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Transmission of Binding Information across Lipid Bilayers

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Abstract: A synthetic transmembrane receptor that is capable of transmitting binding information across a lipid bilayer membrane is reported. The binding event is based on aggregation of the receptor triggered by copper(II) complexation to ethylenediamine functionalities. By labelling the receptor with fluorescent dansyl groups, the copper(II) binding event could be monitored by measuring the extent of fluorescence quenching. Comparing the receptor with a control receptor lacking the transmembrane linkage revealed that the transmembrane receptor binds copper(II) ions more tightly than the non-spanning control receptor at low copper(II) concentrations. Since the intrinsic binding to copper(II) is the same for both receptors, this effect was attributed to synergy between the connected interior and exterior binding sides of the transmembrane receptor. Thus, this is the first reported artificial signalling event in which binding of a messenger on one side of the membrane leads to a cooperative binding event on the opposite side of the membrane, resembling biological signalling systems and helping us to get a better understanding of the requirements for more effective artificial signalling systems.

Keywords: cooperative binding · fluorescence · receptors · transmembranes · vesicles

Introduction

Cell membranes constitute an important barrier between cells' internal fluid and the external medium and regulate many important biological events.[1] They are dynamic, non-covalent, fluid assemblies and many biological membrane processes, mostly regulated by membrane proteins and carbohydrates, depend on the fluidity of the membrane lipids.[2] Cell membranes provide a unique platform to sense, respond to, and transduce signals and information. In these biological signalling processes, cooperativity is believed to play a crucial role. For many ligand/receptor interactions, however, the strength of cooperative binding affinities is still not well understood. In particular, cooperative binding of receptors constrained at the cell membrane surface is still largely unexplored.[3] In this case, the receptors bound at a membrane surface can only move in two dimensions, thus the binding interactions within membranes are expected to be thermodynamically more favourable than binding interactions in bulk solution where movement occurs in three dimensions.[4] Also the polarity at the membrane interface is significantly different from bulk solution, and this microenvironment can have a dramatic effect on binding interactions.[5] Natural cell membrane receptors often fully span lipid bilayers, for example, tyrosine kinase receptors and G-protein coupled receptors, which have seven transmembrane units.[6] These transmembrane receptors are crucial to functions such as initiating signal transduction pathways. In signal transduction, the messenger binds to the external domain of the transmembrane receptor on the cell surface thereby causing significant receptor reorganization and a change in the transmembrane conformation. This process consequently leads to aggregation of the interior receptor sites, thereby stimulating an (often cooperative) intracellular response that in turn triggers a signal cascade inside the cell.[7] For example, bacteria chemotaxic receptors, a family of transmembrane receptors, can detect tiny changes in the concentration of specific chemicals. Recent studies show that in these receptors, binding of ligands at the external sur-

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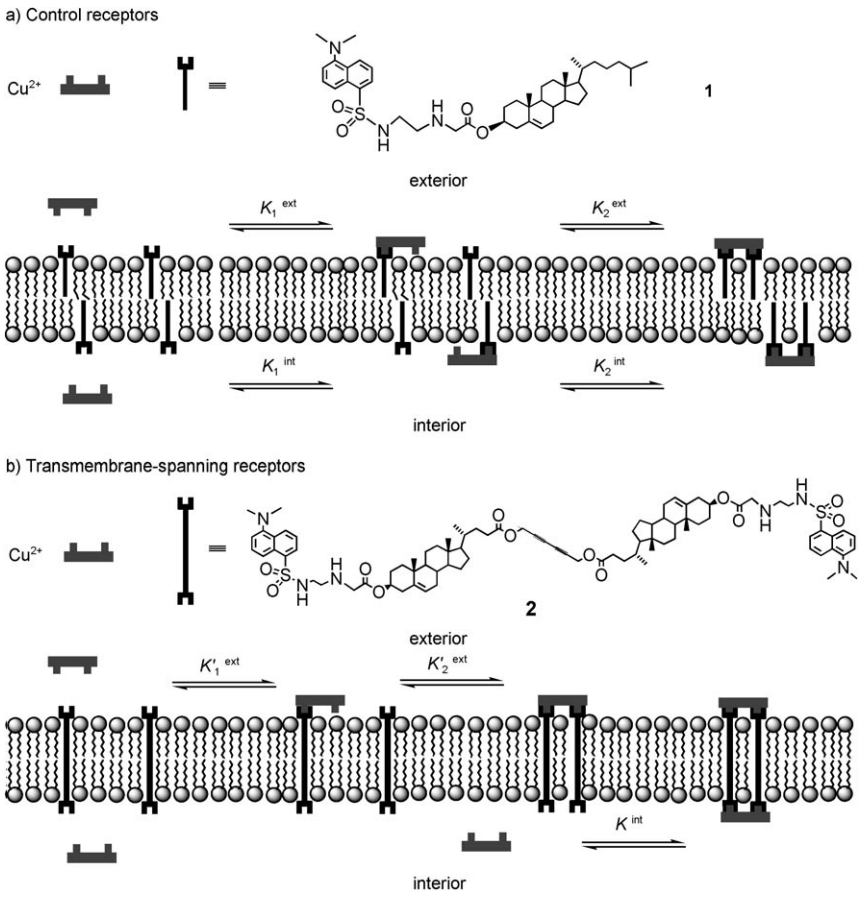
face of the membrane can cause receptor aggregation and such receptor clusters in turn affect the lateral packing interactions of the vertical signalling pathway at the opposing surface of the membrane.[8] As a consequence of the clustering, the chemotaxic receptors elicit changes in their internal environment which leads to whole-cell movement. It is clear that trimers of dimers play a key role in controlling this transmembrane signalling rather than isolated dimeric receptors.[9]

An ongoing research theme in our group is the development of synthetic membrane-bound receptors and the study of their aggregation behaviour upon addition of a messenger. Although these systems represent simplified models of biological membrane receptors, they can give us valuable insight into the mechanisms by which biological signalling processes may take place. For this purpose, a cholesterol based receptor **1** was synthesised containing a fluorescent dansyl ethylenediamine head group (Scheme 1a).[10] Cholesterol inserts well into lipid bilayers and serves as a membrane anchor, and when copper(II) was used as a messenger its coordination to the ethylenediamine group led to the formation of receptor aggregates. The fluorescent properties of the dansyl head group were used in this study as a probe to monitor the aggregation process in situ as copper coordination to the membrane-bound receptors leads to a strong decrease of the fluorescent signal.[11] By constraining the membrane-bound receptors to a membrane surface where they can only move in two dimensions, the binding interactions between the membrane-bound receptors were expected to be very different from those of the receptors in solution. The surprising assembly of a 4:1 receptor/copper(II) complex was deduced and the observed binding constants to copper(II) were significantly larger at the membrane surface relative to binding by similar amines in bulk solution. The affinity for copper(II) ions was strongly dependent on the membrane concentration of the receptors **1** and higher aggregates were strongly favoured with increased concentration of the receptors in the membrane. These observations are due to the higher effective concentration of the ligands achieved by constraining them to the membrane volume and to medium effects at the interface, rather than entropic benefits from pre-organising the receptors at the bilayer surface. Lehn and co-workers reported a similar concentration dependent binding behaviour for a membrane bound diketone ligand in binding studies with Eu^{3+}, obtaining larger observed equilibrium constants at higher receptor loadings.[12] As the size of receptor-ligand clusters can have a significant effect on the binding processes, these data suggest that by changing the number of receptors at the membrane surface, cells can finely control their biological responses to external changes.[13]



Scheme 1. Representation of the binding interactions in receptor: messenger complexes of a) receptor **1** and b) transmembrane receptor **2** in the lipid bilayer of vesicles.

For receptor **1**, the extra- and intravesicle binding activities are separated with no synergy between the two sides of the lipid bilayer, implying that the internal and external binding events are the same and lack transmembrane communication. Therefore, to study how information can be transmitted across lipid bilayers by binding of a messenger to the receptor on the outside of a cell leading to an intravesicular receptor change, we decided to investigate a synthetic receptor that spans the membrane. Such a system resembles biological signalling events and can give us valuable information on their mechanisms. Here, we report a novel transmembrane spanning receptor **2** (Scheme 1b) based on the dansyl ethylenediamine cholesterol moiety and its aggregation behaviour in vesicles upon addition of Cu^{2+} ions (messengers). More specifically,

we studied transmembrane cooperativity by utilising the fluorescence properties of the dansyl functionalities of receptor **2** at both sides of the membrane and by comparing the results with the control receptor **1** which lacks the transmembrane orientation.

Results and Discussion

The transmembrane receptor **2** is based on a synthetic transmembrane signalling system that we recently developed to mimic the tyrosine kinase receptor.[14] The membrane-spanning molecule **2** consists of two cholesterol units that are linked together via a rigid dialkyne bridge. The incorporation of cholesterol assures insertion of this molecule into the lipid bilayers whereas the linear, rigid structure and the polar head groups favour a transmembrane orientation of **2**. Notably, these receptors can potentially also adopt a U-shaped conformation, meaning that both polar head groups are situated on the same side of the membrane and are thus not spanning the membrane. From the current study it is very difficult to distinguish between both forms and therefore a certain percentage of U-shaped receptors cannot be fully excluded. However, it has been shown several times by others that introducing rigidity, for example, a dialkyne unit, into the core of specific lipids force them almost exclusively in a transmembrane orientation,[15]even more flexible bolaamphiphilic molecules have been shown to adopt a completely transmembrane conformation.[16] Furthermore, exactly the same transmembrane unit has been reported recently in another transmembrane spanning system[17] and the transmembrane signalling system we reported recently is based on the same dialkyne–cholesterol scaffold as **2** and results obtained with that system clearly suggested a transmembrane orientation.[14]

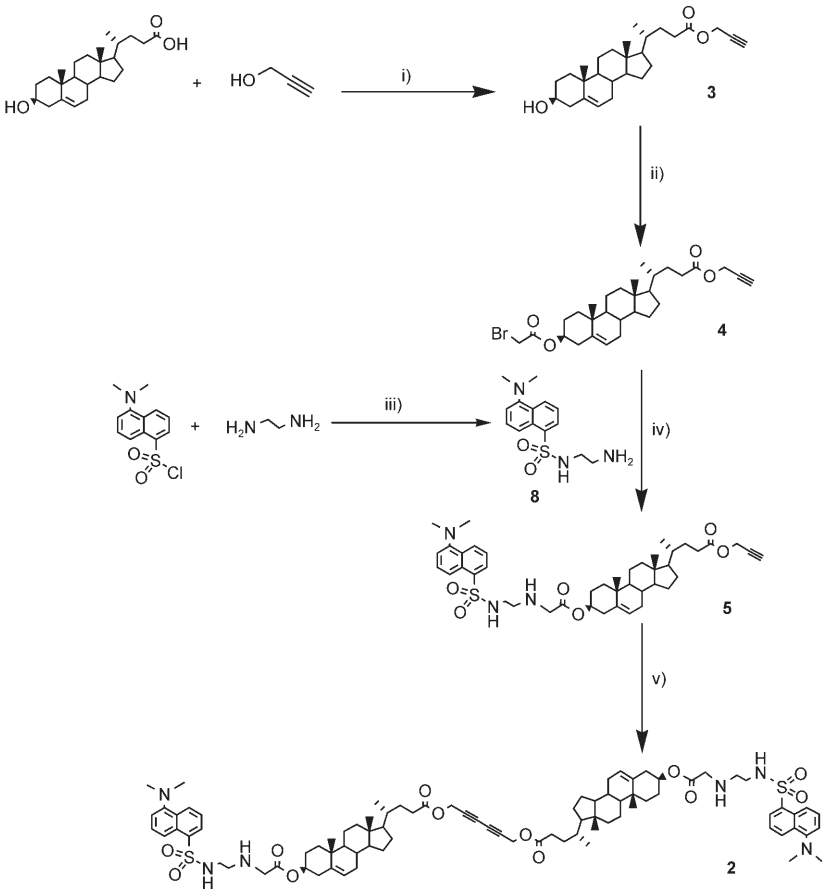
In support of this assumption, we observed that vesicles prepared with the longer molecules have notably different physical properties compared with those that contained only a cholesterol anchor—they were far harder to extrude through the polycarbonate filters that we used, to the extent that on one occasion the force required caused mechanical failure of the extrusion apparatus (bursting the syringe barrel). The shorter embedded molecules did not exhibit this behaviour, which is apparent even at low loadings (0.5 % of **2**). We quantified this observation by measuring the pressure required to pass 800 nm vesicles through 200 nm filter once. That is, the molecules were incorporated into lipids, then extruded to form homogeneous unilamellar 800 nm vesicles. These were then passed through a 200 nm filter using a syringe pump and monitoring the force exerted on the barrel using a pressure traducer. This revealed that the spanning molecules required five-fold higher pressure (extruding 20 mM lipid at a flow rate of 2 mL per minute) than vesicles containing the same fraction of cholesterol derivative but where they are not linked by the dialkyne unit. If the embedded molecules adopt a U-shape, then it is difficult to envisage that the physical properties should be greatly different. Therefore, we conclude that the majority of receptors **2** adopt a transmembrane orientation when included in vesicular bilayers.

Following our earlier results with half-spanning membrane receptor **1**, we again chose dansyl ethylenediamine as the sensing moiety and copper(II) ions as messenger molecules. The dansyl group is a well-developed environmentally sensitive fluorophore and coordination of copper(II) ions to the ethylenediamine ligands quenches the dansyl fluorescence. From our previous studies we know that copper(II) chloride freely crosses the membrane in the presence of **1** or **2**,[10] therefore we know that, after equilibrating the solution, binding of copper(II) ions to the dansyl head groups occurs on both sides of membrane. The aggregation behaviour of **2** upon binding to copper(II) ions can then be effectively studied by monitoring the quenching of the dansyl fluorescence. Non-membrane spanning receptor **1** serves as a useful control system and comparing the results obtained with **1** and **2** gives us direct information about the presence of synergy (and thus cooperativity) between the interior and exterior copper(II)-coordination events in the transmembrane spanning receptor **2**.

The synthesis of transmembrane receptor **2** is depicted in Scheme 2. It was synthesised from cholenic acid, a bifunctional analogue of cholesterol, in four steps. The coupling of cholenic acid with propargyl alcohol mediated by dicyclohexylcarbodiimide, gave cholenic acid propargyl ester **3** in near quantitative yield. Heating of the ester **3** with bromoacetyl chloride under reflux in dry THF afforded the bromoacetylated propargyl ester of cholenic acid **4**. Subsequent displacement of the bromide from **4** with dansyl ethylenediamine **8**, available through the condensation of dansyl chloride with ethylenediamine in dry acetonitrile in the presence of sodium carbonate as a base, resulted in the formation of **5**. A tail-to-tail dimerisation of **5** was achieved using Glaser–Hay coupling conditions[18] with freshly prepared copper(I) chloride[19] in the presence of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. This coupling reaction had to be conducted in air, allowing free diffusion of molecular oxygen into the reaction. The excess copper(II) ions were removed from the crude product by thoroughly washing with EDTA and water several times. Purification by silica gel chromatography yielded receptor **2** as a pale yellow solid.

Compound **1** was synthesized in two steps from cholesterol using previously reported methods.[10] Membrane-bound receptor **1** was used here as a control for membrane-spanning receptor **2** in the membrane aggregation studies.

Preparation of vesicle solutions: In this study we used large unilamellar egg yolk phosphatidylcholine (PC) vesicles with diameters of 800 nm. Unilamellar PC vesicles containing receptors **1** and **2** in MES buffer (pH 6.0) were prepared by extrusion of a mixture of the lipids and the appropriate receptor through 800 nm polycarbonate membranes. The total concentration of dansyl head groups was always kept the same; since **2** has twice as many dansyl head groups as **1**, in each comparison experiment the concentration of $[\mathbf{1}]=[\mathbf{2}]\times$



Scheme 2. Synthesis of transmembrane receptor **2**. i) DMAP, DCC, CH_2Cl_2, RT, 48 h (90%); ii) Bromoacetyl chloride, THF, reflux, 1.5 h (69%); iii) CH_2Cl_2, 0°C→RT, 1 h (92%); *iv.* Na_2CO_3, MeCN, reflux, 20 h (49%); v) i) CuCl, TMEDA, O_2, CH_2Cl_2, RT, 3 d, ii) EDTA wash (45%).

2. This allows us to make a direct comparison between the aggregation behaviour of **1** and **2** upon addition of the messenger, copper(II) chloride. Furthermore, we kept the bulk concentration of the lipids constant at 2.0 mM,[20] but varied the percentage of receptors in the vesicles. The next step was monitoring the fluorescence quenching of each receptor–vesicle solution after addition of copper(II) chloride and allowing the reaction mixture to equilibrate for 30 minutes. The latter was necessary to allow copper(II) chloride to diffuse through the lipid bilayer assuring a homogeneous intra- and extravesicular distribution of copper(II) ions.[10]

Control experiments: We initially performed some control experiments to evaluate background processes that might lead to fluorescence quenching which is not related to copper(II) binding. Firstly, we checked for self-quenching of the fluorescent signal, for example due to spontaneous receptor aggregation, by monitoring the fluorescence of both receptors embedded in vesicles at various concentrations without the addition of $CuCl_2$. The fluorescent signal of vesicles containing receptor **1** and **2** and with dansyl head group loadings ranging from 1.00–7.50 mol% was measured by exciting the samples at 337 nm. The data was plotted as emission fluorescence intensity at 520 nm versus mol% of dansyl head groups in the vesicles (data not shown). As expected, the fluorescence intensity for receptor **1** increased linearly with increasing receptor loading in the vesicles. Since dansyl groups dissolved in aqueous solvents display very low intensity emission compared with the interfacial dansyl groups of membrane-embedded lipid **1** this linearity shows that lipid **1** was fully incorporated into the vesicles over this concentration range. However for transmembrane receptor **2**, we observed incomplete incorporation at high receptor loading (3.75 mol% **2**, equal to 7.50 mol% dansyl head groups). As this would not allow a fair comparison between the aggregation behaviour of **1** and **2** upon addition of $CuCl_2$, we decided only to use low receptor loadings (**2** = 2.50 mol% and **1** = 5.00 mol%) for the comparison studies. Secondly, we also investigated the possibility of vesicle aggregation in solution as this would also potentially affect the outcome of the receptor aggregation experiments. Lehn and coworkers when investigating the coordination behaviour of a membrane-anchored bispyridine ligand with Co^{2+} and Ni^{2+} ions have recently reported receptor-dependent vesicle aggregation, induced by metal-receptor coordination.[21] To rule out this possibility with our system, the fluorescence of receptor **2** (0.50, 1.25 and 2.50 mol%) embedded vesicles at different vesicle concentrations (0.1–2.0 mM lipid concentration) in MES buffer (pH 6.0) was investigated. We found no shift in the maximum emission wavelength of the diluted vesicles and also the emission fluorescence intensity at 520 nm vs. vesicle concentration gave a linear fit, showing that there is no vesicle aggregation in this concentration range. Finally, as it is known that cholesterol and related steroids affect many biological processes[22] such as membrane rigidity, we decided to investigate whether added cholesterol in the membrane will significantly influence the aggregation behaviour of our receptors. Therefore, we conducted a number of experiments in which various amounts of cholesterol (CL) were incorporated into vesicles (800 nm diameter, 2.0 mM lipid) containing receptor **1**. In these experiments, the total concentration of cholesterol groups in the membrane was kept constant (thus [CL] + [**1**] = 20.0 mM), and only the [CL]/[**1**] ratio was varied. Comparing the fluo-

rescence intensity of these experiments at 520 nm with parallel control experiments in which there was no added cholesterol revealed that in the concentration range of [**1** + CL] = 7.50 mol% there was no influence of added cholesterol on the optical properties and thus on the aggregation behaviour of the receptors in the vesicles. Thus, the influence of varying the cholesterol concentration was neglected in the aggregation comparison studies of receptors **1** and **2**.

Binding comparison studies: To study the aggregation behaviour of **1** and **2** in the lipid bilayer upon addition of a Cu^{II} messenger at various receptor loadings, different vesicular solutions were prepared containing 0.50, 1.25, and 2.50 mol% of embedded **2** and 1.00, 2.50, and 5.00 mol% of embedded **1**, respectively. Titration of a 10 mM copper(II) chloride solution into these solutions and equilibrating the resulting solution for 30 minutes resulted in a dramatic decrease in the fluorescent intensity from 400–650 nm with each addition, as shown in Figure 1 for a typical experiment

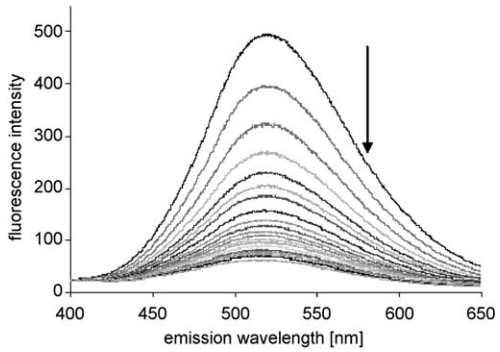


Figure 1. Typical fluorescent spectra for a titration of receptor **2** in phospholipid vesicles with copper(II) ions. Aliquots of 10 mM $CuCl_2$ solution were added to a vesicle solution (2.0 mM lipid concentration) containing 1.25 mol% receptor loading and the fluorescence spectrum was measured after each addition. The arrow indicates the direction of the change upon addition of copper(II) ions.

with 1.25 mol% receptor **2** loading. Comparing the results obtained with **1** and **2** indicated that transmembrane-spanning receptor **2** showed a similar response to its half-spanning analogue **1** upon Cu^{II} coordination to the ethylenediamine dansyl head groups. It should be noted that at higher loadings of receptor **2** (5.00 and 7.50 mol% dansyl groups), reproducibility of the titration experiments became unreliable. Therefore, to determine whether there is synergy between the interior and exterior domains of receptor **2**, we decided to only compare the aggregation results obtained for **1** and **2** at lower receptor loadings, that is, 1.00 and 2.50 mol% dansyl groups.

The results of the copper(II) titration experiments with vesicles containing **1** and **2** at dansyl head group loadings of 1.0 and 2.5 mol% are summarised in Figure 2. In all comparison experiments receptor **2** embedded vesicles showed a more efficient fluorescence quenching upon addition of Cu^{II} ions than those containing **1** (Figure 2, grey dots for **2** and

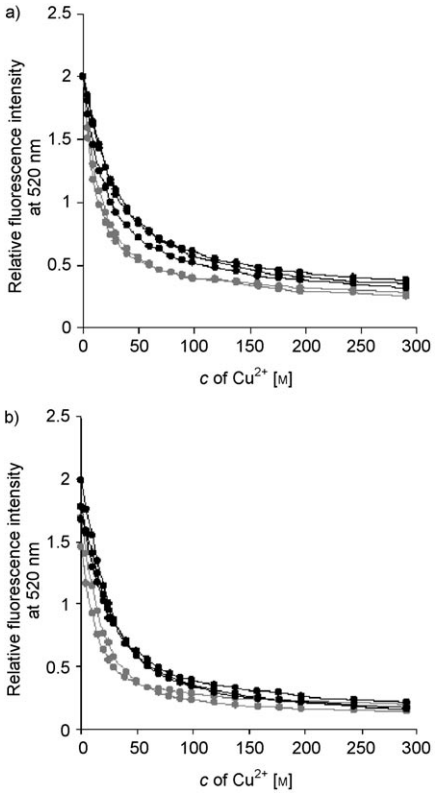


Figure 2. Titration curves showing the decrease in the fluorescence of vesicle-bound receptor **2** (grey) and **1** (black) with a) 1.00 mol% and b) 2.50 mol% dansyl head group loading upon the addition of Cu^{II} ions. Control experiments with added cholesterol in receptor **1** containing vesicles are also shown for comparison but the data are practically identical. Aliquots of 10 mM $CuCl_2$ solution were added to the appropriate solutions and the fluorescence spectrum was measured after equilibration.

black dots for **1**). For example, at 1.00 mol% dansyl head group loading (Figure 2a) only 14 μM Cu^{II}-ions are required to quench 50% of the initial fluorescence for **2** whereas in the case of control receptor **1** a much higher concentration of Cu^{II}-ions (30 μM) is needed for the same amount of quenching. In addition, fluorescence quenching is more efficient at higher receptor loading, that is, 2.5 mol% dansyl head group loadings (Figure 2b), for both receptors (as can be deduced from the steepness of the curves by comparing Figure 2a and b), confirming that higher copper(II) binding affinities are obtained with increasing receptor loadings. This behaviour was reported earlier for **1**[10] and again emphasises that both receptors behave similarly with respect to copper(II) coordination.

Cooperative binding: The fact that receptor **2** has a transmembrane orientation means that receptor **2** can potentially couple binding events across the lipid bilayer. Previously, we have already determined that receptor **1** upon addition of Cu^{II} ions can form 1:2 and 1:4 coordination complexes ($Cu\mathbf{1}_2$ and $Cu\mathbf{1}_4$, respectively) in the membrane depending on the concentration of embedded **1**.[10] In addition, it was found that the observed first (Cu**1** complex) and second binding

constants ($Cu\mathbf{1}_2$ complex) for copper(II) binding to the ethylenediamino dansyl head group are higher for binding at the membrane surface (observed K_1 and $K_2 \approx 1–2\times10^4\,M^{-1}$, Scheme 1a) as compared to bulk solution, ensuring efficient receptor aggregation induced by Cu^{II} binding. As the intrinsic copper(II) binding events for receptors **1** and **2** should be similar, receptor **2** will also show strong aggregation around copper(II), as found with **1**.[10] Furthermore, as the individual binding actions between the ethylenediamine ligand and the Cu^{II} ion are the same in both receptor systems, these results suggest that linking the extra- and intravesicular copper(II)-binding domains together in **2** leads to increased copper(II)-binding affinity and thus to more efficient fluorescence quenching. We propose that in the case of **2** the complexation of copper(II) ions on one side of the membrane causes receptor aggregation thereby leading to increased copper(II)-binding affinities on the other side of the membrane as a result of increased effective molarities of the linked ethylene diamine Cu^{II}-ligand sites (see Scheme 1b). Since these comparison experiments were performed at low receptor loadings (=2.5 mol% dansyl head group loading), a 2:1 dansyl/Cu complex is expected as was earlier deduced for the non-spanning receptor **1** at these receptor concentrations (see above). The more efficient copper(II) affinity of **2** is especially pronounced at $[Cu^{2+}] < 150$ mM. Thus, whereas the first copper(II) complexation leads to dimerisation of the receptor **2** in a two-step sequence similar to receptor **1** (K_1' and K_2', Scheme 1b and K_1 and K_2, Scheme 1a, respectively), the copper(II) binding event at the other side of the membrane is proposed to be a single binding event to a pseudo intramolecular tetradentate bis(ethylenediamine) ligand system. Such a tetradentate ligand system can only be formed in case of receptor **2** in which the external and internal copper(II) binding domains are connected, explaining the cooperative binding of copper(II) to receptor **2** as compared to **1**. At higher copper(II) concentrations (>150 mM) all ethylenediamine sites are bound to copper(II) ions and thus full fluorescence quenching is observed for both receptor systems. Thus by linking the extra- and intravesicular binding sites together in one receptor system (**2**), we can obtain clear cooperativity in copper(II) complexation experiments thereby transmitting binding information across a lipid bilayer. This synthetic transmembrane receptor system provides an artificial signalling mechanism that resembles signalling events occurring in nature.

Conclusion

Biological signalling events in which information is transmitted from the exterior to the interior of cells are of crucial importance in life. Developing synthetic signalling systems that allow the controlled and detailed investigation of such processes may provide us with valuable information about how nature regulates these events. Here, we have reported the first example of a synthetic transmembrane receptor system that shows synergy between the interior and exterior domains of the receptor when embedded in vesicle bilayers, providing an artificial transmembrane signalling mechanism. Moreover, the design of the transmembrane spanning receptor **2** and non-spanning membrane control receptor **1** has allowed us to demonstrate the existence of cooperativity in a synthetic transmembrane signalling system upon binding to a messenger (copper(II)).

Earlier we reported that non-spanning receptor **1** showed enhanced affinity for copper(II) ions with increasing concentration of **1** in the membrane, illustrating how cells might finely control the formation of certain receptor–ligand clusters by either increasing or decreasing the receptor concentration in the membrane. This in combination with the cooperativity observed for transmembrane spanning receptor **2**, shows how cells can control receptor aggregation and transmission of binding information across lipid bilayers. This particular setup closely resembles biological signalling systems in which a messenger on the outside of a cell triggers a reorganisation of the transmembrane domains thereby initiating subsequent secondary events on the cell interior. Therefore, the research described here is an illustrative example of how artificial signalling systems can be developed that can help us to shine more light on the complex mechanisms by which biological signalling events operate.

Experimental Section

NMR spectra were recorded on Bruker AC 250 or AMX 400 spectrometers and chemical shifts are given relative to residual solvent signal. UV/Vis spectra were monitored on a Varian Cary 1 Bio spectrophotometer. Fluorescence spectra were recorded on Shimadzu RF-5301PC or Hitachi F-4500 fluorescence spectrophotometers. ES+ and EI+ mass spectra were obtained on Micromass Prospec and Micromass Platform spectrometers. Column chromatography was carried out on 60 mesh silica gel. Dansyl ethylenediamine **8** and control receptor **1** were prepared following literature protocols.[10] Egg yolk phosphatidylcholine (EYPC, type XVI, 99% TLC, M_W 768) was used as purchased from Sigma; $CuCl_2$ (99.999% purity) was purchased from Aldrich. All the other chemicals were used without any further purification. Water was distilled twice before use.

3-Hydroxychol-5-en-24-oic acid propargyl ester (3): Cholenic acid (333 mg, 889 μmol) was suspended in dichloromethane (25 mL). To the stirred suspension was added 4-dimethylaminopyridine (112 mg, 894 μmol), followed by the addition of propargyl alcohol (4.6 mL) and dicyclohexylcarbodiimide (189 mg, 894 μmol). The yellow mixture was stirred at room temperature for 48 h. The final mixture was washed with 0.1 M HCl (3×20 mL), the organic layer dried over $MgSO_4$, filtered, and the solvent removed under reduced pressure. The crude product was purified by column chromatography on silica gel (chloroform/ethyl acetate 4:1). Removal of the solvent gave **3** as a white solid (330 mg, 90%). ^{1}H NMR ($CDCl_3$, 250 MHz): δ= 0.60–2.45 (m, 34H, cholesterol protons), 2.47 (t, 1H, ^4J(H,H)=2.4 Hz, alkyne-CH), 3.52 (m, 1H, ^3J(H,H)=4.3 Hz, 3-CH-cholesterol), 4.65 (d, 2H, ^4J(H,H)=2.4 Hz; d, CH_2-alkyne), 5.32 ppm (d, 1H, ^3J(H,H)=5.2 Hz, 6-CH-cholesterol); ^{13}C NMR (CDCl3, 63 MHz): δ=173.4, 140.7, 121.6, 77.8, 74.7, 71.7, 56.7, 55.7, 51.8, 50.1, 42.4, 42.2, 39.7, 37.2, 36.5, 35.3, 31.9, 31.8, 31.6, 30.9, 30.8, 28.1, 24.2, 21.1, 19.4, 18.3, 11.9 ppm; MS (EI+): *m*/*z*: 412 $[M+H]^+$; HRMS (EI+): *m*/*z*: calcdfor $C_{27}H_{40}O_3$: 412.297746; found 412.297245$[M+H]^+$.

3-*O*-(Bromoacetyl)cholenic acid prop-2-ynyl ester (4): Propargyl cholenate **3** (220 mg, 534 μmol) was dissolved in dry THF (15 mL) and bromoacetyl chloride (150 μL, 1.8 mmol) was added dropwise. The reaction

mixture was heated at reflux for 2 h. The solution was concentrated under reduced pressure affording an oil and recrystallisation of this residue from hexane gave the product **4** as a white solid (195 mg, 69%). ^{1}H NMR ($CDCl_3$, 250 MHz): $\delta = 0.60$–2.45 (m, 34H, cholesterol protons), 2.48 (t, H, $^4J(H,H) = 2.4$ Hz, alkyne-CH), 3.83 (s, 2H, -CH_2Br) 4.68 (m + d, 1H, $^3J(H,H) = 4.3$ Hz, 3-CH-cholesterol; 1H, $^4J(H,H) = 2.4$ Hz, CH_2-alkyne), 5.40 ppm (d, 1H, $^3J(H,H) = 4.3$ Hz, 6-CH-cholesterol); ^{13}C NMR ($CDCl_3$, 63 MHz): $\delta = 173.4$, 166.7, 139.2, 123.0, 76.1, 74.7, 56.6, 55.7, 51.8, 49.9, 42.4, 39.7, 37.8, 36.9, 36.5, 35.3, 31.8, 31.0, 30.8, 28.1, 27.5, 26.4, 24.2, 21.0, 19.3, 18.3, 11.9 ppm; MS (ES+): *m/z*: calcd for $C_{29}H_{41}BrO_4Na$: 555; found: 555 $[M+Na]^+$.

3-*O*-(2-(2-Aminoethyl)-dansylamide)acetyl)cholenic acid prop-2-ynyl ester (5): Dansyl ethylenediamine **8** (70 mg, 238 μmol), bromoacetyl propargyl cholenate **4** (112 mg, 211 μmol) and sodium carbonate (60 mg, 566 μmol) were suspended in dry acetonitrile (ca. 10 mL). The reaction was heated at reflux overnight. The resulting solution was concentrated, dissolved in chloroform, filtered, and concentrated again. The crude product was purified by column chromatography on silica gel (chloroform/ethyl acetate 4:1). After removal of the solvent, the product **5** was obtained as a pale green solid (78 mg, 49%). ^{1}H NMR ($CDCl_3$, 250 MHz): $\delta = 0.60$–2.45 (m, 34H, cholesterol protons), 2.48 (t, 1H, $^4J(H,H) = 2.4$ Hz, alkyne-CH), 2.63 (t, 2H, $^3J(H,H) = 5.2$ Hz, ethylenediamine CH_2), 2.88 (s, 6H, $N(CH_3)_2$), 2.95 (t, 2H, $^3J(H,H) = 5.2$ Hz, ethylenediamine CH_2), 3.15 (s, 2H, $NHCH_2COO$), 4.60 (m, 1H, $^3J(H,H) = 4.0$ Hz, 3-CH-cholesterol), 4.66 (d, 2H, $^4J(H,H) = 2.4$ Hz, CH_2-alkyne), 5.32 (d, 1H, $^3J(H,H) = 4.3$ Hz, 6-CH-cholesterol), 7.18 (d, 1H, $^3J(H,H) = 8.2$ Hz, 6-CH-dansyl), 7.53 (dd, 2H, $^3J(H,H) = 8.5$, $^5J(H,H) = 1.2$ Hz, 3- and 7-CH-dansyl), 8.25 (m, 2H, $^3J(H,H) = 7.6$, $^5J(H,H) = 1.2$ Hz, 4- and 8-CH-dansyl), 8.53 ppm (d, 1H, $^3J(H,H) = 7.6$ Hz, 2-CH-dansyl); ^{13}C NMR ($CDCl_3$, 63 MHz): $\delta = 173.4$, 171.3, 152.0, 139.3, 134.6, 130.4, 129.9, 129.7, 128.4, 123.2, 122.9, 118.9, 115.2, 74.8, 74.7, 56.6, 55.7, 51.8, 49.9, 47.7, 45.4, 42.4, 42.3, 39.7, 38.0, 36.9, 36.5, 35.3, 31.8, 31.0, 30.8, 29.7, 28.1, 27.7, 24.2, 21.0, 19.3, 18.3, 11.9 ppm; MS (ES+): *m/z*: 747 $[M+H]^+$; HRMS (ES+): *m/z*:calcd for $C_{43}H_{59}N_3O_6S$: 746.4203; found: 746.4174 $[M+H]^+$.

1,6-Bis(3-*O*-(2-(2-aminoethyl)dansylamide)acetyl) cholenic acid hexa-2,4-diynyl ester (2): Compound **5** (78 mg, 104 μmol) was dissolved in dry dichloromethane (50 mL). Next, the freshly prepared copper(I) chloride (11 mg, 82 μmol) was added to the solution, followed by the dropwise addition of *N,N,N′,N′*-tetramethylethylenediamine (15.8 μL, 105 μmol). The reaction was stirred at room temperature for 3 d. The resulted solution was washed with water (3×50 mL), saturated EDTA (aq) (3×50 mL), and water (3×50 mL), respectively. The organic layer was collected, dried with $MgSO_4$ and concentrated. The crude product was purified by column chromatography on silica gel (eluting initially with 4:1 chloroform/ethyl acetate and then with 9:1 chloroform/methanol). The solvent was removed to give the product **1** as a pale yellow solid. The purity of **1** was determined by ^{1}H NMR spectroscopy (400 MHz) using an internal standard (1,1,2,2-tetrachloroethane) and was found to be > 95%. Yield: 34 mg, 45%. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 0.60$–2.45 (m, 68H, cholesterol protons), 2.58 (t, 4H, $J = 4.8$ Hz, ethylenediamine CH_2), 2.88 (s + t, 16H, $^3J(H,H) = 4.8$ Hz, $N(CH_3)_2$ + ethylenediamine CH_2), 3.07 (s, 4H, $NHCH_2COO$), 4.60 (m, 2H, $^3J(H,H) = 3.7$ Hz, 3-CH-cholesterol), 4.72 (s, 4H, CH_2-alkyne), 5.35 (d, 2H, $^3J(H,H) = 4.0$ Hz, 6-CH-cholesterol), 7.17 (d, 2H, $^3J(H,H) = 7.3$ Hz, 6-CH-dansyl), 7.55 (m, 4H, $^3J(H,H) = 8.5$, $^5J(H,H) = 1.2$ Hz 3- and 7-CH-dansyl), 8.27 (m, 4H, $^3J(H,H) = 8.5$, $^5J(H,H) = 1.2$ Hz, 4- and 8-CH-dansyl), 8.53 ppm (d, 2H, $^3J(H,H) = 8.5$ Hz, 2-CH-dansyl); ^{13}C NMR ($CDCl_3$, 63 MHz): $\delta = 173.2$, 171.7, 151.7, 148.1, 139.3, 134.6, 130.4, 129.9, 129.7, 128.3, 123.2, 122.8, 118.8, 115.2, 74.7, 73.7, 70.2, 56.6, 55.7, 52.0, 50.1, 50.0, 48.9, 47.6, 42.4, 41.6, 39.7, 38.1, 36.9, 36.6, 35.3, 31.8, 30.9, 28.1, 27.7, 24.2, 21.0, 19.3, 18.3, 11.9 ppm; MS (ES+): *m/z*: 1490$[M+H]^+$; HRMS (ES+): *m/z*: calcd for $C_{86}H_{116}N_6O_{12}S_2$: 1489.8171; found 1489.8103 $[M+H]^+$.

Preparation of vesicles: Unilamellar vesicles were prepared by combining the required amount of egg yolk phosphatidylcholine (from a 25 mg per mL stock solution in $CHCl_3$) and receptor **1** (0.50, 1.25, 2.50 and 3.75 mol%) or **2** (1.00, 2.50, 5.00 and 7.50 mol%) into a 10 mL flask; both receptors were transferred from 1 mg per mL stock solution in $CHCl_3$. Next, the solvent was evaporated under atmospheric pressure while applying gentle heating and slow rotation of the flask to give a thin film of phospholipid on the interior of the flask. The film was dried under high vacuum overnight, buffer was added (50 mM MES buffer, pH 6.0) and the thin film was detached by vortex mixing to give a suspension of vesicles. These suspensions were extruded 29 times through a single 800 nm polycarbonate membrane using an Avestin Liposofast extrusion apparatus to give unilamellar 800 nm vesicles containing varying concentrations of receptors **1** or **2**. Vesicle size was characterized by static light scattering and membrane integrity confirmed through carboxyfluorescein encapsulation.

Titrations with copper(II) chloride: Aliquots of a 10 mM copper(II) chloride solution dissolved in double distilled water were added to the appropriate solutions. The solution was equilibrated for 30 minutes after each addition and subsequently the fluorescence spectrum was recorded. Typically, 2.5 mL of sample was placed in the fluorescent cuvette, the sample excited at 337 nm, and the emission monitored from 400 nm to 650 nm (slit widths of 5 nm, 25°C).

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