Supplementary Information

Application in Enzyme Encapsulation of Fluorinated Dendritic Amphiphiles and their Stomatosome Aggregates

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2 DLS

Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd.) in backscattering mode (detection angle = 173°) at a constant temperature of T = 22.5 °C using quartz Hellma® QL Macro cells (Hellma GmbH & Co., Müllheim, Germany). Water was used from a Milli-Q station from Millipore (Merck, Millipore) with minimum resistivity of 18.0 M Ω cm. At least 1 ml of sample was used. Each sample was measured 3 times the quality of the data was evaluated by the software and outliers were excluded from the average. 11 runs were done for each measurement, each lasting 10 s (i.e., total of 33 runs for each sample). Data evaluation was performed using the Zetasizer software (Malvern Panalytical Ltd., Malvern, United Kingdom). The cumulant method was used: the initial part of the autocorrelation function is fitted into a single exponential decay where the first and the second cumulant terms provide the *z*-average size and PDI, respectively.[1] All samples were filtered through 0.45 µm regenerated cellulose membrane syringe filters to homogenize the dispersions and remove dust particles. Diluted solutions were left at room temperature for a few hours to equilibrate.

Table S1: DLS results in PBS buffer for stomatosomes formed from amphiphile **1** encapsulating urease. These samples were not filtrated through a membrane to preserve the stomatosome structure.

z-average (d.nm)		Intensity		Volume		Number	
358		Size (d.nm)	%	Size (d.nm)	%	Size (d.nm)	%
PDI		700 ± 300	69	1000 ± 300	84	110 ± 40	98
0.503		150 ± 50	29	140 ± 50	13	600 ± 300	2
		5200 ± 500	2	5300 ± 700	3	-	-

2.1 CAC determination

The CAC was determined by following the increase in count rate with increase in concentration. The formation of aggregates results in an increase of derived count rate with concentration, while below the CAC the concentration has no effect. Two linear regressions can be drawn, and their intercept corresponds to the CAC.

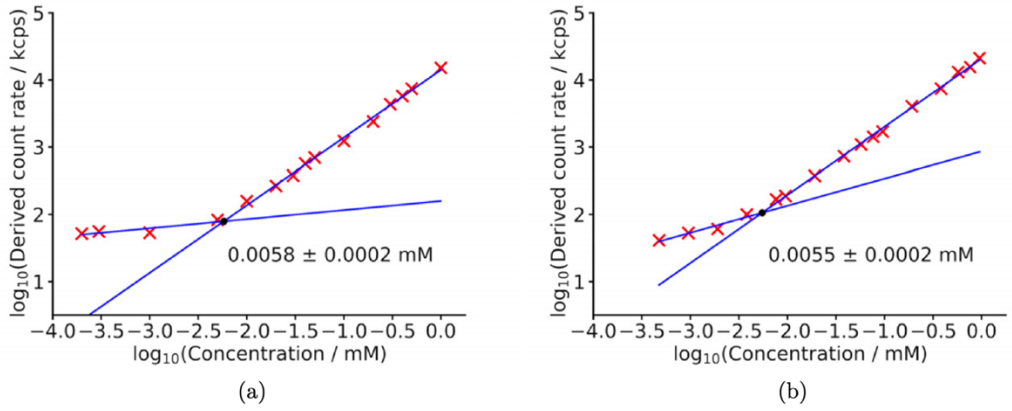

Figure S1 : CAC of (a) **1** and (b) **2** in ultrapure water from the derived count rate from DLS measurements. The data analysis and error calculation was done using the scipy.stats module. The same was done in for measurements in PBS.

3 Microscopy

3.1 (Cryogenic) transmission electron microscopy

The samples for cryo-TEM were prepared in a vitrobot Mark IV (ThermoFisher Scientific Inc., Waltham, MA, USA). The chamber's climate was set to 22 °C and 100% humidity. After placing a droplet (4 µL) of the sample solution on a hydrophilized (60 s Plasma treatment at 8 W using a BALTEC MED 020 device) perforated Quantifoil grid (Quantifoil Micro Tools GmbH, Großlöbichau, Germany) excess fluid is automatically blotted off to create an ultrathin layer (typical thickness of 100 nm) of the solution spanning the holes of the film. The grids are immediately propelled into liquid ethane at its freezing point (–184 °C). This ultra-fast cooling of the aqueous solution (vitrification) is necessary to produce artifact-free cryo-samples by avoiding crystallization of the solvent or rearrangement of the assemblies. The vitrified grids were assembled to so-called autogrids, which can be stored under liquid nitrogen before use. Autrogrids were then transferred into a Talos® Arctica transmission electron microscope (ThermoFisher Scientific Inc., Waltham, MA, USA) using the microscope's autoloader transfer routine. The microscope is illuminated by an X-FEG cathode operated at 200 kV acceleration voltage. Micrographs were recorded on a Falcon 3CE direct electron detector at full size (4K) at a magnification of 28.000× using the microscopes low-dose protocol. The defocus was chosen to be 4 µm in all cases to create sufficient phase contrast.

3.2 Confocal laser scanning microscopy

Fluorescence images were taken using Leica confocal microscope (TCS SP8, Germany). The 3D images were generated from z-stacks using the Leica Application Suite X Software. The oil immersion objective was used (HC PL APO 63x/1,40 OIL CS2), setting the pinhole aperture of the confocal system at 999.55 mAU. Cy5 dye was purchased from FEW Chemicals GmbH (index S 0944). The lasers used were a diode laser (405 nm), argon (488 nm, power emission at objective 19.0 W), and HeNe (633 nm). The detectors used were HyD (494 – 581 nm and 640 – 720 nm). Hybrid detectors or HyD use two technologies: vacuum tubes (like in PMTs), and semiconductor microelectronics. Images were taken in a sequential order and not simultaneously, which increases the image quality by avoiding cross-talk. The transmission microscopy channel used the PMT detector. The scan speed was 600 Hz for 2608 × 2608 pixel images. Cy5 was encapsulated by the thin-film hydration method with a concentration of 5 µM.

3.3 STED

Time-gated STED (gSTED) microscopy was performed using an Abberior Instruments Expert Line STED setup equipped with an inverted IX83 microscope (Olympus, Japan), two pulsed STED lasers for depletion at 775 nm (0.98 ns pulse duration, up to 80 MHz repetition rate) and at 595 nm (0.52 ns pulse duration, 40 MHz repetition rate) and pulsed excitation lasers (at 488 nm, 561 nm and 640 nm). Multi-channel 2D confocal and gSTED images were acquired with a 100× oil-immersion objective lens (UPLSAPO100XO, Olympus, NA = 1.4), with a pixel dwell time of 2 µs, with 10x and 30x line accumulation, respectively, at 16-bit sampling and a field of view of 10 µm x 10 µm. Lateral pixel size was set to 20 nm. Time gating was set at 750 ps with a width of 8 ns. Fluorescence signals were detected sequentially by line by avalanche photodiode detectors at appropriate spectral regions. ATTO 565 confocal and gSTED images were acquired following acquisition of the other channels.

These procedures were operated by the software Imspector (version 16.3.15507, Abberior Instruments, Germany).
Raw gSTED images were processed for Richardson-Lucy deconvolution with default settings using the Imspector software (version 16.3.15507, Abberior Instruments, Germany). The point spread function was automatically computed with a 2D Lorentzian function having a full- width half- maximum of 40 nm, based on measurements with 40 nm Crimson beads.

4 Labelling of urease

4.1 ATTO 565 NHS-ester

ATTO 565 NHS-ester (ATTO-TEC GmbH) was used to label urease following a procedure from ATTO-TEC. A 0.2 M sodium bicarbonate solution was made, which was adjusted to pH 9.0 with 2 M NaOH. A labelling buffer (pH = 8.3) was made using 20 parts PBS buffer and 1 part sodium bicarbonate solution. The dye (1.0 mg) was dissolved in 200 µL of anhydrous DMSO. Urease (TCI, 5 mg) was dissolved in 1 ml of labelling buffer. A dye to protein molar ratio of 9 was used. Dye was added as dissolved in DMSO to the enzyme while gently shaking, and the mixture was incubated protected from light for 18 hours at ambient temperature. The labelled urease was separated from free ATTO 565 and dye aggregates or hydrolysis products on a Sephadex® G-25 column (PBS as buffer mobile phase). Labelled enzyme was stored in the dark at 4 °C.

The labelling efficiency was determined by UV–Vis and expressed as the dye to protein ratio. Quartz Hellma® QL Macro cell were used for the measurements. The urease extinction coefficient was calculated by taking UV–Vis absorption spectra of urease solutions at different concentrations. They were measured between 200 and 1100 nm on the UV–Vis spectrometer Agilent Cary 8454. The urease was purified on a PD-10 column before measuring, which contains the same stationary phase as Sephadex® G-25 but allows a known volume to exit the column. This volume contains all the material that was added initially and so the concentration is known. Before encapsulation experiments, the ATTO 565–urease solution was concentrated on Vivaspin® 3 kDa MWCO to the desired volume (1000 RCF).

The degree of labelling (DOL), i.e., the dye to protein molar ratio, was calculated using the following formula:

$$DOL = \frac{A_{564}}{\varepsilon' \times protein\ concentration\ /\ M}$$

Where:

$$Protein\ concentration\ /\ M\ = \frac{A_{280} - (A_{564} \times CF)}{\varepsilon}$$

A_{564} is the maximum absorbance of ATTO 565 dye and CF is the correction factor as ATTO 565 also absorbs at 280 nm. ε' is the extinction coefficient of FITC, which is 1.2 x 10^5 M^{-1} cm^{-1}.[2] The CF is 0.12 for ATTO 565 at 280 nm. The DOL was determined to be 0.340 ± 0.005.

5 Enzyme encapsulation

5.1 Thin-film hydration method

All experiments with urease used phosphate buffered saline (PBS) buffer as a solvent. For 1.00 L of buffer, NaCl (8.00 g, 0.1370 M), KCl (0.20 g, 0.0027 M), sodium phosphate dibasic (1.44 g, 0.0100 M), potassium phosphate monobasic (0.25 g, 0.0018 M) were all dissolved in 1.00 L distilled water and the pH was adjusted to 7.4.

To make the film, amphiphile was dissolved in MeOH and solvent was evaporated in vacuo at 250 mbar with slow stirring to incite a homogenous distribution in the round-bottom flask. Residual solvent was removed on the high vacuum line for 1 – 2 h (1×10^{-3} mbar). The amphiphile was hydrated with either enzyme-containing solution or PBS buffer and sonicated (10 min) then vortexed (20 s, room temperature). This was repeated 4 times for the amphiphile to be fully dissolved. The concentrations used were 5.0 mg mL^{-1} of urease and 20 mg mL^{-1} of amphiphile.

5.2 Separation of free and encapsulated urease

The centrifuge Rotina 380R (Andreas Hettich GmbH & Co. KG) was used. After hydrating the amphiphile film, the samples were centrifuged at room temperature (30 min, 6300 RCF). The supernatant was removed, and the same volume of PBS was added. This was repeated three times, as all free enzyme was removed after this point, which was verified by BCA assays.

5.3 BCA assays

Pierce BCA Protein Quantification Kit was purchased from ThermoFisher. Urease samples of known concentrations were used to make a standard curve. The working reagent was prepared by mixing reagant A and B (50:1). The microplate (Sarstedt TC plate 96 well, standard, F) procedure was followed: 25 µL was used for each sample and 200 µL of working reagent was added to each well then mixed. They were incubated at 37 °C for 30 min and the absorbance was measured at 562 nm after cooling to room temperature. Protein concentration was determined from the standard curve. The standard curve was found as a 3 degree polynomial using numpy.polyfit from the NumPy Python package. The mass of urease was measured using the microbalance Sartorius CPA2P.

5.4 Pyrene

Pyrene was encapsulated using the thin-film hydration method. Pyrene was dissolved in acetone and amphiphile **1** in MeOH. Only pyrene or both pyrene and the amphiphile were made into a dry film. The films were hydrated using PBS buffer with final concentrations of 2 µM for pyrene and 20 mg mL^{-1} for the amphiphile. Fluorescence spectroscopy was used to obtain the spectra of pyrene in different microenvironments. Samples were diluted to avoid saturation the detector and were analyzed in quartz Hellma® QL Macro cells using a fluorescence spectrometer Jasco FP-6500 at room temperature. The excitation wavelength used was 317 nm. Intensity ratios can be used to determine the micro-environment of pyrene, as the pattern of pyrene fluorescence fine structure varies with solvent polarity.[3] The signal I_1 can be found at λ_{em} = 373 nm and I_3 at λ_{em} = 384 nm. This $\frac{I_1}{I_3}$ ratio

decreases with increasing hydrophobicity or decreasing dielectric constant. This is observed here where $\frac{I_1}{I_3}$ = 1.17 when encapsulated and $\frac{I_1}{I_3}$ = 1.51 when free inaqueous solution.

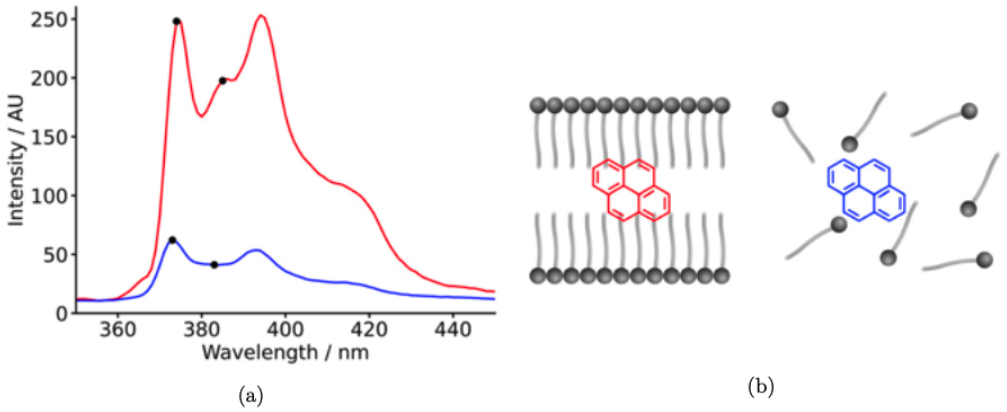

Figure S2: In (a), fluorescence emission spectrum of pyrene monomers in presence of stomatosomes (red) and without (blue), and in (b) a drawing of pyrene in apolar (red) and polar (blue) environments.

6 Urease activity assays

The urease activity assay kit from Sigma-Aldrich (MAK120) was used to determine urease enzymatic activity. An ammonium chloride standard curve was made for each assay, and standard errors were obtained from the linear regression uncertainty. Urea solution (10 µL, concentration not given in technical bulletin) was added to urease samples of equivalent concentration and incubated for 10 min at room temperature. Then 100 µL of the sample was added to wells in a 96-well plate (Sarstedt TC plate 96 well, standard, F), 100 µL of reagent A was added then 50 µL of B, mixing each time. The reaction was incubated in the dark at room temperature for 30 min, and the absorbance was measured at 670 nm. The urease activity was calculated using the following equation:

$$Urease\ activity\ /\ units\ L^{-1} = \frac{(A_{670})_{sample} - (A_{670})_{blank}}{Slope \times t}$$

Where A_{670} is the absorbance at 670 nm of either the investigated sample or the blank, t is the incubation time and the slope is found from a linear regression analysis of the standard curve, which was done using Microsoft Excel data analysis tools. One unit of urease is the amount of enzyme that catalyzes the formation of 1.0 mmol of ammonia per minute at pH 7.0. The activity was normalized when plotted with other results for comparison.

The activity was measured from supernatants after separation of free and encapsulated enzyme. Urease was encapsulated in the same way as presented before, with the only difference being that each supernatant was assayed for urease activity. Before centrifugation, samples were incubated with urea for 10 min. They were then centrifugated and the supernatant was removed and used in an activity assay as described above. The same amount of PBS buffer was then added, the pellet was redissolved by vortexing, and the same procedure was repeated three times, so when no more free enzyme remains in the supernatant after centrifugation.

To compare free and encapsulated urease activity, enzyme was again encapsulated within stomatosomes or closed vesicles in the same way as before, or simply dissolved in PBS buffer. The EE% was determined with BCA assays and samples were all diluted to the same concentration. Samples were assayed for urease activity.

To reuse urease and evaluate its activity over multiple reuse cycles, it was either dissolved free in PBS solution or encapsulated within stomatosomes. The EE% was determined with BCA assays and samples were diluted to the same concentration. Urea was incubated in the samples, and the reaction products were removed either by centrifugation (for encapsulated enzyme) or by ultrafiltration (Vivaspin® 50 kDa MWCO) and assayed as described previously.

In figure S3, enzyme activity was measured by assaying the supernatant collected when separating free and encapsulated enzyme. The reaction products that are assayed are also present in the supernatant. In the first supernatant, both free and encapsulated enzyme are active and so activities are comparable for perforated and closed vesicles. Upon assaying the supernatant after a second round of centrifugation, there is less free enzyme and so there is now a differentiation between the closed and perforated systems. In the last supernatant, effectively all free enzyme has been removed (confirmed by BCA assays) and the enzyme in closed vesicles clearly shows reduced activity compared to enzyme in stomatosomes. In this experiment, EE% were not measured after each stage, and so part of the activity difference may be due to a difference in amount of enzyme in closed and perforated vesicles. However, the EE% was not very different for closed vesicles: we observed a 7% difference, which is not large enough to justify the 26% loss in activity. We cannot say for sure that here the activity difference is only because of the perforations, but it is interesting to see the differentiation between the two systems after consecutive centrifugation cycles.

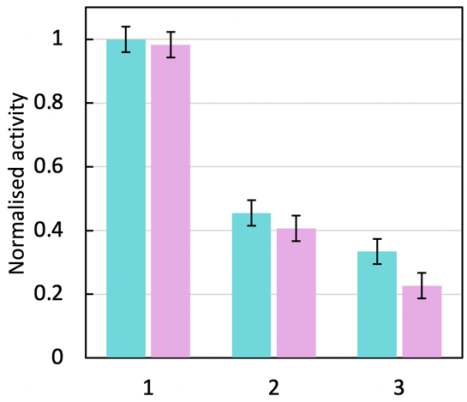

Figure S3: Normalised activity at room temperature measured from the supernatant after separation of free and encapsulated enzyme (closed vesicles in pink and stomatosomes in blue) with consecutive centrifugation cycles (1 to 3). As free enzyme is removed, difference in activity of the two systems can be seen.

7 References

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