Fluorescent Artificial Receptor-Based Membrane Assay (FARMA) for Spatiotemporally Resolved Monitoring of Biomembrane Permeability

Frank Biedermann, Garima Ghale, Ghale, Andreas Hennig, Werner M. Nau

Submitted date: 27/08/2019 • Posted date: 28/08/2019
Licence: CC BY-NC-ND 4.0
Citation information: Biedermann, Frank; Ghale, Garima Ghale; Hennig, Andreas; Nau, Werner M. (2019): Fluorescent Artificial Receptor-Based Membrane Assay (FARMA) for Spatiotemporally Resolved Monitoring of Biomembrane Permeability. ChemRxiv. Preprint.

The spatiotemporally resolved monitoring of membrane translocation, e.g., of drugs or toxins, has been a long-standing goal. Herein, we introduce the fluorescent artificial receptor-based membrane assay (FARMA), a facile, label-free method. With FARMA, the permeation of more than hundred organic compounds (drugs, toxins, pesticides, neurotransmitters, peptides, etc.) through vesicular phospholipid bilayer membranes has been monitored in real time (µs-h time scale) and with high sensitivity (nM-µM concentration), affording permeability coefficients across an exceptionally large range from $10^{-9}$ to $10^{-3}$ cm s$^{-1}$. From a fundamental point of view, FARMA constitutes a powerful tool to assess structure-permeability relationships and to test biophysical models for membrane passage. From an applied perspective, FARMA can be extended to high-throughput screening by adaption of the microplate reader format, to spatial monitoring of membrane permeation by microscopy imaging, and to the compartmentalized monitoring of enzymatic activity.

File list (2)

| Name                                | Size          | Link Information |
|-------------------------------------|---------------|------------------|
| Membrane-Manuscript-final.pdf       | (1.66 MiB)    | view on ChemRxiv | download file |
| SI-Membrane-Manuscript-final.pdf    | (1.74 MiB)    | view on ChemRxiv | download file |
Fluorescent Artificial Receptor-based Membrane Assay (FARMA) for Spatiotemporally Resolved Monitoring of Biomembrane Permeability

Frank Biedermann,1,2 Garima Ghale,2 Andreas Hennig,2 and Werner M. Nau2

The spatiotemporally resolved monitoring of membrane translocation, e.g., of drugs or toxins, has been a long-standing goal. Herein, we introduce the fluorescent artificial receptor-based membrane assay (FARMA), a facile, label-free method. With FARMA, the permeation of more than hundred organic compounds (drugs, toxins, pesticides, neurotransmitters, peptides, etc.) through vesicular phospholipid bilayer membranes has been monitored in real time (µs-h time scale) and with high sensitivity (nM-µM concentration), affording permeability coefficients across an exceptionally large range from $10^{-9}$-$10^{-3}$ cm s$^{-1}$.

From a fundamental point of view, FARMA constitutes a powerful tool to assess structure-permeability relationships and to test biophysical models for membrane passage. From an applied perspective, FARMA can be extended to high-throughput screening by adaption of the microplate reader format, to spatial monitoring of membrane permeation by microscopy imaging, and to the compartmentalized monitoring of enzymatic activity.

1 Institute of Nanotechnology, Karlsruhe Institute of Technology (KIT), Hermann-von-Helmholtz Platz 1, 76344, Eggenstein-Leopoldshafen, Germany
2 Department of Life Sciences and Chemistry, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany.
Correspondence should be addressed to F.B. (frank.biedermann@kit.edu) and W.M.N. (w.nau@jacobs-university.de).
The permeability of molecules through biological membranes is a fundamental physicochemical property, e.g., it allows cells to regulate the influx/efflux of nutrients, neurotransmitters, and pharmaceutical drugs as well as of xenobiotics.\textsuperscript{1-3} Several assays for screening membrane permeability of potentially bioactive compounds have been developed,\textsuperscript{4-7} two of which have become routine in pharmaceutical-industrial and academic settings: the parallel artificial membrane (PAMPA)\textsuperscript{8-10} and the Caco-2 cell permeability assay.\textsuperscript{11} The cost-efficient PAMPA assay quantifies the passive diffusion of substances through macroscopic and flat synthetic membranes, whose composition has been optimized to model the permeability behavior of phospholipid-based biomembranes.\textsuperscript{10} The Caco-2 assay aims to identify substances that can pass through a monolayer of colon epithelial cells which is highly relevant for the gastrointestinal uptake. Obstacles are its slow turnaround time, high cost, and the potential involvement of metabolic pathways, active transporters or efflux systems.\textsuperscript{4} Importantly, both the PAMPA and Caco-2 set-ups are usually restricted to single-point measurements and the results depend strongly on extrinsic factors, such as the rate of stirring,\textsuperscript{9} which complicates access to elementary kinetic information.

The permeability of aromatic molecules is of pivotal importance because aromatic moieties are ubiquitously occurring in peptides, hormones, neurotransmitters, toxins, biocides, as well as colorants and drugs. For instance, approximately 80% of oral drugs contain at least one aromatic moiety.\textsuperscript{12} While aromatic compounds are chromophoric, and in principle detectable by spectrophotometry, about 40% of all substances fail UV/Vis detection because of low solubility (typically 50-100 μM are required)\textsuperscript{9} or insufficient absorbance in the near UV/Vis region.\textsuperscript{13} Consequently, the development of a sensitive fluorescence-based permeability assay has been a long-standing goal.\textsuperscript{14} Frequently, fluorescently labelled analytes have been used but the attachment of fluorescent tags can drastically influence the permeability characteristics.\textsuperscript{14, 15} Thus, label-free fluorescence-based permeation assays are sought for but
currently limited to purpose-selected analytes. For instance, dynamic fluorescence quenching of an encapsulated reporter dye was used for permeation monitoring of redox-active analytes at very high (millimolar) concentrations.\textsuperscript{16} We showed that an indicator displacement assay\textsuperscript{17} can be adopted to monitor membrane translocation of a label-free highly charged antimicrobial peptide and selected charged amino acid derivatives through membrane pores.\textsuperscript{18} For this purpose, an environment-responsive dye is precomplexed with a host to yield a chromophoric or emissive reporter pair. In the presence of a host-binding analyte, the dye is competitively displaced, giving rise to a quantifiable signal change. The limitations of indicator displacement assays, most notably its limited scope to strongly binding and slowly translocating analytes, are described in detail in the Supplementary Information.

Supramolecular chemists have in recent years designed or discovered several artificial hosts for biorelevant organic analytes, \textit{e.g.} calixarenes, cyclodextrins, cucurbit[\textit{n}]urils and their acyclic congeners, pillar[\textit{n}]arenes, deep cavitands, and molecular tweezers.\textsuperscript{19-24} Of particular interest for sensing applications are fluorescent artificial receptors (FAR) with a wide analyte scope, a high binding affinity in water, and a rapid analyte-binding kinetics.\textsuperscript{23} We hypothesized that through encapsulation of such FARs in liposomes, a fluorescent artificial receptor membrane assay (FARMA) can be established that will be capable of monitoring the membrane passage of a very wide range of label-free, biologically relevant analytes in real time. The schematic FARMA concept is depicted in Fig. 1a. In this study, we demonstrate its utility for self-assembled FARs composed of the macrocycle cucurbit[8]uril and a tightly bound dicationic reporter dye as the co-factor (Fig. 1b).\textsuperscript{23} This system is excellently suited for monitoring the permeation of aromatic analytes (>100 compounds tested, see Fig. 2) through a phospholipid bilayer membrane in the biologically most relevant nM-\textmu M range.
Fig. 1 | Operational principle of the fluorescent artificial receptor membrane assay (FARMA). (a) Encapsulation of membrane-impermeable fluorescent artificial receptors (FAR) into liposomes spatially separates the FARs from the subsequentially added analyte. Upon analyte permeation through the membrane into the liposome, and subsequent rapid analyte-complexation by FAR, a readily observable change in the fluorescence intensity of the FAR can be observed (typically emission quenching). (b) Expected emission-readout for a permeable vs. an impermeable analyte. (c) Structures of the synthetic host CB8 and dyes (D1-D3), from which FAR-1, FAR-2, and FAR-3 were assembled.

RESULTS

Binding of analytes in aqueous media to fluorescent artificial receptors (FARs)

Fluorescent artificial receptors (FARs) were self-assembled in aqueous buffer from the macrocycle cucurbit[8]uril (CB8) and fluorescent, dicationic dyes D1-D3, forming discrete 1:1 CB8•dye complexes (Fig. 1a).23,25 These FARs possess residual space in their cavity that serves as a binding pocket for aromatic moieties, e.g., phenyl, indoyl and naphthyl species, see Fig. 1c. Crucial for their use in FARMA, the herein utilized CB8-dye-based FARs are phospholipid membrane-impermeable and photo-stable (see Supplementary Information, e.g. Fig. 9).
The signal response (generally emission quenching)\textsuperscript{23} of FAR-1 and FAR-2 towards selected analytes with an aromatic recognition motif was quantified in homogeneous aqueous solution by fluorescence titration experiments, see the Supplementary Fig. 1 and Supplementary Table 1. Some analytes show the appearance of an exciplex emission band when binding to FAR-3, see Supplementary Fig. 4. Importantly, the response is “immediate” even in stopped-flow experiments, suggesting that FARs are suitable for time-resolved ($t \geq 100$ $\mu$s) monitoring of analyte permeation through phospholipid membranes.

Our analyte test library (Fig. 2) contained more than 100 bio- and drug-relevant compounds with aromatic moieties such as phenols, anilines, indoles, naphthalenes, polyaromatic hydrocarbons, benzimidazoles, alkylated benzenes, halogenated aryl-species, quinolines, pyridines, and furans (Fig. 2). The analytes carried a wide spectrum of functional groups, ranging from electron-donating to electron-withdrawing groups, e.g. –NR$_2$, –NH$_2$, –OR, –OH, –F, –Cl, –Br, –I, –SH, –OP(O)$_3$\textsuperscript{2–}, –COR, –CONHR, –COOR, –COOH, , –SO$_2$NH$_2$, –SO$_3$H, –CN, –NO$_2$. Representative analytes with immediate biological relevance are aromatic amino acids (e.g., tryptophan), metabolites (tryptophanamide), neurotransmitters (e.g., serotonin), antibiotics (e.g., penicillin G), drugs (e.g., omeprazole), herbicides (e.g., propanil), fungicides (e.g., thiabendazole), carcinogens (e.g., anthracene), food additives (e.g., raspberry ketone), and bioactive peptides (e.g., somatostatin). We are unaware of any alternative method, which allows the direct real-time assaying of membrane permeability of such a structurally diverse library. In fact, an adaption of the method to a dye-displacement format allows for the monitoring of additional aliphatic analytes, e.g., adamantane derivatives such as the drug memantine, and alkyl amines, such as the metabolite cadaverine, see Figs. 14 to 17 in the Supplementary Information.
Fig. 2 | Chemical structures of compound library investigated in this study. (a) Benzene derivatives, (b) amino acid derivatives and peptides, (c) polycyclic aromatic and heterocyclic compounds, (d) pesticides, insecticides, fungicides, herbicides, and antibiotics, (e) drugs, and (f) vitamin B1 as well as selected non-aromatic compounds, for which the alternative dye-displacement strategy was adopted (see the Supplementary Information). The compounds are represented in their predominant charge state at pH 7. Color code: green = rapidly permeable, black = slowly permeable, and red = impermeable.
Fig. 3 | Temporally and spatially resolved FARMA experiments. (a) Emission spectra of FAR-1-loaded liposomes prior and after addition of naphthalene (ethanolic stock); addition of neat ethanol is shown as the control. (b) Emission intensity of FAR-2-loaded liposomes upon addition of indole (aq. stock); control exp. with D1-loaded liposomes are shown in blue. (c) Time-resolved translocation monitoring of tryptophan (Trp) and tryptophanamide (TrpNH₂), both 8 µM from aq. stock, with FAR-1-loaded liposomes (d) Translocation monitoring of tryptamine (aq. stock, 16 µM) with FAR-1- and FAR-2-loaded liposomes, and with two membrane-encapsulated indicator displacement ensembles (blue and green, see SI). (e-f) Kinetic
traces from stopped-flow experiments for rapid mixing (1:1 v/v) of phenol (aq. stock) with FAR-2-loaded liposomes. (f) Plot of $k_{obs}$, from monoexponential fits of kinetic traces, vs. phenol concentration. (g) Time series of fluorescence microscopy images of a FAR-1-loaded GUV after addition of 5 µL tryptophan methyl ester (TrpOMe, 800 µM stock) to the medium, as real-color images (top) and as intensity-coded images (bottom).

**Implementation of FARs into membrane translocation assays**

Most established drug permeation assays are based on single-point determinations where the signal is recorded before and after a fixed time period. To demonstrate that FARMA can effectively complement these state-of-the-art single-point assays the following sequence of steps was executed: 1) FARs were encapsulated into phospholipid liposomes formed in aqueous buffer by a rehydration and freeze-thaw procedure, followed by chromatographic separation of FAR-containing liposomes from non-encapsulated FARs, see the online methods. 2) The purified FAR-containing liposome suspension was transferred to fluorescence cuvettes or into microwells and the emission spectra at $t = 0$ were recorded. 3) Aliquots of the test analyte were added, and the emission spectra were recorded again after fixed time intervals (5-60 min).

Comparison of the fluorescence spectra before and after analyte addition after a fixed time yielded a pattern that is in full accordance with the graphical depiction of the FARMA principle in Fig. 1a: A quenching of the emission intensity of the FARs was observed for permeating analytes (*e.g.*, naphthalene or indole), Fig. 3a-b and Supplementary Fig. 2-4, whereas non-permeating analytes (*e.g.*, zwitterionic tryptophan) caused no significant changes in the fluorescence signal, Fig. 3c and Supplementary Fig. 2 and 3. The FARMA method is transferable to microplate reader format in disposable and cost-economic plastic wells (Supplementary Fig. 2).

Several control experiments were carried out to ensure that the observed fluorescence changes were not due to a disruption of liposomes caused by the analytes and not due to the leakage of the FARs, see Fig. 3a-b and the Supplementary Information.
Assay sensitivity
The series of FARMA experiments showed that an analyte concentration of 10 µM is generally sufficient to differentiate between permeating and non-permeating analytes. In fact, for selected analytes such as indole, phenol, tryptophan methyl ester (TrpOMe) and tryptamine even nM to low µM concentrations were sufficient (Fig. 3b & 4 and Supplementary Fig. 5). Converted to 96-well microplate reader format, the FARMA sensitivity corresponds to 4-400 ng/well, depending on analyte. Thus, the sensitivity of FARMA is comparable to established MS-coupled permeation assay formats, and superior to common absorbance-based permeation assay formats requiring typically 50-100 µM analyte concentration.4, 8-11 We observed that with liposome-encapsulated FARs, analytes can be detected at least an order of magnitude more sensitively compared to experiments with non-encapsulated FARs in homogeneous solution. This sensitivity enhancement through FAR encapsulation will be also relevant for additional sensing applications besides permeation monitoring, e.g., for environmental monitoring.

Time-resolved analyte translocation monitoring by FARMA
When carrying out the measurements in a time-resolved manner, i.e., with a continuous recording of the emission intensity, the permeation process of the analytes through the phospholipid bilayer membrane becomes observable in real-time (see the schematics in Fig. 1b and the plotted experimental data in Fig. 3c-g). This constitutes a major advancement compared to PAMPA or Caco-2 assays. Depending on the absolute permeation rates, FARMA kinetics can be either monitored upon manual mixing with standard fluorometers or microplate readers (min-hours) or by stopped-flow techniques with fluorescence detection (ms-s). The CB8-dye-based FARs are useful for monitoring of both slow and fast analyte translocation kinetics because FAR•analyte complex formation is very rapid (up to diffusion limited23) and, thus, not rate-determining.
As an example of slow translocation kinetics, the permeation of tryptamine (Fig. 3d) and tryptophanamide (TrpNH₂, Supplementary Fig. 6) was measured with FAR-1 and FAR-2 as well as with two different dye-displacement strategies. Reassuringly, the normalized kinetic profiles were superimposable, within error. As an example of very fast translocation kinetics, a representative stopped-flow data set is shown in Fig. 3e for the addition of phenol to FAR-2 liposomes. It was found that the observed kinetic traces could be well fitted by monoexponential decay model, affording pseudo-unimolecular rate constants (k_{obs}). As expected, the k_{obs} values were found to be linearly proportional to the analyte concentration, at least in a low concentration range (see the Discussion). The observed kinetic rate constants for 28 structurally related, non-charged, and rapidly permeating phenol- and aniline-type analytes were obtained analogously (Supplementary Table 2). The recovered rate constants span more than three orders of magnitude in range. Such data can be utilized to compare the permeation characteristics of different analytes and to uncover structure-property relations, and to derive permeation rate constants (k_p) and apparent permeability coefficients (P_{app}), see the Discussion.

When compared to PAMPA and Caco-2, our FARMA method offers access to the entire time course of the permeation process, which can be employed for detailed mechanistic investigations of the permeation process. Kinetic investigation of the more complex case of charged analytes illustrate how the FARMA method can potentially be used to obtain novel mechanistic information. Unlike the findings for small, neutral analytes (Fig. 3), the permeation of charged tryptamine, TrpNH₂, and serotonin led to significant deviations from monoexponential kinetic traces, see Fig. 3c-d and Supplementary Fig. 6 & 8g. This is in line with translocation models for charged species, which predict deviations from simple monoexponential kinetics on account of co-transport of counterions or the neutralization of the charge by a prior deprotonation step. The lipid composition of the bilayer membrane can also strongly influence the permeation rates. FARMA can be employed to study these ef-
ferts, e.g., the influence of cholesterol as a membrane component, see Supplementary Fig. 7.

**Spatially and temporally resolved FARMA with giant unilamellar vesicles**

For specialized permeation assays, it would be desirable to monitor the permeation kinetics of single membrane-compartmentalized entities instead of the ensemble average obtained with small FAR-encapsulated liposomes. We therefore tested whether a spatially and temporally resolved permeation monitoring is possible with FARMA. To this end, giant unilamellar vesicles (GUVs), which can be studied by conventional fluorescence microscopy, were loaded with FAR-1 using electroformation (see the online methods). To allow direct imaging, we skipped a potential separation step to remove the non-encapsulated FAR-1 from the buffer medium. Instead, the non-permeating analyte tryptophan was added to the media, which saturates the binding sites of the non-encapsulated FAR-1 and, thus, quenches the extravesicular emission. The inner compartment of the FAR-1-encapsulated GUVs is not accessible to the analyte Trp, and, thus, it remains emissive (Fig. 3g) – and available for binding of a subsequently added, membrane-permeable analyte. Indeed, when TrpOMe as a rather quickly permeating charged analyte (Table 1), was added to the medium with FAR-1 encapsulated GUV, a loss of the fluorescence emission from the interior of the GUVs was observed over time, indicating that TrpOMe reaches the GUV-encapsulated FAR-1 target (Fig. 3g). This sequence of experiments also serves as an independent, visual verification that the FARs are permanently encapsulated inside the liposomes and that lysis of the membrane upon analyte addition does not occur (see also Supplementary Fig. 8).

**Selective analyte detection and monitoring of enzymatic reactions by FARMA**

Beyond its use for permeation monitoring of drugs and other biorelevant analytes, FARMA opens up new sensing opportunities, overcoming standing issues related to
the low selectivity of FARs. For instance, selective detection of permeable species (e.g., TrpOMe) is possible even in the presence of aromatic amino acids such as tryptophan that would, in homogeneous solution, quench the emission of the FAR (see Fig. 3g). Furthermore, analytes can be also distinguished from each other when their permeation rates are sufficiently different, see for instance the examples in Supplementary Fig. 9 - 10.

Several charged, non-permeable analytes can be selectively detected when a suitable enzyme is used in combination with FARMA (Fig. 4a). For instance, negatively charged, impermeable aryl-phosphates such 2-naphthyl phosphate (Fig. 4b) are selectively converted to permeable phenols (e.g., 2-naphthol) upon addition of the enzyme alkaline phosphatase (ALKP). This new compartmentalized variant of a supramolecular tandem enzyme assay allows for the detection of down to 300 nM aryl phosphates, while the magnitude of the response is considerably lower in the absence of the protective membrane (Supplementary Fig. 11). Similarly, the affinities of phenol and phenyl-β-D-galactopyranoside for FAR-1 are comparable, $K_d = 1.8$ mM and 3.3 mM, respectively, such that they cannot be readily distinguished in a homogeneous sensing-format. However, with FARMA, phenol gives an “immediate” signal ($t < 5$ s, see Fig. 3e) whereas phenyl-β-D-galactopyranoside provides a slow response ($t > 5$ min, see Fig. 4c). Upon addition of the enzyme β-galactosidase (β-gal), a fast hydrolysis of phenol-substituted β-D-galactopyranoside occurs, upon which the phenol product quickly permeates through the membrane and binds to FAR-1, see Fig. 4a. Besides, information about the enzymatic reaction rate can be derived at the same time from the enzyme-coupled FARMA experiments. The membrane-mediated spatial separation of FARs from the enzymes ensures native functionality and catalytic activity of the enzyme. Finally, with FARMA, enzymatic reaction monitoring is feasible at lower substrate concentration than in homogeneous FAR-based sensing formats.
DISCUSSION

Classification of analytes according to their kinetic permeability profiles

The FARMA method affords information-rich kinetic permeation profiles as the primary output. These can be directly compared to each other to rank the membrane permeation characteristics of different analytes. The relative permeation characteristics extracted in this way suffice for the majority of envisioned practical applications of FARMA, e.g., when testing drug candidates.

From the permeation screening experiments, analytes can be divided into non-permeating, slowly permeating, and rapidly permeating, see color codes in Fig. 2 and in Supplementary Table 3. Almost all neutral and positively charged species were found to be readily membrane-permeable, unless they are polar and large (such as peptides), very hydrophilic (such as dopamine), or dicationic, such as para-
quat (viologen), or the dyes D1-D3 themselves. Representative kinetic traces are shown in Fig. 3 and Supplementary Figs. 5 & 9-10.

The positively charged neurotransmitters tryptamine, tyramine, and serotonin were shown to pass the membrane within several minutes to hours. Expectedly, the more hydrophilic species permeate more slowly, e.g., phenethylamine > tyramine (Supplementary Fig. 9f) & tryptamine > serotonin (Supplementary Fig. 5 vs. 9g). The catecholamine neurotransmitters dopamine and adrenaline (epinephrine) are very slowly permeating through the biomembrane, while the parent catechol is “instantaneously” permeating (Supplementary Fig. 9f).

Almost all anionic species were found to be phospholipid membrane-impermeable (Supplementary Table S2 and Supplementary Fig. 9 & 10), which is in agreement with expectation (e.g., for aromatic amino acid derivatives30 or ampicillin31) and rationalized by the Columbic repulsion between the analyte and the negatively charged bilayer biomembrane. The “hydrophobic anion” 2-adamantyl-carboxylate is a noteworthy exception; it is membrane-permeable, albeit at a slower rate than its non-charged (2-adamantanol) and positively charged (2-ammonium-adamantane) analogues, see Supplementary Fig. 16 & 17. In fact, lipidization of drugs through connection to adamantyl-moieties is a known approach to increase their membrane permeability and, thus, bioavailability.32

In order to rationalize the permeation rate trends of structurally simple, non-charged aromatic species (Supplementary Table 2), the molecular van-der-Waals volume (vdW volume, \( V_W \)) of the analyte and the \( \log P \) values – a measure for the lipophilicity of an analyte – were employed as descriptors. For example, the permeability of phenol (\( \log P = 1.64 \)) is 20 times lower than that of toluene (\( \log P = 2.52 \)) which is in line with lipophilicity differences.33 Furthermore, an inverse relationship between the vDW volume and the permeability is observed for the subset of \( \text{para} \)-alkylated or halogenated phenols, \( i.e., \), the \( k_{obs} \) are ordered as \( H > Me > Et > tBu \) and \( F \geq H > Cl > Br > I \). However, interesting exceptions were also observed; 4-\( \text{ tert-butyl} \)phenol is
more lipophilic and smaller than propanil but permeates three orders of magnitude more slowly. Indeed, it has been proposed that highly lipophilic molecules (log $P > 3$) can be retained in the lipid membrane and, therefore, exit the membrane slowly, causing an overall decrease in the observed analyte translocation rate.$^{34}$

When attempting to correlate the permeation rates with the log $P$ and $V_W$ values for the whole set of 28 small-molecule aromatics, it becomes immediately obvious that a simplified structure-activity relationship using the lipophilicity and size of the permeating species does not exist, see Fig. 5. Such counterintuitive behavior exemplifies the complexity of the passive diffusion through a membrane for which FARMA can provide useful experimental benchmark data.

![Fig. 5](image)

**Fig. 5** | Observed permeation rate constants ($k_{obs}$) for small aromatics, e.g., phenols and anilines (colour-coded from blue = fastest to red = slowest permeating on a logarithmic scale) correlated to their log $P$ values (x-axis) and their van der Waals volumes ($V_W$, y-axis). The numbering of the compounds follows their order of permeation speed from 1 = fastest to 28 = slowest. See Supplementary Table 2 for the numerical $k_{obs}$, log$P$, and $V_W$ values.

### Determination of permeation rate constants and permeability coefficients

Elementary physical parameters, such as permeability coefficients, can be extracted from the full kinetic FARMA data. This kinetic profile → parameter mapping requires some assumptions to be made in regard to the permeation mechanism. We applied a reported permeation model for liposomes$^{28}$ to arrive at permeation rate constants ($k_p$) and apparent permeability coefficients ($P_{app}$). The derivation of the mathematical rela-
tion (1), linking the fundamental permeability rate constant and permeability coefficient to experimental measurable \( k_{\text{obs}} \) rate constants, the known total analyte and total FAR concentration, and the experimentally determined liposome radius (\( r \)), is shown in the Supplementary Information.

\[
P_{\text{app}} = k_p \cdot (r/3) = k_{\text{obs}}/c_{\text{analyte}} \cdot c_{\text{FAR}} \cdot (r/3)
\]

The volume-to-surface correction factor \( r/3 \) accounts for the fact that the observed rates depend on the size, \( i.e. \) radius \( r \), of the liposomal assembly.\(^{28}\) Experimentally, the ratio \( k_{\text{obs}}/c_{\text{analyte}} \) can be obtained as the slope of the plot of \( k_{\text{obs}} \) against \( c_{\text{analyte}} \), as is shown in Fig. 3f for phenol as the analyte, and in Supplementary Fig. 12 for indole, tryptamine, tryptophan methyl ester (TrpOMe), and \( N \)-acetyl tryptophanamide (NATA). The initial rate method can also be applied, see the Supplementary Information. Moreover, \( k_{\text{obs}}/c_{\text{analyte}} \) can be estimated by single-point measurements at selected \( c_{\text{analyte}} \) concentration.

Representative permeation rate constants (\( k_p \)) and permeability coefficients (\( P \)) obtained through equation 1 are listed in Table 1 for selected analytes for which literature \( P \) values were available. The \( k_{\text{obs}} \), \( k_p \), and \( P_{\text{app}} \) values for a series of 28 phenols and anilines is given in Table S2 in the Supplementary Information.

The obtained permeability coefficients shown in Table 1 compare favorably with ranges of literature values obtained by other methods for neutral analytes with comparable molecular weight.\(^{25}\) For instance, the \( P_{\text{app}} \) value (all in \( 10^{-6} \) cm s\(^{-1}\)) for indole determined by FARMA in liposomal POPS:POPC bilayer membranes (106) lies within the range of values extracted from PAMPA in synthetic membranes (32)\(^{36}\) from Caco-2 assay in liposomal POPS:POPC bilayer membranes (57),\(^{37}\) and for a macroscopic bilayer of brain phospholipid (250).\(^{27}\) Likewise, when comparing the \( P_{\text{app}} \) values for other non-charged analytes, a good qualitative agreement is found, \( e.g. \) for NATA, where the FARMA value (1.0) lies between that determined by fluorescence quenching (0.1), PAMPA (1.9) and Caco-2 (2.5). For the drug memantine, the FAR-
MA value (24) is close to the reported Caco-2 based value (43). However, for charged and rather hydrophilic substances, our set-up yields consistently lower permeabilities than PAMPA and Caco-2. For instance, for tryptamine, our value determined by FARMA in liposomal POPS:POPC bilayer membranes (0.19) comes close to that obtained by fluorescence quenching experiments (0.33) but falls one order of magnitude short to that of PAMPA and other flat lipid membranes (Table 1). Similar findings were made for TrpNH₂, ranitidine, and serotonin, which all show an order of magnitude faster permeation under PAMPA and Caco-2 conditions than in our POPS:POPC bilayer membranes. Conversely, for non-charged lipophilic aromatics, we mostly observed faster permeation in our POPS:POPC bilayer membranes than reported under PAMPA conditions, e.g., compare the series of phenols and anilines. These differences point to a specific mechanistic involvement of the different membrane lipids in the permeation process.

It transpires that the FARMA method affords absolute permeability coefficients, which are comparable with those determined by established methods. Differences may be traced back to the use of flat hexadecane membranes (PAMPA) vs. spherical phospholipid bilayers (FARMA), potential complications arising from transporters (Caco-2), and saturation effects occurring at higher analyte concentration (usually not considered for PAMPA and Caco-2 but uncovered by FARMA).

CONCLUSION

In conclusion, the FARMA method allows real-time optical monitoring of the permeation of a large variety of drugs, toxins, and other organic compounds, circumventing the need for labeled analytes,14, 15 by-passing methodologies limited to analyte-induced pH jumps,38 circumventing single-point mass-spectrometric detection,13 and complementing alternative assays in membrane research such as PAMPA8-10 or Caco-2.11 Important to note, FAR-based membrane assays allow accessing the entire kinetic traces even for the most rapid permeation events. Furthermore, the FARMA
procedure can be modified to allow real spatial resolution to microscopically follow analyte uptake. Owing to the use of fluorescence for detection, flexible implementation into microplate and confocal imaging formats are readily performed. Furthermore, different FARs can be adopted that vary with respect to analyte scope, sensitivity, selectivity, and excitation as well as emission wavelengths. We therefore contend that FARMA will become a complementary tool both in fundamental and applied membrane permeation research.

ACKNOWLEDGEMENTS
This work was supported by the German Academic Exchange Service (DAAD, F.B.) and the Deutsche Forschungsgemeinschaft (DFG Grants BI-1805/2-1 for F.B., NA-686/11 for W.M.N., and HE 5967/4-1 for A.H.). The authors thank Mathias Winterhalter for instrument usage, Denisa Hathazia for performing the enzyme-coupled FARMA assay, and Solène Collin for testing reproducibility.

AUTHOR CONTRIBUTION
F.B. and W.M.N. designed the experiments and prepared the manuscript. F.B. prepared the FARs and liposomes, and carried out the FARMA experiments. G.G. performed the complementary dye-displacement-based assays and the GUV studies. A.H. contributed to the fluorescence data analysis by developing the initial-rate method.

Supplementary Information Available. Any Supplementary Information and Source Data files are available in the online version of the paper.
METHODS

**Materials.** Analytes, buffers and lipids were purchased from Alfa Aesar and Sigma Aldrich and used as received. Peptides were purchased from BIOSYNTAN GmbH. Hosts CB7 and CB8 were purchased from Strem. Dyes D1-D3 were prepared according to literature procedures, see ref. 23 and references therein. Dyes 2,6-ANS and DapoxyS, which were utilized for the dye-displacement assay described in the Supplementary Information, were purchased from Invitrogen. All experiments were carried out at ambient temperature (295 K).

**Preparation of FARs.** The chemosensors FAR-1, FAR-2, and FAR-3, were self-assembled from the host CB8 and the dyes D1, D2 and D3, respectively, by dissolving the solid materials together in HEPES buffer (10 mM, adjusted to pH 7.0) to reach 500 µM in CB8 and 550 µM in the dye component (a slight excess of dye was used to ensure full complexation of the host). The dissolution process was assisted by heating to 40-50°C and use of a sonication bath.

**Determination of binding constants for FAR-analyte pairs.** Fluorescence titrations were carried out in aqueous HEPES buffer (10 mM, pH 7) unless stated otherwise. To a solution of the FAR (typically at 10 to 30 µM) was stepwise added a solution of the analyte (typically up to 2 mM) and the fluorescence spectra were recorded. The normalized emissions at 450 nm (FAR-1) or at 370 nm (FAR-2) were fitted with an equation for a 1:1 binding by a least-square fit. The resulting affinity constants are reported in Table S1 in the Supplementary Information. Representative titration plots are shown in Supplementary Fig. 1. Note that the high CB8-dye binding affinities correspond to a near quantitative degree of host-dye complexation, *i.e.*, quantitative formation of the FAR, such that the subsequent binding of the analyte can be treated independently.
**Representative liposome preparation and FAR encapsulation procedure.** A solution of 2.5 mg/mL of POPC and 0.33 mg/mL of POPS in chloroform was purged with nitrogen and dried overnight under high-pressure vacuum. The lipid film was rehydrated with 1 mL HEPES buffer (10 mM) containing FAR (0.5 mM, prepared from 500 µM in CB8 and 550 µM in the dye component in HEPES buffer, see above) followed by 13-15 freeze-thaw cycles (freeze in liquid nitrogen, thaw at 40 °C in a water bath). The resulting FAR-loaded liposomes were separated from non-encapsulated species by size-exclusion chromatography (NAP-25 column) while maintaining the same buffer. The absence of non-encapsulated FAR was confirmed by adding a non-membrane-permeable species such as tryptophan to a fluorescence cuvette containing 25 µL of liposomes diluted in 1 mL HEPES (10 mM) buffer. Complete removal of non-encapsulated FAR is indicated by the absence of a significant fluorescence change verified upon Trp addition. The size of the liposomes (r ~ 100 nm) was measured by dynamic light scattering (Zetasizer Nano from Malvern Instruments).

**FARMA procedure.** In a typical experiment, 20 µL of liposome solution loaded with FAR was diluted in 1 mL HEPES buffer (10 mM, pH 7.0) in a 1-mL quartz cuvette. An emission spectrum was recorded after 10 min of “equilibration time” on a Varian Eclipse spectrofluorometer, using λ_{exc} = 400 nm for FAR-1, λ_{exc} = 310 nm for FAR-2, and λ_{exc} = 330 nm for FAR-3. In the time-resolved experiments, the emission intensity at λ_{abs} = 450 nm for FAR-1, λ_{abs} = 370 nm for FAR-2, and λ_{abs} = 370 nm & 500 nm for FAR-3 (the latter is the emerging excimer band for certain FAR-3•analyte complexes) was recorded with an averaging time of 0.5 s. Once the signal had stabilized, 8 µL of a 1 mM analyte solution in the same buffer (HEPES, 10 mM) was added (c_{final} = 8 µM) to the cuvette and the emission monitoring was continued until no significant change occurred. For single-point experiments, a full spectrum was recorded after a fixed time or several time intervals after analyte addition. Control experiments confirmed that the autofluorescence of each analyte at the given excitation and emission
wavelength for a FAR was unnoticeable or small and could be corrected for. It was also confirmed by UV/Vis spectroscopy that the absorbance of the analyte at the given excitation wavelength of the FAR is low (Abs < 0.05), such that inner filter effects are also negligible. Only FAR-analyte combinations were used for FARMA for which no significant autofluorescence and competitive light absorption of the analyte occurred.

Microplate measurements were performed with a JASCO FP-8500 spectrofluorometer coupled with a JASCO FMP-825 microplate reader accessory in 96-well microplates, using flat-bottom black microplates with a nonbinding surface. After filling with liposome solution (200 μL) the microplate was placed into the reader and equilibrated for 10 min. Then fluorescence intensity of each well was recorded, followed by analyte addition and subsequent fluorescence recording at specific time intervals.

**Time-resolved FARMA experiments for rapidly permeating analytes.** Stopped-flow experiments were performed with a Bio-Logic stopped-flow SFM-20 module coupled to a JASCO FP-8500 spectrofluorometer. In a standard experimental setup, 400 μL of a FAR-2-loaded liposome solution was diluted in 10 mL of 10 mM buffer in Syringe 1, while Syringe 2 contained phenol at different concentrations, between 2-80 μM. Fluorescence measurements were initiated by mixing the contents of the two syringes in equal volumes (total volume = 200 μL, flow speed of 4.5 mL/s) in the stopped-flow chamber, such that the final phenol concentration range for measurement was 1-40 μM. All experiments were carried out in 10 mM HEPES buffer, pH 7.0, at 20 °C. Fluorescence intensities were recorded with an excitation wavelength of 310 nm and emission at 350 nm. For each experiment, measurements from 6 injections were accumulated and the average of these traces was used for data analysis.
**Spatially and temporally resolved FARMA experiments with GUVs.** Giant unilamellar vesicles (GUVs) were prepared using Vesicle Prep Pro from Nanion Technologies. Specifically, a mixture of a 30 µL POPC solution (25 mg/mL in CHCl₃) and 10 µL POPS solution (5 mg/mL in CHCl₃) was spread as a thin film on ITO-coated glass slides. After the solvent had evaporated and the film had dried, it was rehydrated with 300 mM of sucrose solution containing FAR-1 and covered with another ITO slide. After 2 h of preparation in Vesicle Prep Pro, GUVs had formed and the suspension was collected. All subsequent measurements were carried out on the same day: A drop of the GUV suspension was pipetted on a glass slide and the formation of GUVs was confirmed by bright-field microscopy (Supplementary Fig. 8). To this suspension, 5 µL of tryptophan (1 mM stock) was added to quench the fluorescence of the nonencapsulated FAR-1 chemosensing ensemble. Fluorescence images of such treated GUVs were taken with a fluorescence microscope (Axiovert 200, Carl Zeiss, filter set 02, i.e., G365 nm, FT 395 nm and BP 420 nm), equipped with a digital camera (Evolution QEi monochrome). The first image was taken immediately after the addition of 5 µL of TrpOMe (800 µM stock) to the suspension. Subsequent images were taken at regular intervals (1 min) thereafter. Constant illumination was avoided to reduce potential photobleaching, i.e., the sample was illuminated only when the images were taken. Exposure time and camera settings were constant across all images. Images were analyzed by using the Image J software. In order to ensure that the apparent decrease in fluorescence over time was not due to photobleaching of the dye, experiments were carried out exactly as described above, except for the addition of TrpOMe. Indeed, in the absence of TrpOMe, no noticeable change in fluorescence intensity was observed (Supplementary Fig. 8).

**FARMA-coupled enzymatic experiments.** The hydrolysis of 2-naphthyl phosphate by alkaline phosphatase (ALKP) from bovine intestinal mucosa (activity of approx. 2000 units/mg according to supplier) was monitored by emission spectroscopy (λ_{exc} =
420 nm, $\lambda_{em} = 450$ nm) with FAR-1 at 37 °C following literature procedures. In analogy to the FARMA procedure, 20 µL of liposome solution loaded with FAR-1 was diluted in 1 mL HEPES buffer (10 mM, pH 7.5) in a 1-mL quartz cuvette. Once the emission signal had stabilized, the required volume of a 1 mM 2-naphthyl phosphate solution in the same buffer was added to reach a final substrate concentration between 310 nM to 5 µM in the cuvette. After a ca. 1 min equilibration time, ALKP stock solution was added to reach an enzyme concentration of 16 µg ml$^{-1}$ in the cuvette. The emission recording was continued until no significant change occurred anymore (after ca. 5 min). The control experiments in homogeneous solution, i.e., in the absence of a protective membrane, were conducted analogously, employing 5 µM FAR-1 and 5 µM to 30 µM 2-naphthyl phosphate, see also Supplementary Fig. 11a.

The hydrolysis of phenyl-β-D-galactopyranoside by β-galactosidase (β-gal) from A. oryzae (activity of 8 units/mg according to supplier) was conducted analogously, but at 22 °C. 1 mM phenyl-β-D-galactopyranoside solution was added as substrate to reach a final substrate concentration between 0 to 2.5 µM in the cuvette and, after ca. 1 min equilibration time, β-gal stock solution was added to reach an enzyme concentration of 43 µg ml$^{-1}$ in the cuvette. The recording was continued until no significant change occurred anymore (after ca 10 min). The control-experiments in homogeneous solution employed 5 µM FAR-1 and 10 µM to 50 µM phenyl-β-D-galactopyranoside, see also Supplementary Fig. 11b.
References

1. Sugano, K. et al. Coexistence of passive and carrier-mediated processes in drug transport. *Nat. Rev. Drug Discov.* 9, 597-614 (2010).
2. Dobson, P.D. & Kell, D.B. Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nat. Rev. Drug. Discov.* 7, 205-220 (2008).
3. Pardridge, W.M. Drug transport across the blood-brain barrier. *J. Cereb. Blood Flow Metab.* 32, 1959-1972 (2012).
4. Avdeef, A. Absorption and drug development: solubility, permeability, and charge state. (John Wiley & Sons, 2012).
5. Yu, P., Liu, B. & Kodadek, T. A high-throughput assay for assessing the cell permeability of combinatorial libraries. *Nat. Biotechnol.* 23, 746-751 (2005).
6. Tsamaloukas, A.D., Keller, S. & Heerklotz, H. Uptake and release protocol for assessing membrane binding and permeation by way of isothermal titration calorimetry. *Nat. Protoc.* 2, 695-704 (2007).
7. Jentzsch, A.V. et al. Transmembrane anion transport mediated by halogen-bond donors. *Nat. Commun.* 3, 905 (2012).
8. Kansy, M., Senner, F. & Gubernator, K. Physicochemical High Throughput Screening: Parallel Artificial Membrane Permeation Assay in the Description of Passive Absorption Processes. *J. Med. Chem.* 41, 1007-1010 (1998).
9. Woehnsland, F. & Faller, B. High-Throughput Permeability pH Profile and High-Throughput Alkane/Water log P with Artificial Membranes. *J. Med. Chem.* 44, 923-930 (2001).
10. Di, L., Kerns, E.H., Fan, K., McConnell, O.J. & Carter, G.T. High throughput artificial membrane permeability assay for blood–brain barrier. *Eur. J. Med. Chem.* 38, 223-232 (2003).
11. Hubatsch, I., Ragnarsson, E.G.E. & Artursson, P. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat. Protoc.* 2, 2111-2119 (2007).
12. Bickerton, G.R., Paolini, G.V., Besnard, J., Muresan, S. & Hopkins, A.L. Quantifying the chemical beauty of drugs. *Nat. Chem.* 4, 90-98 (2012).
13. Mensch, J. et al. Novel generic UPLC/MS/MS method for high throughput analysis applied to permeability assessment in early Drug Discovery. *J. Chromatogr. B* 847, 182-187 (2007).
14. Marks, J.R., Placone, J., Hristova, K. & Wimley, W.C. Spontaneous Membrane-Translocating Peptides by Orthogonal High-Throughput Screening. *J. Am. Chem. Soc.* 133, 8995-9004 (2011).
15. Szeto, H.H., Schiller, P.W., Zhao, K. & Luo, G. Fluorescent dyes alter intracellular targeting and function of cell-penetrating tetrapeptides. *FASEB J.* 19, 118-120 (2005).
16. Berry, M.D., Shitut, M.R., Almousa, A., Alcorn, J. & Tomberli, B. Membrane permeability of trace amines: Evidence for a regulated, activity-dependent, nonexocytotic, synaptic release. *Synapse* 67, 656-667 (2013).
17. You, L., Zha, D. & Anslyn, E.V. Recent Advances in Supramolecular Analytical Chemistry Using Optical Sensing. *Chem. Rev.* 115, 7840-7892 (2015).
18. Ghale, G. et al. Chemosensing Ensembles for Monitoring Biomembrane Transport in Real Time. *Angew. Chem. Int. Ed.* **53**, 2762-2765 (2014).
19. Xu, Z. et al. Heteromultivalent peptide recognition by co-assembly of cyclodextrin and calixarene amphiphiles enables inhibition of amyloid fibrillation. *Nat. Chem.* **11**, 86-93 (2019).
20. Murray, J., Kim, K., Ogoshi, T., Yao, W. & Gibb, B.C. The aqueous supramolecular chemistry of cucurbit[n]urils, pillar[n]arenes and deep-cavity cavitands. *Chem. Soc. Rev.* (2017).
21. Barrow, S.J., Kasera, S., Rowland, M.J., del Barrio, J. & Scherman, O.A. Cucurbituril-Based Molecular Recognition. *Chem. Rev.* **115**, 12320-12406 (2015).
22. Ma, D. et al. Acyclic cucurbit[n]uril molecular containers enhance the solubility and bioactivity of poorly soluble pharmaceuticals. *Nat. Chem.* **4**, 503-510 (2012).
23. Sinn, S. & Biedermann, F. Chemical Sensors Based on Cucurbit[n]uril Macrocycles. *Isr. J. Chem.* **58**, 357-412 (2018).
24. Bier, D. et al. Molecular tweezers modulate 14-3-3 protein–protein interactions. *Nat. Chem.* **5**, 234 (2013).
25. Biedermann, F., Hathazi, D. & Nau, W.M. Associative chemosensing by fluorescent macrocycle-dye complexes - a versatile enzyme assay platform beyond indicator displacement. *Chem. Commun.* **51**, 4977-4980 (2015).
26. Saparov, S.M., Antonenko, Y.N. & Pohl, P. A New Model of Weak Acid Permeation through Membranes Revisited: Does Overton Still Rule? *Biophys. J.* **90**, 86-88 (2006).
27. Bean, R.C., Shepherd, W.C. & Chan, H. Permeability of Lipid Bilayer Membranes to Organic Solutes. *J. Gen. Physiol.* **52**, 495-508 (1968).
28. Chakrabarti, A.C. & Deamer, D.W. Permeability of lipid bilayers to amino acids and phosphate. *Biochim. Biophys. Acta — Biomembranes* **1111**, 171-177 (1992).
29. Hennig, A., Bakirci, H. & Nau, W.M. Label-free continuous enzyme assays with macrocycle-fluorescent dye complexes. *Nat. Meth.* **4**, 629-632 (2007).
30. Chakrabarti, A.C. Permeability of membranes to amino acids and modified amino acids: Mechanisms involved in translocation. *Amino Acids* **6**, 213-229 (1994).
31. Nestorovich, E.M., Danelon, C., Winterhalter, M. & Bezrukov, S.M. Designed to penetrate: time-resolved interaction of single antibiotic molecules with bacterial pores. *Proc. Natl. Acad. Sci. USA* **99**, 9789-9794 (2002).
32. Witt, K.A., Gillespie, T.J., Huber, J.D., Egleton, R.D. & Davis, T.P. Peptide drug modifications to enhance bioavailability and blood-brain barrier permeability. *Peptides* **22**, 2329-2343 (2001).
33. Potts, R.O. & Guy, R.H. A predictive algorithm for skin permeability: the effects of molecular size and hydrogen bond activity. *Pharm. Res.* **12**, 1628-1633 (1995).
34. Alvarez-Figueroa, M.J. et al. Evaluation of the membrane permeability (PAMPA and skin) of benzimidazoles with potential cannabinoid activity and their relation with the Biopharmaceutics Classification System (BCS). *AAPS PharmSciTech* **12**, 573-578 (2011).
35. Fujikawa, M., Nakao, K., Shimizu, R. & Akamatsu, M. QSAR study on permeability of hydrophobic compounds with artificial membranes. *Biorg. Med. Chem.* **15**, 3756-3767 (2007).
36. Fujikawa, M., Ano, R., Nakao, K., Shimizu, R. & Akamatsu, M. Relationships between structure and high-throughput screening permeability of diverse drugs with artificial membranes: Application to prediction of Caco-2 cell permeability. *Biorg. Med. Chem.* **13**, 4721-4732 (2005).

37. Ano, R. et al. Relationship between structure and permeability of dipeptide derivatives containing tryptophan and related compounds across human intestinal epithelial (Caco-2) cells. *Biorg. Med. Chem.* **12**, 249-255 (2004).

38. Li, S., Hu, P.C. & Malmstadt, N. Imaging molecular transport across lipid bilayers. *Biophys. J.* **101**, 700-708 (2011).

39. Urakami, M. et al. Relationship between structure and permeability of tryptophan derivatives across human intestinal epithelial (Caco-2) cells. *Z. Naturforsch. C* **58**, 135-142 (2003).

40. Cardenas, A.E. et al. Unassisted Transport of N-Acetyl-l-tryptophanamide through Membrane: Experiment and Simulation of Kinetics. *J. Phys. Chem. B* **116**, 2739-2750 (2012).

41. Beconi MG, H.D., Park L, Lyons K, Giuliano J, Dominguez C, Munoz-Sanjuan I, Pacifici R. Pharmacokinetics of memantine in rats and mice. *PLOS Currents Huntington Disease* 10.1371/currents.RRN1291 (2012).
Figure Captions

Fig. 2 | Operational principle of the fluorescent artificial receptor membrane assay (FARMA). (a) Encapsulation of membrane-impermeable fluorescent artificial receptors (FAR) into liposomes spatially separates the FARs from the subsequentially added analyte. Upon analyte permeation through the membrane into the liposome, and subsequent rapid analyte-complexation by FAR, a readily observable change in the fluorescence intensity of the FAR can be observed (typically emission quenching). (b) Expected emission-readout for a permeable vs. an impermeable analyte. (c) Structures of the synthetic host CB8 and dyes (D1-D3), from which FAR-1, FAR-2, and FAR-3 were assembled.

Fig. 2 | Chemical structures of compounds investigated in this study. (a) Benzene derivatives, (b) amino acid derivatives and peptides, (c) polycyclic aromatic and heterocyclic compounds, (d) pesticides, insecticides, fungicides, herbicides, and antibiotics, (e) drugs, and (f) vitamin B1 as well as selected non-aromatic compounds, for which the alternative dye-displacement strategy was adopted (see the Supplementary Information). The compounds are represented in their predominant charge state at pH 7. Color code; green: rapidly permeable, black: slowly permeable, and red: impermeable.

Fig. 3 | Temporally and spatially resolved FARMA experiments. (a) Emission spectra of FAR-1-loaded liposomes prior and after addition of naphthalene (ethanolic stock); addition of neat ethanol is shown as the control. (b) Emission intensity of FAR-2-loaded liposomes upon addition of indole (aq. stock); control exp. with D1-loaded liposomes are shown in blue. (c) Time-resolved translocation monitoring of tryptophan (Trp) and tryptophanamide (TrpNH2), both 8 µM from aq. stock, with FAR-1-loaded liposomes (d) Translocation monitoring of tryptamine (aq. stock, 16 µM) with FAR-1- and FAR-2-loaded liposomes, and with two membrane-encapsulated indicator displacement ensembles (blue and green, see SI). (e-f) Kinetic traces from stopped-flow experiments for rapid mixing (1:1 v/v) of phenol (aq. stock) with FAR-2-loaded liposomes. (f) Plot of kobs, from monoexponential fits of kinetic traces, vs. phenol concentration. (g) Time series of fluorescence microscopy images of a FAR-1-loaded GUV after addition of 5 µL TrpOMe (800 µM stock) to the medium, as real-color images (top) and as intensity-coded images (bottom).

Fig. 4 | Enzyme-coupled FARMA method. (a) Schematic operational principle and representative enzymatic reactions. (b) Time-resolved emission of liposome-encapsulated FAR-1 with the membrane-impermeable substrate 2-naphthyl phosphate and the enzyme alkaline phosphatase (ALKP, 16 µg ml⁻¹). (c) Time-resolved emission of liposome-encapsulated FAR-1 and the slowly permeating substrate phenyl-β-D-galactopyranoside, with and without the enzyme β-galactosidase (β-gal, 43 µg ml⁻¹).
Fig. 5 | Observed permeation rate constants ($k_{obs}$) for small aromatics, e.g., phenols and anilines (colour-coded from blue = fastest to red = slowest permeating on a logarithmic scale) correlated to their log $P$ values (x-axis) and their van der Waals volumes ($V_W$, y-axis). The numbering of the compounds follows their order of permeation speed from 1 = fastest to 28 = slowest. See Supplementary Table 2 for the numerical $k_{obs}$, log$P$, and $V_W$ values.
### Tables

**Table 1.** Permeation rates ($k_p$) and apparent permeability coefficients ($P_{app}$) for the permeation of charged analytes through liposomal POPS:POPC bilayer membranes (r ca. 100 nm), ordered from slowest to fastest permeability coefficient.

| Analyte  | $k_p/(10^{-2} \text{ s}^{-1})$ | $P_{app}/(10^{-6} \text{ cm s}^{-1})$ | Literature | Assay/lipid type |
|----------|-------------------------------|--------------------------------------|------------|------------------|
|          | this work<sup>b</sup>         | this work<sup>b</sup>                 | literature<sup>f</sup> |                   |
| Trp<sup>c</sup> | < 0.01                     | < 0.0003                             | 0.00041    | end-point analysis/EPC liposomes |
| serotonin | 0.12<sup>a</sup>            | 0.004<sup>a</sup>                   | 1.1        | aliquot analysis/flat lipid bilayer |
| ranitidine | 0.39<sup>a</sup>            | 0.013<sup>a</sup>                   | 0.88       | PAMPA             |
| TrpNH<sub>2</sub> | 5.2                      | 0.17                                 | 2.7        | PAMPA             |
| tryptamine | 5.6                        | 0.19                                 | 5.4        | PAMPA             |
| (initial rates) |                          |                                      | 6.7        | aliquot analysis/flat lipid bilayer |
| NATA     | 31                           | 1.0                                  | 1.9        | PAMPA             |
| memantine| 720                          | 24                                   | 43         | Caco-2            |
| indole   | 3200                         | 106                                  | 32         | PAMPA             |
| phenol   | 3400                         | 112                                  | 47         | PAMPA             |
| 4Cl-aniline | 5000<sup>a</sup>   | 170<sup>a</sup>                     | 42         | PAMPA             |
| aniline  | 9400<sup>a</sup>            | 310<sup>a</sup>                     | 76         | PAMPA             |
| 4CN-phenol | 50000<sup>a</sup>     | 1700<sup>a</sup>                    | 17         | PAMPA             |

<sup>a</sup> Obtained by the FARMA method with a receptor concentration of ca. 500 μM (500 μM CB8 and 550 μM dye); entries 4Cl-aniline, aniline, and 4CN-phenol correspond to Supplementary Table 2. <sup>b</sup> Value obtained by eq. 1, taking the slopes $k_{obs}/c_{analyte}$ from the linear fit of $k_{obs}$ versus $c_{analyte}$ in the linear range, i.e., $c_{analyte} \leq 20 \text{ μM}$, unless stated otherwise, 20% error (reproducibility). <sup>c</sup> No permeation observed up to 100 μM. <sup>d</sup> No passive permeation but active transport. <sup>e</sup> Values from single-point measurements according to eq. 1 at 40 μM analyte concentrations, except for serotonin (16 μM) and ranitidine (100 μM). <sup>f</sup> Literature values taken from the following references: end-point analysis/EPC liposomes, aliquot analysis/flat lipid bilayer, Caco-2, PAMPA, and fluorescence quenching. For memantine, $P_{app}$ was taken from ref. for Caco-2.

---

29
Supplementary Information

Fluorescent Artificial Receptor-based Membrane Assay (FARMA) for Spatiotemporally Resolved Monitoring of Biomembrane Permeability

Frank Biedermann,1,2 Garima Ghale,2 Andreas Hennig,2 and Werner M. Nau2

Table of Contents
Supplementary Figures .................................................................................................................. 2
Calculation of the unimolecular permeation rate constants (kp) and the apparent permeability coefficients (Papp) ........................................................................................................ 9
Dye Displacement Membrane Assay ......................................................................................... 10
Supplementary Tables ................................................................................................................. 14
Supplementary References ......................................................................................................... 22

1 Institute of Nanotechnology (INT), Karlsruhe Institute of Technology (KIT), Hermann-von-Helmholtz Platz 1, 76344, Eggenstein-Leopoldshafen, Germany
2 Department of Life Sciences and Chemistry, Jacobs University Bremen, Campus Ring 1, 28759 Bremen, Germany.
Correspondence should be addressed to F.B. (frank.biedermann@kit.edu) or W.M.N. (w.nau@jacobs-university.de).
Supplementary Fig. 1 | Emission spectra and fittings (1:1 binding model) for the titration of FAR-1 ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 450$ nm) with (a) catechol, (b) 4-hydroxyphenylpyruvic acid, (c) phenyl-$\beta$-D-galactopyranoside, (d) serotonin, (e) paracetamol, (f) tryptamine, (g) propanil, (h) dopamine. Emission spectra and fittings (1:1 binding model) for the titration of FAR-2 ($\lambda_{ex} = 310$ nm, $\lambda_{em} = 370$ nm) with (i) 2-hydroxybenzonitrile, (j) 4-hydroxybenzonitrile, (k) hydroquinone, (l) $p$-anisylboronic acid, (m) 4-methoxyphenol and (n) 4-ethoxyphenol. All titrations were carried out in homogenous solution, 10 mM HEPES buffer, pH 7.
Supplementary Fig. 2 | FARMA assay recorded on a microplate reader (96-well plates) with FAR-1. (a) In the absence of analyte, no significant change in the emission intensity of FAR-1 occurred during 60 min, demonstrating their stability in the microplate wells. (b-d) Upon addition of different analytes, fluorescence readings at 0, 5 and 60 minutes (normalized to initial intensities) revealed a rapid decrease in fluorescence for (b) indole and (c) tryptamine, but no significant change for (d) tryptophan (Trp). Note that the 5-min (red) and 60-min (blue) traces for indole and tryptophan are essentially superimposed. The results are consistent with the permeability of the same analytes obtained from quartz cuvette-based assays, i.e., instantaneous permeation of indole, slow permeation of tryptamine, and no permeation of Trp.

Supplementary Fig. 3 | FARMA assay recorded in quartz cuvettes with FAR-2 and selected analytes, (a) indole (10 µM), (b) tryptamine (8 µM), (c) Trp (20 µM), (d) benzimidazole (8 µM), (e) 4-cresol (20 µM), (f) tyramine (10 µM), (g) Phe (20 µM), and (h) phenethylamine (10 µM). The spectra (λex = 310 nm) were recorded prior to analyte addition (black trace) and after analyte addition (red trace), once the system had equilibrated. Note that the apparent slight increase of the emission for the FARMA experiment with Trp (panel c) is due to the weak autofluorescence of Trp at 310 nm excitation (green trace).
Supplementary Fig. 4 | FARMA assay with FAR-3 and representative analytes, each at 16 µM, (a) TrpNH₂, (b) TrpOMe, (c) tryptamine, (d) benzimidazole, (e) quinoline, and (f) indole. The spectra (λ_ex = 330 nm) were recorded prior to analyte addition (black trace) and after analyte addition (red trace), once the system had equilibrated. In some cases, both the quenching of the emission band of FAR-3 around 370 nm, but also the appearance of an excimer band from 450-550 nm is observed. The insets show the time-resolved monitoring of the relative emission intensity at 500 nm. Panel c shows also the weak autofluorescence of quinoline at 330 nm excitation under the same measurement conditions (green trace).

Supplementary Fig. 5 | FARMA assay with FAR-2 (λ_ex = 310 nm) and (a) TrpOMe and (b) tryptamine at different concentrations in order to demonstrate the high sensitivity of the method.
Supplementary Fig. 6 | Translocation monitoring of TrpNH₂ (aq. stock, 16 µM) with FAR-1- and FAR-2-loaded liposomes, and with two membrane-encapsulated indicator displacement ensembles (blue and green, see SI).

Supplementary Fig. 7 | Translocation monitoring of tryptamine (aq. stock, 16 µM) with FAR-2 loaded liposomes (1000 µL of the same aqueous liposome stock), which have been pre-treated for 20 min with 10 µL of a 10 mM ethanolic cholesterol solution, or with 10 µL of ethanol as the control.
**Supplementary Fig. 8** | (a) Phase-contrast image of FAR-1-loaded GUVs. The red arrow points to the GUV, whose fluorescence we selected to follow after the addition of the analyte, tryptophan methyl ester (TrpOMe), see Fig. 3g in main text. (b) Normalized fluorescence intensities of GUVs in the absence and presence of TrpOMe monitored at different times (after addition). In the absence of TrpOMe no noticeable change in fluorescence intensity was observed, which further signifies that the decrease in intensity upon addition of TrpOMe cannot be attributed to photobleaching.

**Supplementary Fig. 9** | FARMA assay with FAR-1 ($\lambda_{ex} = 400 \text{ nm}$, $\lambda_{em} = 450 \text{ nm}$) and representative analytes, each at 8 µM in 10 mM aqueous HEPES buffer, pH 7. (a) Hydrophobic aromatics that were titrated from an ethanolic stock solution and the corresponding blank control, (b-c) structurally related substances for investigating biomembrane permeability of non-charged vs. negatively charged species, (d-e) representative drugs,
(f) catecholamine neurotransmitter and related substances, (g) slow permeation trace of the neurotransmitter serotonin and photostability control in the absence of analyte. The titration of the analytes was conducted from an aqueous stock with the exception of analytes shown in (a).

**Supplementary Fig. 10** | FARMA assay with FAR-2 ($\lambda_{ex} = 310$ nm, $\lambda_{em} = 370$ nm) and representative analytes. (a) Trp and related species (each at 8 µM), traces were corrected for autofluorescence of Trp-species. (b) $p$-substituted phenols (8 µM), the inset shows the control experiments with D2 dye-only loaded liposomes. (c) Consecutive addition of aliquots (each step 8 µM) for weakly binding analytes. The control shows the addition of analyte to liposomes that were loaded only with dye D2. (d) Hydrophobic aromatics (80 µM) that were titrated from an ethanolic stock solution and the corresponding blank control (e) Dye-displacement variant with FAR-2 to monitor the membrane-permeation of aliphatic adamantylamine (Ada) via (i) addition of 10 µM of rapidly permeating indole, causing emission quenching of FAR-2. (ii) Addition of Ada, causing a displacement of both indole and the disassembly of FAR-2, such that the emission of free dye D2 is observed. See further below for a detailed description of the dye displacement approach. The experiments were carried out in 10 mM aqueous HEPES buffer, pH 7.
Supplementary Fig. 11 | Monitoring of enzymatic reactions with FAR-1 in homogenous solution, i.e., in the absence of a protective membrane. See Fig. 4 in the main text for the corresponding FARMA assays.

Supplementary Fig. 12 | Observed concentration dependent permeation rate of (a) indole, (b) TrpOMe, (c) tryptamine and (d) NAcTrpNH₂ into FAR-2-loaded liposomes (λₐₓ = 310 nm, λₑₘ = 370 nm). For indole, the rates were extracted from monoexponential fits of the time-resolved fluorescence traces. For the other analytes, the initial rate method was used because of the observed non-exponential shape of the kinetic traces. The error bars denote the estimated fitting errors of the initial rates. The dotted line is a fit of the data points were the rate-concentration dependence is approximately linear. Note the saturation behavior at higher analyte concentrations.
Calculation of the unimolecular permeation rate constants ($k_p$) and the apparent permeability coefficients ($P_{app}$).

The passive diffusion (permeation) of small non-charged compounds follows a modified Fick’s law and can be expressed by a first order kinetics. Similar to first order chemical reactions, the kinetics curve can therefore be fitted monoexponentially to obtained the observed permeation rates ($k_{obs}$). In our assay set-up, such an exponential fit is an approximation, since it neglects that a certain fraction of transported analyte is bound to the FAR and cannot exit the liposome. Nevertheless, the fit of the kinetic curves of the phenol and aniline series (see Supplementary Table S2) with a simple monoexponential decay equation generally yielded very good fits ($R^2 > 0.95$), indicating that the approximation is justified.

In our assay, we assumed a quantitative formation of the FAR receptor (experimental concentrations: 500 μM CB8 and 550 μM dye) inside the liposome and we assumed that each analyte molecule permeating into the interior of the liposome is being complexed by the artificial receptor (strong binding) and gives rise to a signal.

The analyte-specific unimolecular permeation rate constants ($k_p$) can be obtained by a linear regression of the experimentally $k_{obs}$ with $c_{analyte}$

$$k_{obs} = \left(\frac{k_p}{c_{FAR}}\right)c_{analyte} \quad (S1)$$

with $c_{FAR}$ as the concentration of the FAR receptor that is encapsulated inside the liposome. Alternatively, the initial transport rates ($v_i$) can be obtained by a linear fit of the initial part of the concentration-normalized fluorescent signal curves and $k_p$ can be obtained as the slope when plotting $v_i$ versus $c_{analyte}$. The initial rate method may be best choice for analytes that show a strongly non-monoexponential kinetic permeation profile and whose permeation is slow enough such that the initial part of the kinetic profile is reasonably linear given the experimental signal resolution. From these

For a unimolecular transport process, $P_{app}$ and $k_p$ are in a first approximation mathematically connected by

$$P_{app} = k_p \cdot (r/3) \quad (S2)$$

where the volume-to-surface correction factor $r/3$ accounts for the fact that the observed rates depend on the size of the liposomal assembly (characterized by radius $r$), and that smaller liposomes show apparently faster permeation rates. Combing equations S1 and S2, one arrives at

$$P_{app} = k_p \cdot (r/3) = k_{obs}/c_{analyte} \cdot c_{FAR} \cdot (r/3) \quad \text{(equation 1 in main text)}$$
Dye Displacement Membrane Assay

Dye displacement membrane assays rely on selective encapsulation of reporter pairs inside liposomes and the reversible interaction between the receptor and the translocated analyte. To conduct the assay, liposomes containing the chemosensing ensemble composed of a macrocyclic host and a dye are prepared and purified, such that a subsequently added analyte affects the dye fluorescence only if it can enter the vesicle and displaces the dye from the macrocycle. Until now, only the dye displacement has been used as the signaling mechanism for tandem membrane assays, \(^3,^4\) whereby the competitive displacement of the dye from such host-dye complexes upon analyte addition restores the fluorescence features of the dye in solution, Supplementary Fig. 13 - 14. Specifically, this signaling mechanism has been mostly employed with macrocyclic hosts with cavity space that is capable of forming 1:1 complexes, \(e.g.,\) cucurbit[7]uril (CB7) and the dyes berberine (BER) or palmatine (PAL), see Supplementary Fig. 13a, or with \(p\)-sulfonato calix[4]arene (CX4) and lucigenin. \(^3,^4\) Expanding the repertoire of chemosensors for tandem membrane assays, we exploit herein also the ability of a larger macrocyclic host such as cucurbit[8]uril (CB8) to form 1:1:1 ternary complexes, \(^5\)-\(^7\) and utilize here the quencher methyl viologen (MV) in combination with the fluorescent dyes 5-hydroxy tryptophan (5HO-Trp), 2-anilinonaphthalene-6-sulfonic acid (2,6-ANS) or dapoxyl sulfonic acid, sodium salt (DapoxS), Supplementary Fig. 13b. Upon addition of an analyte, the fluorescent dye, the quencher or both the fluorescent dye and quencher are displaced from the CB8 cavity, which in each case is reflected by an increase in the emission intensity of the dye (switch-on displacement assay), Supplementary Fig. 14-16. Notably, upon displacement, the emission of amphiphilic 2,6-ANS and DapoxS is likely further enhanced by their partitioning into the phospholipid membrane.

The dye displacement signal strategy has two important disadvantages compared to the preferred fluorescent artificial receptor (FAR) assays:

(i) The dye displacement strategy is limited to strongly binding analytes, \(i.e.,\) \(K(\text{analyte}) \geq K(\text{dye})\), otherwise a large excess concentration of analyte is needed to generate significant emission change. For instance, non-charged analytes at 10 \(\mu\text{M}\) concentration such as indole were generally not suitable to displace the dyes berberine (BER) or palmatine (PAL) from CB7. In other words, the CB7•BER and CB7•PAL are generally only applicable to analytes with a positively charged moiety such as tryptamine, but were not suitable for most analytes shown in Fig. 2 in the main text.
Similarly, the membrane-encapsulated chemosensing ensemble CB8•MV•2,6-ANS can detect the permeation of indole (conc. ≥ 1 µM) but FAR-2 is at least 10x more sensitive, see Fig. 3b in the main text. Likewise, weakly binding analytes at 10 µM analyte concentration such as p-cresol were unable to displace significant amounts of dye from CB8•MV•2,6-ANS.

(ii) The dye displacement strategy may result in apparent kinetic rates if the dye-dissociation kinetic is slow compared to the analyte permeation through the membrane. For instance, the dissociation rate of BER from CB7 was reported to be 0.8 s⁻¹ at 298 K,⁸ which implies that analyte-permeation kinetics that are comparably fast or faster cannot be monitored by the dye displacement approach (for instance, \( k_p = 2.7 \cdot 10^6 \text{ s}^{-1} \) for phenol, section “Time-resolved analyte translocation monitoring by FARMA” in the main text & see Table 1 in the main text). Conversely, associative binding of aromatic analytes by CB7 and ternary complex formation with CB8 show rates of \( >10^6 \text{ M}^{-1} \text{ s}^{-1} \),⁸⁻¹⁰ in agreement with our observations for the FARs. This very fast rate of analyte-receptor binding ensures that the FARs are capable to “immediately” respond to the concentration changes resulting from analyte diffusion through the membrane, even for the fastest membrane-permeable analytes such as toluene (Table 1 in the main text).

For purely aliphatic guests that are non-detectable with the FARs, the dye displacement method remains to date the best choice, e.g., see Supplementary Fig. 16 & 17.

Supplementary Fig. 13 | Graphical representation of membrane assays using dye displacement as a signaling mechanism. Diffusion of analytes through a lipid membrane is detected by displacement of the dye from a (a) host•dye or (b) host•dye•quencher complex, leading to a reduction or enhancement in the emission intensity.
**Supplementary Fig. 14** | Membrane permeation monitoring of analytes through the dye displacement approach. (a) Time-resolved changes in fluorescence intensity of CB7•BE-loaded liposomes ($\lambda_{\text{exc}} = 347$ nm, $\lambda_{\text{obs}} = 500$ nm) upon addition of the membrane permeable analyte tyramine and membrane destabilizing peptide melittin. No significant fluorescence changes other than photobleaching of BER, verified by control experiments is observed upon addition of spermine, cadaverine and phenylalanine, implying that these multiply charged species are membrane impermeable. (b) Evolution of fluorescence intensity after addition of dimethylbenzylamine (DBA) to CB7•PAL-loaded liposomes ($\lambda_{\text{exc}} = 347$ nm, $\lambda_{\text{obs}} = 500$ nm). (c) Fluorescence response after addition of slowly membrane permeable analyte tryptamine to liposomes loaded with CB8•MV•5OH-Trp ($\lambda_{\text{exc}} = 310$ nm, $\lambda_{\text{obs}} = 337$ nm). The black trace shows the control for the addition of the same volume of blank buffer. (d) Fluorescence response after addition of membrane permeable analytes propranolol and of impermeable Trp to liposomes loaded with CB8•MV•2,6-ANS ($\lambda_{\text{exc}} = 380$ nm, $\lambda_{\text{obs}} = 463$ nm).

**Supplementary Fig. 15** | Dye displacement-based membrane permeation assay recorded on a microplate reader (96-well plates) with CB8•MV•DapoxS. (a) In the absence of analyte, no significant change in the emission intensity occurred during 60 min, demonstrating the stability of the chemosensing ensemble in the microplate wells. (b-d) Normalized fluorescence readings prior to analyte (16 $\mu$M) addition (0 min) and after analyte addition (60 min equilibration) revealed an increase in fluorescence for (b) indole and (c) tryptamine, but no significant change for (d) tryptophan (Trp).
**Supplementary Fig. 16** | Dye-displacement-based membrane permeation assay for memantine ($K_a$ for CB8 approx. $10^{11}$ M$^{-1}$)$^{11,12}$ with CB8•MV•5HO-Trp-loaded liposomes. Unlike the examples shown in Supplementary Fig. 14c-d & 15 where the aromatic analyte displaced only the dye from the CB8•quencher•dye complex, in this example both the quencher and the dye are displaced from CB8 by the large, aliphatic guest memantine.

**Supplementary Fig. 17** | Addition of 50 µM adamantane derivatives to CB8•MV•5OHTrp-loaded liposomes. On account of the comparably small changes in relative fluorescence intensity for the negatively charged analyte 2-adamantylcarboxylate, multiple additions were performed to undoubtedly verify its membrane-permeation. See further below for a detailed description of the dye displacement approach.
### Supplementary Tables

**Supplementary Table 1** | Quenching efficiencies and binding constants for the formation of FAR-analyte complexes in 10 mM HEPES buffer (pH 7), determined by fluorescence titrations in homogenous solution.

| FAR    | analyte                                | QE\(^a\) | \(K_a / (10^3 \text{ M}^{-1})\)\(^b\) |
|--------|----------------------------------------|----------|-------------------------------------|
| FAR-1  | 1,2-dihydroxybenzene                   | >0.9     | 20                                  |
| FAR-1  | 1,4-benzoquinone                       | >0.9     | 0.5                                 |
| FAR-1  | 1,4-dihydroxybenzene                   | >0.9     | 13                                  |
| FAR-1  | 4-ethoxyphenol                         | >0.9     | 1                                   |
| FAR-1  | 4-ethylphenol                          | 0.6      | 1.2                                 |
| FAR-1  | 4-hydroxyphenylpyruvic acid (HPP)      | >0.9     | 17                                  |
| FAR-1  | SHO-Trp                                | >0.9     | 7.7                                 |
| FAR-1  | adrenaline                             | - - -    | <1                                  |
| FAR-1  | aniline                                | 0.9      | 33                                  |
| FAR-1  | benzene                                | <0.3     | 4                                   |
| FAR-1  | benzimidazole                          | 0.8      | 7.5                                 |
| FAR-1  | ceftazidime                            | - - -    | <1                                  |
| FAR-1  | chloramphenicol                        | - - -    | <1                                  |
| FAR-1  | dopamine                               | >0.9     | 0.10                                |
| FAR-1  | homogentisic acid (HGA)                | >0.9     | 0.14                                |
| FAR-1  | hydroquinone                           | >0.9     | 13                                  |
| FAR-1  | NAc-Trp                                | >0.9     | 50                                  |
| FAR-1  | NAc-TrpNH\(_2\)                        | >0.9     | 110                                 |
| FAR-1  | naphthalene                            | >0.9     | >50                                 |
| FAR-1  | naproxen                               | 0.5      | 2                                   |
| FAR-1  | N-phenylsuccinimide                    | >0.9     | 30                                  |
| FAR-1  | paracetamol                            | >0.9     | 7                                   |
| FAR-1  | Phe                                    | 0.5      | 5                                   |
| FAR-1  | phenol                                 | >0.9     | 56                                  |
| FAR-1  | phenylethylamine                       | 0.4      | 8                                   |
| FAR-1  | phenyl-β-D-galactopyranoside           | >0.9     | 30                                  |
| FAR-1  | prochloraz                             | 0.7      | 2.8                                 |
| FAR-1  | propanil                               | >0.9     | 8                                   |
| FAR-1  | propranolol                            | >0.9     | 3                                   |
| FAR-1  | quinoline                              | 0.6      | 2.2                                 |
| FAR-1  | ranitidine                             | 0.2      | 2                                   |
| FAR-1  | rotenone                               | 0.7      | 4.8                                 |
| FAR-1  | serotonin                              | >0.9     | 5                                   |
| FAR-1  | Trp                                    | >0.9     | 150                                 |
| FAR-1  | TrpNH\(_2\)                           | >0.9     | 460                                 |
| sensing ensemble | analyte                | QE$^a$ | $K_a$ / (10$^3$ M$^{-1}$)$^b$ |
|------------------|------------------------|--------|-----------------------------|
| FAR-1            | TrpOMe                 | >0.9   | 14                          |
| FAR-1            | tryptamine             | >0.9   | 50                          |
| FAR-2            | 2,6-dihydroxynaphthalene | >0.9   | 72$^c$                      |
| FAR-2            | 2,7-dihydroxynaphthalene | >0.9   | 66$^c$                      |
| FAR-2            | 2-cyanophenol          | >0.9   | 5.8                         |
| FAR-2            | 2-naphthol             | >0.9   | 140$^c$                     |
| FAR-2            | 4-cyanophenol          | >0.9   | 5.2                         |
| FAR-2            | 4-ethoxyphenol         | >0.9   | 1.0                         |
| FAR-2            | 4-iodophenol           | >0.9   | 4.1$^c$                     |
| FAR-2            | 4-methoxyphenol        | >0.9   | 2.4                         |
| FAR-2            | $p$-anisylboronic acid | >0.9   | 4.8                         |
| FAR-2            | aniline                | >0.9   | 25                          |
| FAR-2            | GlyGlyTrp              | >0.9   | 4.1$^c$                     |
| FAR-2            | GlyTrpGly              | >0.9   | 17$^c$                      |
| FAR-2            | hydroquinone           | >0.9   | 2.9                         |
| FAR-2            | indole                 | >0.9   | 180$^c$                     |
| FAR-2            | phenol                 | >0.9   | 15$^c$                      |
| FAR-2            | sesamol                | >0.9   | 4.2$^c$                     |
| FAR-2            | sodium benzoate        | n.a.   | <<1                         |
| FAR-2            | Trp                     | >0.9   | 34$^c$                      |
| FAR-2            | TrpGlyGly              | >0.9   | 120$^c$                     |
| CB8•MV           | 2,6-dihydroxynaphthalene | n.a.   | 590$^e$                     |
| CB8•MV           | 2,7-dihydroxynaphthalene | n.a.   | 160$^e$                     |
| CB8•MV           | 2-naphthol             | n.a.   | 610$^e$                     |
| CB8•MV           | 4-iodophenol           | n.a.   | 13$^e$                      |
| CB8•MV           | GlyGlyTrp              | n.a.   | 3.1$^d$                     |
| CB8•MV           | GlyTrpGly              | n.a.   | 21$^d$                      |
| CB8•MV           | NAc-TrpNH$_2$          | n.a.   | 3.1$^d$                     |
| CB8•MV           | Phe                    | n.a.   | 5.3$^d$                     |
| CB8•MV           | phenol                 | n.a.   | 22$^e$                      |
| CB8•MV           | Trp                    | n.a.   | 43$^d$                      |
| CB8•MV           | TrpGlyGly              | n.a.   | 130$^d$                     |
| CB8•MV           | TrpOMe                 | n.a.   | 63$^d$                      |
| CB8•MV           | tryptamine             | n.a.   | 54$^d$                      |
| CB8•MV           | Tyr                    | n.a.   | 2.2$^d$                     |
| CB7              | cadaverine             | AO, >0.9$^f$ | 4500$^i$                 |
| CB7              | $N,N$-dimethylbenzylamine(DMBA) | PAL, >0.9$^f$ | 3040$^g$               |
| CB7              | Phe                    | AO, >0.9$^f$ | 170$^j$                   |
| CB7              | phenylethylamine       | AO, >0.9$^f$ | 6800$^i$                 |
| CB7              | tryptamine             | DAPI, 0.8$^f$ | 130$^h$                 |
| sensing ensemble | analyte                    | QE\textsuperscript{a} | $K_a / (10^3 \text{ M}^{-1})$\textsuperscript{b} |
|------------------|---------------------------|------------------------|-----------------------------------------------|
| CB7              | tyramine                  | DAPI, 0.8\textsuperscript{f} | 3800\textsuperscript{g}                     |
| CB7              | vitamin B1                | PAL, >0.9\textsuperscript{f} | 6920\textsuperscript{g}                     |
| CB7              | nicotine                  | BE, 0.9\textsuperscript{f} | 160\textsuperscript{g}                      |
| CB7              | spermine                  | BE, 0.9\textsuperscript{f} | 2170\textsuperscript{g}                     |
| CB7              | 1-adamantanol             | k                      | $2.3 \times 10^7$                           |
| CB7              | 1-adamantylamine          | k                      | $1.7 \times 10^{11}$                       |
| CB7              | 1-adamantane-carboxylic acid | i                  | $3.2 \times 10^5$                           |
| CB7              | Tyr                       | m                      | 22                                            |

\textsuperscript{a} Quenching efficiency. \textsuperscript{b} Obtained by nonlinear least-square fitting to a 1:1 binding model in 10 mM phosphate buffer, taken from ref. 13. \textsuperscript{c} From ref. 13, in 10 mM sodium phosphate buffer (pH 7). \textsuperscript{d} From ref. 14, in 10 mM sodium phosphate buffer (pH 7), by isothermal titration calorimetry (ITC). Note that MV is not emissive. \textsuperscript{e} From ref. 15, in 10 mM sodium phosphate buffer (pH 7), by ITC; for other representative binding constants of CB8•MV with aromatic compounds see the same reference. \textsuperscript{f} From dye-displacement titrations; here, the quenching efficiency is reported as the total change in fluorescence intensity upon complete displacement of the dye from the host cavity, which is irrespective of the photophysical properties, e.g., quenching ability, of the dye. \textsuperscript{g} In 10 mM sodium phosphate buffer (pH 7). \textsuperscript{h} From ref. 16. \textsuperscript{i} From ref. 17. \textsuperscript{j} From ref. 18. \textsuperscript{k} From ref. 19, by ITC. \textsuperscript{l} From ref. 11, in 50 mM NaO\textsubscript{2}CCD\textsubscript{3}-buffered D\textsubscript{2}O (pD 4.74). \textsuperscript{m} From ref. 16, by ITC.
**Supplementary Table 2** | Experimentally observed rates ($k_{\text{obs}}$) and apparent permeability coefficients ($P_{\text{app}}$) for permeation of non-charged, rapidly permeating aromatic analytes through liposomal POPS:POPC bilayer membranes (r ca. 100 nm), their corresponding octanol-water partition coefficients ($\log P$), and van der Waals volumes ($V_W$); ordered from slowest (top) to fastest permeation rate.

| entry | analyte         | $k_{\text{obs}}$/s$^{-1}$ | $\log P$ | $V_W$/Å$^3$ |
|-------|-----------------|---------------------------|----------|-------------|
| 1     | toluene         | 75                        | 2.5      | 103         |
| 2     | propanil        | 42                        | 2.7      | 179         |
| 3     | 4CN-phenol      | 40                        | 1.7      | 111         |
| 4     | 4NO$_2$-phenol  | 38                        | 1.4      | 112         |
| 5     | $p$-xylene      | 17                        | 3.0      | 120         |
| 6     | 2CN-phenol      | 10                        | 1.7      | 111         |
| 7     | aniline         | 7.5                       | 1.2      | 98          |
| 8     | 4CN-aniline     | 6.5                       | 1.3      | 116         |
| 9     | 4F-aniline      | 4.4                       | 1.4      | 100         |
| 10    | 4Cl-aniline     | 4.0                       | 1.8      | 112         |
| 11    | 4Br-aniline     | 4.0                       | 2.1      | 119         |
| 12    | 4I-aniline      | 3.1                       | 2.6      | 127         |
| 13    | 4F-phenol       | 2.8                       | 1.8      | 96          |
| 14    | benzimidazole   | 2.7                       | 1.2      | 112         |
| 15    | phenol          | 2.7                       | 1.6      | 93          |
| 16    | indazole        | 2.4                       | 1.4      | 112         |
| 17    | 4Cl-phenol      | 2.2                       | 2.2      | 107         |
| 18    | indole          | 1.8                       | 1.6      | 117         |
| 19    | 4Br-phenol      | 1.6                       | 2.5      | 115         |
| 20    | N-phenylsuccinimide | 1.1           | 0.87     | 175         |
| 21    | 4I-phenol       | 0.69                      | 3.0      | 122         |
| 22    | DMABN           | 0.68                      | 2.4      | 150         |
| 23    | pentafluoroaniline | 0.51                  | 2.0      | 111         |
| 24    | 4Me-phenol      | 0.40                      | 2.1      | 110         |
| 25    | 2Me-phenol      | 0.33                      | 2.1      | 110         |
| 26    | 4Et-phenol      | 0.24                      | 2.6      | 127         |
| 27    | phenethylamine  | 0.22                      | 1.4      | 134         |
| 28    | 4FBu-phenol     | 0.062                     | 3.4      | 160         |

$^a$ Observed pseudo-unimolecular rates ($k_{\text{obs}}$), obtained by fitting of the time-resolved fluorescence intensities with a monoexponential decay function. Experiments were carried out at an analyte concentration of 40 µM (in the bulk) and a FAR-2 concentration of ca. 500 µM in the liposome interior; 20% error (reproducibility).

$^b$ $\log P$ (octanol) was obtained by using the software ChemDraw.

$^c$ van der Waals volume of analytes was calculated by using the software HyperChem.
### Supplementary Table 3 | Qualitative assessment of membrane permeability for more than 90 different organic compounds investigated by the FARMA-assay with receptors FAR-1, FAR-2, and FAR-3, and, for comparison, investigations by the dye displacement signal generation strategy with the sensing ensembles (CB8•MV•2,6-ANS, CB8•MV•5HO-Trp, CB7•BE, and CB7•PAL).

| analyte<sup>a</sup>                                                                 | FAR-1<sup>b</sup> | FAR-2<sup>c</sup> | FAR-3<sup>d</sup> | CB8•MV•2,6-ANS<sup>e</sup> | CB8•MV•5HO-Trp<sup>f</sup> | CB7•BE<sup>g</sup> | CB7•PAL<sup>h</sup> |
|--------------------------------------------------------------------------------------------|-------------------|-------------------|-------------------|-----------------------------|-----------------------------|-------------------|-------------------|
| 1,2-dihydroxybenzene (catechol)                                                            | +                 | + i               |                   |                             |                             |                   |                   |
| (R)-phenylephrine                                                                           | +                 |                   |                   |                             |                             |                   |                   |
| 1-adamantylamine (1-ADA)                                                                    |                   | +                 |                   |                             |                             |                   |                   |
| 1-adamantanecarboxylic acid (1-ADC)                                                        | +                 |                   |                   |                             |                             |                   |                   |
| 1-adamantanol                                                                               |                   | +                 |                   |                             |                             |                   |                   |
| 1,2-dichlorobenzene                                                                         |                   |                   | + k               |                             |                             |                   |                   |
| 1,4-benzoquinone                                                                            |                   |                   | + k               |                             |                             |                   |                   |
| 1,4-dihydroxybenzene (hydroquinone)                                                        | +                 |                   |                   |                             |                             |                   |                   |
| 1,4-dimethylbenzene                                                                         |                   |                   |                   |                             |                             |                   |                   |
| (p-xylene)                                                                                   |                   |                   |                   |                             |                             |                   |                   |
| 1-naphthyl phosphate                                                                        |                   |                   |                   |                             |                             |                   |                   |
| 2-cyanophenol                                                                               | +                 |                   |                   |                             |                             |                   |                   |
| 2-methylnaphthalene (o-cresol)                                                              | +                 |                   |                   |                             |                             |                   |                   |
| 2-naphthol (2-NpOH)                                                                         | +                 |                   |                   |                             |                             |                   |                   |
| 2-naphthyl phosphate                                                                        |                   |                   |                   |                             |                             |                   |                   |
| 2-naphthylsulfonamide                                                                       |                   |                   | + o               |                             |                             |                   |                   |
| 2-phenylbenzimidazole                                                                       |                   |                   |                   |                             |                             |                   |                   |
| (Ph-benzimidazole)                                                                           |                   |                   |                   |                             |                             |                   |                   |
| 2-phenylbenzimidazole-5-sulfonic acid (O<sub>2</sub>SPh-benzimidazole)                     |                   |                   |                   |                             |                             |                   |                   |
| 2,7-dihydroxynaphthalene (2,7-Np(OH)<sub>2</sub>)                                           | +                 |                   |                   |                             |                             |                   |                   |
| 3-phenylpropanoic acid (hydrocinnamic acid)                                                  |                   |                   |                   |                             |                             |                   |                   |
| 3,5-dimethyladamantan-1-amine (memantine)                                                    |                   |                   |                   |                             |                             |                   |                   |
| 4-(N,N-dimethyl amino) benzonitrile (DMABN)                                                  | +                 |                   |                   |                             |                             |                   |                   |
| 4-bromoaniline                                                                              | +                 |                   |                   |                             |                             |                   |                   |
| 4-bromobenzene                                                                              |                   | + k               |                   |                             |                             |                   |                   |
| 4-bromophenol                                                                               | +                 |                   |                   |                             |                             |                   |                   |
| 4-chloroaniline                                                                             | +                 |                   |                   |                             |                             |                   |                   |
| 4-chlorophenol                                                                              | +                 |                   |                   |                             |                             |                   |                   |
| 4-cyanoaniline                                                                              | +                 |                   |                   |                             |                             |                   |                   |
| 4-cyanophenol                                                                               | +                 |                   |                   |                             |                             |                   |                   |
| analyte                                          | FAR-1 | FAR-2 | FAR-3 | CB8•MV•2,6-ANS | CB8•MV•5HO-Trp | CB7•BE | CB7•PAL |
|-------------------------------------------------|-------|-------|-------|----------------|----------------|--------|--------|
| 4-ethoxyphenol                                  |       |       |       |                |                |        |        |
| 4-ethylphenol                                   |       |       |       |                |                |        |        |
| 4-fluoroaniline                                 |       |       |       |                |                |        |        |
| 4-fluorophenol                                  |       |       |       |                |                |        |        |
| 4-hydroxybenzyl acetone (raspberry ketone)      |       |       |       |                |                |        |        |
| 4-hydroxyphenylpyruvic acid (HPPA)              |       |       |       |                |                |        |        |
| 4-idoaniline                                    |       |       |       |                |                |        |        |
| 4-iodophenol                                    |       |       |       |                |                |        |        |
| 4-methoxyphenol                                 |       |       |       |                |                |        |        |
| 4-methoxythiophenol                             |       |       |       |                |                |        |        |
| 4-methoxyphenylboronic acid (p-anisylboronic acid) |       |       |       |                |                |        |        |
| 4-methylaniline                                 |       |       |       |                |                |        |        |
| 4-methylphenol (p-cresol)                       |       |       |       |                |                |        |        |
| 4-nitrophenol                                   |       |       |       |                |                |        |        |
| 4-tert-butylphenol                              |       |       |       |                |                |        |        |
| 5-hydroxytryptophan (5HO-Trp)                   |       |       |       |                |                |        |        |
| adamantane                                      |       |       |       |                |                |        |        |
| adrenaline                                      |       |       |       |                |                |        |        |
| ampicillin                                      |       |       |       |                |                |        |        |
| aniline                                         |       |       |       |                |                |        |        |
| anthracene                                      |       |       |       |                |                |        |        |
| bentazon                                        |       |       |       |                |                |        |        |
| benzene                                         |       |       |       |                |                |        |        |
| benzimidazole                                   |       |       |       |                |                |        |        |
| bisphenol A                                     |       |       |       |                |                |        |        |
| cadaverine                                      |       |       |       |                |                |        |        |
| carbendazim                                     |       |       |       |                |                |        |        |
| N,N-dimethylbenzylamine (DMBA)                  |       |       |       |                |                |        |        |
| dopamine                                        |       |       |       |                |                |        |        |
| ethyl 4-aminobenzoate (benzocaine)              |       |       |       |                |                |        |        |
| imidacloprid                                    |       |       |       |                |                |        |        |
| indazole                                        |       |       |       |                |                |        |        |
| indole                                          |       |       |       |                |                |        |        |
| lansoprazole                                    |       |       |       |                |                |        |        |
| melatonin                                       |       |       |       |                |                |        |        |
| melittin                                        |       |       |       |                |                |        |        |
| analyte                      | FAR-1<sup>b</sup> | FAR-2<sup>b</sup> | FAR-3<sup>d</sup> | CB8•MV•2,6-ANS<sup>e</sup> | CB8•MV•5HO-Trp<sup>f</sup> | CB7•BE<sup>g</sup> | CB7•PAL<sup>h</sup> |
|-----------------------------|------------------|-----------------|------------------|---------------------------|-------------------------|-----------------|------------------|
| benzene                     |                  |                 |                  |                           |                         |                 |                  |
| benzimidazole               | +                | +               | +                |                          |                         |                 |                  |
| methyl benzoate             | +<sup>k</sup>    |                 |                  |                          |                         |                 |                  |
| N-acetyl tryptophan (NAc-Trp) |                  |                 |                  |                          |                         |                 |                  |
| N-acetyl tryptophan amide (NAc-TrpNH<sub>2</sub>) | +    | +          | +<sup>m</sup> |                          |                         |                 |                  |
| N-phenylsuccinimide         | +                |                 |                  |                          |                         |                 |                  |
| naphthalene                 | +<sup>k</sup>    |                 |                 |                         |                         |                 |                  |
| nicotine                    |                 | +               |                  |                          |                         |                 |                  |
| omeprazole                  | +<sup>k</sup>    |                 |                  |                          |                         |                 |                  |
| paracetamol                 | +                | +              |                  |                          |                         |                 |                  |
| penicillin G                | +                |                 |                  |                          |                         |                 |                  |
| pentafluoroaniline          | +                |                 |                  |                          |                         |                 |                  |
| phenanthroline              | +<sup>k</sup>    |                 |                  |                          |                         |                 |                  |
| phenol                      | +                |                 |                  |                          |                         |                 |                  |
| phenyl-β-D-galactopyranoside| +                |                 |                  |                          |                         |                 |                  |
| phenylalanine (Phe)         | +                |                 |                  |                          |                         |                 |                  |
| phenylethylamine            | +                |                 |                  |                          |                         |                 |                  |
| primaquine                  | +                |                 |                  |                          |                         |                 |                  |
| propanil                    | +                |                 |                  |                          |                         |                 |                  |
| propranolol                 | +                |                 |                  |                          |                         |                 |                  |
| quinoline                   | +                | +              |                  |                          |                         |                 |                  |
| rabenzazole                 | +<sup>k</sup>    |                 |                  |                          |                         |                 |                  |
| ranitidine                  | +                |                 |                  |                          |                         |                 |                  |
| serotonin                  | +                |                 |                  |                          |                         |                 |                  |
| somatostatin                | +                |                 |                  |                          |                         |                 |                  |
| spermine                    | +                |                 |                  |                          |                         |                 |                  |
| thiacendazole               | +<sup>k</sup>    |                 |                  |                          |                         |                 |                  |
| toluene                     | +                |                 |                  |                          |                         |                 |                  |
| tropicamid                   | +<sup>k</sup>    |                 |                  |                          |                         |                 |                  |
| Trp-(Ala)<sub>6</sub>-NH<sub>2</sub> |                  |                 |                  |                          |                         |                 |                  |
| Trp-(Leu)<sub>6</sub>-NH<sub>2</sub> |                  |                 |                  |                          |                         |                 |                  |
| Trp-(Lys)<sub>6</sub>-NH<sub>2</sub> |                  |                 |                  |                          |                         |                 |                  |
| tryptamine                  | +                | +              |                  |                          |                         |                 |                  |
| tryptophan (Trp)            | +                |                 |                  |                          |                         |                 |                  |
| tryptophan amide (TrpNH<sub>2</sub>) | +          | +            |                  |                          |                         |                 |                  |
| tryptophan methyl ester (TrpOMe) | +         | +            |                  |                          |                         |                 |                  |
| tyramine                    | +                |                 |                  |                          |                         |                 |                  |
| tyrosine (Tyr)              | +                |                 |                  |                          |                         |                 |                  |
| vitamin B1                  | +                |                 |                  |                          |                         |                 |                  |

<sup>a</sup> <sup>b</sup> <sup>c</sup> <sup>d</sup> <sup>e</sup> <sup>f</sup> <sup>g</sup> <sup>h</sup>
See also Fig. 2 in main text for chemical structures. \( \lambda_{\text{exc}} = 400 \text{ nm}, \lambda_{\text{obs}} = 450 \text{ nm}. \)

\( \lambda_{\text{exc}} = 310 \text{ nm}, \lambda_{\text{obs}} = 350 \text{ nm}. \)

\( \lambda_{\text{exc}} = 330 \text{ nm}, \lambda_{\text{obs}} = 370 \text{ nm}. \)

\( \lambda_{\text{exc}} = 380 \text{ nm}, \lambda_{\text{obs}} = 463 \text{ nm}. \)

\( \lambda_{\text{exc}} = 310 \text{ nm}, \lambda_{\text{obs}} = 337 \text{ nm}. \)

\( \lambda_{\text{exc}} = 347, \lambda_{\text{obs}} = 500 \text{ nm}. \)

\( \lambda_{\text{exc}} = 347, \lambda_{\text{obs}} = 500 \text{ nm}. \)

Small changes (higher conc.).

Analyte was dissolved in ethanol (10 mM stock).

Emerging excimer band at \( \lambda_{\text{obs}} = 500 \text{ nm} \) was monitored.

Control experiment in the absence of host (CB8) was also carried out.

\( \lambda_{\text{obs}} = 500 \text{ nm} \) was chosen to by-pass the self-fluorescence band from the analyte.

Melittin is a membrane-disrupting peptide; the fluorescence response in this case is due to the release of the reporter pairs from liposomes, shifting the complexation equilibria towards the un-complexed fluorescent dye on account of dilution.
Supplementary References

1. M. Beals LG, S. Harrell. DIFFUSION THROUGH A CELL MEMBRANE. [cited 11.08.2019] Available from: http://www.tiem.utk.edu/~gross/bioed/webmodules/diffusion.htm
2. Chakrabarti, A.C. & Deamer, D.W. Permeability of lipid bilayers to amino acids and phosphate. Biochim. Biophys. Acta - Biomembranes 1111, 171-177 (1992).
3. Ghale, G. et al. Chemosensing Ensembles for Monitoring Biomembrane Transport in Real Time. Angew. Chem. Int. Ed. 53, 2762-2765 (2014).
4. Norouzy, A., Azizi, Z. & Nau, W.M. Indicator Displacement Assays Inside Live Cells. Angew. Chem. Int. Ed. 54, 792-795 (2015).
5. Biedermann, F. & Scherman, O.A. Cucurbit[8]uril Mediated Donor–Acceptor Ternary Complexes: A Model System for Studying Charge-Transfer Interactions. J. Phys. Chem. B 116, 2842-2849 (2012).
6. Biedermann, F., Vendruscolo, M., Scherman, O.A., De Simone, A. & Nau, W.M. Cucurbit[8]uril and Blue-Box: High-Energy Water Release Overwhelms Electrostatic Interactions. J. Am. Chem. Soc. 135, 14879-14888 (2013).
7. Kim, H.-J. et al. Selective Inclusion of a Hetero-Guest Pair in a Molecular Host: Formation of Stable Charge-Transfer Complexes in Cucurbit[8]uril. Angew. Chem. Int. Ed. 40, 1526-1529 (2001).
8. Miskolczy, Z. & Biczók, L. Kinetics and Thermodynamics of Berberine Inclusion in Cucurbit[7]uril. J. Phys. Chem. B 118, 2499-2505 (2014).
9. Miskolczy, Z. & Biczok, L. Sequential Inclusion of Two Berberine Cations in Cucurbit[8]uril Cavity: Kinetic and Thermodynamic Studies. Phys. Chem. Chem. Phys. 16, 20147-20156 (2014).
10. Tang, H. et al. Guest Binding Dynamics with Cucurbit[7]uril in the Presence of Cations. J. Am. Chem. Soc. 133, 20623-20633 (2011).
11. Liu, S. et al. The Cucurbit[n]uril Family: Prime Components for Self-Sorting Systems. J. Am. Chem. Soc. 127, 15959-15967 (2005).
12. Cao, L. et al. Cucurbit[7]uril-Guest Pair with an Attomolar Dissociation Constant. Angew. Chem. Int. Ed. 53, 988-993 (2014).
13. Biedermann, F. et al. Benzobis(imidazolium)–Cucurbit[8]uril Complexes for Binding and Sensing Aromatic Compounds in Aqueous Solution. Chem. Eur. J. 16, 13716-13722 (2010).
14. Bush, M.E., Bouley, N.D. & Urbach, A.R. Charge-Mediated Recognition of N-Terminal Tryptophan in Aqueous Solution by a Synthetic Host. J. Am. Chem. Soc. 127, 14511-14517 (2005).
15. Rauwald, U., Biedermann, F., Deroo, S., Robinson, C.V. & Scherman, O.A. Correlating Solution Binding and ESI-MS Stabilities by Incorporating Solvation Effects in a Confined Cucurbit[8]uril System. J. Phys. Chem. B 114, 8606-8615 (2010).
16. Bailey, D.M., Hennig, A., Uzunova, V.D. & Nau, W.M. Supramolecular Tandem Enzyme Assays for Multiparameter Sensor Arrays and Enantiomeric Excess Determination of Amino Acids. Chem. Eur. J. 14, 6069-6077 (2008).
17. Nau, W.M., Ghale, G., Hennig, A., Bakirci, H.s. & Bailey, D.M. Substrate-Selective Supramolecular Tandem Assays: Monitoring Enzyme Inhibition of Arginase and Diamine Oxidase by Fluorescent Dye Displacement from Calixarene and Cucurbit[8]uril Macrocycles. J. Am. Chem. Soc. 131, 11558-11570 (2009).
18. Ghale, G., Kuhnert, N. & Nau, W.M. Monitoring stepwise proteolytic degradation of peptides by supramolecular domino tandem assays and mass spectrometry for trypsin and leucine aminopeptidase. Nat. Prod. Commun. 7, 343-348 (2012).
19. Moghaddam, S. et al. New Ultrahigh Affinity Host-Guest Complexes of Cucurbit[7]uril with bicyclo[2.2.2]octane and adamantane guests: Thermodynamic analysis and evaluation of M2 affinity calculations. J. Am. Chem. Soc. 133, 3570-3581 (2011).
