **5**) did not affect the binding affinity of the vesicular receptor **VR-5** to PP_i and ATP (Table 1).

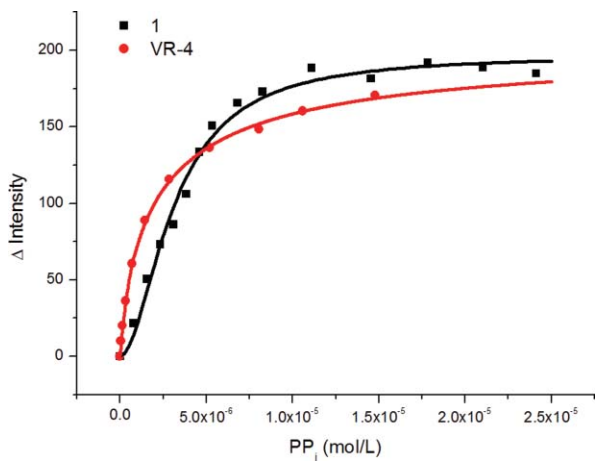


Fig. 6 Comparison of binding isotherms obtained by indirect IDA methods of **1** and **VR-4** to PP_i.

Monitoring of phosphate binding to compounds **2** and **3** by changes of their luminescence was not possible in aqueous solution, most likely due to the flexible linkage of binding and signalling sites. Compounds **5** and **6** are expected to be embedded with their alkyl chain and the coumarin dye into the vesicle bilayer, as reported for similar coumarin derivatives.[30] This should significantly restrict their movement in the highly ordered vesicle

bilayer, which might be beneficial for the sensing properties.[31] In addition, the local polarity change at the fluorophore, which is a crucial factor for the response to phosphate binding, is expected to be larger at the vesicle membrane–water interface compared to bulk water.[30] Coumarin dye derivatives are known for their solvatochromism in various solvents of different polarity.[32]

Subsequently to the binding studies with low molecular weight phosphate species, binding towards larger analytes bearing several phosphate anions was investigated to potentially take advantage of the numerous binding sites on the surface of the nanometre-sized vesicles. To demonstrate this, the affinities towards proteins with and without multiple phosphorylated sites were determined: α-S1-Casein from bovine milk bearing eight phosphorylated serine residues was expected to specifically interact with the zinc cyclen receptors on the vesicle surface. A (for the most part) dephosphorylated sample of the same protein and BSA were used as control to exclude non-specific vesicle–protein interactions.

The vesicular receptors (**VR-6**) exhibited, as expected, a significant non-specific interaction to all tested proteins regardless of the presence of phosphorylated sites (data not shown). Such non-specific interactions between proteins and liposome surfaces, for example due to electrostatic attraction, are well documented.[33]

To suppress the non-specific interactions between the surfaces of liposome and protein and to gain selectivity for the phosphate anion zinc-cyclen receptor interaction, the vesicular surface was shielded by polyethylene glycol (PEG) residues. Therefore, vesicles were prepared from mixtures of DSPC and DSPC-PEG350 having oligoethylene glycol residues attached to its polar head groups (see ESI†).

At an optimum composition of about 6 : 4 DSPC to DSPC-PEG350 (**VR-6P**) the non-specific binding is suppressed sufficiently to allow a selective recognition of α-S1-Casein (Fig. 5 right). Due to its multiple phosphorylations, the protein's affinity is in the nanomolar range and about two orders of magnitude higher than the affinity determined for a single phosphate group (Table 1). BSA shows only a very small relative change in fluorescence intensity, as does the dephosphorylated α-S1-Casein sample (considering the fact that it still contains residual phosphate moieties). Furthermore the two control proteins do not give meaningful binding isotherms. Vesicles with a lower PEG-content showed reduced or no selectivity, whereas a higher PEG-concentration on the vesicular surface decreases the binding affinity to the phosphate groups significantly (data not shown).

Conclusions

Indicator displacement assays revealed the binding of bis-zinc(II)-cyclen complex **1** or the vesicle-embedded amphiphilic complex **4** to phosphate anions in buffered aqueous solution. On the contrary, bis-zinc(II)-cyclen derivatives **2** and **3**, which are covalently modified with coumarin or dansyl labels, respectively, show no response to the presence of phosphate anions in homogeneous aqueous solution. Only if the amphiphilic coumarin-labelled derivatives **5** and **6** are embedded into vesicular membranes do they show a luminescent response to the presence of phosphate anions. We explain this by the more confined environment of the fluorophore in the membrane if compared to homogeneous solution and the change of this environment and the localization of the fluorophore upon phosphate anion binding. Such membrane-embedded luminescent metal complexes responding to the presence of anions have not been reported before and may find applications in sensory surfaces or particles. The binding affinity of all bis-zinc(II)-cyclen complexes towards phosphate anions correlates with the overall charge of the anions and their steric demand. The discrimination of low molecular phosphate species by the vesicular receptors is not sufficient for practical analytical applications, but they provide an ideal scaffold for the molecular recognition of biologically important multivalent targets. This is illustrated by their specific interaction with a phosphorylated protein. To suppress non-specific interactions, an optimized amount of PEG-modified amphiphiles was used to prepare the functionalized vesicles, which showed a selective response and nanomolar affinity to the phosphorylated protein α-S1-Casein. The simple method of their preparation may allow the development of more complex vesicles responding with even higher affinity and selectivity by the combination of different synthetic receptors into the membrane surface. Our ongoing investigations focus on the preparation of new receptor building blocks and chromophores and the recognition of multivalent ligands by multi-receptor vesicles.

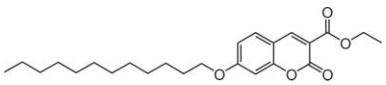
Experimental part

General methods and material

Fluorescence measurements were performed with UV-grade solvents (Baker or Merck) in 1 cm quartz cuvettes (Hellma) and recorded on a Varian 'Cary Eclipse' fluorescence spectrophotometer with temperature control. Absorption spectra were recorded on a Varian Cary BIO 50 UV/VIS/NIR Spectrometer with temperature control by use of a 1 cm quartz cuvette (Hellma) and Uvasol solvents (Merck or Baker). PCS measurements were performed on a Malvern Zetasizer 3000 at 25 °C using 1 cm disposable polystyrene fluorescence cuvettes (VWR). Three subsequent measurements of 60 s each were performed for each sample. Data analysis was performed using the Malvern PCS software. NMR Spectra: Bruker Avance 600 (^{1}H: 600.1 MHz, ^{13}C: 150.1 MHz, $T = 300$ K), Bruker Avance 400 (^{1}H: 400.1 MHz, ^{13}C: 100.6 MHz, $T = 300$ K), Bruker Avance 300 (^{1}H: 300.1 MHz, ^{13}C: 75.5 MHz, $T = 300$ K). The chemical shifts are reported in δ[ppm] relative to external standards (solvent residual peak). The spectra were analyzed by first order, the coupling constants are given in Hertz [Hz]. Characterization of the signals: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad singlet, psq = pseudo quintet, dd = double doublet, dt = double triplet, ddd = double double doublet. Integration is determined as the relative number of atoms. Assignment of signals in ^{13}C spectra was determined with DEPT-technique (pulse angle: 135°) and given as (+) for CH_3 or CH, (–) for CH_2 and (C_q) for quaternary C_q. Error of reported values: chemical shift: 0.01 ppm for ^{1}H-NMR, 0.1 ppm for ^{13}C-NMR and 0.1 Hz for coupling constants. The solvent used is reported for each spectrum. Mass Spectra: Varian CH-5 (EI), Finnigan MAT 95 (CI; FAB and FD), Finnigan MAT TSQ 7000 (ESI). Xenon serves as the ionisation gas for FAB. IR spectra were recorded with a Bio-Rad FTS 2000 MX FT-IR and Bio-Rad FT-IR FTS 155. Melting Points were determined on Büchi SMP or a Lambda Photometrics OptiMelt MPA 100. Thin layer chromatography (TLC) analyses were performed on

silica gel 60 F-254 with a 0.2 mm layer thickness. Detection *via* UV light at 254 nm/366 nm or through staining with ninhydrin in EtOH. Column chromatography was performed on silica gel (70–230 mesh) from Merck. Starting materials were purchased from either Acros or Sigma-Aldrich and used without any further purification. Commercially available solvents of standard quality were used. Dry THF was prepared by distillation from potassium. Unless otherwise stated, purification and drying was done according to accepted general procedures.[34] Elemental analyses were carried out by the centre for chemical analysis of the Faculty of chemistry and pharmacy of the University of Regensburg.

Synthesis

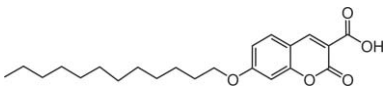


7-Dodecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid ethyl ester (8). Under nitrogen atmosphere hydroxy-coumarin ethylester **7** (779 mg, 3.3 mmol) was dissolved in dry DMF (12 mL) and K_2CO_3 (2.68 g, 11.6 mmol) was added. Subsequently 1-bromododecane (1.2 mL, 5.0 mmol) was given dropwise to the stirred suspension. The reaction mixture was stirred over night (20 h) at 80 °C. The reaction progress was monitored by TLC (chloroform). K_2CO_3 was filtered off and the filtrate was concentrated. The crude product was purified by flash column chromatography on flash silica gel (chloroform; R_f 0.48) yielding compound **8** (537 mg, 1.33 mmol, 40%) as yellow solid.

MP: 71 °C. – **^{1}H-NMR** (400 MHz; $CDCl_3$): δ (ppm) = 0.87 (t, ^3J = 7.1 Hz, 3 H, COSY: $C^{24}H_3$), 1.17–1.36 (m, 16 H, COSY: $C^{16}H_2$ – $C^{23}H_2$), 1.38 (t, ^3J = 7.1 Hz, 3 H, COSY: C^1H_3), 1.43–1.51 (m, 2 H, COSY: $C^{15}H_2$), 1.81 (quin, 2 H, COSY: $C^{14}H_2$), 4.03 (t, ^3J = 6.1 Hz, 2 H, COSY: $C^{13}H_2$), 4.39 (q, ^3J = 7.1 Hz, 2 H, COSY: C^2H_2), 6.78 (d, ^3J = 1.9 Hz, 1 H, HMBC: C^7H), 6.87 (dd, ^3J = 1.9 Hz, 8.5 Hz, 1 H, COSY: $C^{10}H$), 7.48 (d, ^3J = 8.5 Hz, 1 H, COSY: C^9H), 8.49 (s, 1 H, HMBC: $C^{12}H$). – **^{13}C-NMR** (100 MHz; $CDCl_3$): δ (ppm) = 14.1 (+, 1 C, HSQC, COSY: $C^{24}H_3$), 14.3 (+, 1 C, HSQC, COSY: C^1H_3), 22.6, 29.26, 29.29, 29.47, 29.52, 29.59, 31.9 (–, 8 C, HSQC, COSY: $C^{16}H_2$ – $C^{23}H_2$), 25.9 (–, 1 C, HSQC, COSY: $C^{15}H_2$), 28.8 (–, 1 C, HSQC, COSY: $C^{14}H_2$), 61.3 (–, 1 C, HSQC, COSY: C^2H_2), 70.0 (–, 1 C, HSQC, COSY: $C^{13}H_2$), 100.8 (+, 1 C, HSQC, HMBC: C^7H), 114.0 (+, 1 C, HSQC, COSY: $C^{10}H$), 130.6 (+, 1 C, HSQC, COSY: C^9H), 149.0 (+, 1 C, HSQC, HMBC: $C^{10}H$), 111.4 (C_q, 1C, HMBC: C^{11}), 113.9 (C_q, 1 C, HMBC: C^6), 157.2 (C_q, 1 C, HMBC: C^5), 157.6 (C_q, 1 C, HMBC: C^4), 163.5 (C_q, 1 C, HMBC: C^3), 164.7 (C_q, 1 C, HMBC: C^8). – **IR** (KBr) [cm^{-1}]: $\tilde{\nu}$ = 2918, 2847, 1748, 1693, 1598, 1553, 1469, 1434, 1378, 1301, 1213, 1110, 1027, 793, 722. – **UV**($CHCl_3$): λ_{max} (lg ε) = 352 nm (4.423). – **MS** (CI (NH_3): *m/z* (%) = 403.2 (100) [MH^+], 420.2 (38) [M + NH_4^+]. – **HRMS** Calcd for $C_{24}H_{34}O_5$: 402.2406; Found: 402.2398. – **MF**: $C_{24}H_{34}O_5$ – **FW**: 402.54 g mol^{-1}

7-Dodecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid (9). Under nitrogen atmosphere hydroxy-coumarin ethylester **7** (1.3 g, 5.6 mmol) was dissolved in dry DMF and K_2CO_3 (2.7 g, 19.5 mmol) was added. Subsequently 1-octadecylamine (2.8 g, 8.4 mmol) was given dropwise to the stirred suspension. The reaction mixture was stirred over night (20 h) at 60 °C. The reaction progress was monitored by TLC (chloroform). K_2CO_3 was filtered off and the filtrate was concentrated. The crude product was purified by flash column chromatography on flash silica gel (chloroform; R_f 0.34) yielding compound **9**(1.4 g, 2.8 mmol, 50%) as yellow solid.

MP: 84 °C. – **^{1}H-NMR** (300 MHz; $CDCl_3$): δ (ppm) = 0.87 (t, ^3J = 6.7 Hz, 3 H, CH_3), 1.12–1.35 (m, 28 H, CH_2), 1.39 (t, ^3J = 7.1 Hz, 3 H, CH_3), 1.43-1.54 (m, 2 H, CH_2), 1.81 (quin, 2 H, CH_2), 3.63 (t, ^3J = 6.6 Hz, 1.7 H, CH_2), 4.02 (t, ^3J = 6.6 Hz, 0.3 H, CH_2), 4.39 (t, ^3J = 7.1 Hz, 2 H, CH_2), 6.79 (d, ^3J = 2.2 Hz, 1 H, CH), 6.87 (dd, ^3J = 2.3 Hz, 8.6 Hz, 1 H, CH), 7.48 (d, ^3J = 8.8 Hz, 1 H, CH), 8.49 (s, 1 H, CH). – **^{13}C-NMR** (75 MHz; $CDCl_3$): δ (ppm) = 14.1 (+, 1 C, CH_3), 14.3 (+, 1 C, CH_3), 22.7 (–, 1 C, CH_2), 25.8 (–, 0.2 C, CH_2), 25.9 (–, 0.8 C, CH_2), 28.9 (–, 1 C, CH_2), 29.32 (–, 1 C, CH_2), 29.38 (–, 1 C, CH_2), 29.46 (–, 0.2 C, CH_2), 29.54 (–, 0.8 C, CH_2), 29.59 (–, 1 C, CH_2), 29.67, 29.71 (–, 8 C, CH_2), 31.9 (–, 0.9 C, CH_2), 32.8 (–, 0.1 C, CH_2), 61.7 (–, 0.8C, CH_2), 63.1 (–, 0.2 C, CH_2), 69.0 (–, 1 C, CH_2), 100.8 (+, 1 C, CH), 111.5 (C_q, 1C), 113.8 (C_q, 1 C), 114.0 (+, 1 C, CH), 130.7 (+, 1 C, CH), 149.1 (+, 1 C, CH), 157.3(C_q, 1 C), 157.7 (C_q, 1 C), 163.5 (C_q, 1 C), 164.8 (C_q, 1 C). – **IR** (ATR) [cm^{-1}]: $\tilde{\nu}$ = 2916, 2849, 1746, 1702, 1624, 1510, 1472, 1376, 1228, 1180, 1040, 847, 793, 718. – **UV**($CHCl_3$): λ_{max} (lg ε) = 352 nm (4.246). – **MS** (EI): *m/z* (%) = 486.3 (20) [$M^{+\bullet}$], 440.4 (12) [$M^{+\bullet}$ - EtOH], 247.0 (50) [$M^{+\bullet}$ - $C_{17}H_{35}^{\bullet}$], 234.0 (100) [$M^{+\bullet}$ - $C_{18}H_{36}$], 189.0 (90) [$M^{+\bullet}$ - $C_{18}H_{36}$ - EtO$^{\bullet}$]. – **HRMS** Calcd for $C_{30}H_{46}O_5$ 486.3345; Found: 486.3347. – **MF**: $C_{30}H_{46}O_5$ – **FW**: 486.70 g mol^{-1}



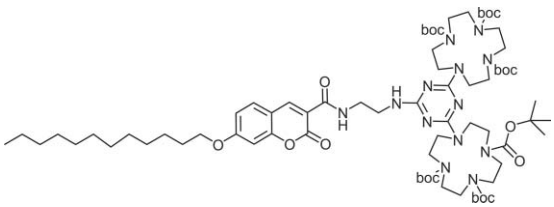
7-Dodecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid (10). Ethyl ester of compound **8** (386 mg, 0.96 mmol) was dissolved in THF (6.0 mL) and heated to reflux. Subsequently 2 M NaOH (15.4 mL) was added and the solution was refluxed for 5 h. Reaction control was performed by TLC (chloroform). The reaction mixture was cooled to room temperature and further to 0 °C by an ice bath. The yellow solution was acidified with 1 M HCl until a white precipitate was formed which was isolated by filtration and washed with cold water. Compound **10** was obtained as a white solid (360 mg, 0.96 mmol, 100%).

MP: 126 °C. – **^{1}H-NMR** (600 MHz; $CDCl_3$): δ (ppm) = 0.87 (t, ^3J = 7.0 Hz, 3 H, HSQC, COSY: C^1H_3), 1.16-1.33 (m, 14 H, HSQC, COSY: C^2H_2 – C^8H_2), 1.33-1.42 (m, 2 H, HSQC, COSY: C^9H_2), 1.47 (quin, ^3J = 7.4 Hz, 2 H, HSQC, COSY: $C^{10}H_2$), 1.84 (quin, ^3J = 7.6 Hz, 2 H, HSQC, COSY: $C^{11}H_2$), 4.08 (t, ^3J = 6.5 Hz, 2 H, HSQC, COSY: $C^{12}H_2$), 6.89 (d, ^4J = 2.0 Hz, 1 H, HSQC, COSY: $C^{14}H_2$), 6.99 (dd, ^3J = 8.8 Hz,^4J = 2.2 Hz, 1 H, HSQC, COSY: $C^{15}H_2$), 7.62 (d, ^3J = 8.7 Hz, 1 H, HMBC, HSQC: $C^{21}H_2$), 8.84 (s, 1 H, HMBC, HSQC: $C^{17}H_2$), 12.16 (bs, 1 H, HSQC: $COOH^{23}$). – **^{13}C-NMR** (150 MHz; $CDCl_3$): δ (ppm) = 14.1 (+, 1 C, HSQC, COSY: C^1H_3), 22.6, 29.29, 29.46, 29.51, 29.58, 29.59, 31.9 (–, 7 C, HMBC, HSQC: C^2H_2 – C^8H_2), 25.8 (–, 1 C, HSQC, COSY: $C^{10}H_2$), 28.8 (–, 1 C, HSQC, COSY: $C^{11}H_2$), 29.23 (–, 1 C, HSQC, COSY: C^9H_2), 69.4 (–, 1 C, HSQC, COSY: $C^{12}H_2$), 101.2 (+, 1 C, HSQC, COSY: $C^{14}H$), 110.6 (C_q, 1 C, HMBC, HSQC: C^{18}), 112.1 (C_q, 1 C, HMBC, HSQC: C^{16}), 115.4 (+, 1 C, HSQC, COSY: $C^{15}H$), 131.6 (+, 1 C, HMBC, HSQC: $C^{21}H$), 151.2 (+, 1C, HMBC, HSQC: $C^{17}H$), 157.1 (C_q, 1 C, HMBC, HSQC: C^{20}), 163.1 (C_q, 1 C, HMBC, HSQC: C^{19}), 164.6 (C_q, 1 C, HMBC, HSQC: C^{22}), 165.9

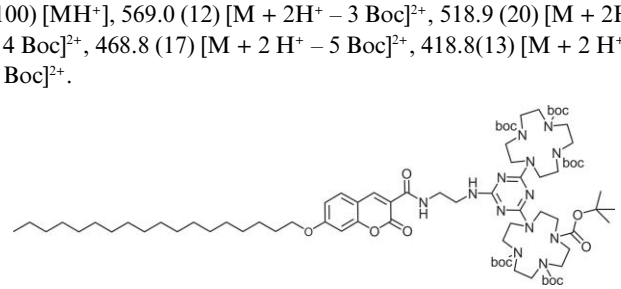
(C_q, 1 C, HMBC, HSQC: C^{13}). – **IR**(ATR) [cm^{-1}]: $\tilde{\nu}$ = 2914, 2846, 1734, 1686, 1619, 1560, 1504, 1466, 1429, 1384, 1258, 1217, 1121, 922, 807, 720. – **UV** ($CHCl_3$): λ_{max} (lg ε) = 277 (4.305), 358 nm (4.418). – **MS** (ESI(–), EE/MeOH + 10 mmol L^{-1} NH_4Ac): *m/z* (%) = 373.1(100) $[M - H^+]^-$, 329.1 (13) $[M - CO_2]^-$, 747.4 (8) $[2M - H^+]^-$. – **HRMS** Calcd for $C_{22}H_{30}O_5$: 374.2093; Found: 374.2088. – **MF**: $C_{22}H_{30}O_5$ – **FW**: 374.48 g mol^{-1}

7-Octadecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid (11). Ethyl ester of compound **9** (297 mg, 0.61 mmol) was dissolved in THF (5.0 mL) and heated to reflux. Subsequently 2 M NaOH (10.3 mL) was added and the solution was refluxed for 4 h. Reaction control was performed by TLC (chloroform). The reaction mixture was cooled to room temperature and further to 0 °C by an ice bath. The yellow solution was acidified with 1 M HCl until a white precipitate was formed which was isolated by filtration and washed with cold water. After drying in vacuum 278 mg (0.61 mmol, 100%) of compound **11** was obtained as a white solid.

MP: 125 °C. – **^{1}H-NMR** (300 MHz; $CDCl_3$): δ (ppm) = 0.86 (t, ^3J = 6.7 Hz, 3 H, CH_3), 1.03-1.38 (m, 28 H, CH_2), 1.41-1.58 (m, 2 H, CH_2), 1.68-1.97 (m, 2 H, CH_2), 3.64 (t, ^3J = 6.7 Hz, 0.2 H, CH_2), 4.08 (t, ^3J = 6.4 Hz, 1.8 H, CH_2), 6.89 (d, ^4J = 2.5 Hz, 1 H, CH), 6.99 (dd, ^3J = 8.8 Hz,^4J = 2.5 Hz, 1H, CH), 7.62 (d, ^3J = 8.8 Hz, 1 H CH), 8.84 (s, 1 H, CH). – **^{13}C-NMR** (75 MHz; $CDCl_3$): δ (ppm) = 14.1 (+, 1 C, CH_3), 22.7 (–, 1 C, CH_2), 25.9 (–, 1 C, CH_2), 28.8 (–, 1 C, CH_2), 29.3 (–, 1 C, CH_2), 29.4, (–, 1 C, CH_2), 29.53 (–, 1 C, CH_2), 29.58 (–, 1 C, CH_2), 29.67 (–, 2 C, CH_2), 29.71 (–, 6 C, CH_2), 31.9(–, 1 C, CH_2), 69.4 (–, 1 C, CH_2), 101.2 (+, 1 C, CH), 110.6 (C_q, 1 C), 112.2 (C_q, 1C), 115.5 (+, 1 C, CH), 131.7 (+, 1 C, CH), 151.3 (+, 1 C, CH), 157.1 (C_q, 1 C), 163.2(C_q, 1 C), 164.6 (C_q, 1 C), 166.0 (C_q, 1 C). – **IR** (ATR) [cm^{-1}]: $\tilde{\nu}$ = 2915, 2850, 1733, 1686, 1622, 1560, 1505, 1471, 1383, 1256, 1221, 1122, 1005, 820, 798. – **UV**($CHCl_3$): λ_{max}(lg ε) = 358 nm (4.009). – **MS** (ESI(+), DCM–MeOH + 10 mmol L^{-1} NH_4Ac): *m/z*(%) = 459.3 (100) $[MH^+]$, 476.3 (25) $[M + NH_4^+]$. – **HRMS** Calcd for $C_{28}H_{42}O_5$ 458.3032; Found: 458.3026. –**MF**: $C_{28}H_{42}O_5$ – **FW**: 458.64 g mol^{-1}



7-Dodecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid {2-[4,6-bis-(1,4,7,10 tetraaza-cyclododec-1-yl)-[1,3,5]triazin-2-yl]-ethyl}-1,4,7-tricarboxylic acid tri-*tert*-butyl ester (13). 7-Dodecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid **10** (200 mg, 0.53 mmol), DIPEA (368 µL, 2.14 mmol), TBTU (189 mg, 0.59 mmol), and HOBt (90 mg, 0.59 mmol) were dissolved under nitrogen atmosphere in dry DMF–THF (2 mL/4 mL) under ice cooling and stirred for 1 h. Subsequently **12** (635 mg, 0.59 mmol) dissolved in DMF (2 mL) was added dropwise. The reaction was allowed to warm to room temperature and was stirred for 30 min at rt and 2.5 h at 40 °C. The reaction progress was monitored by TLC (ethyl acetate–petrol ether). After completion of the reaction the solvent was removed and the crude product was purified by flash column chromatography on flash silica gel (ethyl acetate–petrol ether 1 : 1; R_f 0.25) yielding compound **13** (608 mg, 79%) as a lightly yellow solid. **MP**: 113 °C. – **^{1}H-NMR** (400 MHz; $CDCl_3$): δ = 0.85 (t, ^3J = 6.9 Hz, 3 H, HSQC, COSY: C^1H_3), 1.20-1.33 (m, 18 H, HSQC, COSY: C^2H_2 – $C^{10}H_2$), 1.41 (s, 18H, HSQC, COSY: $C^{33}H_3$), 1.42 (s, 36 H, HSQC, COSY: $C^{33}H_3$), 1.80 (quin, ^3J = 7.3 Hz, 2 H, HSQC, COSY: $C^{11}H_2$), 3.02-3.89 (m, 36 H, HSQC, COSY: $C^{24}H_2$, $C^{25}H_2$, $C^{30}H_2$), 4.02 (t, ^3J = 6.5 Hz, 2 H, HSQC, COSY: $C^{12}H_2$), 4.99 (bs, 1 H, HMBC, HSQC: NH^{23}), 6.81 (d, ^4J = 2.2 Hz, 1 H, HMBC, HSQC, COSY: $C^{21}H_2$), 6.90 (dd, ^4J = 2.3 Hz, ^3J = 8.8 Hz, 1 H, HMBC, HSQC, COSY: $C^{14}H$), 7.54 (d, ^3J = 8.8 Hz, 1 H, HMBC, HSQC, COSY: $C^{15}H$), 8.79 (s, 1 H, HMBC, HSQC: $C^{17}H$), 8.87 (m, 1 H, HMBC, HSQC: NH^{26}). – **^{13}C-NMR** (100 MHz; $CDCl_3$): δ = 14.0 (+, 1 C, HSQC, COSY: C^1H_3), 22.6, 25.9, 29.23, 29.26, 29.46, 29.49, 29.55, 29.57, 31.8 (–, 9 C, HSQC, COSY: C^2H_2 – $C^{10}H_2$), 28.43, 28.47 (+, 18 C, HSQC, COSY: $C^{33}H_3$), 28.8 (–, 1 C, HSQC, COSY: $C^{11}H_2$), 39.8, 40.6 (–, 2C, HSQC, COSY: $C^{24}H_2$, $C^{25}H_2$), 50.2 (–, 16 C, HSQC, COSY: $C^{30}H_2$), 69.0 (–, 1 C, HMBC, HSQC: $C^{12}H_2$), 79.7 (C_q, 6 C, HMBC, HSQC: C^{32}), 100.7 (+, 1 C, HMBC, HSQC, COSY: $C^{21}H$), 112.1 (C_q, 1 C, HMBC, HSQC: C^{16}), 114.35 (+, 1 C, HMBC, HSQC, COSY: $C^{14}H$), 114.41 (C_q, 1 C, HMBC, HSQC: C^{19}), 130.8 (+, 1 C, HMBC, HSQC, COSY: $C^{15}H$), 148.3 (+, 1 C, HMBC, HSQC, COSY: $C^{17}H$), 156.3 (C_q, 6 C, HMBC, HSQC: C^{31}), 156.7 (C_q, 1 C, HMBC, HSQC: C^{20}), 161.7 (C_q, 1 C, HMBC, HSQC: C^{18}), 162.7 (C_q, 1 C, HMBC, HSQC: C^{22}), 164.5 (C_q, 1 C, HMBC, HSQC: C^{13}), 165.9 (C_q, 3 C, HMBC, HSQC: C^{27}, C^{28}, C^{29}). – **IR**(KBr) [cm^{-1}]: $\tilde{\nu}$ = 2973, 2929, 2878, 1686, 1603, 1535, 1501, 1466, 1408, 1247, 1160, 1026, 971, 858, 776. – UV ($CHCl_3$): λ_{max} (lg ε) = 351 nm (4.336). – **MS** (ESI(+), EE/MeOH + 10 mmol L^{-1} NH_4Ac): *m/z* (%) = 1437.3 (100) $[MH^+]$, 569.0 (12) $[M + 2H^+ - 3\ Boc]^{2+}$, 518.9 (20) $[M + 2H^+ - 4\ Boc]^{2+}$, 468.8 (17) $[M + 2\ H^+ - 5\ Boc]^{2+}$, 418.8(13) $[M + 2\ H^+ - 6\ Boc]^{2+}$.



7-Octadecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid {2-[4,6-bis-(1,4,7,10 tetraaza-cyclododec-1-yl)-[1,3,5]triazin-2-ylamino]-ethyl}-1,4,7-tricarboxylic acid tri-*tert*-butyl ester (14). 7-Octadecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid **11** (160 mg, 0.35 mmol), DIPEA (360 µL, 2.09 mmol), TBTU (123 mg, 0.38 mmol), and HOBt (59 mg, 0.38 mmol) were dissolved under nitrogen atmosphere in dry DMF–THF (2 mL/4 mL) under ice cooling and stirred for 1 h. Subsequently **12** (415 mg, 0.38 mmol) dissolved in DMF (2 mL) was added dropwise. The reaction was allowed to warm to room temperature and was stirred 30 min at rt and 4.5 h at 40 °C. The reaction progress was monitored by TLC (ethyl acetate–petrol ether). After completion of the reaction the solvent was removed and the crude product was purified by flash column chromatography on flash silica gel (ethyl acetate–petrol ether 1 : 1; R_f 0.25) yielding compound **14** (375 mg, 71%) as a colourless solid.

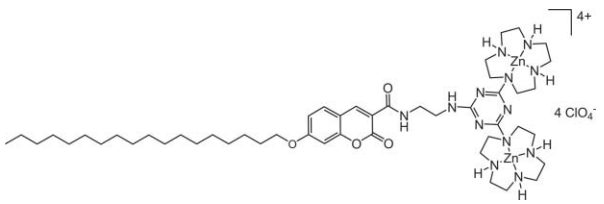
MP: 108 °C. – **^{1}H-NMR** (600 MHz; $CDCl_3$): δ = 0.86 (t, ^3J = 7.2 Hz, 3 H, HSQC, HMBC: C^1H_3), 1.24 (m, 28 H, HSQC, HMBC: C^2H_2 – $C^{15}H_2$), 1.31-1.64 (m, 56 H, HSQC, HMBC: boc-CH_3, $C^{16}H_2$), 1.78-1.83 (m, 2 H, HSQC, HMBC: $C^{17}H_2$), 3.00-3.92 (m, 36H, HSQC, HMBC: cyclen-$C^{36}H_2$, $C^{30}H_2$, $C^{31}H_2$), 4.02

(t, ^3J = 6.5 Hz, 2 H, HSQC, HMBC: $C^{18}H_2$), 5.04 (bs, 1 H, HSQC, HMBC: $N^{32}H$), 6.82 (d, ^4J = 2.3 Hz, 1 H, HSQC, HMBC: $C^{27}H$), 6.90 (dd, ^3J = 8,7 Hz, ^4J = 2.3 Hz, 1 H, HSQC, HMBC: $C^{20}H$), 7.55 (d, ^3J = 8,7 Hz, 1 H, HSQC, HMBC: $C^{21}H$), 8.79 (s, 1 H, HSQC, HMBC: C^{23}), 8.88 (m, 1H, HSQC, HMBC: $N^{29}H$). – **^{13}C-NMR** (150 MHz; $CDCl_3$): δ = 14.1 (+, 1 C, HSQC, HMBC: C^1H_3), 22.6 (–, 1 C), 25.9 (–, 1 C), 29.26 (–, 1 C), 29.30 (–, 1 C), 29.52(–, 1 C), 29.59 (–, 2 C), 29.61 (–, 1 C), 29.63 (–, 5 C), 31.9 (–, 1 C) HSQC, HMBC: C^2H_2 – $C^{16}H_2$), 28.4, 28.5 (+, 18 C, HSQC, HMBC: $C^{39}H_3$), 28.8 (–, 1 C, HSQC, HMBC: $C^{17}H_2$), 39.7 (–, 1 C, HSQC, HMBC: $C^{30}H_2$), 40.6 (–, 1 C, HSQC, HMBC: $C^{31}H_2$), 50.3 (–, 16 C, HSQC, HMBC: $C^{36}H_2$), 69.0 (–, 1 C, HSQC, HMBC: $C^{18}H_2$), 79.7 (C_q, 6 C, HSQC: C^{37}), 100.7 (+, 1 C, HSQC, HMBC: $C^{27}H$), 112.4 (C_q, 1C, HSQC, HMBC: C^{22}), 114.37 (+, 1 C, HSQC, HMBC: $C^{20}H$), 114.39 (C_q, 1 C, HSQC, HMBC: C^{24}), 130.9 (+, 1 C, HSQC, HMBC: $C^{21}H$), 148.3 (+, 1 C, HSQC, HMBC: $C^{23}H$), 156.7 (C_q, 7 C, HSQC, HMBC: C^{26}, C^{38}), 161.7 (C_q, 1 C, HSQC, HMBC: C^{25}), 162.7 (C_q, 1 C, HSQC, HMBC: C^{28}), 164.5 (C_q, 1 C, HSQC, HMBC: C^{19}), 165.9 (C_q, 3C, HSQC, HMBC: C^{33}, C^{34}, C^{35}). – **UV** ($CHCl_3$): λ_{max} (lg ε) = 350 nm (4.333). – **MS** (ESI(+), DCM–MeOH + 10 mmol L^{-1} NH_4Ac): m/z (%) = 1521.4 (100) [MH^+], 769.7 (46) [$MH^+ + NH_4^+]^{2+}$, 761.2 [M + 2 $H^+]^{2+}$.

7-Dodecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid {2-[4,6-bis-(1,4,7,10 tetraaza-cyclododec-1-yl)-[1,3,5]triazin-2-ylamino]-ethyl}-amide (15). Compound **13** (200 mg, 0.14 mmol) was dissolved in DCM (4 mL) and cooled to 0 °C. Subsequently TFA (901 µL, 11.7 mmol) was added. The solution was stirred 15 min at 0 °C and additionally 20 h at room temperature. The solvent was removed *in vacuo*, yielding quantitatively the protonated TFA salt of compound **15** as a pale yellow solid. To obtain the free base of compound **15** a weak basic ion exchanger resin was swollen for 15 min in water and washed neutral with water. A column was charged with resin (1.1 g, 40 mmol hydroxy equivalents at a given capacity of 5 mmol g^{-1}). The hydrochloride salt was dissolved in water–MeOH (8 : 2), applied to the column and eluted with water–MeOH (8 : 2). The elution of the product was controlled by pH indicator paper (pH > 10) and was completed when pH became neutral. The eluate was concentrated and lyophilised to yield 110 mg (93%) of free base **15**, as pale yellow solid. **MP**: 174 °C. – **^{1}H-NMR** (400 MHz; $CDCl_3$): δ = 0.85 (t, ^3J = 6.9 Hz, 3 H, CH_3), 1.19-1.37 (m, 16 H, CH_2), 1.39-1.49 (m, 2 H, CH_2), 1.80 (quin, ^3J = 7.2 Hz, 2 H, CH_2), 2.82-3.84 (m, 36 H, CH_2), 4.02 (t, ^3J = 6.2 Hz, 2 H, CH_2), 6.80 (d, ^4J = 1.8 Hz, 1 H, CH), 6.93 (dd, ^3J = 8.7 Hz, ^4J = 1.7 Hz, 1 H, CH), 7.02 (bs, 1H, NH), 7.66 (d, ^3J = 8.8 Hz, 1 H, CH), 8.73 (s, 1 H, CH), 9.18 (bs, 1H, NH). – **^{13}C-NMR** (100 MHz; $CDCl_3$): δ = 14.0 (+, 1 C, CH_3), 22.6 (–, 1 C, CH_2), 25.9 (–, 1 C, CH_2), 28.8 (–, 1C, CH_2), 29.27 (–, 2 C, CH_2), 29.46 (–, 1 C, CH_2), 29.51 (–, 1 C, CH_2), 29.56 (–, 2 C, CH_2), 31.8 (–, 1 C, CH_2), 39.2 (–, 1 C, CH_2), 39.3 (–, 1 C, CH_2), 42.5 (–, 1 C, CH_2), 43.3 (–, 2 C, CH_2), 43.7 (–, 1 C, CH_2), 44.8 (–, 4 C, CH_2), 45.8 (–, 4 C, CH_2), 46.9 (–, 4C, CH_2), 69.1 (–, 1 C, CH_2), 100.8 (+, 1 C, CH), 111.9 (C_q, 1 C), 113.4 (C_q, 1 C), 114.5 (+, 1 C, CH), 131.3 (+, 1 C, CH), 148.5 (+, 1 C, CH), 156.8 (C_q, 1 C), 161.6 (C_q, 1 C), 163.7 (C_q, 1 C), 164.9 (C_q, 1 C), 165.9 (C_q, 1 C), 167.2 (C_q, 1 C), 168.0 (C_q, 1 C). – **IR** (ATR) [cm^{-1}]: $\tilde{\nu}$ = 2926, 2855, 1676, 1597, 1536, 1496, 1418, 1366, 1297, 1198, 1174, 1120, 1017, 795, 719. – **UV** ($CHCl_3$): λ_{max} (lg ε) = 352 nm (4.197). – **LC-MS** (+ c ESI Q1MS): m/z (%) = 418.7.0 (100) [M + 2 $H^+]^{2+}$, 836.6 (10) [MH^+]. – **HRMS** Calcd for $C_{43}H_{74}N_{13}O_4$ 836.5987; Found: 836.5960.

7-Octadecyloxy-2-oxo-2*H*-chromene-3-carboxylic acid {2-[4,6-bis-(1,4,7,10 tetraaza-cyclododec-1-yl)-[1,3,5]triazin-2-ylamino]-ethyl}-amide (16). Compound **14** (375 mg, 0.26 mmol) was dissolved in DCM (4 mL) and cooled to 0 °C. Subsequently HCl/ether (14 mL) was added. The solution was stirred 15 min at 0 °C and additionally 24 h at room temperature. The solvent was removed *in vacuo*, yielding quantitatively the protonated HCl salt of compound **16** as a pale yellow solid. To obtain the free base of compound **16** a weak basic ion exchanger resin was swollen for 15 min in water and washed neutral with water. A column was charged with resin (1.9 g, 40 mmol hydroxy equivalents at a given capacity of 5 mmol g^{-1}). The hydrochloride salt was dissolved in water–MeCN (5 : 1), loaded onto the column and eluted with water–MeCN (5 : 1). The elution of the product was controlled by pH indicator paper (pH > 10) and was completed when pH again was neutral. The eluate was concentrated and lyophilised to yield 200 mg (85%) of free base **16**, as colourless solid. **MP**: 179 °C. – **IR** (ATR) [cm^{-1}]: $\tilde{\nu}$ = 3347, 2921, 2850, 1708, 1594, 1538, 1496, 1418, 1362, 1274, 1222, 1142, 1017, 809, 739. – **UV** ($CHCl_3$): λ_{max} (lg ε) = 354 nm (4.360). – **LC-MS** (+ c ESI Q1MS): m/z (%) = 460.8 (100) [M + 2 $H^+]^{2+}$, 920.7 (5) [MH^+]. – **HRMS** Calcd. for $C_{49}H_{86}N_{13}O_4$ 920.6926; Found: 920.6901. No meaningful NMR data were obtained most likely due to slow molecular motion of parts of the molecule on the NMR timescale.

Binuclear Zn(II)-cyclen-coumarin C12 (6). A solution of compound **15** (50 mg, 60 µmol) in MeOH (1 mL) was heated to 65 °C and subsequently a methanolic solution of $Zn(ClO_4)_2$ (0.1 M, 1.2 mL, 120 µmol) was added dropwise. After stirring the reaction mixture for 20 h at 65 °C, the methanol was removed *in vacuo*. The residue was dissolved in water and was lyophilized yielding complex **5** as a colourless solid in quantitative yield (82 mg). **MP**: 232 °C. – **IR** (ATR) [cm^{-1}]: $\tilde{\nu}$ = 3537, 3303, 2929, 2854, 1702, 1599, 1546, 1424, 1347, 1283, 1224, 1058, 964, 848. – **UV** ($CHCl_3$): λ_{max} (lg ε) = 353 nm (3.740). – **MS**(ESI(+), DCM–MeOH + 10 mmol L^{-1} NH_4Ac): m/z (%) = 540.9 (100) [M^{4+} + $2CH_3COO^-]^{2+}$, 561.9 (26) [M^{4+} + ClO_4^- + $CH_3COO^-]^{2+}$, 510.9 (20) [M^{4+} – H^+ + $CH_3COO^-]^{2+}$.



Binuclear Zn(II)-cyclen-coumarin C18 (5). Compound **16** (100 mg, 0.11 mmol) was dissolved in 5 mL of water and heated to 65 °C yielding a clear yellow solution. Subsequently zinc(II)-perchlorate (81 mg, 0.22 mmol) dissolved in 5 ml of water was added slowly to the stirred reaction mixture, which was stirred for additional 24 h at 65 °C. The solvent was removed *in vacuo* and the residue was redissolved in water and lyophilized to yield 158 mg (quantitative) of **6** as a lightly yellow solid. **MP**: 209 °C. – **^{1}H-NMR** (600 MHz; $CDCl_3/CD_3CN$ 1 : 1): δ = 0.86 (t, ^3J = 7.1 Hz, 3 H, HSQC, HMBC: C^1H_3), 1.20-1.31 (m, 26 H, HSQC, HMBC: C^2H_2 – $C^{14}H_2$), 1.31-1.37 (m, 2 H, HSQC, HMBC, ROESY: $C^{15}H_2$), 1.41-1.46 (2 H, HSQC, HMBC, ROESY: $C^{16}H_2$), 1.73-1.80 (m,

2 H, HSQC, HMBC, ROESY: $C^{17}H_2$), 2.51-3.57 (m, 36 H, HSQC, HMBC: cyclen-CH_2, $C^{30}H_2$, $C^{31}H_2$), 4.06 (t, ^3J = 6.5 Hz, 2 H, HSQC, HMBC: $C^{18}H_2$), 6.11 (s, 1 H, HSQC, ROESY: cyclen-NH), 6.20 (s, 1 H, HSQC, ROESY: cyclen-NH), 6.29 (s, 1 H, HSQC, ROESY: cyclen-NH), 6.44 (s, 1 H, HSQC, ROESY: cyclen-NH), 6.87 (d, ^4J = 2.2 Hz, 1 H, HSQC, HMBC, ROESY: $C^{27}H$), 6.95(dd, ^3J = 8.7 Hz, ^4J = 2.3 Hz, 1 H, HSQC, HMBC, ROESY: $C^{20}H$), 7.74 (d, ^3J = 8.7 Hz, 1 H, HSQC, HMBC, ROESY: $C^{21}H$), 8.85 (s, 1 H, HSQC, HMBC, ROESY: $C^{23}H$), 9.22 (m, 1 H, HSQC, ROESY: NH^{29}). – **^{13}C-NMR** (150 MHz; $CDCl_3/CD_3CN$ 1 : 1): δ = 14.3 (+, 1 C, HSQC, HMBC: C^1H_3), 26.3 (–, 1 C, HSQC, HMBC, ROESY: $C^{16}H_2$), 23.1, 29.7, 29.94, 29.97, 30.1, 32.3 (–, 13 C, HSQC, HMBC: C^2H_2 – $C^{14}H_2$), 29.3 (–, 1 C, HSQC, HMBC, ROESY: $C^{17}H_2$), 29.75 (–, 1 C, HSQC, HMBC: $C^{15}H_2$), 39.9, 42.6, 44.3, 45.8, 45.9, 46.3, 46.9 3 (–, 18 C, HSQC, HMBC, ROESY: cyclen-CH_2, $C^{30}H_2$, $C^{31}H_2$), 69.8 (–, 1 C, HSQC, HMBC, ROESY: $C^{18}H_2$), 101.4 (+, 1 C, HSQC, HMBC, ROESY: $C^{27}H$), 112.8 (C_q, 1 C, HSQC, HMBC: C^{22}), 113.8(C_q, 1 C, HSQC, HMBC: C^{24}), 115.1 (+, 1 C, HSQC, HMBC, ROESY: $C^{20}H$), 132.3(+, 1 C, HSQC, HMBC, ROESY: $C^{21}H$), 149.8 (+, 1 C, HSQC, HMBC, ROESY: $C^{23}H$), 157.5 (C_q, 1C, HSQC, HMBC: C^{26}), 162.1 (C_q, 1 C, HSQC, HMBC: C^{25}), 164.8(C_q, 1 C, HSQC, HMBC: C^{28}), 165.6 (C_q, 1 C, HSQC, HMBC: C^{19}). – **IR**(ATR)[cm^{-1}]: $\tilde{\nu}$ = 3355, 2919, 2851, 1705, 1655, 1612, 1534, 1426, 1276, 1225, 1051, 926. – **UV** ($CHCl_3$): λ_{max} (lg ε) = 355 nm (3.523). – **MS** (ESI(+), EE/MeOH + 10 mmol L^{-1} NH_4Ac): m/z (%) = 583.0 (100) [M^{4+} + 2 CH_3COO^-]$^{2+}$.

Vesicle preparation

In a small round-bottom flask 2–12 mg (2.5–15 μmol) of DSPC were dissolved in 5–10 mL of chloroform and 10 mol% of the respective amphiphilic receptors were added. After warming to 75 °C under vigorous shaking, the solvent was slowly removed under reduced pressure to yield a thin lipid film. Traces of solvent were removed by high vacuum. An appropriate amount of buffer (HEPES 25 mM, pH 7.4) was added to obtain lipid concentrations of 1.5–2.5 mM and heating to 75 °C for 15–30 min yielded a turbid MLV-suspension. SUV-dispersions were obtained by extrusion through 100 nm-pore size polycarbonate membranes with a LiposoFast liposome extruder from Avestin.[19,35]

Size exclusion chromatography

Vesicle dispersions were separated from low molecular weight solutes on minicolumns of Sephadex LH-20 gel filtration media as described in literature.[23]

Dynamic light scattering

Photon correlation spectroscopy (PCS) measurements were performed on a Malvern Zetasizer 3000 at 25 °C using 1 cm disposable polystyrene fluorescence cuvettes (VWR). Three subsequent measurements of 60 s each were performed for each sample. Data analysis was performed using the Malvern PCS software.

Binding studies

All titrations were carried out at 25 °C in HEPES buffer (25 mM, pH 7.4) and corrected for dilution. Data analysis was performed with Origin 8 software.

Receptor concentration on vesicle surface

For all binding studies the concentration of vesicular receptors refers to the outer surface exposed binding units. The following equation describes the correction factor f for surface exposed receptor molecules as a fraction its entire quantity of matter:

$$f = \frac{\sigma_o}{\sigma_o + \sigma_i}$$

The ratio of outer surface (σ_o) and inner (σ_i) surface of the respective vesicles was calculated using the hydrodynamic diameters obtained from dynamic light scattering and the assumption that the bilayer thickness for the prepared vesicles generally amounts to 5 nm.[36]

Indicator displacement assays (IDA)

Evaluation of the indicator binding towards receptors **1** and **VR-4** was performed by utilizing Hill plots, whereas for the subsequent displacement titrations a competitive binding model was used.[37] For all titrations the initial indicator concentration was 3.5×10^{-5} M for PV and 5.0×10^{-7} M for CMS. After each addition, the cuvette was shaken for 1 min before the absorption (PV: λ_{max} = 636 nm) or fluorescence spectrum (CMS: λ_{ex} = 396 nm, λ_{em} = 480 nm) was recorded.

Vesicular receptor binding monitored by direct emission

Evaluation of phosphate binding towards the vesicular receptors **VR-5** and **VR-6** was performed by plotting Δ emission values against the analyte concentration and employing non-linear curve fitting using the Hill equation. The initial concentration of vesicular receptors for all titrations was 5.0×10^{-7} M, after each analyte addition, the cuvette was shaken for 1 min before the fluorescence spectrum (λ_{ex} = 348 nm, λ_{em} = 406 nm) was recorded.

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