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Dynamically Analyte-Responsive Macrocyclic Host–Fluorophore Systems

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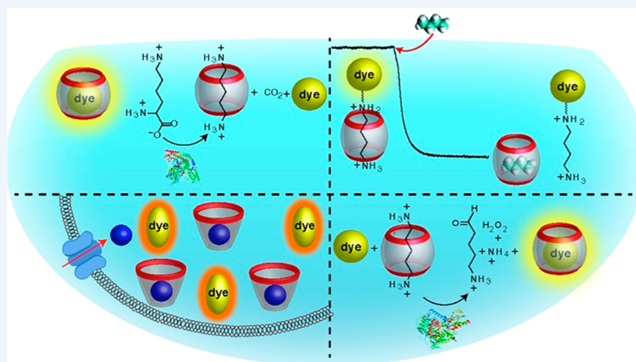
CONSPECTUS: Host–guest chemistry commenced to a large degree with the work of Pedersen, who in 1967 first reported the synthesis of crown ethers. The past 45 years have witnessed a substantial progress in the field, from the design of highly selective host molecules as receptors to their application in drug delivery and, particularly, analyte sensing. Much effort has been expended on designing receptors and signaling mechanism for detecting compounds of biological and environmental relevance. Traditionally, the design of a chemosensor comprises one component for molecular recognition, frequently macrocycles of the cyclodextrin, cucurbituril, cyclophane, or calixarene type. The second component, used for signaling, is typically an indicator dye which changes its photophysical properties, preferably its fluorescence, upon analyte binding. A variety of signal transduction mechanisms are available, of which displacement of the dye from the macrocyclic binding site is one of the simplest and most popular ones. This constitutes the working principle of indicator displacement assays.



However, indicator displacement assays have been predominantly exploited in a static fashion, namely, to determine absolute analyte concentrations, or, by using combinations of several reporter pairs, to achieve a differential sensing and, thus, identification of specific food products or brands. In contrast, their use in biological systems, for example, with membranes, cells, or with enzymes has been comparably less explored, which led us to the design of the so-called tandem assays, that is, dynamically analyte-responsive host–dye systems, in which the change in analyte concentrations is induced by a biological reaction or process. This methodological variation has practical application potential, because the ability to monitor these biochemical pathways or to follow specific molecules in real time is of paramount interest for both biochemical laboratories and the pharmaceutical industry.

We will begin by describing the underlying principles that govern the use of macrocycle-fluorescent dye complexes to monitor time-dependent changes in analyte concentrations. Suitable chemosensing ensembles are introduced, along with their fluorescence responses (switch-on or switch-off). This includes supramolecular tandem assays in their product- and substrate-selective variants, and in their domino and enzyme-coupled modifications, with assays for amino acid decarboxylases, diamine, and choline oxidase, proteases, methyl transferases, acetylcholineesterase (including an unpublished direct tandem assay), choline oxidase, and potato apyrase as examples. It also includes the very recently introduced tandem membrane assays in their published influx and unpublished efflux variants, with the outer membrane protein F as channel protein and protamine as bidirectionally translocated analyte. As proof-of-principle for environmental monitoring applications, we describe sensing ensembles for volatile hydrocarbons.

■ INTRODUCTION

The first competitive binding assay was described by Berson and Yalow in 1960, for measuring plasma insulin.[1,2] Since then, competitive assays have found widespread applications in biomedicine. In addition, nonradioactive techniques using *enzymes* have evolved, specifically enzyme-linked immunosorbent assays (ELISA)[3,4] and enzyme-multiplied assays (EMIT),[5] which frequently exploit chemoluminescence[6] and fluorescence for detection.[7,8] Immunoassays were perhaps the first demonstration of a "biotic" receptor–ligand system responsive to external stimuli such as the addition of an unlabeled analyte.

With the advancement of supramolecular chemistry, conceptually related indicator displacement assays (IDAs)[9–12] have been introduced which exploit the potential of synthetic receptors, particularly macrocyclic hosts, for analyte sensing. IDAs bypass the demanding design of chemosensors containing the (macrocyclic) recognition unit with a covalently linked signaling unit, typically a tethered chromophore.[13,14] The sensing principle of IDAs relies on the competition between a test substance and an indicator for the same binding site on the host. When an analyte is added to a solution containing host·indicator complex, the analyte displaces the indicator from the binding site. Upon displacement of the indicator, a change in

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signal is observed, for example, of its fluorescence (Figure 1). These assays have been popularized in supramolecular chemistry by Anslyn and co-workers;[10,15,16] many other groups have also contributed to this area.[17−22]

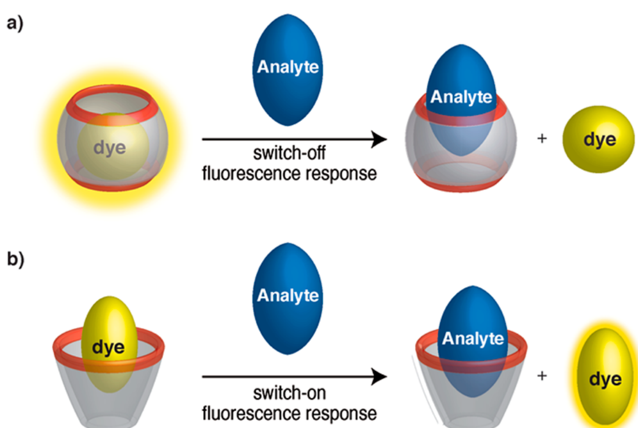

Figure 1. Indicator displacement assays for analyte sensing using a macrocyclic host and a fluorescent dye. The addition of an analyte is signaled by either (a) a fluorescence decrease or (b) a fluorescence increase due to displacement of the dye from the host.

Macrocycles (Figure 2a) are capable of binding and recognizing guest molecules such as fluorescent dyes (Figure 2b). The photophysical properties of a dye are changed upon formation of a macrocycle·dye complex.[23] Specifically, for cyclodextrins and cucurbiturils (Figure 2a), the fluorescence of the encapsulated dye is frequently enhanced due to relocation into a more hydrophobic, solvent-protected environment. In contrast, for the electron-rich cyclophanes and calixarenes (Figure 2a), a charge-transfer induced quenching of the complexed fluorescent dyes generally applies. Conversely, when the dye is displaced from the cavity due to a competitive binding of an analyte, its original fluorescence is restored (Figure 2c). This spectroscopic response of the host·dye complex allows its use as a "reporter pair" or "chemosensing ensemble". Herein, we demonstrate how host·dye systems can be exploited to track, in real time, changes in concentrations of diverse analytes (Figure 3) as they occur in enzymatic reactions, during membrane transport, or upon dissolution of volatile gases.

SUPRAMOLECULAR TANDEM ENZYME ASSAYS

The fundamental principles of supramolecular chemistry dictate that the selectivity of macrocyclic receptors is determined, among others, by the complementary charge and size of their guest molecules.[35,36] Consequently, we projected that any chemical process that alters the overall charge or size of an analyte, such as that resulting from an enzymatic transformation, could be monitored by using a host·dye complex as a dynamically analyte-responsive "reporter pair". This conceptual approach afforded a new label-free and versatile method to monitor enzymatic activity, which we introduced as supramolecular tandem enzyme assays The enzymes, enzyme classes, and the substrate/product combinations for the successfully investigated biotransformations are shown in Table 1, along with the photophysical response. Different variants of tandem assays are discussed next, followed by specific examples as highlights.[24−32]

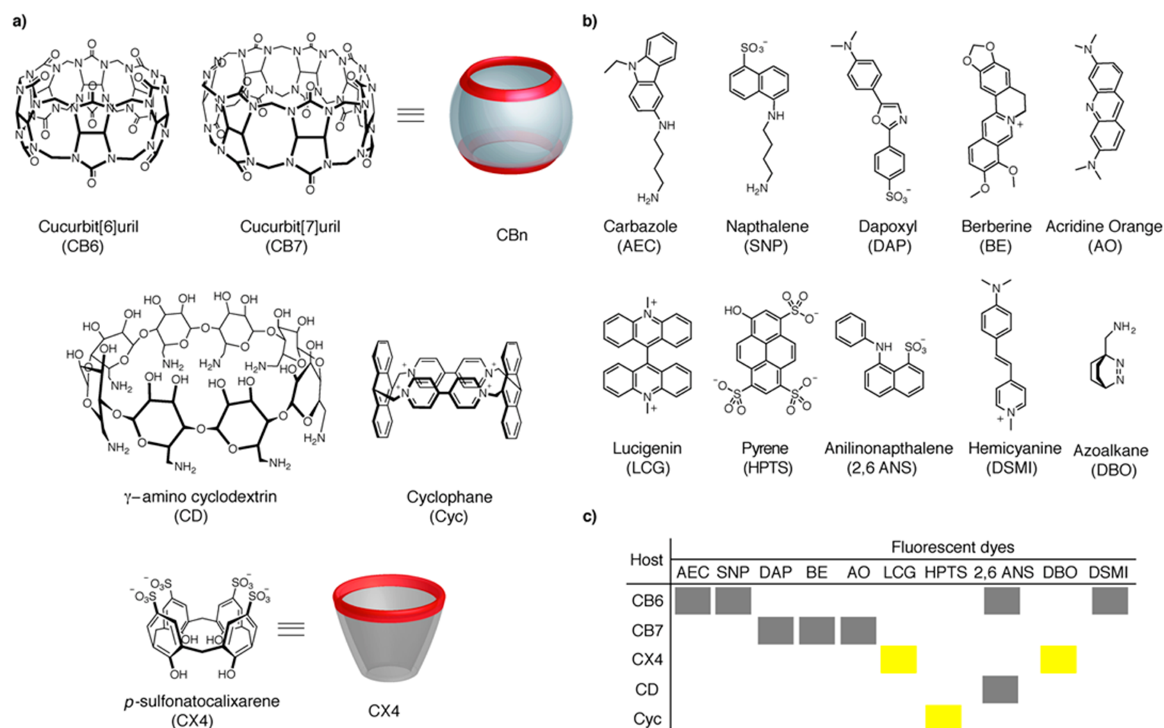

Figure 2. Chemical structures of (a) macrocyclic hosts as synthetic receptors and (b) fluorescent dyes investigated by our group as indicators. (c) Matrix representation for combinations of host and dye complexes suitable as chemosensing ensembles in tandem assays are indicated by a bar, where the photophysical response upon analyte binding is color-coded in yellow and gray, indicating an enhancement and quenching of the fluorescence intensity, respectively.[23]

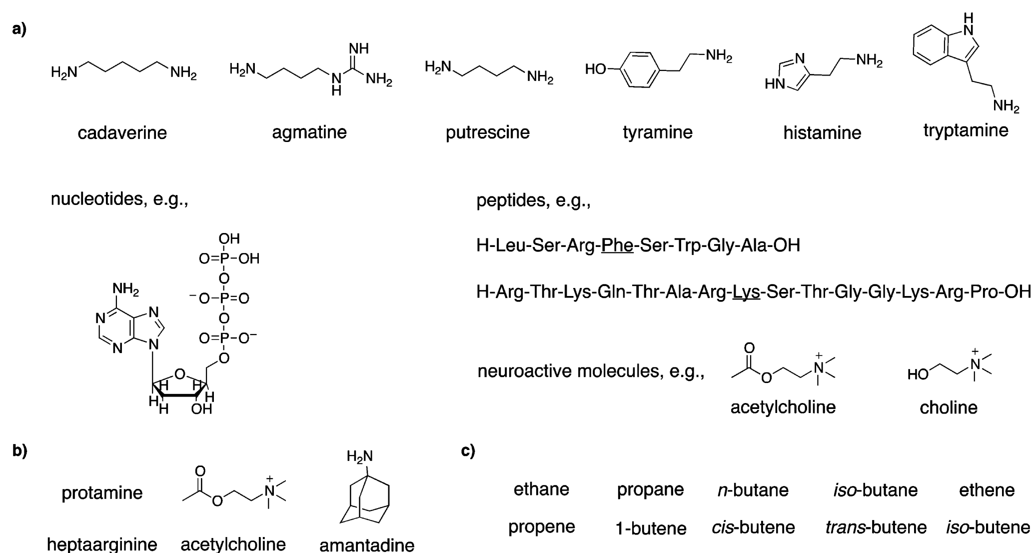

Figure 3. Chemical structures of analytes detected during (a) enzymatic transformations,[24−32] (b) membrane translocation,[33] and (c) gas dissolution.[34] For enzymatic reactions, only the strong competitor from the substrate/product pair is shown. The underlined amino acids indicate the residue where enzymatic cleavage or conversion takes place.

Table 1. Dynamically Analyte-Responsive Host·Dye Systems for the Detection of Analytes Generated or Depleted during Enzymatic Transformations

host·dye[a]	enzyme (enzyme class)[b]	substrate/product[c]	ref
	assays with switch-on fluorescence response		
CX4·DBO	amino acid decarboxylase (EC4)	amino acid/biogenic amine	31
	arginase (EC3)	arginine/ornithine	32
CX4·LCG	choline oxidase (EC1)	choline/betaine	30
	histone methyl transferase (EC2)	unmethylated/methylated peptide	26
Cyc·HPTS	potato apyrase (EC3)	nucleotide tri-/monophosphates	27
OH-β-CD·2,6-ANS	isomerase (EC5)	aromatic/arranged aromatic	37
	assays with switch-off fluorescence response		
CB7·DAP	amino acid decarboxylase (EC4)	amino acid/biogenic amines	31
CB7·AO	lysine decarboxylase (EC4)	lysine/cadaverine	32
	diamine oxidase (EC1)	cadaverine/aminoaldehyde	32
	thermolysin (EC3)	polypeptides/dipeptides	29
	leucine aminopeptides (EC3)	polypeptides/amino acids	28
	trypsin (EC3)	polypeptides/peptide fragments	28
CX4·LCG	butylcholinesterase (EC3)	succinylcholine/choline	38
CB6·DSMI	acetylcholinesterase (EC3)	acetylcholine/choline	[d]
CB6·AEC	lysine decarboxylase (EC4)	lysine/cadaverine	39
CD·2,6-ANS	potato apyrase (EC3)	nucleotide tri-/monophosphates	27

[a]See Figure 2 for chemical structures. [b]EC1, oxidoreductases; EC2, transferases; EC3, hydrolases; EC4, lyases; EC5, isomerases; and EC6, ligases. [c]Analyte that binds more strongly to the host is underlined. [d]This work.

■ ASSAY VARIANTS

Product-Selective Supramolecular Tandem Assays

At the heart of supramolecular tandem assays lies the reversible and competitive binding of a macrocyclic host with a fluorescent dye and an analyte, and its differential binding with the enzymatic substrate or product. The assay setup is particularly straightforward when the product binds more strongly to the macrocycle which leads to a *product*-selective assay. This is the case for amino acid decarboxylases, where a macrocyclic host (for example, CB7) is chosen such that it binds weakly with a substrate (lysine), but strongly with the corresponding enzymatic product (cadaverine). Upon addition of lysine decarboxylase, lysine, the weak competitor, undergoes enzymatic decarboxylation to form the biogenic amine, the strong competitor. This results in a continuous displacement of the fluorescent dye (AO) from the host molecule (Figure 4, left), such that the enzymatic conversion is reported as a decrease in fluorescence (switch-off response, Figure 4, bottom). Ideally, the fluorescent dye is selected such that its affinity to the host (defined as its binding constant times its concentration)[25,32] lies in between that of the substrate and the corresponding product.

Product-selective tandem assays closely resemble fluorescence-based antibody−antigen assays, where a fluorescently tagged antigen is displaced from the antibody by the enzymatic product (the unlabeled antigen) during the course of enzyme reaction. With tandem enzyme assays, however, macrocyclic receptors serve as an economic and less selective substitute for antibodies. For example, using one macrocyclic host, CB7, the

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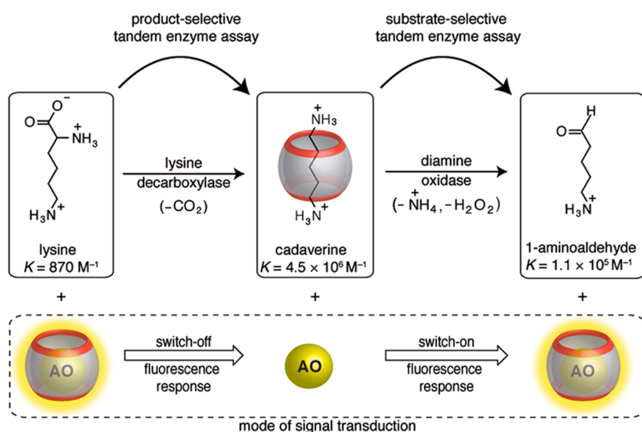

Figure 4. Schematic representation of supramolecular tandem enzyme assays showing the product-selective variant (left), the substrate-selective one (right), and their combination to set up a domino tandem assay. The chemosensing ensemble comprises the macrocyclic host CB7 and the fluorescent dye AO ($K_{CB7·AO} = 2.9 \times 10^5$ M^{-1}).[32]

enzymatic activity of several amino acid decarboxylases can be monitored,[24,31] while an antibody would be specific for a single biogenic amine, if it could be raised for such simple, omnipresent metabolites at all.

Substrate-Selective Supramolecular Tandem Assays

When the host binds more strongly to the substrate rather than to the enzymatic product, a substrate-selective assay can be set up in which the depletion of substrate in the course of the enzymatic transformation is being directly followed through an uptake of fluorescent dye.[32] A substrate-selective enzyme assay can be set up, for example, with cadaverine (strong competitor) as substrate undergoing oxidation (Figure 4, right). In the presence of the preassembled host·dye complex (CB7·AO), cadaverine displaces the dye from the host. Addition of the enzyme diamine oxidase leads to the formation of an amino aldehyde as a weaker competitor. This results in a continuous increase in fluorescence because the strong competitor is diminished, allowing the fluorescent dye to be immersed in the macrocycle. Given that the macrocyclic receptor binds to the substrate, thereby lowering its effective concentration, we needed to consider an apparent inhibitory effect of the receptor on the enzymatic activity.[40,41] However, since the absolute amount of receptor can be tuned down (low μM concentration) compared to the concentration of substrate (mM), the rate of enzymatic conversion was not significantly inhibited by the presence of the reporter pair. As a limitation, while enzyme kinetic parameters (K_M, k_{cat}) can be obtained from product-selective tandem assays, this can be more difficult for the substrate-selective variants due to a lag phase observed at higher substrate concentrations.[25,32] Only in special cases, for example, the tandem enzyme assay for butyrylcholinesterase (BuChE) recently reported by Liu et al. kinetic parameters have been determined.[38] In any case, both methods, substrate- and product-selective tandem assays, are well suited for screening of inhibitors or activators where the concentration of substrates and enzymes can be preoptimized.[25,32]

Domino Tandem Assays

By consecutively combining the product- and substrate-selective tandem enzyme assays to follow a cascade of enzymatic transformations, in the same reaction mixture with a single chemosensing ensemble, a new line of tandem assays was developed, the domino ones (Figure 4).[25,28,32] The only requirement for the operation of a domino tandem assay is an alternating binding affinity of the macrocyclic host with the sequentially formed metabolites. The simplest example is the production and degradation of biogenic amines by using the CB7·AO reporter pair.[32] First, the conversion of lysine (weak competitor) to cadaverine (strong competitor) by lysine decarboxylase was monitored as a fluorescence decrease due to the displacement of AO. If an oxidation of cadaverine is subsequently affected by addition of diamine oxidase to form aminoaldehyde (weak competitor), an increase in fluorescence signal is observed, owing to the recomplexation of CB7 and AO (Figure 4, bottom).

CASE STUDIES

Macrocyclic Host·Fluorescent Dye Sensing Ensembles for Anions

The tandem assays described above are limited to cationic analytes as strong competitors. In order to detect changes in the concentration of anions, macrocycles with anion-receptor properties (Cyc or CD, Figure 2a) need to be employed.[27] A class of analytes that appealed to us was that of nucleotides, particularly adenosine triphosphate (ATP), one of the most common cofactors of enzymatic reactions. Two complementary reporter pairs (in terms of photophysical response), Cyc·HPTS (switch-on)[42] and CD·2,6-ANS (switch-off), were selected for monitoring the activity of potato apyrase, an enzyme which hydrolyzes nucleotide triphosphates to monophosphates. Our selected receptors show preferential binding to ATP over AMP. As a result, the enzymatic dephosphorylation was monitored as a decrease (CD·2,6-ANS) or as an increase in fluorescence (Cyc·HPTS).[27] Screening for activators and assaying dephosphorylation of other nucleotide triphosphates (GTP, CTP, and TTP) further extended the utility and transferability of these substrate-coupled, anion receptor-based tandem assays.

The Quest for Acetylcholine and Choline Sensing Systems

When neither the charge nor the size of an analyte undergoes a sufficiently large change to result in a differential binding of substrate and product to the synthetic receptors, tandem assays reach their limit. The conversion of acetylcholine (ACh) to choline (Ch) is such an example, and, in fact, the quest for supramolecular receptors that can differentiate ACh from Ch has presented a seminal challenge in supramolecular chemistry, owing to its importance for neuroscience.[12,30,43−45] For example, *p*-sulfonatocalixarenes are excellent receptors for ACh and Ch, but unfortunately with virtually identical affinities.[43] In order to make up for this low selectivity of CX4, in particular, we combined, in collaboration with the group of Liu, the enzymatic activity of two enzymes, acetylcholinesterase and choline oxidase, in order to detect and quantify both. This resulted in the setup of an enzyme-coupled tandem assay (Figure 5).[30]

The transformation of acetylcholine to choline by acetylcholinesterase could not be monitored because the receptor CX4 was unable to differentiate between the substrate and the product (Figure 5, left). The underlying idea was that—although ACh and Ch could not be differentiated on account of their identical positive charge and the same NMe_3^+ recognition motif, the oxidation product of Ch, betaine, could be detected, because it is zwitterionic, and therefore much more weakly bound (Figure 5, right). LCG (Figure 2b) was found to be an excellent indicator dye for CX4 (fluorescence enhancement

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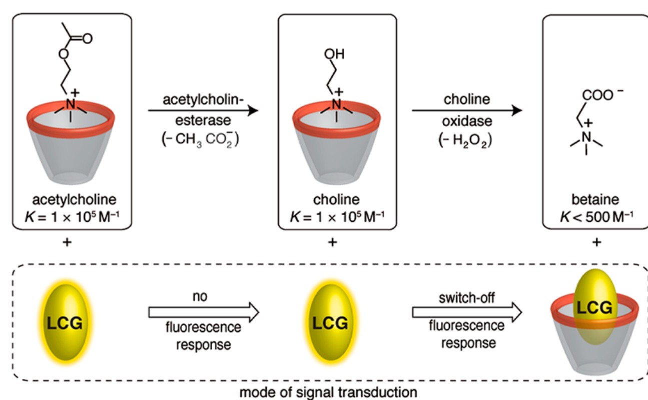

Figure 5. Substrate-selective enzyme-coupled assay for the detection and quantification of acetylcholine and choline by using the supramolecular chemosensing ensemble CX4·LCG ($K_{CX4·LCG}$ = 1.6 × 10^7 M^{-1}). Adapted from ref 30 with permission from The Royal Society of Chemistry.

factor up to 140; this reporter pair has also been successfully used by Hof et al.).[22] Thus, when the reaction was conducted in the presence of an excess of choline oxidase and a rate-determining concentration of acetylcholinesterase, the CX4·LCG reporter pair afforded a decrease in fluorescence signal. This resulted from the formation of betaine as weak competitor, which allowed the dye to be taken up by the macrocycle in the course of the reaction. Through adaptations in the assay setup, it became possible to determine in sequence the absolute concentrations of both, ACh and Ch, in the same reaction mixture with micromolar sensitivity.[30]

From Low Molecular-Weight Metabolites to Biomacromolecules as Substrates

The first-generation of tandem enzyme assays targeted low molecular-weight analytes, whereby the entire analyte was encapsulated in the macrocyclic host. We wanted to expand the applicability of supramolecular sensing ensembles to detect changes in concentration of large biomolecules such as peptides. For this to be achieved, the assay would need to become compatible with the recognition of specific groups or residues rather than the recognition of an entire analyte. Based on earlier reports on the differential binding affinity of CB7 toward the aromatic amino acid Phe residues carrying different neighboring charges,[24,46−48] we hypothesized that CB7·dye systems could be employed to rapidly and conveniently measure many transformations that produce or destroy *N*-terminal aromatic residues. In these second-generation tandem enzyme assays, the aromatic residues of peptides function as recognition motifs. This led to the development of economic and versatile label-free fluorescence-based assays for proteases, which remain prime targets in drug discovery. Indeed, we were able to follow—in collaboration with the group of Urbach and CB7·AO as a fluorescent reporter pair—the enzymatic activity of thermolysin.[29] This metallo-endopeptidase hydrolyzes the *N*-terminal amide bond of hydrophobic amino acids and we tested the tandem enzyme assay principle for enkephalin-based peptides as substrates.

Assays for exopeptidases are particularly scarce,[28] because they evade detection by established assays involving fluorescently labeled peptides.[49] Leucine amino peptidase (LAP) is one of them; it cleaves amino acids in peptides from the *N*-terminus.[28] As a variation of the domino tandem assay concept,[32] we used the Phe-selective CB7·AO complex to monitor the stepwise proteolytic degradation of an extended peptide by LAP. The assay principle is illustrated in Figure 6.

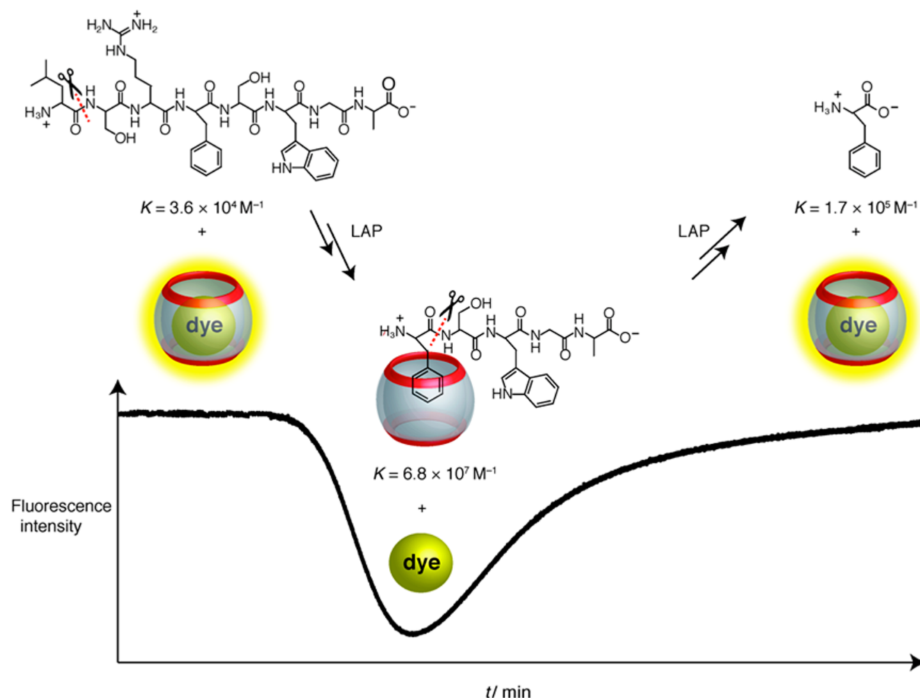

Figure 6. Stepwise proteolytic degradation of an entire peptide monitored via a domino tandem assay.[28] The initial degradation of the substrate to the intermediary peptide with an *N*-terminal Phe residue is indicated by a fluorescence switch-off response. Further hydrolysis leads to the formation of Phe, which is reflected by a fluorescence increase.

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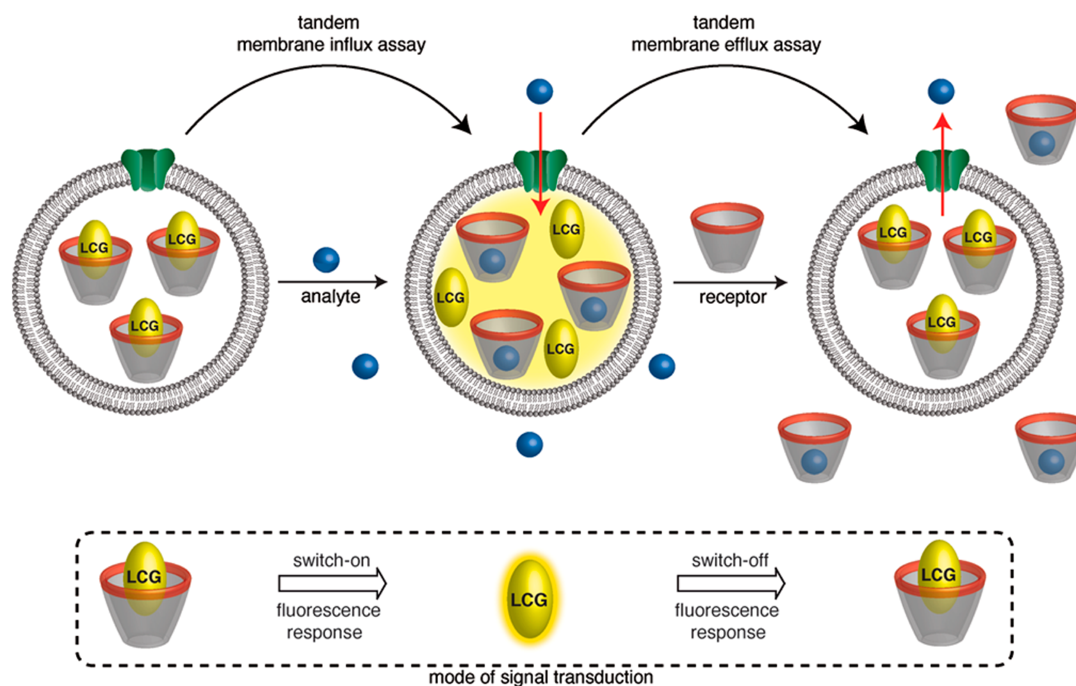

Figure 7. Supramolecular tandem membrane assays to follow the influx (left)[33] and efflux (right) of an analyte (blue) through a channel protein (green). Analytes that enter into the proteoliposomes displace the dye LCG from CX4 which is signaled by an increase in fluorescence.[33] In contrast, the efflux of analytes from proteoliposomes results in a fluorescent dye uptake and associated fluorescence intensity quenching.

Initially, the dye is included inside the CB7 cavity (left). As LAP hydrolyzes the peptide, it cleaves off step-by-step *N*-terminal amino acids. When the enzyme reaches Phe, it exposes the *N*-terminal Phe residue that acts as a strong competitor due to the synergy of hydrophobic and ion-dipole interactions with CB7 (middle). Eventually, LAP continues to hydrolyze the peptide chain, thereby releasing free Phe, which is a weak competitor due to the presence of the negatively charged carboxyl group (right).[24] As a result, the degradation of an entire peptide can be monitored as a down-and-up change in fluorescence.[30] This domino variant includes again a combination of a product- and substrate-selective assay and is subject to the same advantages and limitations (see above).[40]

While CB7 can be used to recognize aromatic amino acids in peptides, CX4 can be used to recognize trimethylated lysine residues in peptides and to differentiate them from unmethylated lysine residues. There are other marcocycles which have similarly selective amino acid recognition features, for example, for arginines.[50] The CX4·LCG system, already used for enzymatic reactions of neurotransmistters (see above), allowed indeed the monitoring of the enzymatic activity of DIM5 from *Neurospora crassa*.[26] This histone lysine methyltransferase specifically trimethylates one lysine residue (Lys9) of the histone H3 tail peptide. The product peptide acts as a strong competitor because the trimethylated lysine residue, in contrast to the unmethylated one in the substrate peptide, has a better size fit with CX4 and is additionally stabilized by CH−π interactions in the host−guest complex.[43] Accordingly, the methylation reaction was followed by the corresponding product-selective tandem assay. A continuous fluorescence increase was observed (switch-on),[26] because in this case the fluorescence of the dye is quenched by the electron-rich macrocycle.

Supramolecular Tandem Membrane Assays

If host·dye reporter pairs can be used to continuously follow the changes in concentration of either a substrate or a product (a chemical reaction), it should also be feasible to monitor the time-resolved change of the concentration of an analyte in general, that is, without a chemical reaction happening. An example is the buildup of an analyte inside compartmentalized structures such as vesicles. This conceptually novel approach resulted in the development of a versatile supramolecular method to monitor biomembrane transport processes: tandem *membrane* assays.[33] Our motivation for exploiting supramolecular chemosensing ensembles for investigating membrane transport stemmed from the fact that fluorescence-based methods to study transport of bioorganic analytes through the lipid bilayer or channel proteins in a label-free fashion in real-time were nonexistent.[51]

The working principle of tandem membrane assays relies on the selective coencapsulation of a membrane impermeable reporter pair (such as CX4·LCG or CB7·BE) inside vesicles and the reversible interaction of the receptor with the translocated analyte (Figure 7). When an analyte that translocates into the liposomes through a channel protein is added to a solution of host·dye-loaded proteoliposomes (channel-protein reconstituted liposomes), a time-resolved change in fluorescence is expected due to the displacement of the dye from the host cavity, whereas addition of a nontranslocating analyte is not expected to affect the signal.

Influx and Efflux Tandem Membrane Assays

To prove the principle of tandem membrane assays, we performed an exploratory investigation on the diffusion of an arginine-rich antimicrobial peptide, protamine, through the bacterial channel outer membrane protein F (OmpF). In the actual experiment, liposomes loaded with CX4·LCG were first prepared, followed by the addition of OmpF to form proteoliposomes (Figure 7, left).[33] When protamine was

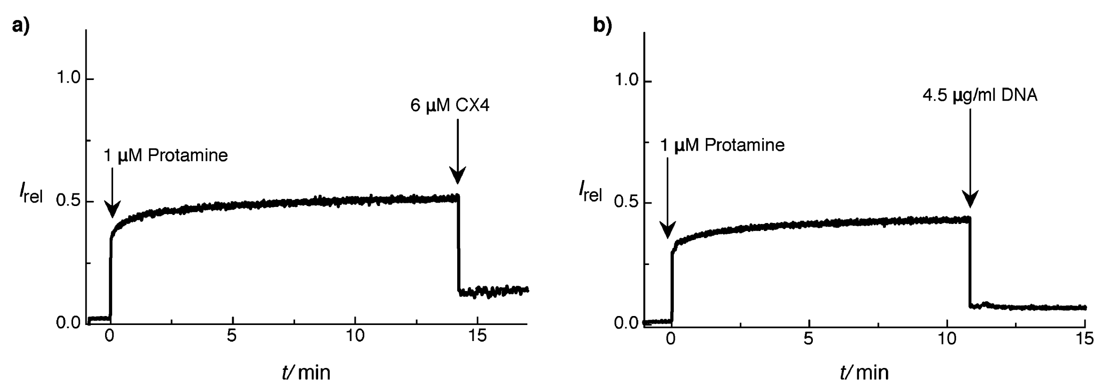

Figure 8. Change in fluorescence of CX4·LCG-loaded liposomes containing 30 nM OmpF upon addition of 1 μM protamine (t = 0 min) and, subsequently, (a) 6 μM CX4, at t = 14 min or (b) 4.5 μg/mL DNA, at t = 11 min. Experiments were performed in 10 mM sodium phosphate buffer, at pH 7.0 and 25 °C in this work.

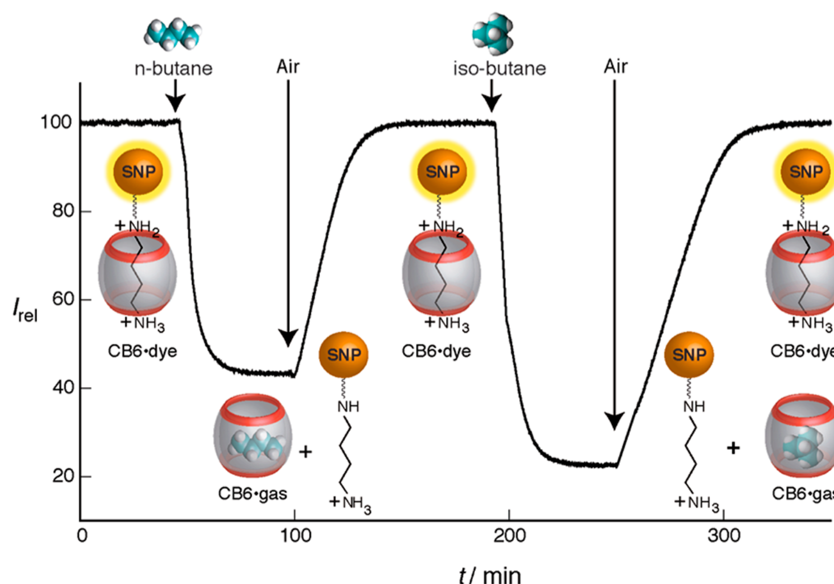

Figure 9. Encapsulation of volatile hydrocarbons (butane and isobutane) by CB6 continuously monitored via fluorescence of the dye SNP. Modified from ref 34 with permission from John Wiley and Sons.

added to the CX4·LCG-loaded proteoliposome solution (Figure 8, t = 0 min), a steep increase in fluorescence was observed. This indicated the displacement of LCG from the CX4 cavity by protamine; this peptide binds strongly to CX4 due to electrostatic and cation−π interactions with the multiple arginine residues.

The translocation of protamine into the liposomes through the channel protein is driven by a concentration gradient. In principle, the process should be reversible; that is, upon inversion of the concentration gradient protamine should translocate out from the vesicles. In order to test this unpublished hypothesis, we added, after protamine had been allowed to diffuse into the liposomes through OmpF, either an excess of CX4 (Figure 8a) or DNA (Figure 8b), as synthetic and natural protamine binders, respectively. In both cases, a steep drop in fluorescence was observed, signaling an effective and fast efflux of protamine from the liposomes. This is due to the fact that both CX4 and DNA bind strongly to protamine, thereby lowering the effective concentration of free protamine in the extravesicular space. In other words, it is possible to first observe the translocation of analytes into the liposomes, and, subsequently, to invert the concentration gradient through addition of a competitive binder to the aqueous bulk. This influx−efflux experiment also unambiguously demonstrated that the reporter pair inside the liposomes had remained intact; that is, it rules out the unlikely possibility that protamine first enters the liposome and subsequently facilitates the efflux of either host or dye, in which case no reversibility can be expected.

Tandem membrane assays constitute not only a label-free method to investigate a bidirectional transport of biomolecules through the lipid bilayer or through membrane proteins, but they have manifold additional functionalities in membrane research. For example, they can also be utilized to screen the activity of channel proteins or channel modulators.[33]

Hydrocarbon Sensing

Other areas, in which the time-resolved detection of analytes with macrocyclic host−dye reporter pairs is of interest, are the monitoring of analytes outside of any biological context, for example, for environmental monitoring. A simple example is the use of macrocyclic host·dye complexes for the monitoring of hydrocarbon gases. Toward this end, we used the anchor dye SNP (Figure 2b) in combination with the macrocyclic host CB6.[34]

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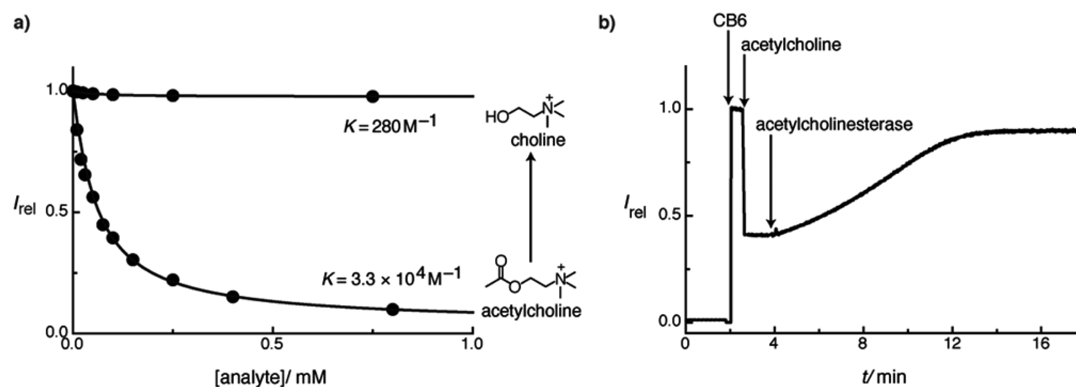

Figure 10. (a) Competitive displacement fluorescence titrations of acetylcholine and choline using 5 μM DSMI and 15 μM CB6. (b) Time-resolved change in fluorescence upon sequential addition of CB6 (10 μM) and then acetylcholine (100 μM) to a solution of 5 μM DSMI. For the substrate-selective tandem assay, 10 μL of 1 mg/mL of acetylcholinesterase was added and the progress of the enzymatic transformation was monitored as an increase in fluorescence. The experiments were carried out in 10 mM sodium phosphate buffer, at pH 7.0 and 25 °C in this work.

When gaseous analytes such as butane or isobutane are bubbled through a solution containing the preassembled, highly fluorescent CB6·SNP reporter pair, a continuous displacement of the dye with an associated decrease in fluorescence is observed (Figure 9). While the decrease in fluorescence reported on the absolute concentration of the dissolved gas, the observation of the plateau region indicated that the water solubility of the hydrophobic gas had been reached. By comparing the plateau regions for butane and isobutane, one could easily infer the difference in binding affinities of CB6 toward these isomeric guests; the latter is more spherical and shows a better size-shape fitting with the host cavity, resulting in a 3 times higher affinity (ca. 10^6 M^{-1}). The most interesting observation, perhaps, was the rapid reversibility of gas encapsulation, which is an essential requirement for potential gas monitoring applications. Thus, by simply purging the solution with air, the volatile analyte was displaced from the CB6 cavity, reflected by the restoration of the initial fluorescence.[34] The method can also be used to determine the affinity of the gases under different conditions, for example, in the presence of salts, where the competitive binding of cations to the CB6 portals lowers the affinity of the encapsulated gases.

Reporter Pairs under Investigation

Inspired by earlier reports on the selective binding of acetylcholine over choline by water-soluble *derivatives* of CB6,[52] we reckoned that the high sensitivity of tandem assays could be exploited to even employ *parent* CB6, a notoriously poorly soluble host in neat water, for time-resolved acetylcholine sensing by using DSMI as an indicator ($K_{CB6\cdot DSMI}$ = 4.2 × 10^4 M^{-1}).[53] DSMI is weakly fluorescent in its uncomplexed, but strongly fluorescent in its CB6-complexed form.

As demonstrated by simple fluorescence titration experiments (Figure 10a), the binding constant of CB6 with acetylcholine is 100 times higher than that with choline. Although the affinity of acetylcholine to CB6 is smaller than that to CX4, the ability of CB6 to differentiate between Ch and ACh can easily be exploited to now directly tandem-assay the enzymatic activity of acetylcholinesterase (Figure 10b), instead of taking the enzyme-coupled route (Figure 5). The direct, CB6-based assay is, however, slightly less sensitive (100 versus 10 μM).[30]

■ CONCLUSIONS

Supramolecular tandem assays exploit dynamically analyte-responsive macrocyclic host−fluorophore systems to achieve a time-resolved monitoring of enzymatic reactions and membrane transport processes. Originally introduced to monitor the formation of biogenic amines by enzymatic decarboxylation of amino acids,[31] they have subsequently been implemented into many more enzymatic transformations and have proven useful for all enzyme types, except ligases (EC6). For those isomerases (EC5) that affect an interconversion of enantiomers or their racemization, we were also not yet able to apply the tandem assay approach, mainly due to the lack of suitable stereo-discriminating macrocycles.[54] The application of tandem assays in membrane transport, translocation, and permeation processes is now beginning to unfold.

The exploitation of the specificity of enzymatic reactions in combination with signaling events resulting from supramolecular analyte recognition is presently also receiving attention in other fields, for multiparameter sensing,[24] for enantiomeric excess determination,[24] in two-component array systems to report simultaneously on the concentrations and identities of enzyme modifications,[22] in chirality sensing and chirogenesis,[55] for dye release from mesoporous hybrid systems,[56] and in allosteric dye release assays.[57,58] The expansion of the library of suitable host·dye combinations to address diverse analytes, to increase the sensitivity, to achieve either a switch-on or switch-off fluorescence response, or to ensure compatibility with lipids presents an ongoing challenge to supramolecular design as well as to combinatorial testing.

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Notes

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