Supramolecular Chemistry Hot Paper

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Chemosensing Ensembles for Monitoring Biomembrane Transport in Real Time**

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Abstract: *The efficacy of drugs and biomolecules relies on their ability to pass through the bilayer. The development of methods to directly and sensitively monitor these membrane transport processes has remained an experimental challenge. A macrocyclic host (p-sulfonatocalix[4]arene or cucurbit[7]uril) and a fluorescent dye (lucigenin or berberine) are encapsulated as a chemosensing ensemble inside liposomes, which allows for a direct, real-time fluorescence monitoring of the passage of unlabeled bioorganic analytes. This in vitro assay is transferable to different channel proteins and analytes, has potential for fluorescence-based screening, e.g., of channel modulators, and yields the absolute kinetics of translocation. Using this new biophysical method, we observed for the first time direct rapid translocation of protamine, an antimicrobial peptide, through the bacterial transmembrane protein OmpF.*

Ion channels and transporter proteins are the third most common drug targets after membrane receptors and enzymes.[1] Robust and sensitive methods to investigate permeation or translocation through channel proteins are consequently of paramount interest. In fact, the lack of biomembrane assays suitable for rapid screening presents a bottleneck in antibiotics and drug discovery.[2] Similarly, many mechanistic questions about the translocation of hydrophilic molecules such as arginine-rich peptides through an intrinsically hydrophobic barrier remain unresolved owing to lack of sensitive in vitro methods for monitoring their translocation.[2d] Whether these polycationic peptides permeate on their own or translocate through channel proteins remains an open but crucial question in regard to their antimicrobial activity and other putative functions.[3]

Only a handful of label-free methods to monitor membrane translocation are at hand. The most prominent biophysical method to monitor analyte translocation through membranes is electrophysiology, which, however, cannot readily differentiate between translocation and binding.[4] Alternative methods involve radioactive uptake, NMR spectroscopy, or isothermal calorimetry.[5] For high-throughput screening in a pharmaceutical-industrial setting, fluorescence-based membrane assays are preferred. The repertoire of these is presently limited to the detection of changes in pH value, detection of chloride influx,[6] or membrane rupture (Supporting Information).

A fluorescence-based method that allows monitoring of the translocation of organic analytes with micromolar sensitivity is still called for. The in vitro method we introduce here—supramolecular tandem membrane assays—affords real-time kinetics, employs unlabeled analytes, and is applicable to a series of structurally related biomolecules. It is based on the co-encapsulation of a fluorescent dye with a macrocyclic host to form a "reporter pair" inside liposomes. The macrocycles in our assay are utilized as receptors for the translocated analyte, which introduces a genuine molecular recognition feature that increases the sensitivity to the micromolar range. It should be noted that macrocycles have already been exploited in membrane research, but for different reasons, that is, to either insert in membranes or to interact directly with membrane proteins.[7]

The working principle of our supramolecular chemical method is illustrated in Figure 1. Liposomes containing the host/dye reporter pair are prepared and purified, such that a subsequently added analyte affects the dye fluorescence only if it is able to enter the vesicle and to displace the dye from the macrocycle (Figure 1b,c). The reporter pair is selected to fulfil the following requirements: a) Neither host nor dye must dissolve in or permeate through the membrane; this is a limiting factor which distinctly raises the physicochemical complexity compared to our previously introduced enzyme assays,[8] a time-resolved variant of indicator displacement assay;[9] b) the macrocyclic host needs to display a high affinity to the target analyte(s), for example, polycationic peptides or cationic neurotransmitters; and c) the dye must show a strong fluorescence response upon release from the host. Consequently, tandem membrane assays can be set up with several reporter pairs for many biological analytes. In fact, a large library of reporter pairs with varying receptor properties is available, many of which commercially.[10] Herein, we first used *p*-sulfonatocalix[4]arene (CX4) and lucigenin (LCG),[8b,11] which are jointly suited to signal the binding of several cationic analytes (Figure 1a).

To document the key steps in the development of a tandem membrane assay for the translocation of a biologically important analyte and to demonstrate the functionality of the assay for measuring the kinetics and identifying ion channel modulators, we have chosen protamine as the analytical target and the outer membrane protein F (OmpF)

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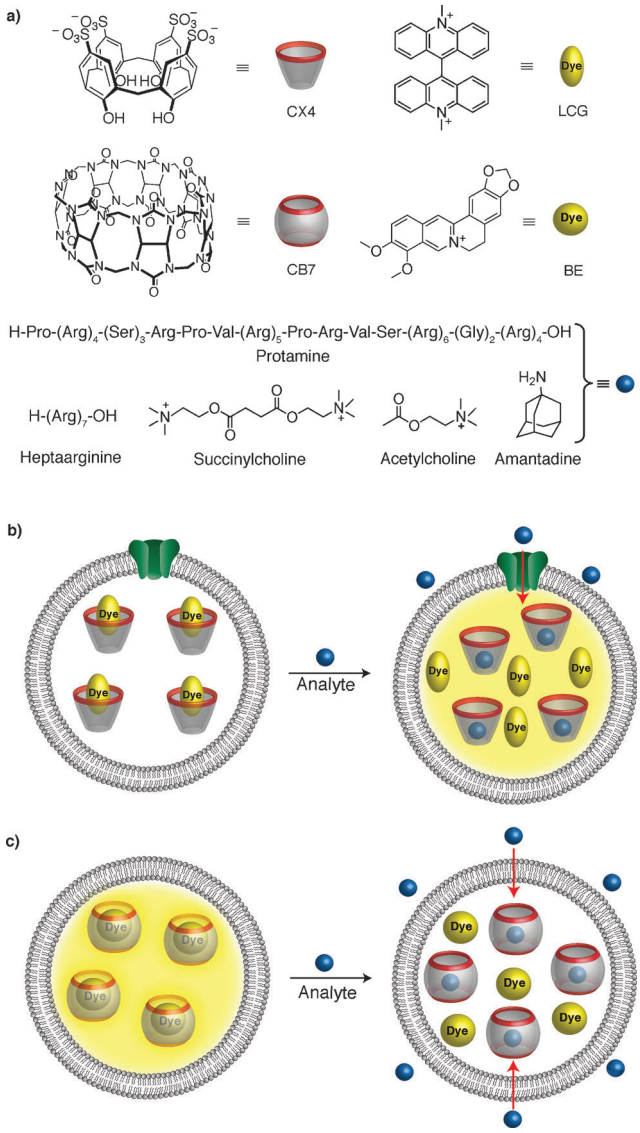

Figure 1. a) Macrocycles and dyes used as reporter pairs as well as the analytes investigated. b,c) Working principle of a supramolecular tandem membrane assay. Illustration of macrocyclic host–dye complexes encapsulated inside a liposome before (left) and after (right) translocation of an analyte (blue) through b) a channel protein (green) or c) directly through the biomembrane; the analyte binds to the macrocycle, thereby displaces the dye, which in turn becomes either b) strongly fluorescent in its uncomplexed form (switch-on fluorescence response) or c) weakly fluorescent (switch-off response).

as a representative channel protein. Cationic antimicrobial peptides, such as protamine (MW ca. 5000 Da), are bactericidal;[3b,12] however, they do not cause any lysis of the outer membrane in gram-negative bacteria. An open, and mechanistically utmost important question is therefore whether the internalization observed for cationic antimicrobial peptides occurs through channel proteins. OmpF, as an example of a cation-selective channel, allows the direct passage of small molecules (MW < 600 Da), including many antibiotics.[1b] Based on this empirical cut-off, passage of protamine is not expected. Nevertheless, the passage of a bacteriocin, colicin E9, has recently been observed, assisted by a membrane surface receptor.[13]

Fluorescence titrations with the pre-formed CX4–LCG reporter pair in homogeneous solution (see the Supporting Information) showed that the addition of micromolar concentrations of protamine led to an efficient fluorescence recovery. This effect is due to a competitive binding, because protamine has a higher affinity ($K_{CX4\text{–protamine}} = (1.24 \pm 0.31) \times 10^9\,M^{-1}$, Figure S3b) to CX4 than the dye ($K_{CX4\text{–LCG}} = (1.03 \pm 0.04) \times 10^7\,M^{-1}$, Figure S3a). Consequently, the reporter pair can be used as a chemosensing ensemble for protamine, while the spatial isolation in the inner liposomal space gives us a handle to directly monitor changes in local protamine concentration.

For the actual assays, CX4–LCG was first encapsulated into the liposomes. An inner-phase concentration of 500 μM LCG allowed for sufficient sensitivity while a slight shortfall of CX4 (450 μM) was needed to produce a linear response to analyte (see the Supporting Information). OmpF was then added to form proteoliposomes (Figure 2a). When protamine was administered, a steep increase in fluorescence was observed (Figure 2b). This was an interesting observation because it immediately demonstrated that this porin (with a putative MW cut-off near 600 Da) facilitated the uptake of this large polycationic peptide (MW 5000 Da) into the interior of the liposome, even in the absence of an auxiliary membrane surface receptor.[13]

Numerous control experiments were performed. In particular, we demonstrated that direct addition of protamine to a solution of liposomes containing the reporter pair did not trigger a fluorescence response (Figure 2c), which provided direct and unequivocal evidence that protamine did not permeate on its own through the biomembrane under our experimental conditions, up to 10 h. Of course, since there was no fluorescence response, it could also be ruled out that protamine caused any pore formation or fusion of the liposomes (Figures S5 and S6). Similarly, we verified that neither host nor dye can escape through the channel (Figure 2d) and that the fluorescence increase is affected only by the presence of both OmpF and protamine, also when added in the reverse order (Figure 2e). Therefore, the fluorescence increase observed in the presence of OmpF provides compulsory evidence for direct channel-mediated translocation of an arginine-rich peptide. This is a critical finding when discussing the potential of antimicrobial peptides as next-generation antibiotics against gram-negative bacteria.[3b,14]

As a unique asset, our method allows real-time measurement of the translocation kinetics. The passage of protamine through OmpF was found to be surprisingly fast, requiring stopped-flow experiments with rapid mixing of the analyte with the proteoliposomes containing the CX4–LCG reporter pair (Figure 3a). Increasing the analyte concentrations resulted in both a faster kinetics and a higher final fluorescence intensity, which reached a plateau at high protamine concentration (Figure 3b). This demonstrated that the translocation rate reached a limiting value and that the displacement became quantitative at high analyte concentration (see the Supporting Information and Figure S11). Hill analysis of the initial rates yielded a half-saturation constant (EC_{50}) of

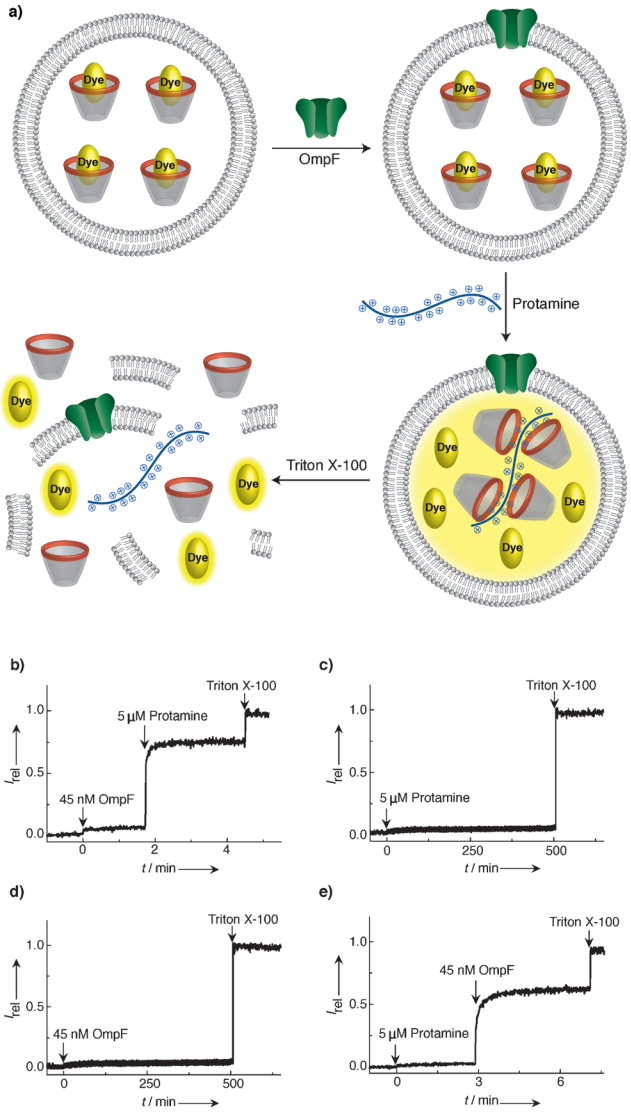

Figure 2. Supramolecular tandem membrane assays to monitor translocation of protamine by fluorescence. a) Schematic representation of spontaneous insertion of OmpF (green) into the membrane of CX4–LCG-loaded liposomes (450 μM/500 μM). Protamine (blue) enters into the liposome through OmpF and displaces LCG from CX4 to result in a switch-on fluorescence response. The addition of Triton X-100 lyses the membrane releasing reporter pairs and analytes into the bulk solution causing supramolecular disassembly and a large dilution (factor of ca. 5000, Figure S9). The supramolecular tandem membrane assay allows monitoring of the translocation of protamine through the LCG fluorescence response. b–e) Fluorescence intensity of CX4–LCG-loaded liposomes upon addition of b) 45 nM OmpF then 5 μM protamine, c) 5 μM protamine, d) 45 nM OmpF, e) 5 μM protamine, then 45 nM OmpF.

450 nM with a Hill slope of approximately 6.[15] The tandem membrane assays therefore complement the electrophysiological measurements, in which the differentiation between analyte binding to the channel or translocation through it, as well as the determination of actual kinetics becomes difficult, in particular for high-molecular-weight analytes and slow translocation rates (see the Supporting Information).[3c,4,16]

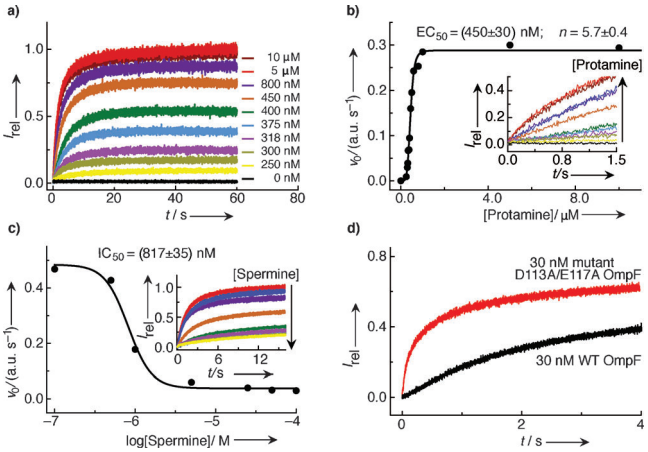

Figure 3. Kinetics of OmpF-mediated translocation of protamine into liposomes, monitored by stopped-flow experiments. a) Fluorescence kinetic traces upon mixing of protamine (0–10 μM) with a solution of CX4–LCG-loaded proteoliposomes (450 μM/500 μM and 30 nM OmpF). b) Fitting of initial rates of translocation at different protamine concentrations according to the Hill equation.[15] Inset: The initial rate increases with protamine concentration. c) Dose-response curve for the inhibition of protamine translocation by spermine. The measurement was initiated by adding 5 μM protamine to a solution of CX4–LCG-loaded liposomes, 45 nM OmpF, and 0–100 μM spermine. Inset: The fluorescence kinetics at various spermine concentrations (red trace: 0 μM, yellow trace: 100 μM). d) Fluorescence kinetics of protamine translocation through wild-type OmpF (30 nM, black trace) and through the double mutant D113A/E117A OmpF (30 nM, red trace). The reaction was initiated by adding 5 μM protamine to the CX4–LCG-loaded proteoliposomes.

Various transmembrane channels are co-regulated by polyamines and, among them, spermine is the most potent modulator of OmpF.[1b,17] When the tandem assays were conducted at different concentrations of spermine, the translocation rate, as monitored by the fluorescence response, decreased (Figure 3c), and the dose-response curve afforded an IC_{50} value of (820 ± 40) nM. With $K_i = IC_{50}/(1+[\text{Protamine}]/EC_{50})$ a K_i value of approximately 70 nM is obtained, which corresponds well to a literature estimate (low nanomolar range).[17] As can be seen, a label-free supramolecular method is now available to pin-point the functions of channels and to screen for modulators, all by fluorescence. Furthermore, since spermine anchors at the constriction region of the OmpF channel, the efficient inhibition of protamine translocation also demonstrates that this analyte indeed passes through the OmpF channel interior and not, to rule out a less-likely detour, along the outer walls of the protein, that is, along the lipid–protein interface.[18]

The constriction region of OmpF is rate-limiting for the translocation of molecules, including hydrophilic antibiotics, such as ampicillin and penicillin.[1b] Electrophysiological studies have pointed to an accelerated uptake of such β-lactam antibiotics through singly substituted OmpF mutants D113A and R132A.[19] Consequently, we studied the effect of mutations that affect the charge and size of the OmpF channel in relation to the flux of protamine. The two negatively charged residues aspartate 113 and glutamate 117 were replaced by alanine through site-directed mutagenesis, and,

indeed, the rate of protamine influx through D113A/E117A OmpF increased threefold (Figure 3d). Again, and in contrast to other experiments, our method allows direct continuous monitoring of the translocation kinetics, a first-time observation for an antimicrobial peptide.[3c] It transpires that the screening of a large library of channel mutants could be readily conducted. The method is transferable to other channel proteins.

Because the method is based on an unselective molecular recognition, it is not limited to protamine, but transferable to other organic analytes (Figure 1a). For instance, we have unambiguously demonstrated the successful translocation of heptaarginine, a membrane transduction peptide, and of acetylcholine, a low-molecular-weight neurotransmitter, both through OmpF (Figure S12). Direct permeation (without channel) of analytes can also be conveniently monitored, for example, of amantadine, an anti-Alzheimer's drug (Figure S13); this assay was additionally performed in liposomes of different lipid composition and by utilizing an alternative chemosensing ensemble composed of cucurbit[7]uril (CB7) as macrocyclic receptor and berberine (BE) as fluorescent dye (Figure 1a).

In summary, the supramolecular chemical method introduced herein (after the necessary adaptations of the reporter pairs) will allow for the screening of diverse classes of analytes, different channel proteins, and channel modulators. It can monitor transport driven either by a concentration gradient, or, potentially, by an electrochemical gradient established by reconstituting highly selective ionophores, such as valinomycin, into liposomes. The micromolar sensitivity and versatility should find ample applications in fundamental and applied membrane research ranging from the simple detection of permeation to the measurement of the real-time transport kinetics of natural metabolites, toxins, and drugs. The applicability of the method to cellular studies is presently being explored.

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