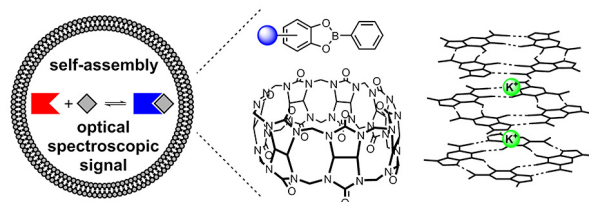


Dynamically Self-Assembled Supramolecular Probes in Liposomes

Andreas Hennig*^a 

^a Center for Cellular Nanoanalytics (CellNanOs) and Department of Biology and Chemistry, Universität Osnabrück, Barbarastrasse 7, 49069 Osnabrück, Germany.


* andreas.hennig@uni-osnabrueck.de



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Abstract Liposomes are artificial vesicles, in which an aqueous inner compartment is separated from its environment by a phospholipid membrane. They have been extensively studied as cell membrane models and offer the possibility to confine molecules and chemical reactions to a small sub-micrometer-sized volume. This short review provides an overview of liposome-encapsulated, dynamically self-assembled, supramolecular structures, in which the assembly and disassembly of the supramolecular structures can be followed by optical spectroscopic methods. This includes self-quenched fluorescent dyes and dye/quencher pairs, helical stacks of guanosine nucleotides, dynamic covalent boronate esters, and supramolecular host-guest complexes. The resulting liposomes are typically used to study membrane transport processes, but the results summarized herein also serve as a potential blueprint for studying dynamic self-assembly in confined spaces by optical spectroscopic methods.

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Key words: self-assembly, host-guest chemistry, liposomes, fluorescence, circular dichroism

1 Introduction

Vesicles play important roles in cells, in which they mediate cellular uptake, secretion of metabolites, or materials trafficking.¹ From a functional point of view, vesicles serve as food and waste containers for the cell, and thus present a ba-

sic level of cellular organization, which enables the cell to spatially confine molecules and reactions at the subcellular level. Liposomes are artificial vesicles, in which an aqueous inner compartment (the vesicle lumen) is enclosed by a spherical bilayer of phospholipids, which spatially separates the interior from its environment. Liposomes have been extensively investigated as drug delivery systems and can be prepared in such a way that there is only one bilayer per vesicle, which is referred to as an unilamellar vesicle.²

Because of their resemblance to an empty cell, liposomes are an extensively studied model system for the cell membrane. They can be prepared in different sizes, among which large unilamellar vesicles (LUVs) with diameters between 100 nm and 1 μm are most popular because they are easy to prepare and very stable, whereas giant unilamellar vesicles (GUVs), which are larger than 1 μm , offer the unique advantage to be directly observable by optical microscopy. The latter enables a straightforward observation of individual events on the single-vesicle level, whereas experiments with smaller vesicles require specialized techniques to analyze their behavior beyond the average of the whole vesicle population.³

Liposomes have also served as simple cell membrane models for supramolecular chemists; in particular, supramolecular systems to transport energy and mass across the lipid bilayer membrane have been studied extensively and remain an active area of research.⁴ More recently, molecular recognition events in the confined environment of the lipid bilayer membrane or on the membrane surface have received increasing attention, which afforded, for example, membrane-based sensors, stimuli-responsive membrane transporters, as well as artificial transmembrane signaling, vesicle adhesion, and fusion systems.⁵

Less attention from supramolecular chemists has so far been devoted to the possibility to confine dynamic self-assembly processes, molecular recognition events, or chemical reactions to the aqueous interior of phospholipid liposomes, the so-called vesicle lumen. Nature uses the volume-confined interior of vesicles for enzymatic reaction cascades,

Biosketch



Andreas Hennig obtained his Diploma (Dipl.-Chem.) from TU Braunschweig (2004) and a Ph.D. (in Chemistry) from Jacobs University Bremen (2007). Subsequently, he had positions at the Université de Genève (2007–2009), the BAM Federal Institute for Materials Research and Testing in Berlin (2009–2014), and Jacobs University Bremen (2014–2020). Since 2020, he is Professor of Organic and Supramolecular Chemistry at the Universität Osnabrück. His research group has a keen interest in the design and synthesis of supramolecular functional systems, their physicochemical characterization by optical spectroscopic methods, and their application, e.g. as sensors and membrane transporters.

and efforts to investigate confinement effects on enzymes have revealed surprising, not yet fully understood, effects; for example, an increased “superactivity” of liposome-encapsulated enzymes such as horseradish peroxidase or chymotrypsin has been noted.⁶ Other self-assembly processes that have been investigated in the vesicle lumen of liposomes aimed towards the creation of artificial cytoskeletons and include actin polymerization,⁷ DNA hybridization,⁸ and nanofiber formation.⁹ Moreover, intravesicular formation of transition metal complexes has been investigated with an interest to enhance drug loading into liposomes.¹⁰

This short review focuses on dynamically self-assembled, supramolecular structures entrapped in the vesicle lumen of phospholipid liposomes, in which the assembly and disassembly of the supramolecular structures can be followed by optical spectroscopic methods. It covers self-quenched fluorescent dyes and dye/quencher pairs, which are commonly encapsulated at high millimolar concentrations, self-assembled helical stacks of guanosine nucleotides, which can be detected by circular dichroism (CD) spectroscopy, boronate esters of pyrocatechol violet (PV), which afford a colorimetric response upon dynamic covalent bond dissociation, and self-assembled supramolecular complexes composed of macrocyclic host molecules and fluorescent dyes. These types of liposomes with internally entrapped optical spectroscopic probes are typically used to study membrane transport processes,¹¹ but the results summarized herein also serve as a potential blueprint for studying dynamic self-assembly in confined spaces by optical spectroscopic methods.

2 Probes Based on Fluorescence Quenching

A comparably simple form of a self-assembled, supramolecular structure is a dimerized dye. The formation of dimers, trimers, and higher aggregates at high concentrations is a common phenomenon for fluorescent dyes, and dye aggregates can be stably encapsulated inside liposomes, when the dyes are membrane-impermeable. Encapsulation is achieved by rehydration of a lipid film with a solution con-

taining the fluorescent dyes at sufficiently high concentrations, such that the liposomes are formed surrounding the aggregated dyes. The external solution can be subsequently exchanged by size exclusion chromatography, which gives liposomes, in which the dye aggregates are encapsulated within the intravesicular aqueous compartment of the liposomes, whereas the external solution contains no dyes.

Liposomes with encapsulated aggregates of fluorescent dyes are very useful in dye efflux assays, which are among the most popular methods to investigate membrane transport processes (Figure 1). The assays are typically set up with dyes at high, self-quenching concentrations or with fluoro-

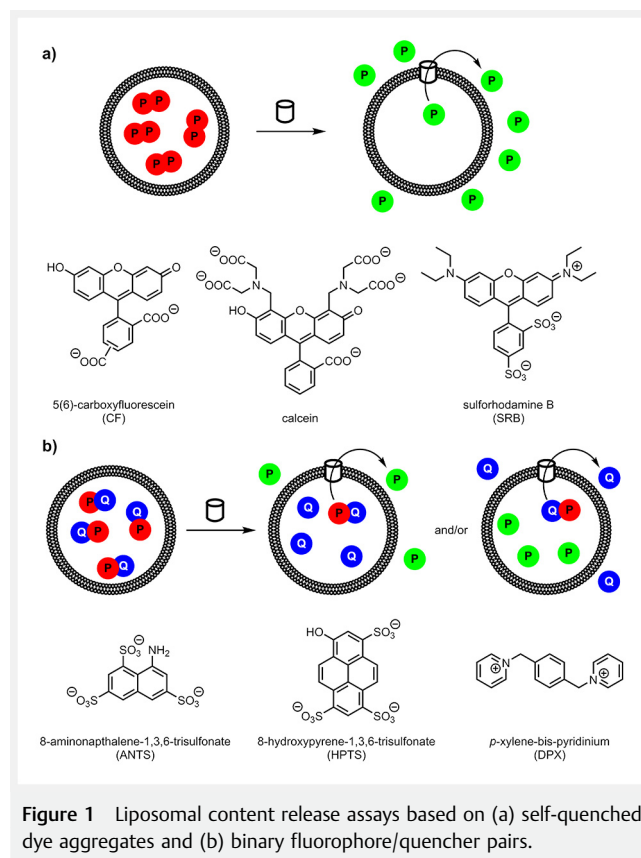


Figure 1 Liposomal content release assays based on (a) self-quenched dye aggregates and (b) binary fluorophore/quencher pairs.

phore/quencher complexes inside the liposomes and a chemical potential in the form of a concentration gradient of the dyes across the lipid bilayer membrane. The membrane activity of an added compound creates a facilitated transport pathway for dissipation of the concentration gradient and escape of the dye and/or the quencher from the liposome. This leads to a dilution of the dye into the bulk solution, where concentrations are too low for dye aggregation such that fluorescence quenching does no longer apply. The membrane activity of the compound is then commonly quantified as the fractional increase in fluorescence intensity after a certain time normalized to complete lysis of the vesicles after addition of a detergent.

The most frequently used self-quenching probes (Figure 1a) in dye efflux assays are carboxyfluorescein (CF),¹² calcein,¹³ and sulforhodamine B (SRB).¹⁴ Intravesicular dye concentrations are typically high (ca. 25–150 mM) and self-quenching of these fluorophores involves the formation of various non-fluorescent aggregates (dimers, trimers, and higher) as well as energy transfer by homo-FRET (fluorescence resonance energy transfer) to these aggregates.¹⁵ Limitations of these probes arise, for example, from the pH-dependent fluorescence properties of CF near physiological pH and the sensitivity of calcein towards transition metal cations.^{14b,16} Noteworthy is also the potential lack of a fluorescence response, which may originate, for example, from an unexpected cation selectivity of the membrane-active compound, such that negatively charged dyes cannot escape the vesicle.

An alternative is the binary ANTS/DPX assay (Figure 1b), which uses the fluorophore/quencher pair 8-aminonaphthalene-1,3,6-trisulfonate (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX).^{12c,17} ANTS is relatively pH-insensitive and its fluorescence is significantly reduced by static quenching in the form of ANTS/DPX complexes.^{17c} A fluorescence increase in the ANTS/DPX assay results from efflux of either the negatively charged dye or the positively charged quencher, such that both, cation-selective as well as anion-selective transport pathways, afford a fluorescence response. Instead of the pH-insensitive ANTS, the pH-sensitive 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) is also efficiently quenched by DPX, which is useful for parallel monitoring of pH changes and dye or quencher efflux.^{11,18}

The simplicity of dye efflux assays is certainly unmet and these types of assays have become very popular to assess the potency of membrane-active compounds. They have been frequently applied with supramolecular membrane transport systems and they are a very well established biophysical research method for the mechanistic investigation of membrane-permeabilizing antimicrobial peptides and protein pores.^{11,19} The assays are commonly straightforward to implement and provided valuable mechanistic insights into how membranes can be destabilized in such a way that the small dye and/or quencher molecules can escape from the

vesicle interior. Efflux assays have been also adapted in many ways. For example, replacing CF with fluorescein-labeled dextrans of different molecular weight allows assessing the size dependence of the permeabilization pathway.²⁰ More sophisticated refinements, such as re-quenching assays by addition of external quenchers, fluorescence lifetime-based measurements, and liposome content release assays involving biomolecular recognition schemes, can provide more detailed mechanistic insights into the still very puzzling mechanisms of membrane permeabilization by membrane-active cell-penetrating and antimicrobial peptides.^{17c,21}

Self-quenched probes:

$$K_d = \frac{[P]^2}{[P \cdot P]}$$

Fluorophore/quencher pairs:

$$K_d = \frac{[P][Q]}{[P \cdot Q]}$$

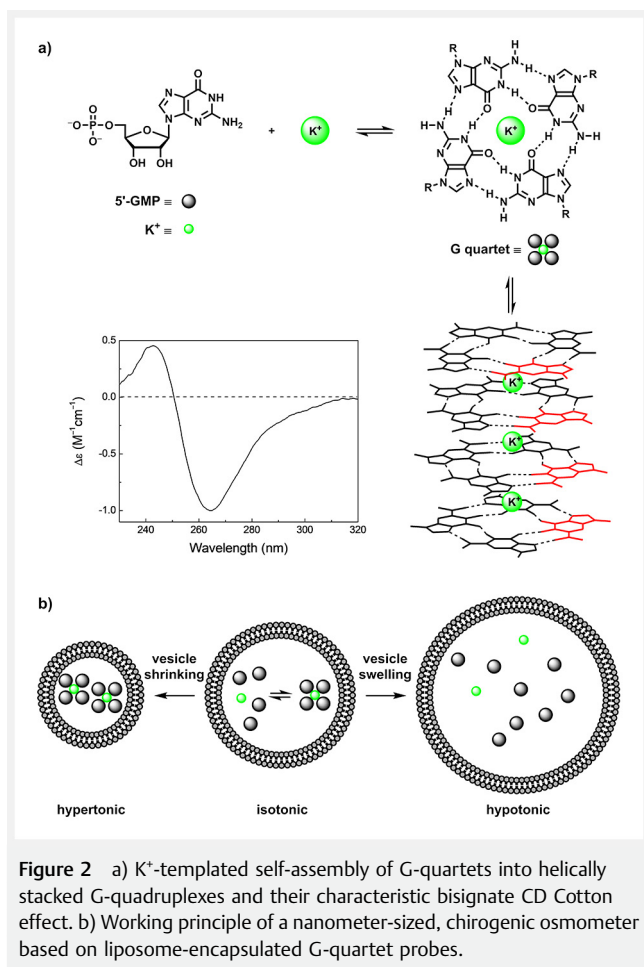
inside: $[P]_{\text{tot}}, [Q]_{\text{tot}} \gg K_d$; outside: $[P]_{\text{tot}}, [Q]_{\text{tot}} \ll K_d$

Equation 1 Equilibrium dissociation constants for probe dimerization and probe/quencher complex formation as well as the desirable prerequisite conditions for the respective content release assays.

The dye efflux and other content release assays rely on the concentration change that is accompanied by the escape from the liposomes into the surrounding bulk solution. The concentrations are typically set up, such that the intravesicular concentrations of the self-assembled species are much higher than the respective equilibrium dissociation constant, K_d , and that the extravesicular concentrations after efflux are much lower than K_d (Eq. 1). In both cases, efflux from the small volume of the vesicle lumen as an intravesicular aqueous compartment into the large volume of the surrounding solution shifts the position of the thermodynamic equilibrium from associated and non-fluorescent towards dissociated and fluorescent. It is, however, noteworthy that the extraction of kinetic rate constants of content release from the time-dependent fluorescence traces may become very involved; accurate physicochemical models are not only complicated by the involvement of different quenching mechanisms, but also require taking different types of release mechanisms into account.^{17c}

3 Chirogenic G-Quartet Probes

An example of more complex structures that can be self-assembled inside liposomes are G-quadruplexes (Figure 2).^{22,23} Alkali metal salts of the 5'-guanosine monophosphate dianion (5'-GMP) form regular ordered structures at high concentrations in aqueous solution, in particular with K^+ .²⁴ Therein, four 5'-GMP molecules form tetrameric G-quartets by Hoogsteen hydrogen bonding, which stack into extended lyotropic liquid crystals by eight-coordination of alkali metal



cations at the central binding sites between two planes of G-quartets. The chirality of 5'-GMP is amplified into the supramolecular assembly leading to a helical twist of the G-quartet planes along the axis of the π -stacked aromatic DNA bases. This gives rise to a characteristic bisignate Cotton effect in CD spectroscopy, which originates from exciton coupling between the DNA bases.²⁵ Since the molar ellipticity of exciton-coupled CD (ECCD) is usually much larger than the induced CD by a chiral center adjacent to the chromophore, assembly and disassembly of G-quadruplexes in liposomes can be followed by the ECCD.^{22,23}

Chirogenic G-quartet probes were initially used to investigate the response of self-assembled intravesicular structures towards osmotic stress.²² When vesicles are exposed to hypertonic conditions, they typically respond with vesicle shrinking to account for the increased concentration of external solutes, whereas hypotonic conditions lead to vesicle swelling. In response to the altered inner volume of the vesicles, vesicle shrinking under hypertonic conditions should lead to assembly of the intravesicular structure and vesicle swelling under hypotonic conditions to disassembly, but

the opposite response is also conceivable. The disassembly of an intravesicular supramolecular structure would increase the number of solutes in the vesicle lumen and thus counterbalance the osmotic stress by external hypertonic conditions, whereas supramolecular assembly would decrease the number of intravesicular solutes in response to external hypotonic conditions. In biological systems, pertinent examples for both directions exist; cells initially swell and shrink when exposed to hypotonic and hypertonic conditions, but then return to the original cell volume by generation or breakdown of organic structures.²⁶ For the chirogenic G-quartet probes, such a regulatory mechanism was not observed. The G-quadruplexes disassembled due to the increased inner volume in a hypotonic environment and assembled when exposed to hypotonic conditions.

This affords a liposome-based, nanometer-sized osmometer that operates by the dynamic disassembly and reassembly of chirogenic G-quartets in vesicles (Figure 2b). Such a supramolecular device may be useful for the investigation of artificial supramolecular water channels as well as natural water channels (aquaporins).^{4f,27} Osmotic swelling and shrinking has also been followed with dynamic light scattering and with encapsulated CF, where the degree of self-quenching is modulated by the accompanied volume changes.²⁸ However, it has been noted that the chirogenic osmometer appeared more robust towards experimental conditions.²²

G-quartet-containing liposomes were also useful in content release assays, in which efflux of K⁺ through the ion channel gramicidin A, efflux of 5'-GMP by counterion-activated polyarginine, or efflux of both through the membrane pore melittin could be followed by CD spectroscopy.²³ As an interesting variation, a vesicle influx assay for K⁺ ions was also demonstrated (Figure 3). Therefore, liposomes were prepared, which contained high intravesicular concentrations

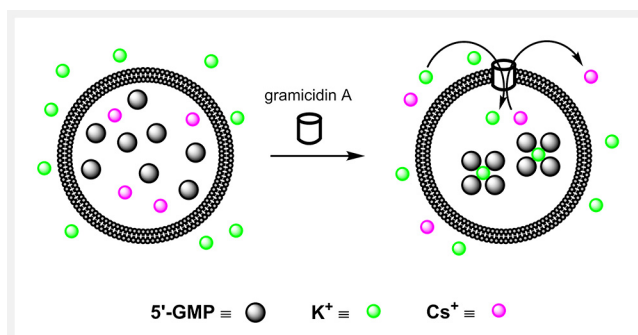


Figure 3 The dynamic self-assembly of intravesicular G-quadruplexes is initiated by addition of the ion channel gramicidin A. Before addition, intravesicular Cs⁺ cannot template G-quadruplexes, whereas Cs⁺/K⁺ antiport through gramicidin A leads to K⁺ influx. The intravesicular K⁺ concentration becomes sufficiently high to enable G-quadruplex assembly.

of 5'-GMP and Cs⁺ ions. The latter are much less effective in templating stacked G-quartets, such that the resulting liposomes were CD-silent since no G-quadruplex structures are formed inside the liposomes. After external buffer exchange to a buffer containing K⁺, the ion channel gramicidin A was added to the liposomes. This caused K⁺ influx and the subsequent intravesicular assembly of the G-quadruplex structures, which was easily detected by the appearance of the bisignate CD Cotton effect in these chirogenic vesicles.

4 Chromogenic Probes Using Dynamic Covalent Bonds

The reversible formation of covalent bonds presents an attractive opportunity for the creation of a large variety of self-assembled structures.²⁹ One of the prototypical reactions used in aqueous dynamic covalent chemistry is the formation of boronate esters from vicinal diols and boronic acids, which proceeds very efficiently with catechols with apparent dissociation constants in the micromolar range at physiological pH.³⁰ When dyes containing catechol groups are engaged in boronate ester formation, a change in color or fluorescence results, which has been exploited in numerous sensing applications.³¹ Boronate esters were also used to transport monosaccharides across lipid membranes,³² to trigger content release from liposomes with surface-exposed boronic acid lipids with carbohydrates,³³ and they have been explored as self-assembled probes in the aqueous inner compartment of liposomes (Figure 4).³⁴

PV forms boronate esters with phenylboronic acids with an apparent dissociation constant of ca. 300 μM around neu-

tral pH leading to a visible color change. This allowed monitoring membrane pore formation by the naked eye when PV efflux from liposomes with encapsulated PV and influx from externally added 4-carboxyphenylboronic acid (CBA) lead to the formation of a PV/CBA boronate ester (Figure 4a). Unfortunately, CBA was found to be also slightly membrane-permeable in the absence of a membrane pore, which prevented the stable encapsulation of PV/CBA esters in liposomes. Moreover, the high extravascular concentrations of CBA also interfered with counterion-activated transport of polyarginine. As a remedy, the more hydrophilic 4-(benzyl-N-glutamate)boronic acid (BGBA) did not cross the lipid membrane such that stable PV/BGBA liposomes could be prepared (Figure 4b). After vesicle export by counterion-activated polyarginine, the PV/BGBA boronate ester was sufficiently diluted to dissociate into PV and the boronic acid.

5 Self-Assembled Host–Dye Reporter Pairs

Supramolecular host–dye reporter pairs are self-assembled complexes of fluorescent dyes with macrocyclic host molecules, e.g. cyclodextrins, calixarenes, and cucurbiturils. The spectroscopic properties of the complexes are typically different from the dyes in solution, which renders them interesting for sensing applications.^{35,36} The complexes are dynamically responsive to a stimulus with typical exchange times in the microsecond to millisecond time regime and have found numerous applications, for example in enzyme assays,³⁷ as optical signal transducers in biosensors,³⁸ for DNA detection,³⁶ for mechanistic investigations of host–guest exchange,³⁹ or for the quantification of surface functional groups.⁴⁰

Host–guest complexes in the form of metal ion–chelates have been used very early to follow content mixing during vesicle fusion with Tb³⁺ and dipicolinic acid⁴¹ or to determine and image biologically relevant metal ions, e.g. Ca²⁺.⁴² However, the first use of a supramolecular complex with a fluorescent dye in biomembrane assays dates back to 2005 and involved the fluorescent dye HPTS and a membrane-impermeable, cyclen-based supramolecular host.⁴³ The host–dye pair formed an efficiently quenched complex with micromolar affinity in water, but the cyclen-based host had a limited solubility in water under physiological conditions (ca. 20–30 μM). Consequently, the host was not encapsulated inside the liposomes, but added externally. This allowed distinguishing liposome-encapsulated HPTS from HPTS in solution after endovesiculation of HPTS as well as after HPTS leakage from vesicles.

More recently, fluorescent dyes have been also encapsulated together with supramolecular hosts in the aqueous interior of liposomes, where the resulting host–dye reporter pairs can serve as intravesicular, dynamically self-assembled probes (Figure 5).⁴⁴ When an externally added analyte can

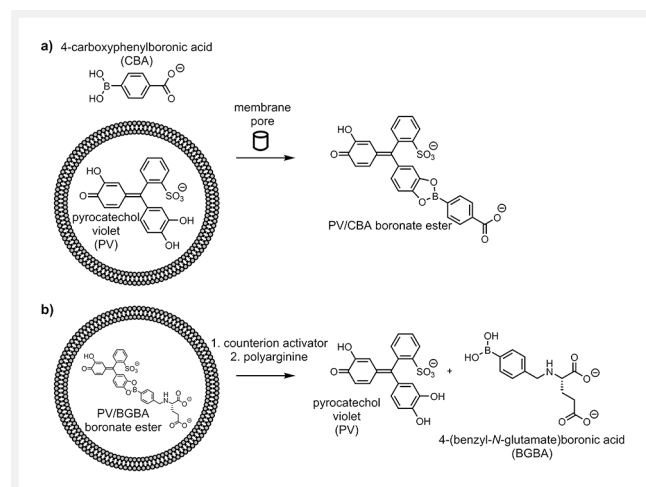


Figure 4 Boronate ester formation with pyrocatechol violet (PV) as a naked eye detection scheme for a) membrane pores and b) counterion-activated peptide transport. Concentrations of catechol and boronic acids are set up to afford either dynamic covalent bond dissociation (a) or formation (b).

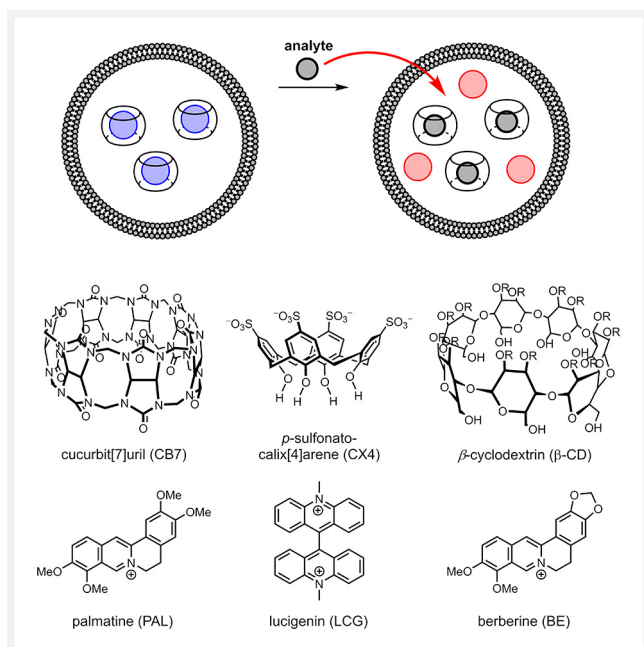


Figure 5 Dynamically self-assembled complexes of macrocyclic hosts and dyes can be encapsulated in the vesicle lumen, where they serve as spatially confined sensors for membrane translocation of analytes.

cross the phospholipid membrane, it will compete with the dye for binding to the host. This affords an intravesicular variant of an indicator displacement assay,⁴⁵ and has been used to follow analyte translocation through membrane protein pores,^{44a,44b} translocation and counterion-activated transport of cell-penetrating peptides,^{44c} membrane permeation of drugs,^{44d} and for the determination of membrane permeation activation energies of amino acid derivatives.^{44e} Dynamic self-assembly of supramolecular host–dye reporter pairs was also successfully used to follow content mixing during fusion of liposomes⁴⁶ as well as vesicle fusion in live cells.⁴⁷

Compared to established transport and fusion assays, the use of host–dye reporter pairs offers several advantages. First, host–dye reporter pairs have typically much higher binding affinities (reaching attomolar affinities in extreme cases)⁴⁸ than self-quenched dimers or fluorescent probe/quenchers pairs (e.g. K_a (HTPS/DPX) = $1.2 \times 10^3 \cdot M^{-1}$).⁴³ The latter require high intravesicular concentrations (> 10 mM), which can cause osmotic stress to the liposomes or which can detrimentally affect the properties of the liposome membrane.^{43,49} High-affinity host–dye complexes can be used at much lower concentrations (typically < 1 mM) and are thus less prone to these complications;⁵⁰ moreover, the high affinity does even enable the mechanistic observation of single to few molecule events.⁴⁶ Second, the molecular recognition properties of host–dye pairs enable the monitoring of a large variety of different organic molecules, which contrasts typical efflux assays, where monitoring is

restricted to transport of the dyes or quenchers. Particularly interesting possibilities include the direct, label-free monitoring of translocation of organic molecules through protein pores,^{44a,44b} of peptides with pore-forming or cell-penetrating properties,^{44c} or of the direct permeation of drugs and other low-molecular-weight biomolecules across the biomembrane.^{44d,44e} In addition, host–dye pairs are compatible with GUV experiments,^{44c,44d} which has been rarely explored with other types of transport assays.⁵¹

6 Conclusions and Outlook

In summary, this short review provided an overview of supramolecular structures and complexes, which have been successfully entrapped in the vesicle lumen of phospholipid liposomes in such a way that their dynamic self-assembly and disassembly can be followed by optical spectroscopic methods. The original motivation for the encapsulation of these probes in the aqueous interior of liposomes was in most cases to follow membrane transport processes indirectly through the efflux of the probes; the latter causes a disassembly of the intravesicular structures by the dilution into the surrounding environment resulting in an observable spectroscopic signal change. Transport assays with liposomes have been summarized previously,¹¹ and the preparation and characterization of LUVs⁵⁰ and GUVs^{44c,44d,51} with internal probes has been reported. The present overview shows additionally that intravesicular assembly or reorganization of dynamically self-assembled supramolecular structures is inducible by extravesicular addition of membrane-permeable molecular building blocks. Since liposomes and other types of vesicles provide unique possibilities for compartmentalization and spatial confinement of dynamic self-assembly processes, molecular recognition events, and reactions of biomolecules,^{6–9} they could also serve as nanometer-sized reaction vessels for the construction of entirely artificial supramolecular structures that could be interfaced with biosystems or could exert orthogonal functions in biological environments. The results reviewed herein could serve as a valuable guide to monitor and further investigate intravesicular self-assembly in confined spaces.

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Conflict of Interest

The author declares no conflict of interest.

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