Signal Transduction

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Entirely Artificial Signal Transduction with a Primary Messenger**

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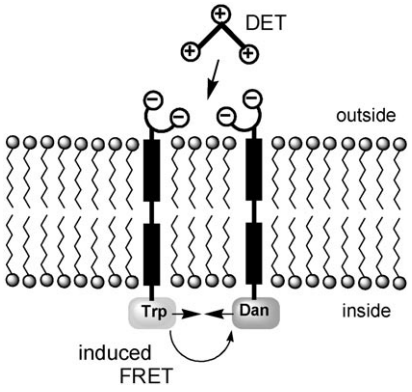
Signal transduction is a natural process of fundamental importance, involving the conversion of a signal from outside the cell to a functional change within the cell.[1] It is typically initiated by a hormone or neurotransmitter which interacts with a receptor at the cell surface, causing a conformational or kinetic change in the membrane-spanning transmitter units and ultimately resulting in release of a second messenger molecule, such as Ca^{2+} ions or cyclic adenosine monophosphate (cAMP). Prominent examples are G-protein coupled receptors (GPCRs) and receptor tyrosine kinases. GPCRs undergo a pivoting motion in one of their transmembrane (TM) helices, and G-protein association initiates a cascade of events resulting in highly efficient energy mobilization.[2] Receptor tyrosine kinases, on the other hand, rely on multivalent binding of growth factors to formerly independent receptor units, whose newly formed active dimers trigger tyrosine phosphorylation on intracellular substrate proteins.[3]

In spite of the eminent importance of transmembrane signaling in nature, very few examples of artificial versions have been constructed by chemists. In early experiments, photochemical switches were combined with impressive conformational changes in attached peptides,[4] and two efficient decalin switches were conformationally coupled by lactam bridges,[5] but only a few of these elegant systems have ever been embedded in membranes. In a related area, very few examples of ligand-gated ion channels exist. One approach uses the intercalation of dialkoxynaphthalene molecules between naphthalenediimide groups of a closed β helix to open the channel,[6] while a cucurbituril-based channel is blocked by acetylcholine.[7] One-side membrane signaling was finally achieved with a lipid signal embedded in a biomembrane model. Copper ions were used to inactivate attached lactate dehydrogenase (LDH); formation of a signal–activator complex translocated all copper ions to the new artificial metal-ion binding site and restored the LDH activity.[8] The first report on true artificial signal transduction involved transmembrane building blocks with attached cysteine and cysteine disulfide moieties for a chemical reaction as output signal. These were, however, brought into close proximity by external thiol oxidation and not by a specific primary messenger molecule.[9] Recently, the Hunter group introduced a somewhat related system based on Cu^{2+} complexation, which featured a dansyl moiety with appended ethylenediamine on both ends of the symmetrical transmembrane units. However, this model produces signals on both sides of the membrane, which are hard to distinguish.[10] Some time ago, we developed *m*-xylylene bisphosphonate dianions as efficient binders for aminoalcohols (such as adrenaline) and especially diammonium cations in aqueous solution.[11] On the basis of these recognition heads, we now present a concept for unidirectional signal transduction triggered by externally added primary messenger molecules.

Our approach involves the construction of two different unsymmetrical transmembrane units with bisphosphonate dianions at one end: a recognition site for compact di- or triammonium cations with K_d values in buffered aqueous solution in the low micromolar range. Covalent elongation of the opposite ends by a FRET pair of fluorescent dyes allows for a highly sensitive and specific spectroscopic readout of the transduction process (FRET = fluorescence resonance energy transfer). After incorporation of both bisamphiphiles into liposomes, a significant decrease in donor emission intensity with simultaneous increase in acceptor emission intensity is expected only if the respective carrier units reside in close proximity during the spectroscopic measurement.[12] This proximity is effected by external addition of the primary messenger, which triggers complex formation between both TM units and stimulates an internal FRET effect (Scheme 1).

The new transmembrane building blocks require considerable synthetic effort. Lithocholic acid *N*-methyl proparglic amide is esterified with the bisphosphonate unit as well as with glycine. The two modified steroids are subsequently covalently fused in a directed Glaser–Hay cross-coupling reaction; this procedure yields the rigid central diacetylene



Scheme 1. Underlying concept of artificial signal transduction with two transmembrane building blocks featuring recognition and effector sites at opposite ends.

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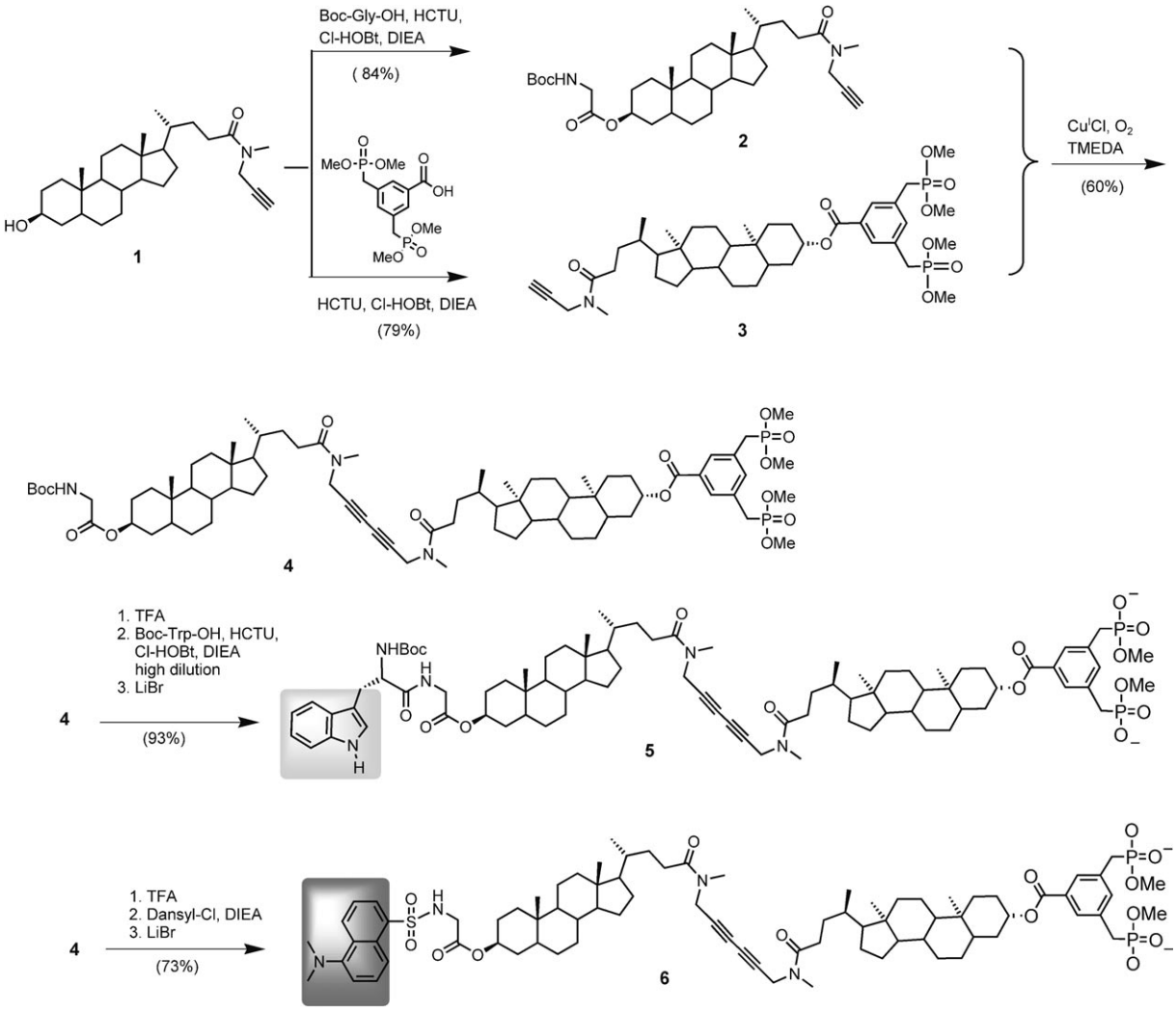
moiety, which further supports the linear incorporation of the whole membrane-spanning unit into the vesicles. Amide coupling attaches either a trpytophan or a dansyl moiety to the free glycine end, and all protecting groups are subsequently removal.[13] Both amphiphilic TM units are soluble in chloroform/methanol (9:1) but not in water and reflect the typical fluorescence properties of their parent dye compounds; they could be easily transferred to unilamellar 200 nm liposomes (Scheme 2).[14]

It should be noted that the starting levels of donor and acceptor emission deviated from the values of the isolated compounds; their presence in the same membrane gives rise to a stable permanent energy transfer.[15] In a preliminary experiment, a large excess of diethylenetriammonium cations was injected into a buffered suspension of dipalmitoylphosphatidylcholine (DPPC) doped with TM units **5** and **6** at 25 °C and produced an immediate moderate additional FRET effect, which slowly approached its maximum value (Figure 1).[16] Since cation diffusion, electrostatic interaction, and FRET are all fast processes, the rate-limiting step must be the lateral movement of the transmembrane building blocks inside the lipid bilayer.[17] Importantly, no changes in emission intensity were observed if only one kind of transmembrane unit was incorporated into the liposome. Lipid bilayers with only trpytophan or only dansyl do not show any alterations in their emission spectra on irradiation into the fluorescence donor absorption maximum (280 nm); both units must be present in the same membrane for the effect. Apparently, complex formation between the anionic headgroups and the cationic messenger led to an overall approach of fluorescence donor and acceptor and produced the observed additional energy transfer. Owing to the small Förster distance for the trpytophan–dansyl pair of 2.1 nm, a transmembrane FRET interaction between oppositely oriented transmembrane units can be excluded.[18] From the observed FRET efficiency, the average donor–acceptor distance induced by messenger recognition can be estimated at 10 Å, well below the Förster radius.[19] An additional interesting feature of this new signal



Scheme 2. New transmembrane building blocks **5** and **6** with recognition sites for a di- or tricationic primary messenger and effector sites for FRET signaling and their synthetic access from known steroidal cores. Boc = *tert*-butoxycarbonyl, DIEA = *N*,*N*-diisopropylethylamine, HCTU = 2-(6-chloro-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylammonium hexafluorophosphate, Cl-HOBt = 6-chloro-1-hydroxy-1*H*-benzotriazole, TFA = trifluoroacetic acid, TMEDA = *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

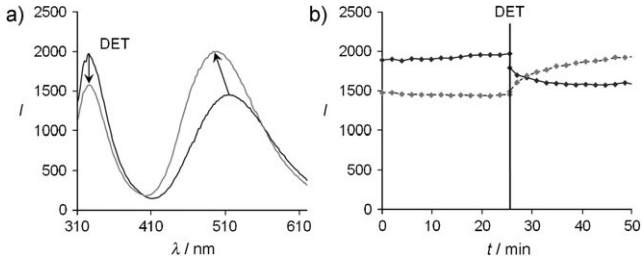


Figure 1. Induced FRET effect on external addition of diethylenetriammonium chloride (100 equiv) to a suspension of mixed DPPC/DMPC vesicles (1:3) doped with 2.5% amphiphilic transmembrane units with bisphosphonate headgroups and donor or acceptor tail groups. Excitation at 280 nm in 375 mM MES buffer. a) Fluorescence spectra at 25 min (DET addition) and at 50 min (full signal). b) Kinetically slow build-up within about 25 min after DET addition (vertical line). DMPC = dimyristoylphosphatidylcholine, MES = 2-(*N*-morpholino)ethanesulfonic acid.

transduction event is the accompanying marked blue shift in the dansyl emission, most likely caused by formation of a charge-transfer complex between the trpytophan–dansyl FRET pair.

A multivalent primary messenger trication could potentially also bridge two neighboring vesicles by simultaneous complexation of their respective surface-exposed bisphosphonate dianions.[20] If this mechanism would bring fluorescence donors and acceptors of two separate vesicles into close proximity, intervesicle FRET might take place. Although the estimated fluorophore distance would be much larger in this case (most likely well beyond the Förster distance), an experiment was devised to check the potential interference of intervesicle FRET with the observed signal transduction. Two liposomes were each equipped with one kind of fluorescent units. An equimolar mixture of both liposomes comprising the same total concentration of fluorophores as before was treated with the same amount of DET. The mixture displayed almost no change in donor and acceptor emission during the time period of the signal transduction process, thus ruling out intervesicle FRET.

It has to be kept in mind that the concept delineated at the outset bears inherent limitations on the maximum possible transduction signal intensity. As the TM units are not oriented inside the membrane, half of all recognition sites are hidden inside the liposome and are therefore unproductive; furthermore, only a statistical third of all ternary bisphosphonate complexes with externally added DET are of the heterodimeric kind useful for FRET. Thus, a complete fluorescence quenching cannot be expected. However, there are some other parameters that can indeed be fine-tuned und thereby shed more light on the whole transduction process.

It is well known that DPPC bilayers undergo a phase transition from the gel to a much more mobile fluidic state at 41 °C.[21] Thus, the lateral movement of TM units inside the liposome can be expected to become greatly facilitated at elevated temperatures. On the other hand, complex ion-pair formation of their headgroups with the primary messenger will be significantly weakened. Experiments directed at 41 °C indeed indicated a greater TM-unit mobility inside the membrane, leading to substantial FRET even before addition of the DET trication; the net transduction signal, however, remained small.

DMPC bilayers, with a lipid length of two carbon atoms less than DPPC, already attain fluidity at ambient temperature, where ion pair formation is still strong; as expected, the induced FRET effect rose significantly when both TM building blocks were incorporated into DMPC vesicles. The most impressive results, however, were produced with mixed vesicles comprising 75% DMPC and 25% DPPC. In a separate investigation, phase transition temperatures were determined for several intermediate binary lipid mixtures. These displayed a constant decrease with a minimum of 23 °C for pure DMPC. Thus, DMPC represents the medium for optimal fluidity, but 20% DPPC is necessary as a perfect geometrical match directly surrounding the synthetic transmembrane units. At room temperature, the induced FRET amounts to a total decrease in maximum donor emission intensity of 25%, accompanied by a simultaneous increase in maximum acceptor emission intensity of 30% (Figure 2a).

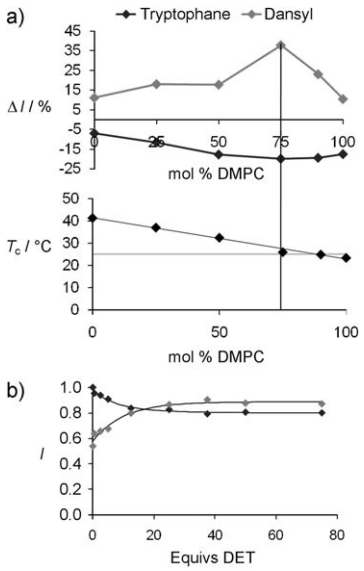
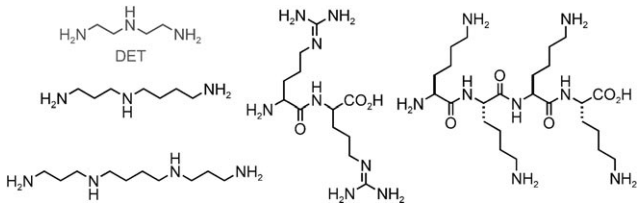


Figure 2. a) Dependence of membrane fluidity and FRET efficiency on the lipid composition. At a 3:1 DMPC/DPPC ratio the optimum is reached, at which maximum fluidity and matching lipids generate maximum FRET intensity. b) Proportional dependence of FRET efficiency on DET excess.

Apart from the lipid composition, the amount of added messenger molecules governs the magnitude of the induced FRET. One equivalent of DET relative to the total number of transmembrane units suffices for unambiguous detection. With increasing messenger concentration, the signal strength increases and reaches its maximum value at approximately 40 equivalents (Figure 2b).

Apart from DET, several other organic and biologically relevant polycations were tested as primary messengers (Scheme 3), but none of them could induce the signaling process. We tentatively assume that the most compact combination of three ammonium ions is mandatory for a sufficient approach of the two transmembrane units, ideally below the Förster distance of 21 Å. Spermine and spermidine



Scheme 3. Structures of DET and selected other tested primary messenger compounds (from left to right: spermidine and spermine, diarginine, tetralysine).

as well as small oligolysines and oligoarginines are all much less compact in this respect, with larger N···N separations. This property renders the system highly selective for a single appropriate external trigger molecule, and it should remain silent in the environment of a cell membrane.[22]

It might be argued that polyamines are in principle able to penetrate cell membranes[23] and that the triammonium salt likewise may cross the vesicle membrane. In this case, it would also reach the internal bisphosphonates and cancel the unidirectional signal transduction. To examine this postulated effect, we filled vesicles with the primary messenger and subsequently filtered them over a Sephadex column.[24] The isolated vesicles were kept in aqueous buffer for one hour and treated with ninhydrin to detect a potential triammonium cation leak. However, all attempts were negative, while the same liposomes furnished strongly purple solutions when they were treated with detergent (Triton X) in the presence of ninydrin. We conclude that within the detection limits, no appreciable amounts of the primary messenger cross the vesicle membrane.

Finally, a close inspection of the propargyl amide centerpiece connecting both steroids reveals several rotatable bonds. It is conceivable that the bisamphiphile may also reside inside the membrane in a U shape, although its steroidal arms would be too short to match the surrounding DMPC or DPPC lipids, and the diacetylene bottom of the "U" would represent a substantial perturbation in the self-assembled bilayer. In this case, however, signaling would only occur on the extracellular face of the liposomes, irrespective of its intracellular contents.[25] To discriminate between the two assumed cases, we added free eosin to the system. This fluorescent dye features an absorption band that overlaps with the dansyl emission, and it produced a remarkable effect at the extracellular face. Even in the absence of primary messenger, both trpytophan and dansyl emission are markedly diminished, whereas an extremely strong eosin emission is turned on.[26] If liposomes only carry a trpytophan or a dansyl TM unit, irradiation into the trpytophan absorption band (280 nm) after external eosin addition leaves the dye silent, while excitation of a dansyl-doped liposome at 330 nm again turns on the superior eosin fluorescence (560 nm). Obviously, a stable multi-FRET system is established, most likely assisted by docking of the negatively charged eosin anion onto the electron-poor dansyl moiety.[27] Now the inner and outer sphere are easily distinguished from each other, and final addition of DET should reveal the location of the signaling event. In the case of U-shaped amphiphiles, the approach of external trpytophan and dansyl units would greatly enhance the multi-FRET. In sharp contrast, however, the eosin emission intensity remained unchanged, and the usual FRET effect was again observed inside, accompanied by inverted dansyl and trpytophan emission changes; this result is exactly the expected scenario for linear transmembrane units (Figure 3).

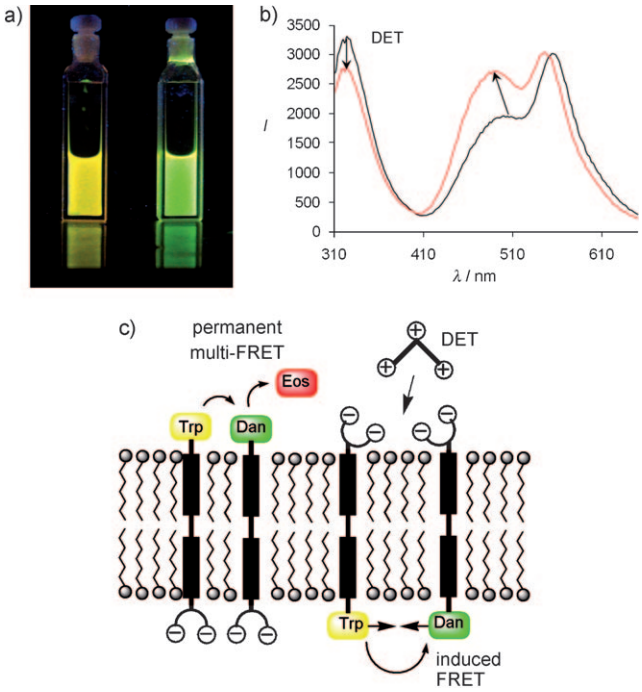


Figure 3. Multi-FRET system with extracellular eosin (0.1 equiv) and trpytophan excitation. Spectral changes on DET addition demonstrate intracellular FRET with unaltered eosin emission intensity; experimental evidence for true signal transduction is visible by the naked eye. a) Cuvettes before and after DET addition (1 μL). b) Fluorescence emission spectrum. c) Permanent multi-FRET (outside) and induced FRET signal (inside).

The external eosin addition has a beneficial side effect. Owing to the permanent multi-FRET, the emitted light takes on another mixed color. This color tone undergoes a hypsochromic shift from orange to green during the signaling event, which is visible by the naked eye (Figure 3). The underlying reason is the substantial blue shift in the dansyl emission, which was already spectroscopically observed in the absence of eosin (Figure 3).

In conclusion, an artificial system has been devised for messenger-induced transmembrane signaling. External addition of a primary messenger molecule thus leads to formation of a heterodimeric complex of two transmembrane units, which in turn stimulates a strong FRET effect on the opposite intracellular side if these units carry a fluorescence donor–acceptor pair. The readout can be enhanced and transferred into the visible range by external eosin addition, thus producing a multi-FRET system. Signaling can thus be detected by the naked eye. In the future, we intend to exchange recognition and signaling sites in order to synthesize

transmembrane units for bioanalytes such as peptides and hormones and also to enable the doped liposomes to trigger a chemical reaction with release of a second messenger molecule. Further challenges include noninvasive detection without irradiation and preorientation of the transmembrane units for inherent unidirectionality.

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