

Thiacalixarene Carboxylic Acid Derivatives as Inhibitors of Lysozyme Fibrillation

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Electronic Supplementary Information (28 pages)

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S1. Materials and Methods

S1.1. General

^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra were obtained on a Bruker Avance-400 spectrometer (Bruker Corp., Billerica, MA, USA) ($^{13}\text{C}\{^1\text{H}\}$ – 100 MHz and ^1H – 400 MHz). The chemical shifts were determined against the signals of residual protons of deuterated solvent (D_2O). The concentrations of the compounds were equal to 3–5% by the weight in all the records. The FTIR spectra were recorded on the Spectrum 400 FTIR ATR spectrometer (Perkin Elmer Inc, Waltham, MA, USA) with a Diamond KRS-5 attenuated total internal reflectance attachment (resolution 0.5 cm^{-1} , accumulation of 64 scans, recording time 16 s in the wavelength range $400\text{--}4000\text{ cm}^{-1}$). ESI-HRMS experiments were performed at Agilent 6550 iFunnel Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA), equipped with Agilent 1290 Infinity II LC. Melting points were determined using Boetius Block apparatus (VEB Kombinat Nagema, Radebeul, Germany).

Chemically pure organic solvents were purified by standard methods. All the aqueous solutions were prepared with the Millipore-Q deionized water ($>18.0\text{ M}\Omega \times \text{cm}$ at 298 K).

S1.2. Dynamic light scattering (DLS)

S1.2.1. Particles' size

The distribution of particles by number, volume, and intensity, the polydispersity index were determined by dynamic light scattering (DLS) on a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK) in quartz cuvettes. The instrument is equipped with the 4 mW He-Ne laser (633 nm). Measurements were performed at a detection angle of 173° . The error in determining the particle size is less than 2%. The results were processed by the DTS program (Dispersion Technology Software 4.20). Deionized water was used to prepare solutions for studying the aggregation of **TOPA** macrocycles. In the course of the experiment, the concentrations of macrocycles varied within 1 – 100 μM . Double systems (thiacalix[4]arene-HEWL) were prepared similarly to those studied by UV-Vis spectroscopy. The particle sizes were measured after 1 h mixing. Measurements were determined after 24 and 178 h three times to evaluate kinetic stability.

S1.3. Electrophoretic light scattering (ELS)

S1.3.1. Zeta potentials

Zeta (ζ) potentials were determined by electrophoretic light scattering (ELS) on a Zetasizer Nano ZS from Malvern Instruments (Worcestershire, UK). Samples

were prepared as for the DLS measurements and were transferred with the syringe to the disposable folded capillary cell for measurement. The zeta potentials were measured using the Malvern M3-PALS method and averaged from three measurements.

*S1.4. Cytotoxicity of **TOPA** on A549 cell line*

The ability of **TOPA** to inhibit the viability and proliferative activity of A549 cells was investigated using the MTT test according to [S1]. A549 – human alveolar adenocarcinoma cell line (ATCC, Rockville, Maryland, USA) was cultured in DMEM (GIBCO, Waltham, MA, USA) medium supplemented with 10% serum and 100 units/mL of penicillin (PanEco, Moscow, Russia) and streptomycin (PanEco, Moscow, Russia) in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were seeded in 96-well plates at a concentration of 10⁴ cells/well. After 24 h of cultivation, the medium was removed from the wells and replaced with a fresh one with the addition of the test substances. The volume of the culture medium in the wells was 100 µL. After 24 h incubation of cells in the presence of substances, the medium in the wells was replaced with a fresh one containing MTT reagent at a final concentration of 0.5 mg/mL. The cells were incubated with MTT for 4 h at 37 °C in an atmosphere of 5% CO₂. Then, the medium was aspirated from the wells and 100 µL of dimethyl sulfoxide was added. Probes were incubated at 37 °C for 15 min in the dark for the formazan crystals to dissolve. The optical density of the formazan solution in the wells was measured using a plate reader (BioRad xMark™ Microplate Spectrophotometer, USA) at a wavelength of 570 nm. Three series of experiments were carried out with at least 8 replications for each variant in the series. For MTT test data significant differences were reported at $p < 0.05$ using the nonparametric Mann-Whitney U-test.

S1.5. UV-Vis spectroscopy

Absorption spectra were recorded on a Shimadzu UV-3600 spectrometer (Kyoto, Japan). Quartz cuvettes with an optical path length of 10 mm were used. Deionized water was used for preparation of the solutions. 300 µL solution of HEWL (10 µM) was added to water solution of **TOPA** (10 µM) in a 1:10 ratio to study the complexation of thiacalix[4]arenes with the guest in solvent. Absorption spectra of mixtures were recorded after an 1 h incubation at 20 °C.

S1.5.1. Determination of the stability constant and stoichiometry of the complex by spectrophotometric titration

The solution (10 μM) of HEWL (100, 200, 300, 400, 500, 600, 700, 800, and 900 μL) in water was added to 300 μL of a solution of **TOPA** (100 μM) in water and diluted to final volume of 3 mL with water. The UV-Vis spectra of the solutions were then recorded. The stability constant of complex was calculated by Bindfit application (Bindfit v0.5, Open Data Fit, <http://app.supramolecular.org/bindfit/>). Three independent experiments were carried out for each series.

S1.6. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco J-1500CD spectrophotometer (Tokyo, Japan) in a quartz cuvette with an optical path length of 10 mm. 20 mM phosphate buffer (PBS) (pH 7.44) containing 20 mM NaCl was used for preparation of the solutions. The experiment was performed at 25 $^{\circ}\text{C}$, scanning wavelength range was 200–250 nm. The scanning rate was 20 nm/min. The concentrations of substances in the CD measurements for HEWL and **TOPA** were equal to 0.5 μM . The CD spectra of mixtures were recorded after an 1 h incubation at 25 $^{\circ}\text{C}$.

S1.7. Transmission and scanning electron microscopy

TEM and SEM measurements were made at Interdisciplinary Center for Analytical Microscopy of Kazan Federal University. Analysis of samples was carried out using a Hitachi HT7700 Exalens atomic transmission electron microscope (Tokyo, Japan) with an Oxford Instruments X-Maxn 80T EDS detector working in STEM mode and a Merlin (Carl Zeiss) universal analytical scanning auto-emission electron microscopy system. Samples of double systems thiacalix[4]arene **TOPA**-HEWL were prepared similarly to those studied by the DLS method. Deionized water was used as a solvent. The suspension (10 μL) was placed on a carbon-coated 3 mm copper grid and dried at room temperature using special holder for microanalysis. After drying, the grid was placed in the transmission electron microscope and analyzed at an accelerating voltage of 100 kV.

S1.8. Preparation of fibrillar solutions of HEWL

HEWL powder (50 mg) was dissolved in 1 mL deionized water. The concentration of the stock HEWL solution was determined spectrophotometrically by HEWL absorbance at 280 nm using a molar extinction coefficient of 37646 $\text{M}^{-1}\times\text{cm}^{-1}$ [S2]. HEWL concentration found to vary in the range of 3.50 ± 0.06 mM (50.5 ± 0.8 mg/mL). HEWL solutions with a concentration of ~ 150 μM were

prepared by diluting the stock solution in 20 mM phosphate buffer (PBS) (pH 7.44) containing 20 mM NaCl and 30% ethanol with and without different concentrations (75, 150, and 300 μ M) of **TOPA**. Incubation of solutions was carried out for 6 h at 65 °C. Then incubated protein solutions were left for 24 hours at room temperature prior to experimental measurements. Aliquots were diluted with 20 mM PBS in each study to maintain the desired concentration in the final working solution.

S1.9. Fluorescence spectroscopy

Fluorescence spectra were recorded on the Fluorolog 3 luminescent spectrometer (Horiba Jobin Yvon, Longjumeau, France). The excitation wavelength was selected as 240 nm. The emission scan range was 460–600 nm. Excitation and emission slits were 3 nm. Quartz cuvettes with an optical path length of 10 mm were used. The cuvette was placed at the front face position to avoid the inner filter effect. Fluorescence spectra were automatically corrected by the Fluorescence program. The fluorescence spectra of ThT (10 μ M) in presence and absence of HEWL fibrillar solutions (5 μ M) were recorded in 20 mM PBS (pH 7.44) at 293 K. Three independent experiments were carried out for each series.

S1.10. FTIR Spectroscopy

The FTIR spectra were recorded on a Spectrum 400 (PerkinElmer, Waltham, MA, USA) FTIR spectrometer, with Diamond KRS-5 ATR accessory. The FTIR spectra from 4000 to 400 cm^{-1} were considered in this analysis. The spectra were measured with 0.5 cm^{-1} resolution and 32-scan accumulation. Thiocalixarenes were used as a baseline in experiments assessing the inhibition of fibril formation to neutralize the effect of amide groups on the fibril α -helices. The aqueous solution was applied directly to the sensor and allowed to air dry naturally. After that, the spectra were recorded. The spectra were cleared of noise by the built-in Perkin Elmer software (Version 10), without baseline correction. To assess the effect of thiocalix[4]arenes on fibril formation, deconvolution of the IR spectra was carried out, with a constant baseline line for all studied spectra. The ratios of α -helices and β -sheets were calculated to evaluate the influence after deconvolution.

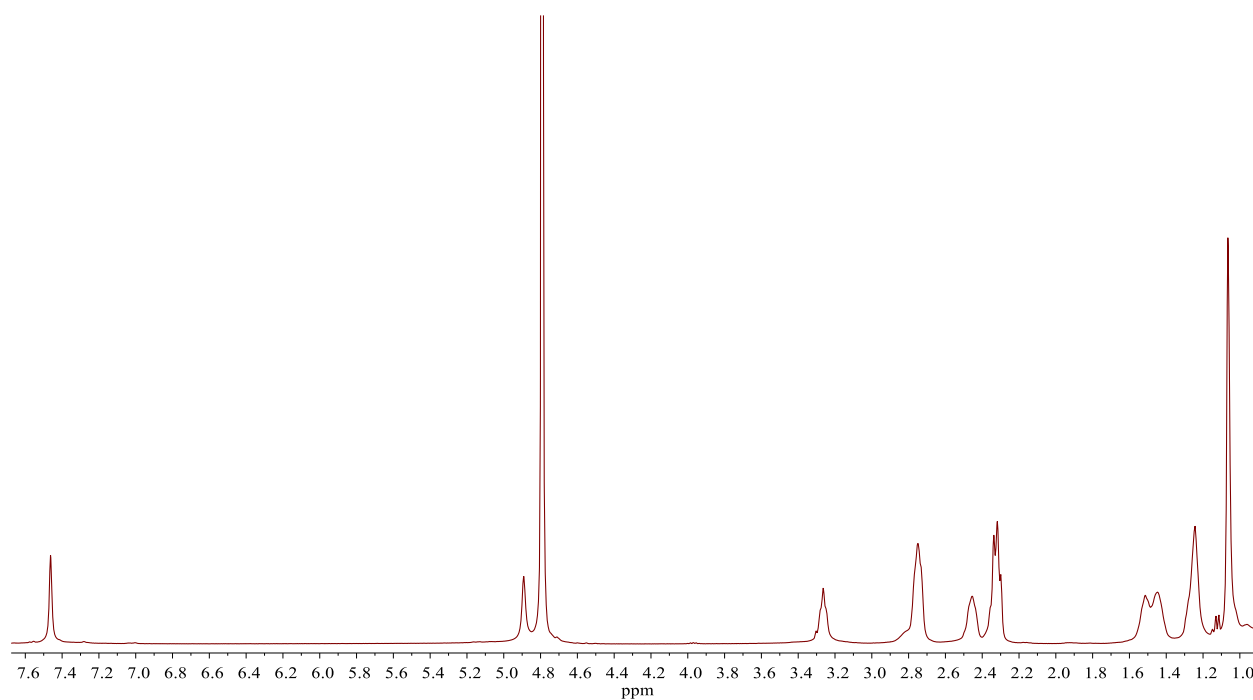


Fig. S1. ^1H NMR spectrum of TOPA-cone, D_2O , 298 K, 400 MHz.

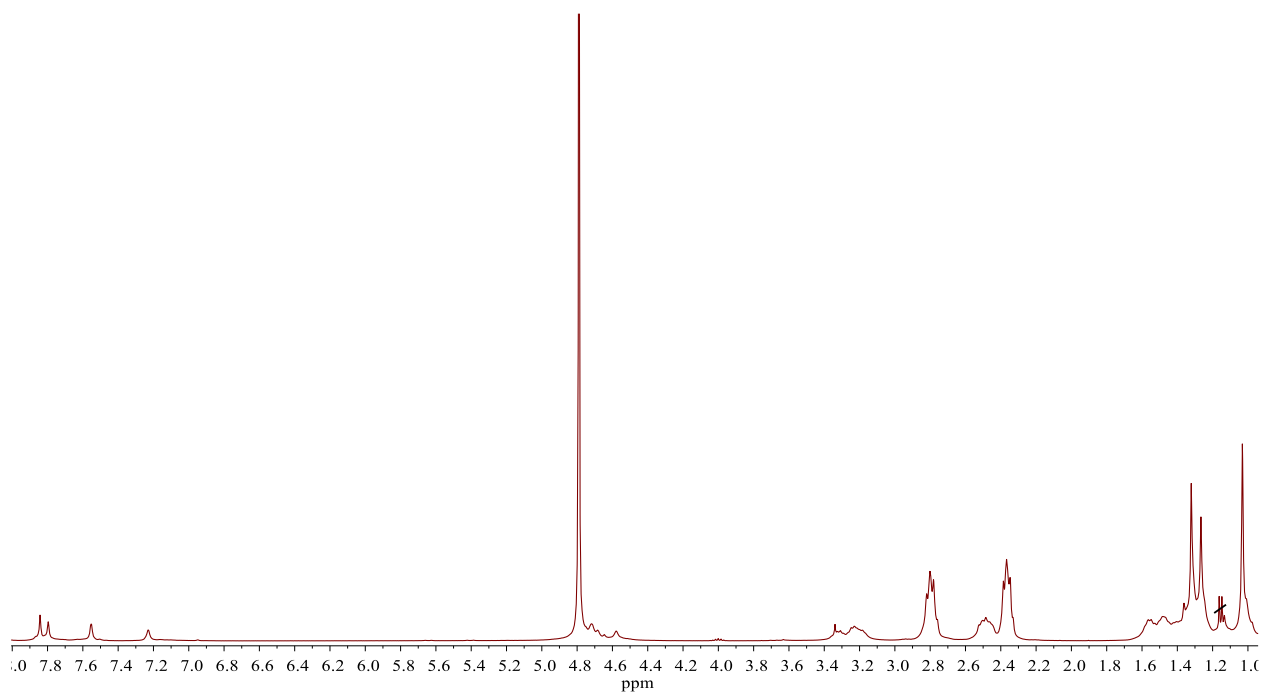


Fig. S2. ^1H NMR spectrum of TOPA-paco, D_2O , 298 K, 400 MHz.

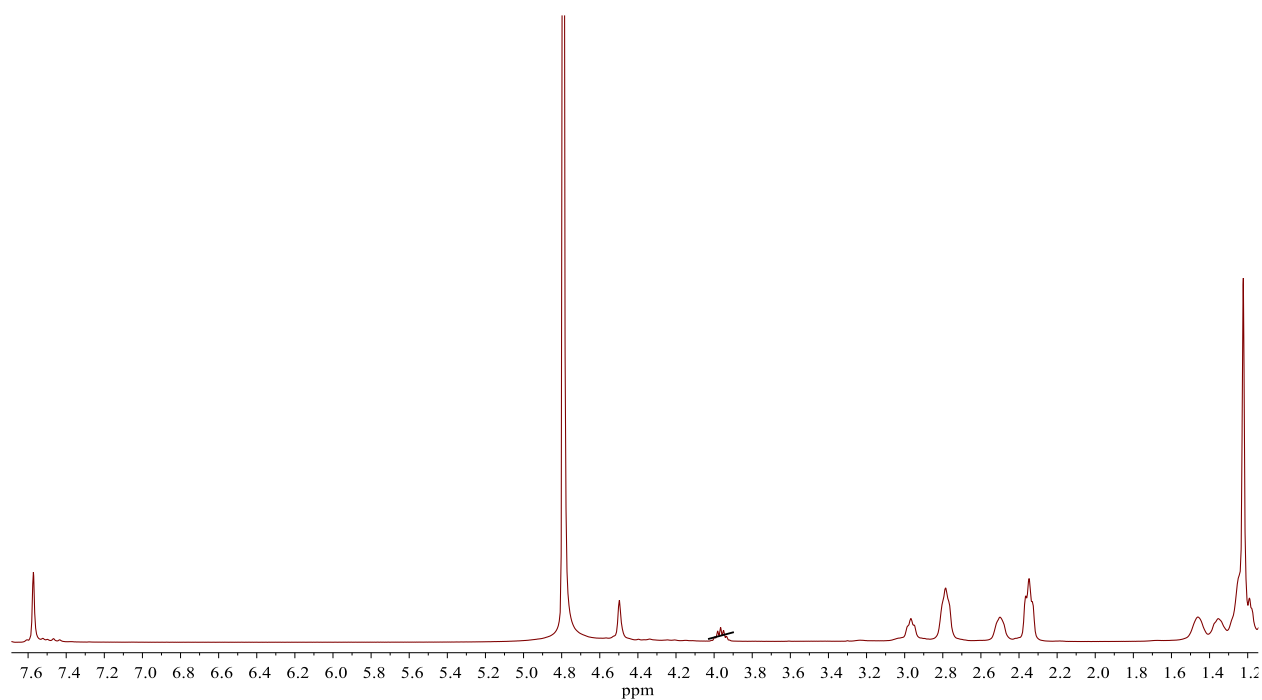


Fig. S3. ^1H NMR spectrum of TOPA-alt, D_2O , 298 K, 400 MHz.

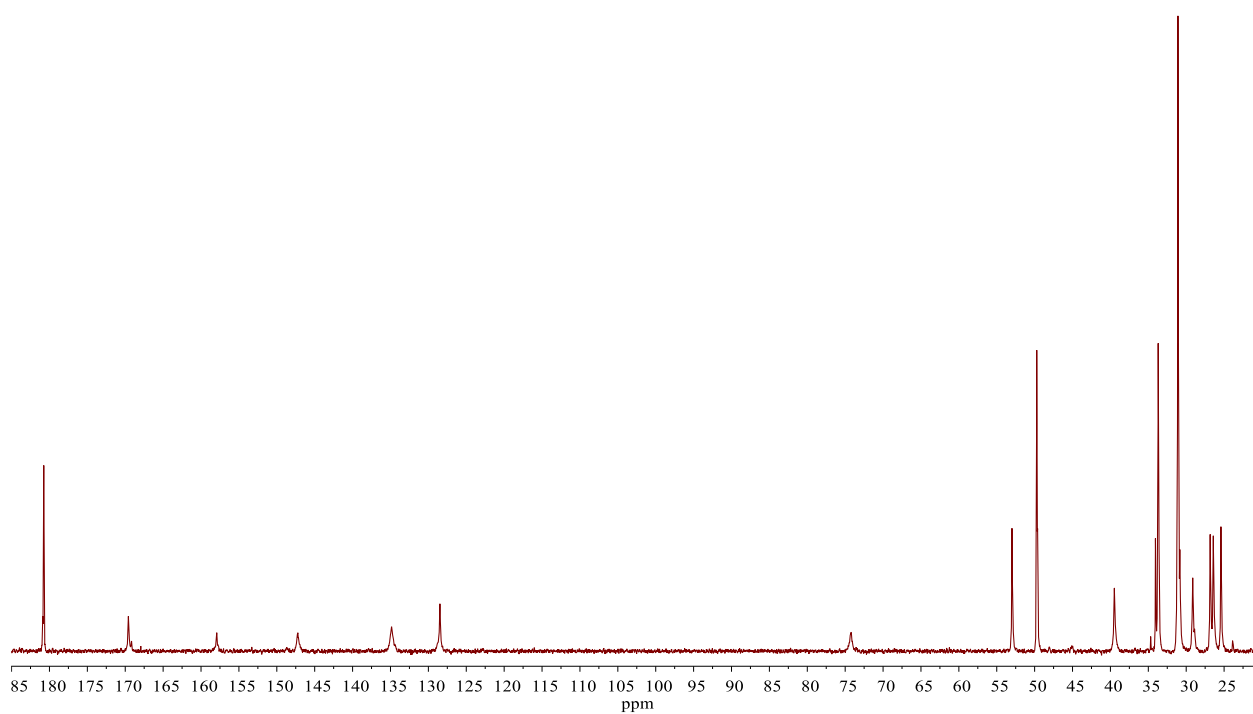


Fig. S4. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of TOPA-cone, D_2O , 298 K, 100 MHz.

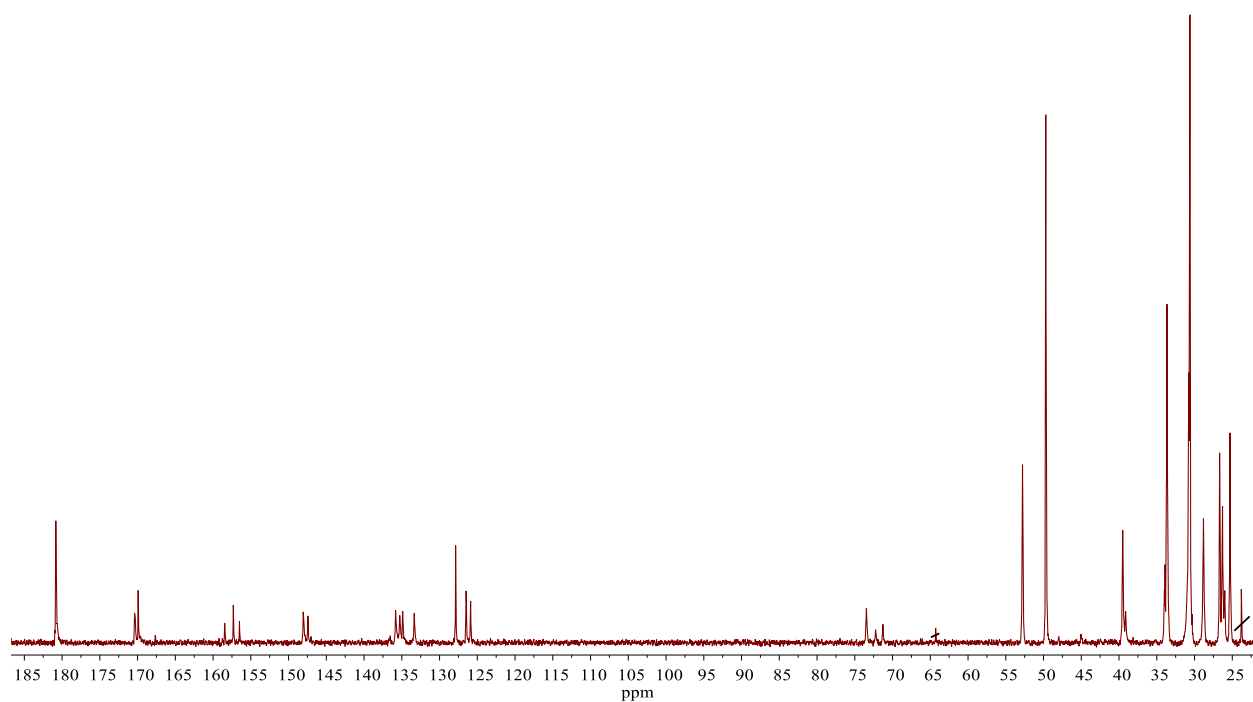


Fig. S5. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of TOPA-paco, D_2O , 298 K, 100 MHz.

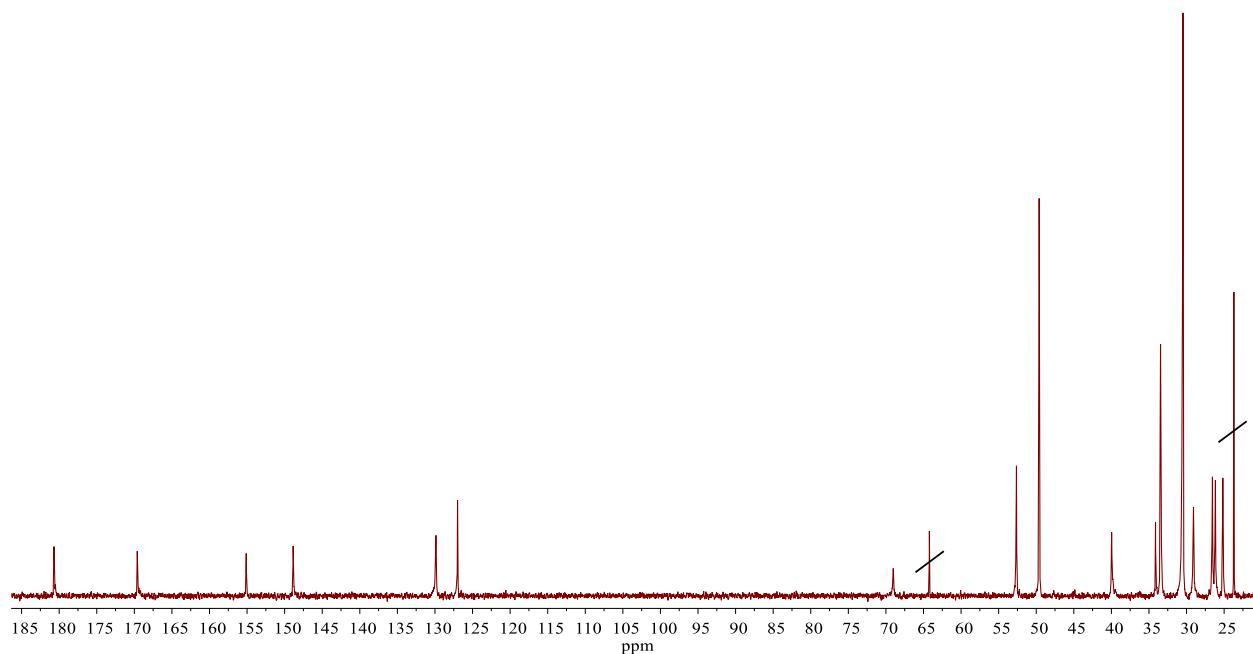


Fig. S6. $^{13}\text{C}\{^1\text{H}\}$ NMR spectrum of TOPA-alt, D_2O , 298 K, 100 MHz.

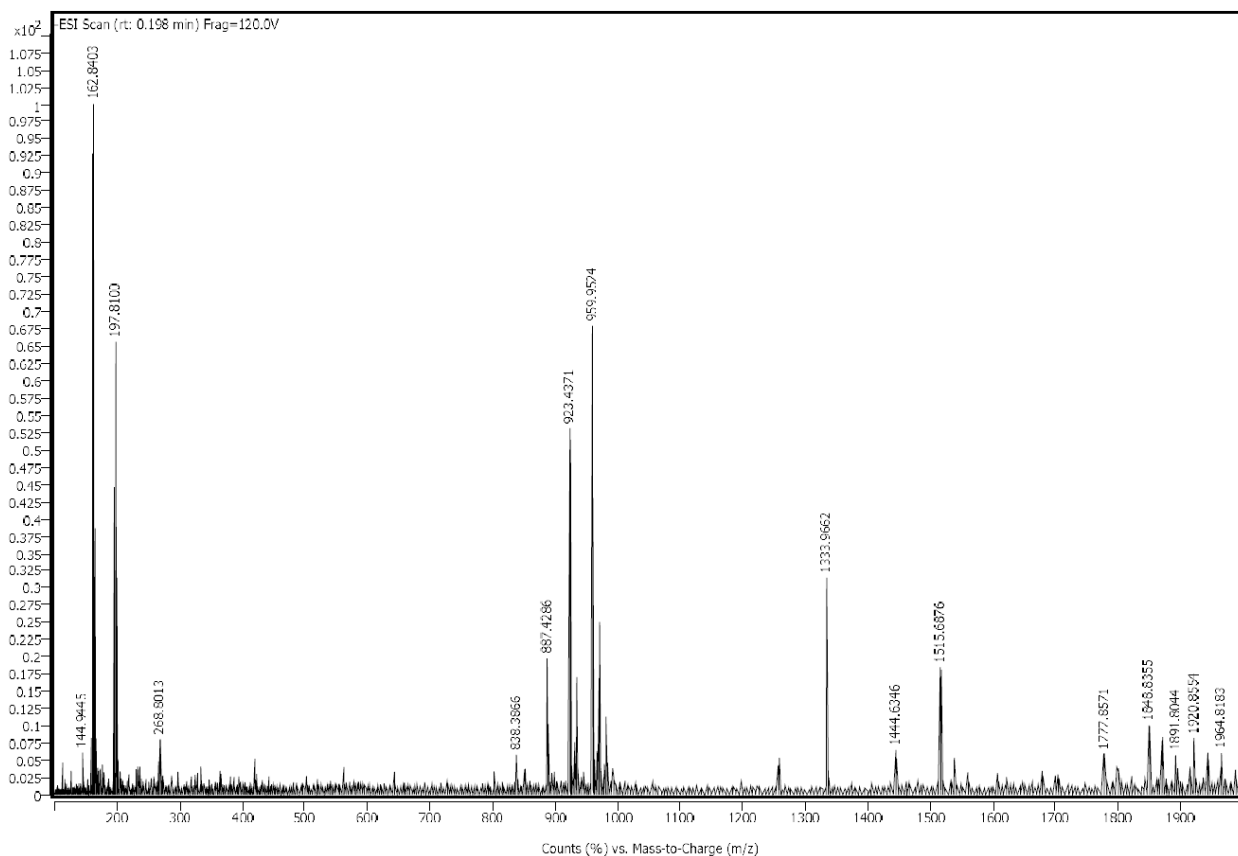


Fig. S7. Mass spectrum (HRMS ESI) of TOPA-cone.

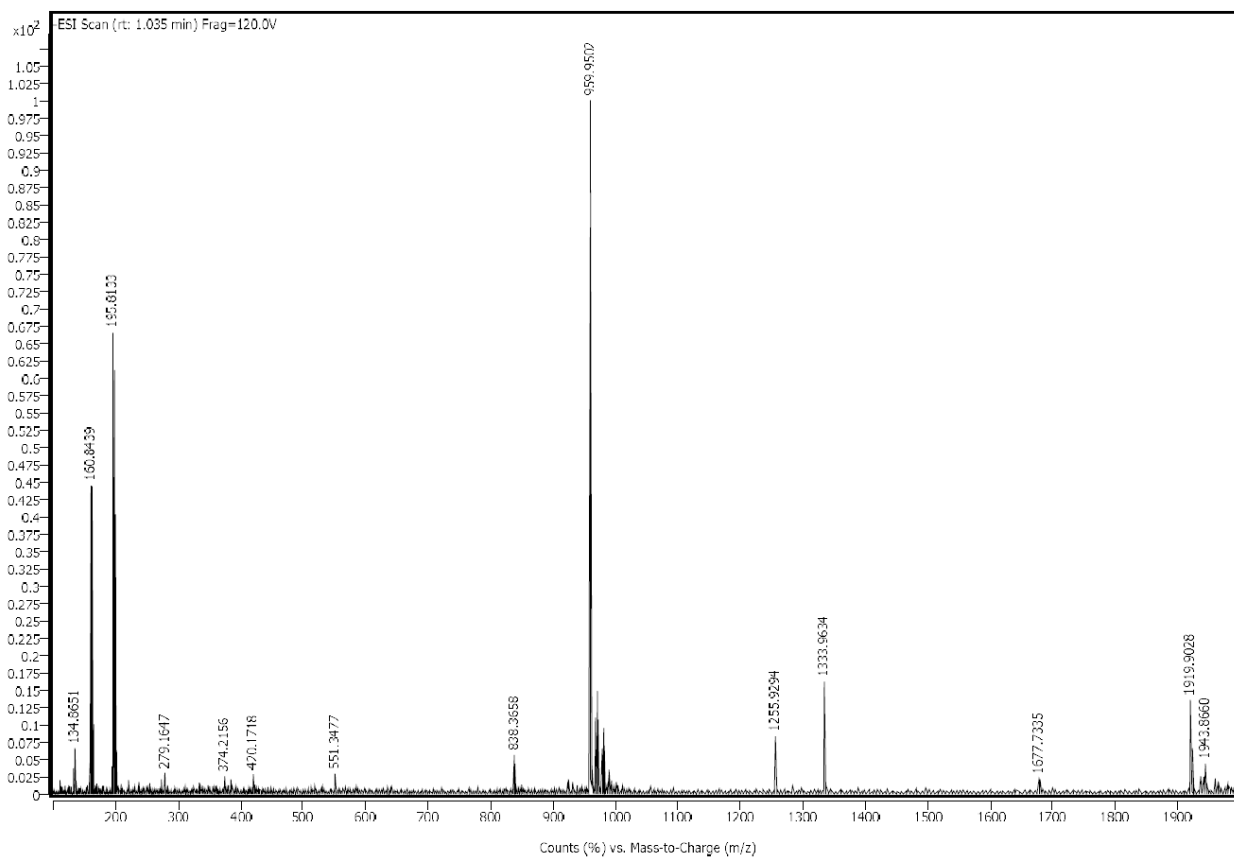


Fig. S8. Mass spectrum (HRMS ESI) of TOPA-paco.

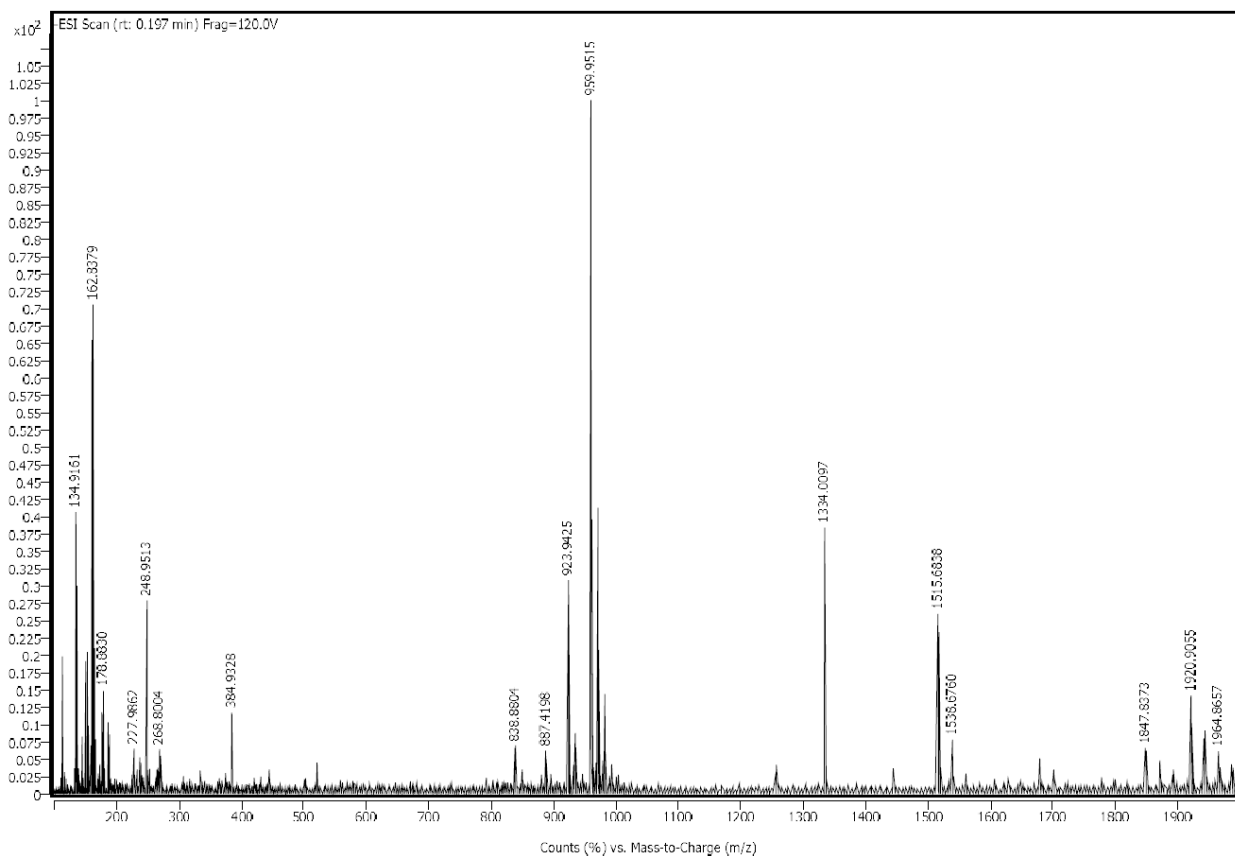


Fig. S9. Mass spectrum (HRMS ESI) of TOPA-alt.

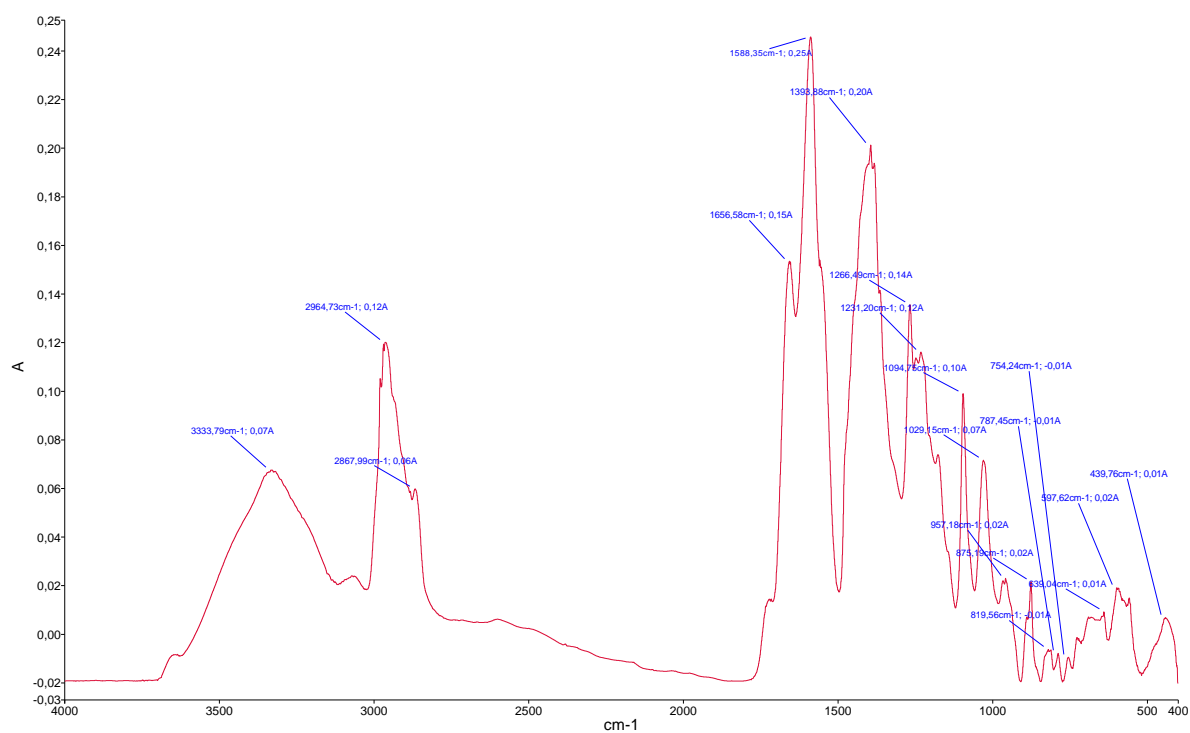


Fig. S10. FTIR spectrum of TOPA-cone.

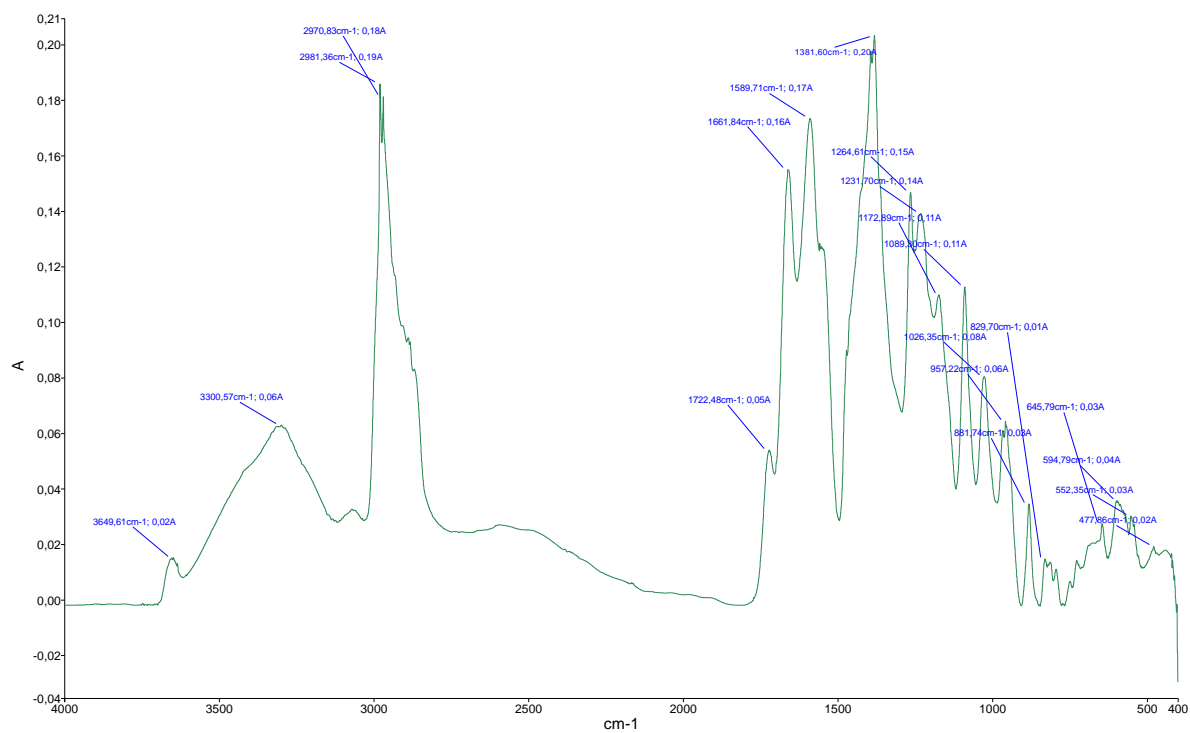


Fig. S11. FTIR spectrum of TOPA-paco.

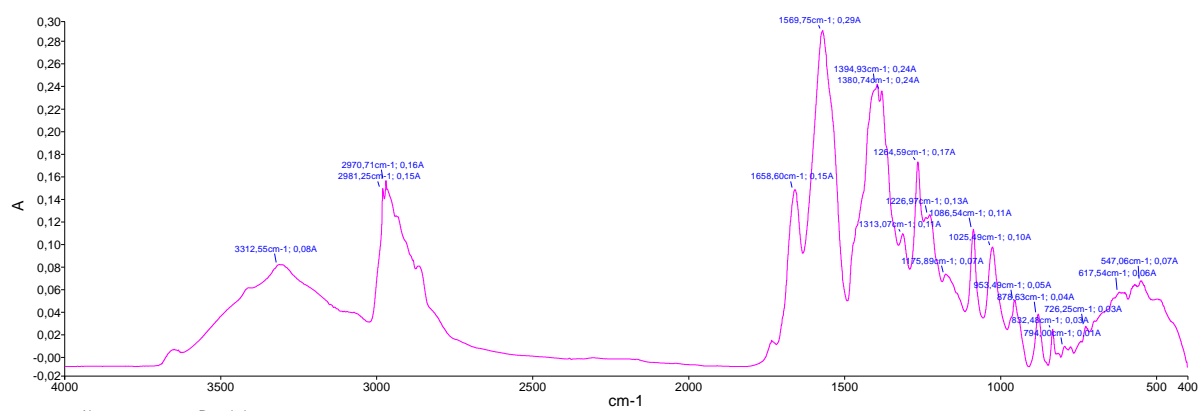


Fig. S12. FTIR spectrum of TOPA-alt.

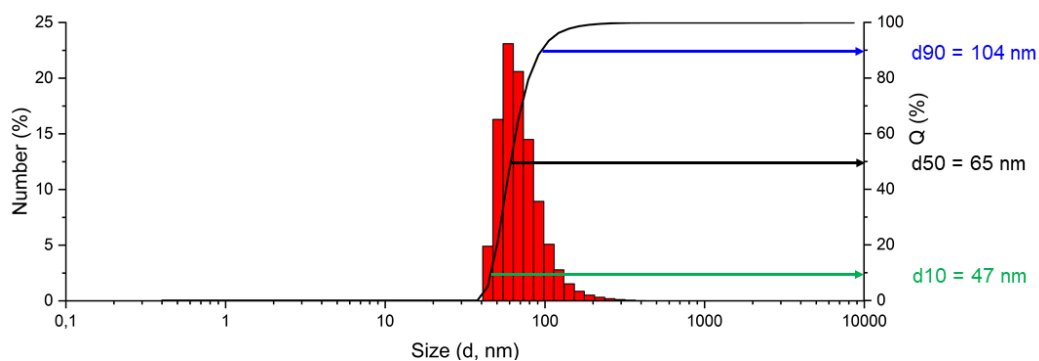


Fig. S13. Size distribution of particles by number for macrocycle TOPA-cone (1 μ M) in H₂O.

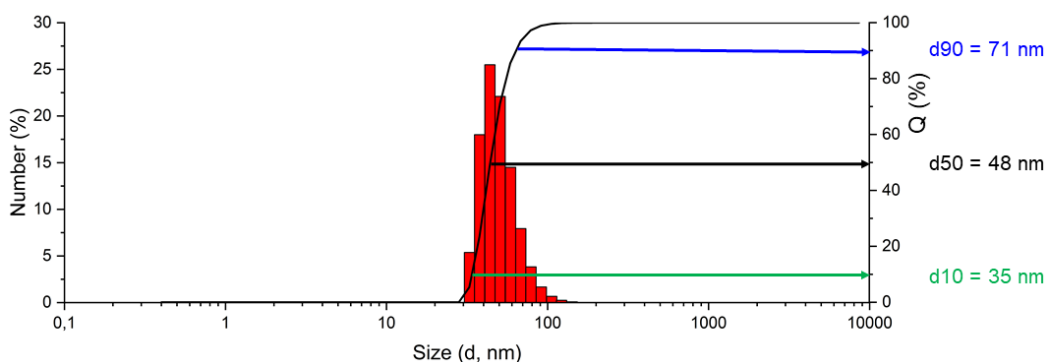


Fig. S14. Size distribution of particles by number for macrocycle TOPA-cone (10 μ M) in H₂O.

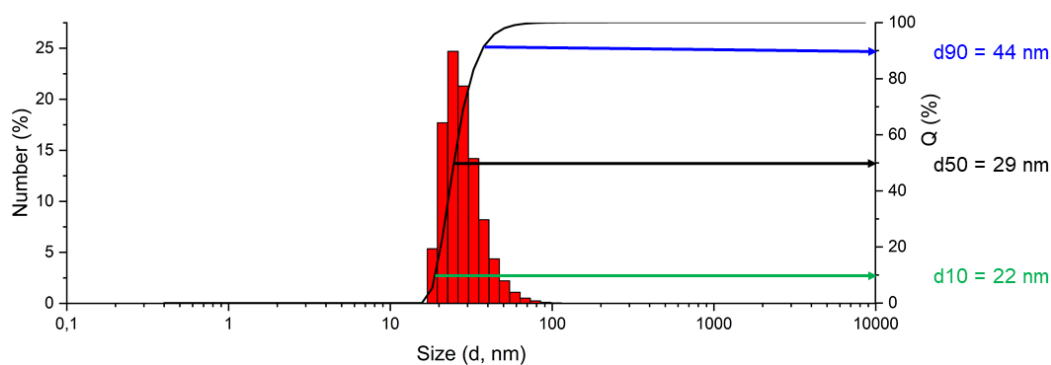


Fig. S15. Size distribution of particles by number for macrocycle TOPA-cone (100 μ M) in H₂O.

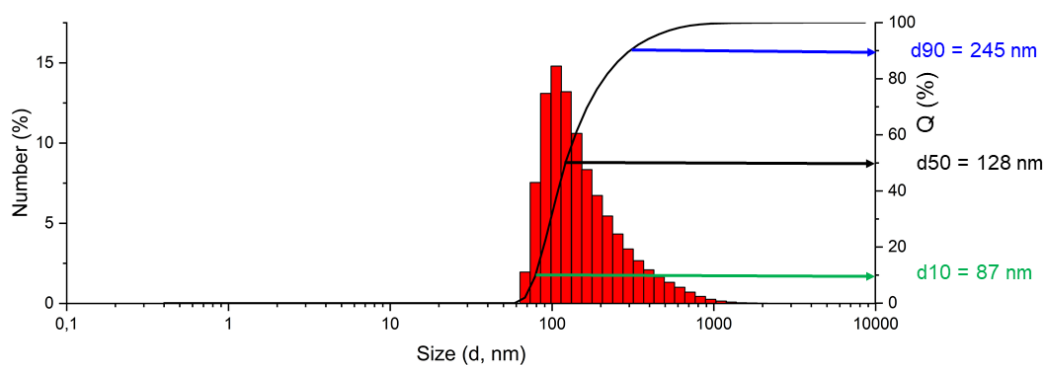


Fig. S16. Size distribution of particles by number for macrocycle TOPA-paco (1 μ M) in H₂O.

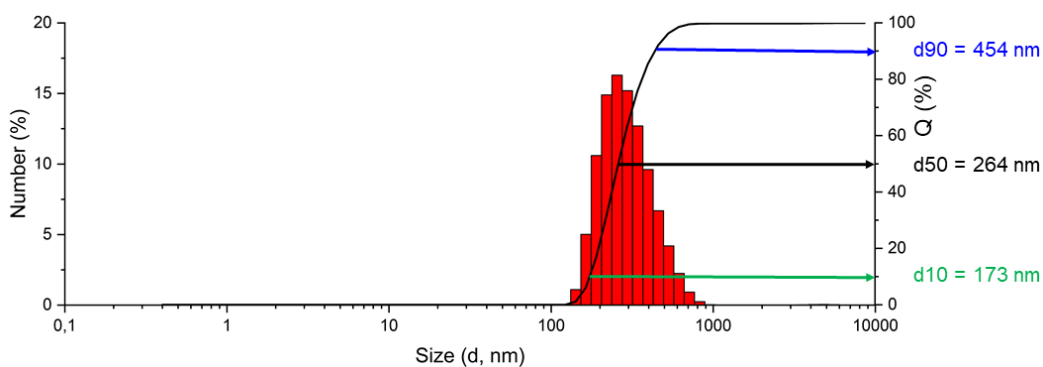


Fig. S17. Size distribution of particles by number for macrocycle TOPA-paco (10 μ M) in H₂O.

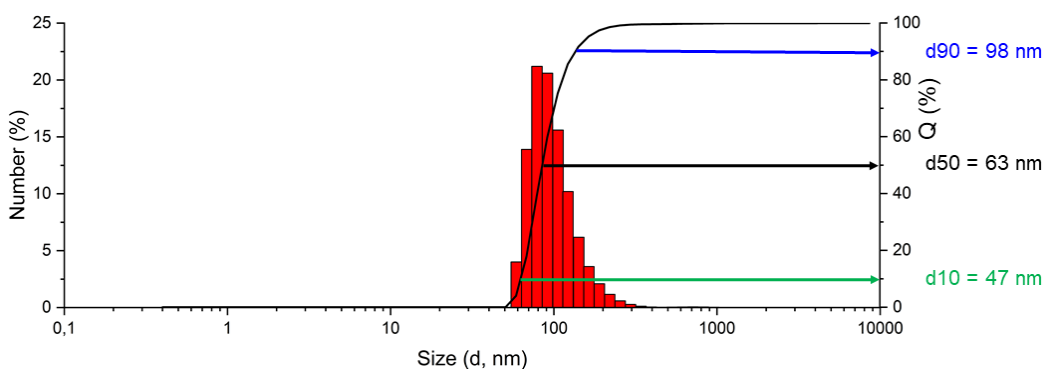


Fig. S18. Size distribution of particles by number for macrocycle TOPA-paco (100 μ M) in H₂O.

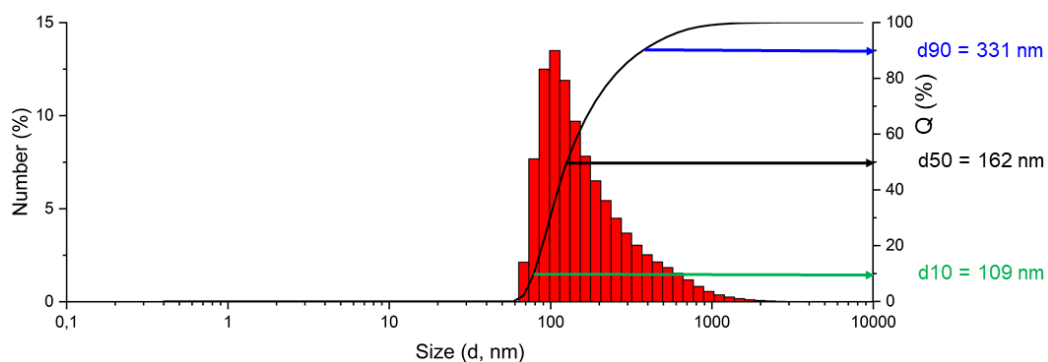


Fig. S19. Size distribution of particles by number for macrocycle TOPA-alt (1 μM) in H_2O .

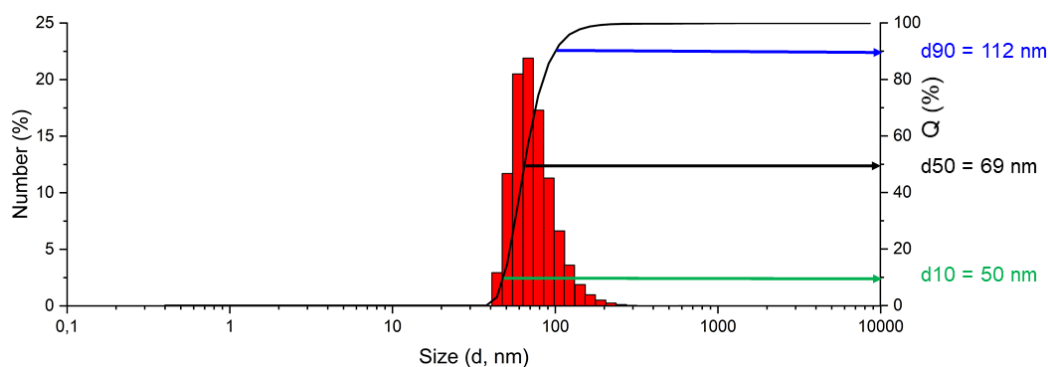


Fig. S20. Size distribution of particles by number for macrocycle TOPA-alt (10 μM) in H_2O .

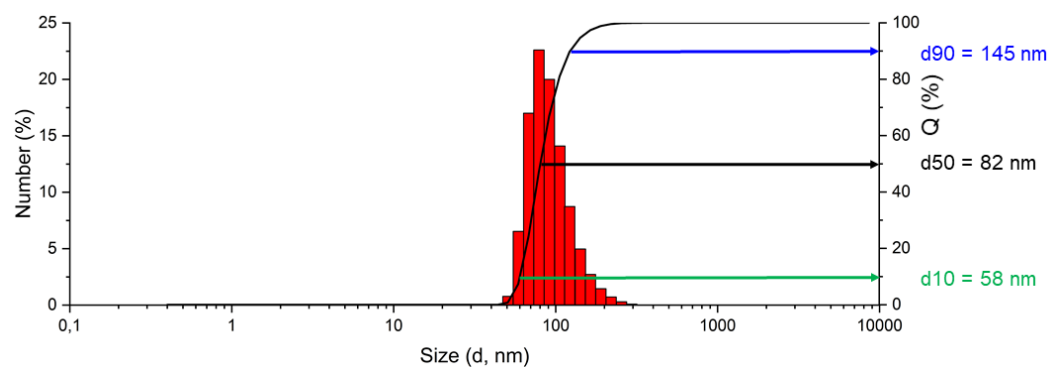


Fig. S21. Size distribution of particles by number for macrocycle TOPA-alt (100 μM) in H_2O .

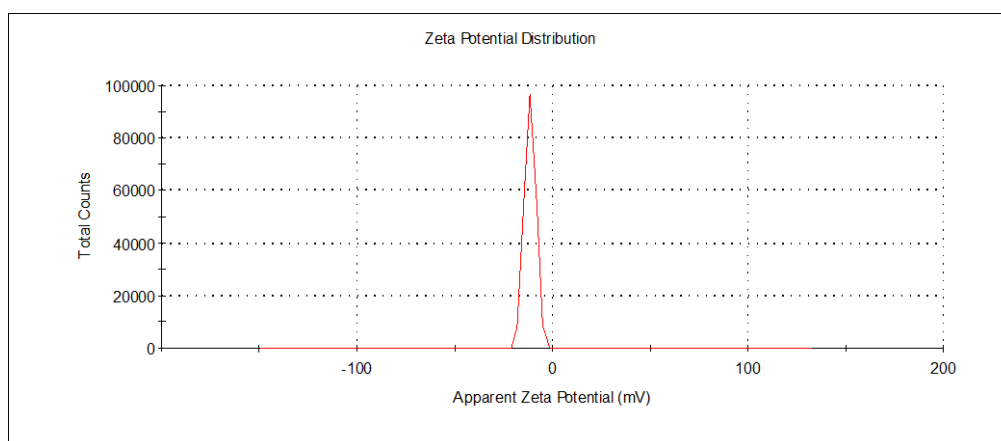


Fig. S22. Zeta potential distribution of the particles for macrocycle TOPA-cone (1 μ M) in H₂O.

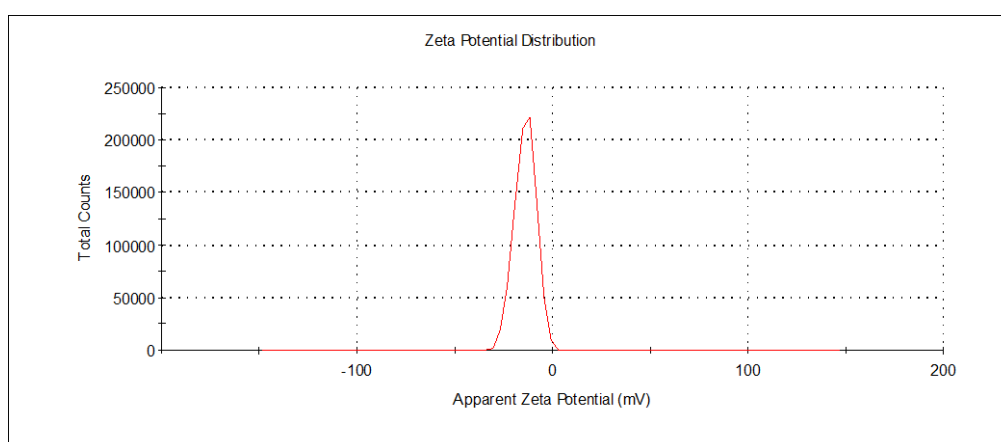


Fig. S23. Zeta potential distribution of the particles for macrocycle TOPA-cone (10 μ M) in H₂O.

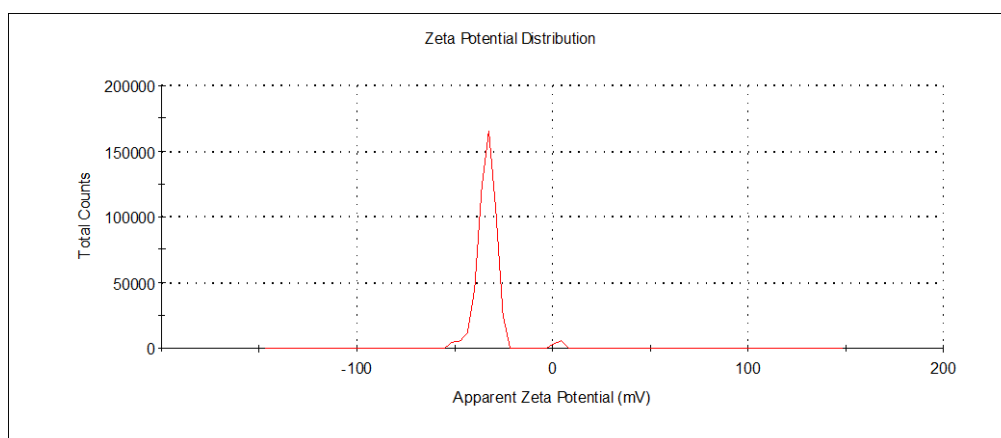


Fig. S24. Zeta potential distribution of the particles for macrocycle TOPA-cone (100 μ M) in H₂O.

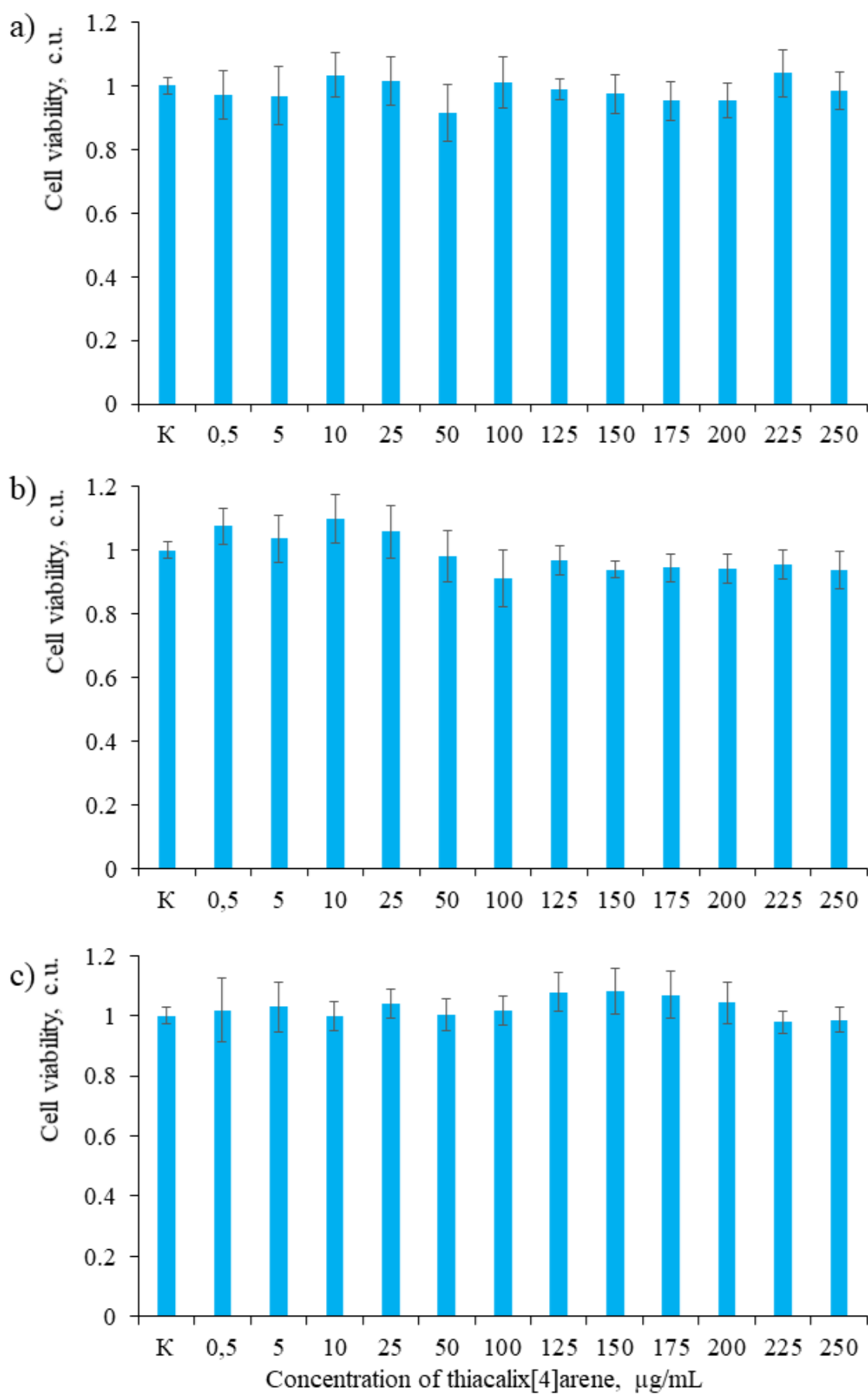


Fig. S25. Cytotoxicity of thiacalix[4]arenes on A549 cell line: a) TOPA-cone; b) TOPA-paco; c) TOPA-alt.

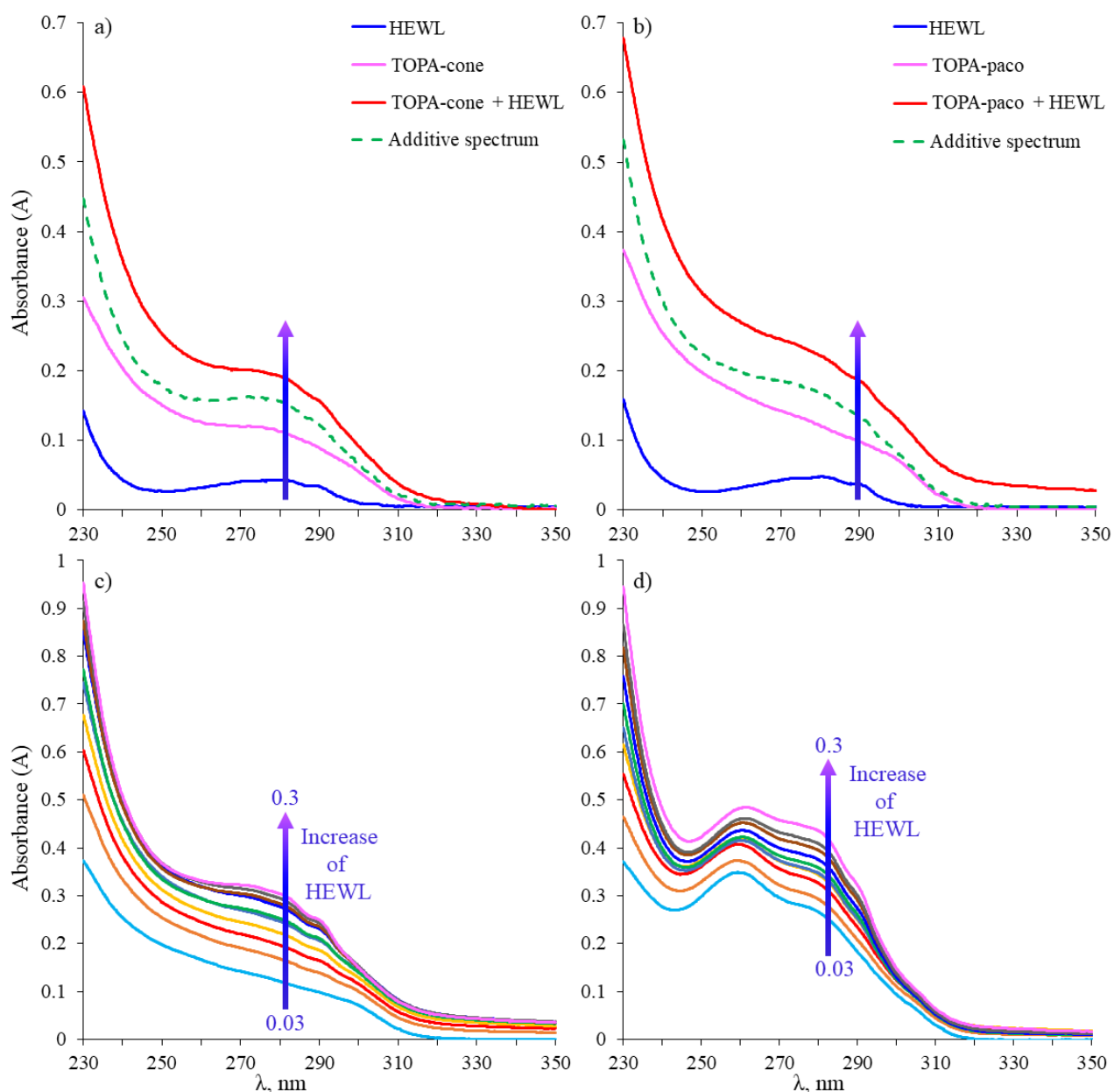


Fig. S26. Electron absorption spectra of: a) 10-fold excess of TOPA-cone with HEWL ($C_{\text{TOPA-cone}} = 10 \mu\text{M}$, $C_{\text{HEWL}} = 1 \mu\text{M}$) in H_2O ; b) 10-fold excess of TOPA-paco with HEWL ($C_{\text{TOPA-paco}} = 10 \mu\text{M}$, $C_{\text{HEWL}} = 1 \mu\text{M}$) in H_2O ; c) TOPA-paco with different concentrations of HEWL (from 0.03 to 0.3 excess) in H_2O ; d) TOPA-alt with different concentrations of HEWL (from 0.03 to 0.3 excess) in H_2O .

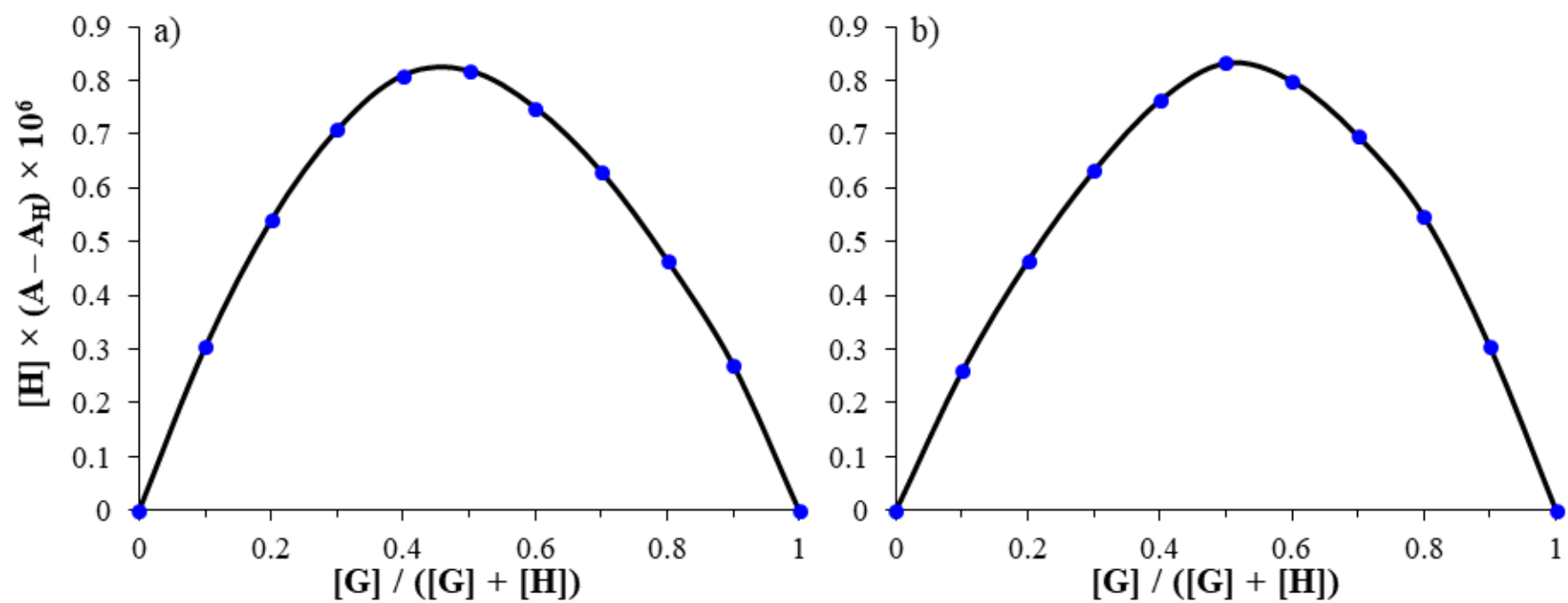


Fig. S27. Job's plot of: a) TOPA-paco and HEWL in H_2O (1:1); b) TOPA-alt and HEWL in H_2O (1:1).

Data Browser Picker ☐ Pager

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Results per page: 5 Jump: 1

Fitter: UV 1:1 Fit Summary Save

Details

Time to fit 0.3025 s
SSR 166.6747
Fitted datapoints 16010
Fitted params 1602

Parameters

| Parameter (bounds) | Optimised | Error | Initial |
|--------------------------------|------------------------------|-------------------|---------------------------|
| K (0 \rightarrow ∞) | 133641.92 M ⁻¹ | \pm 3.7138 % | 100.00 M ⁻¹ |

Back Next

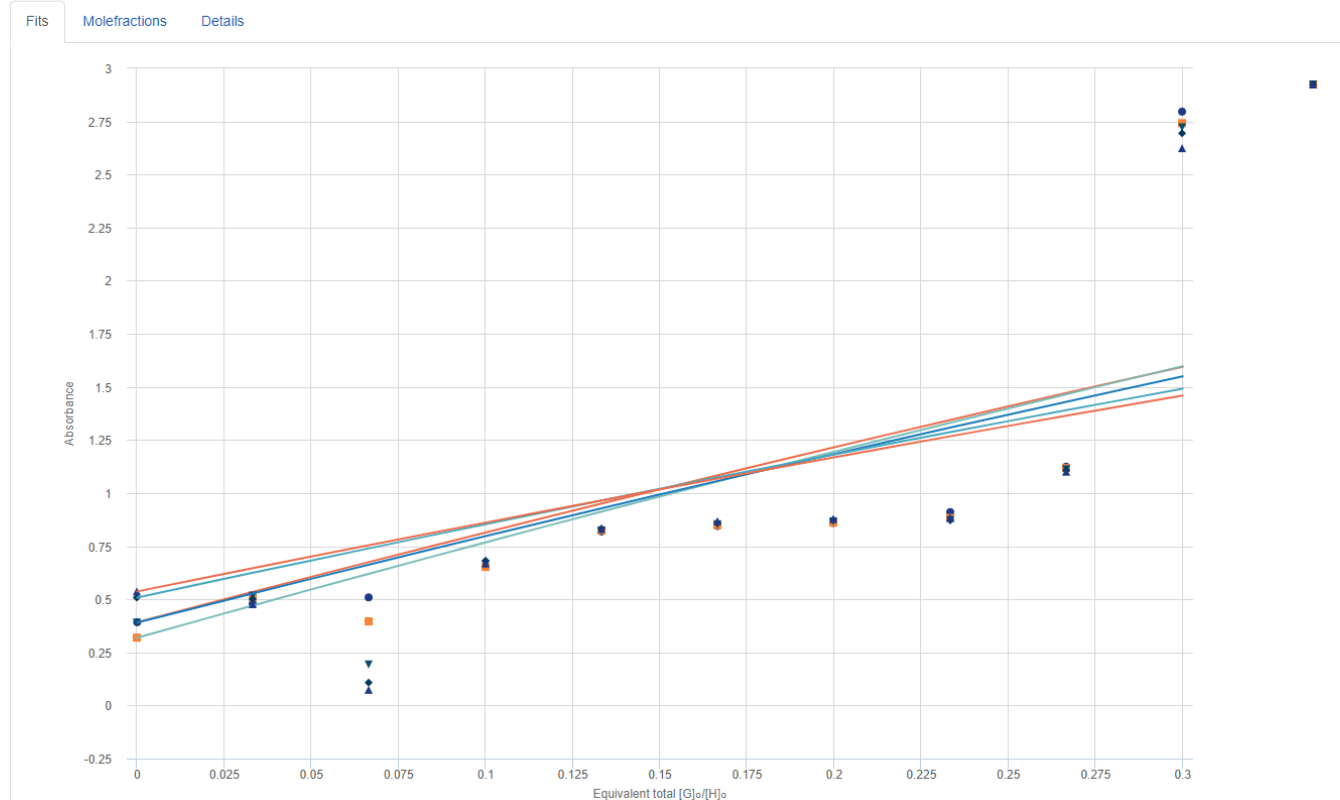


Fig. S28. Bindfit (Fit data to 1:1 Host-Guest equilibria). Screenshots taken from the summary window of the website supramolecular.org. This screenshots shows the raw data for florescence titration of TOPA-cone with HEWL in H₂O, the data fitted to 1:1 binding model.

Data Browser Picker ☐ Pager

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Results per page: 5 Jump: 1

Fitter: UV 1:1 Fit Summary Save

Details

Time to fit 0.2678 s
SSR 5.1323
Fitted datapoints 9606
Fitted params 1602

Parameters

| Parameter (bounds) | Optimised | Error | Initial |
|--------------------|------------------------------|---------------|---------------------------|
| K (0 → ∞) | 112435.75 M ⁻¹ | ± 2.5341 % | 100.00 M ⁻¹ |

Back Next

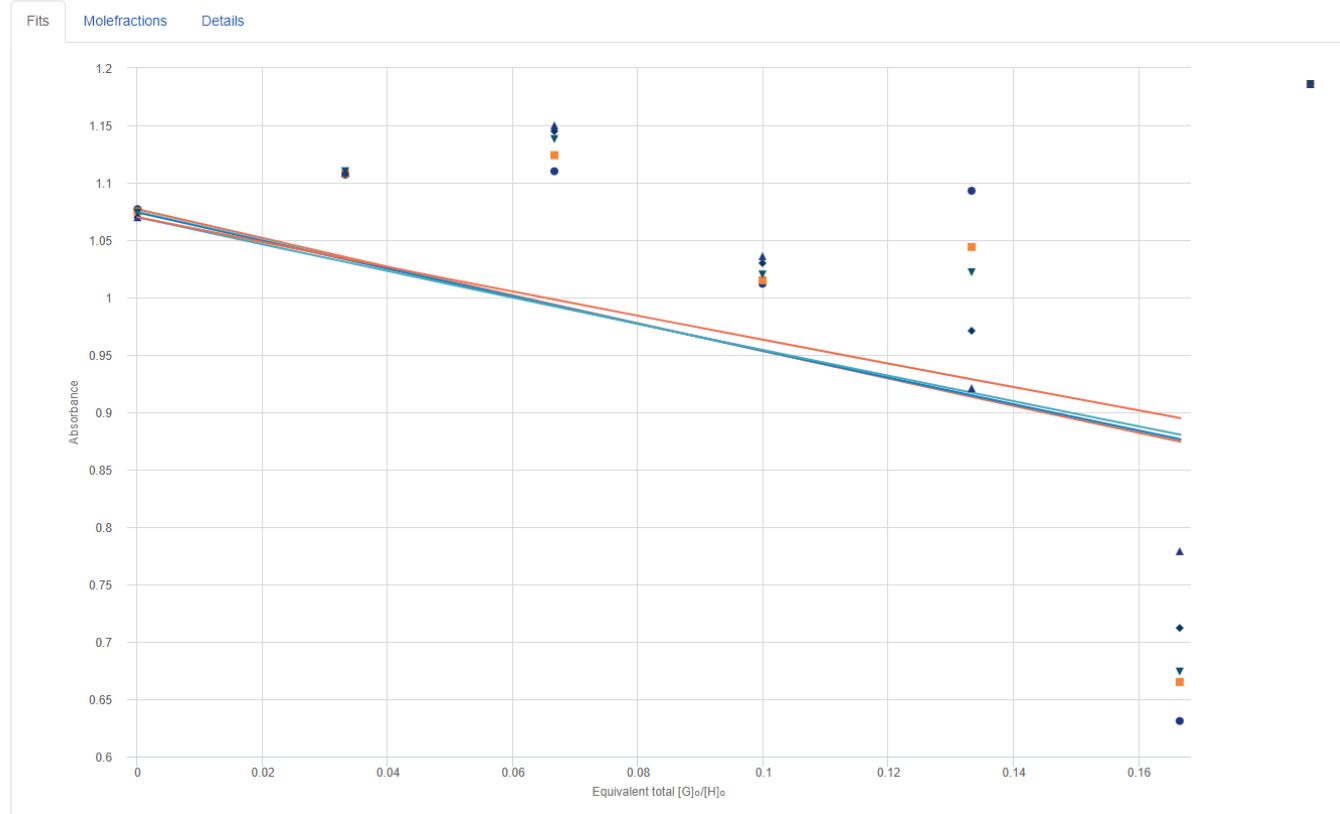


Fig. S29. Bindfit (Fit data to 1:1 Host-Guest equilibria). Screenshots taken from the summary window of the website supramolecular.org. This screenshots shows the raw data for florescence titration of TOPA-paco with HEWL in H₂O, the data fitted to 1:1 binding model.

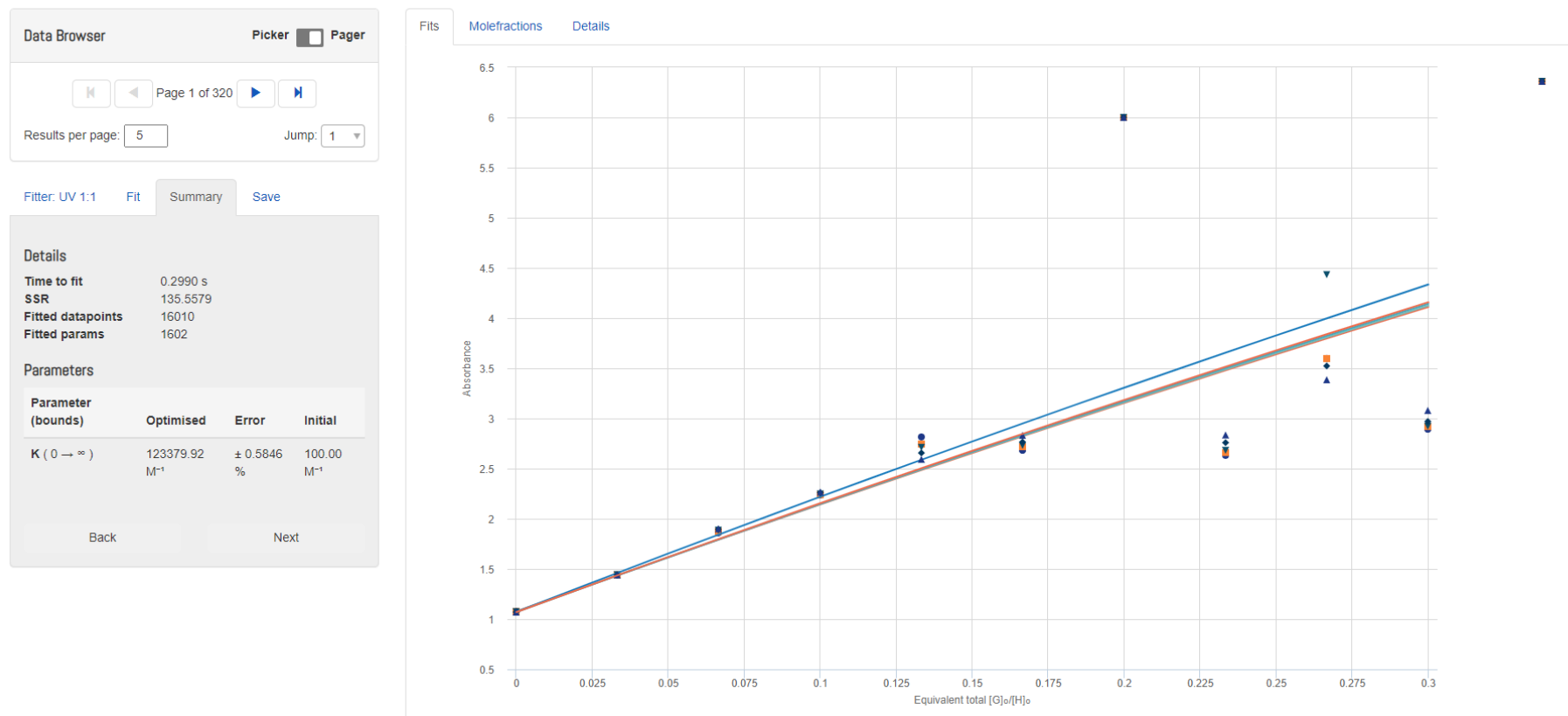


Fig. S30. Bindfit (Fit data to 1:1 Host-Guest equilibria). Screenshots taken from the summary window of the website supramolecular.org. This screenshots shows the raw data for florescence titration of TOPA-alt with HEWL in H₂O, the data fitted to 1:1 binding model.

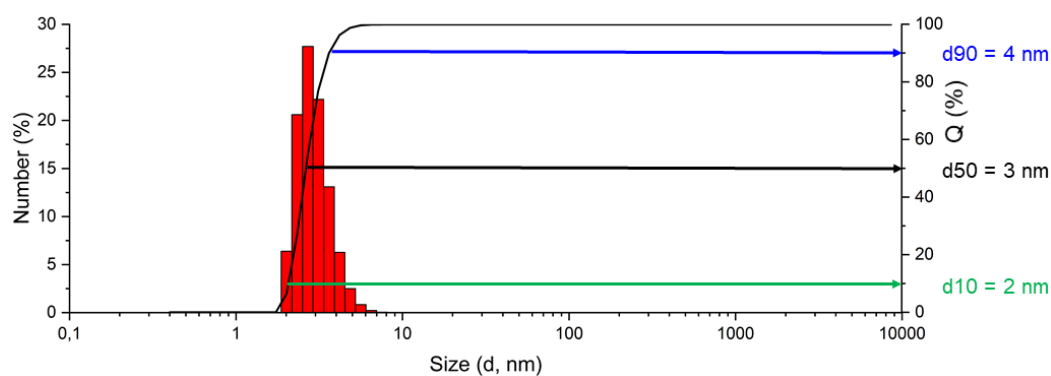


Fig. S31. Size distribution of particles by number for HEWL ($C_{\text{HEWL}} = 10 \mu\text{M}$) in H_2O .

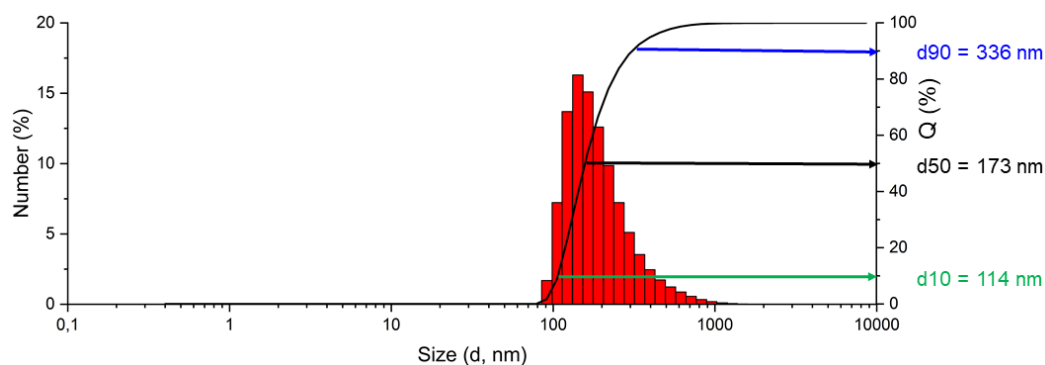


Fig. S32. Size distribution of particles by number for TOPA-cone / HEWL in equimolar ratio ($C_{\text{TOPA-cone}} = C_{\text{HEWL}} = 10 \mu\text{M}$) in H_2O .

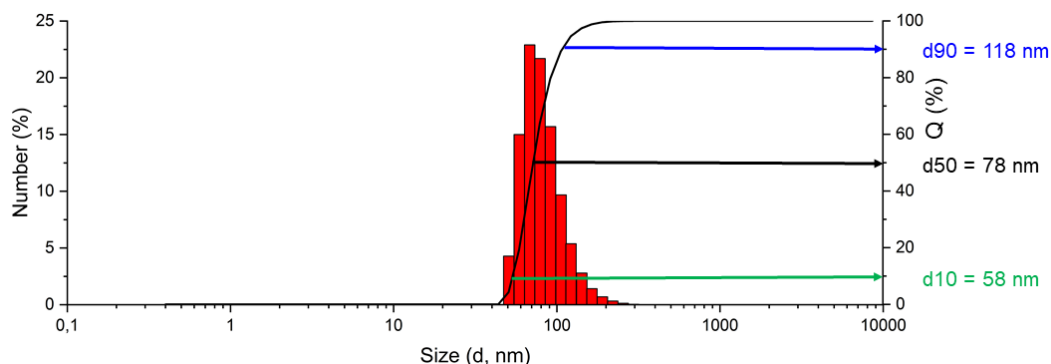


Fig. S33. Size distribution of particles by number for TOPA-paco / HEWL in equimolar ratio ($C_{\text{TOPA-paco}} = C_{\text{HEWL}} = 10 \mu\text{M}$) in H_2O .

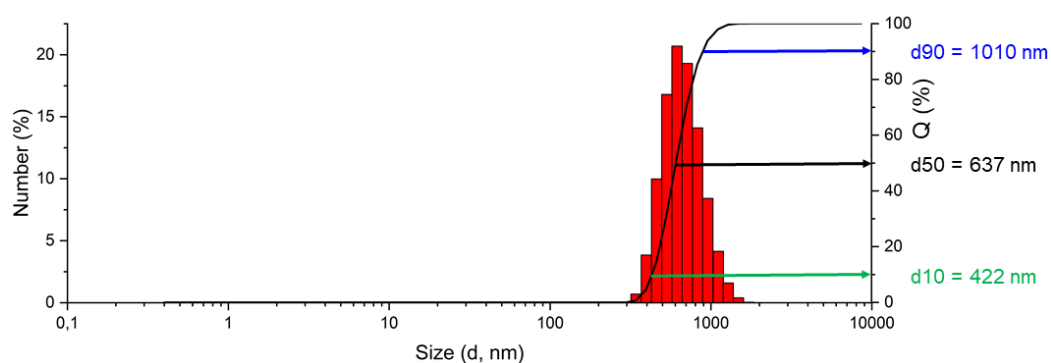


Fig. S34. Size distribution of particles by number for TOPA-alt / HEWL in equimolar ratio ($C_{\text{TOPA-alt}} = C_{\text{HEWL}} = 10 \mu\text{M}$) in H_2O .

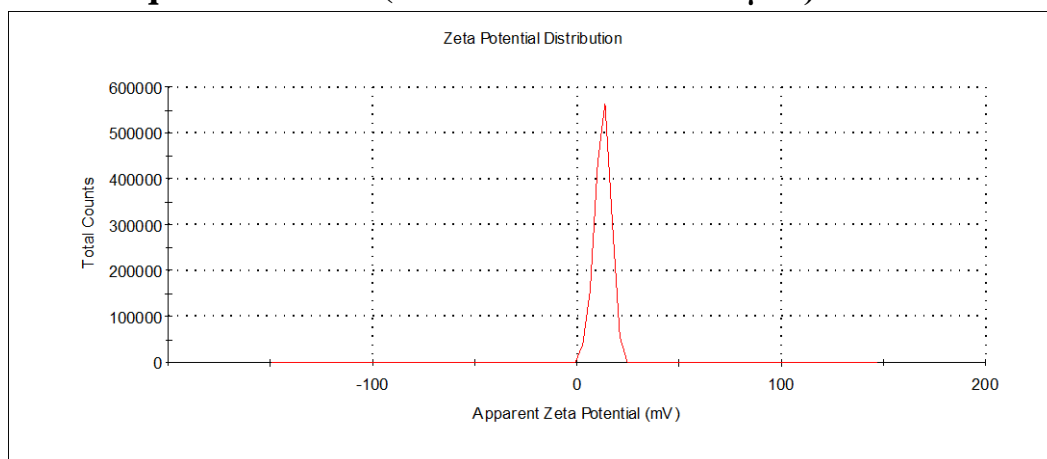


Fig. S35. Zeta potential distribution of the particles for TOPA-cone / HEWL in equimolar ratio ($C_{\text{TOPA-cone}} = C_{\text{HEWL}} = 10 \mu\text{M}$) in H_2O .

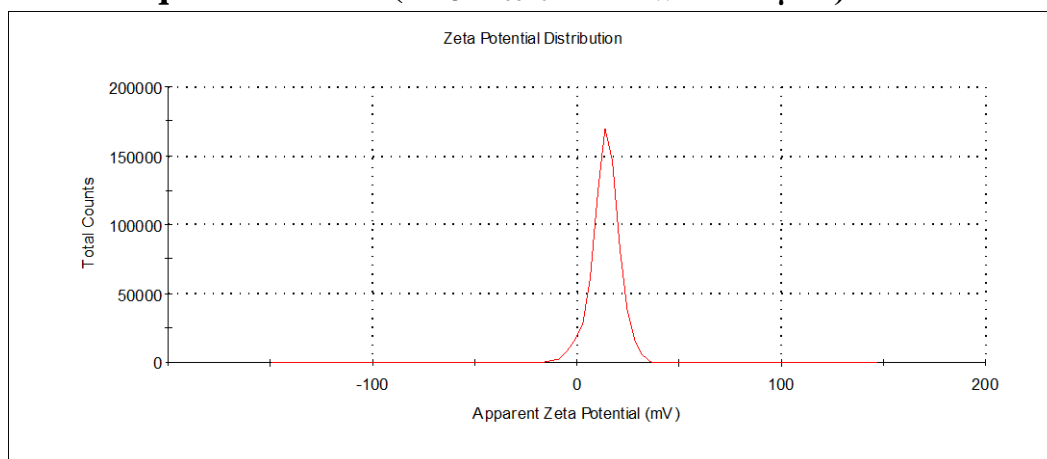


Fig. S36. Zeta potential distribution of the particles for TOPA-paco / HEWL in equimolar ratio ($C_{\text{TOPA-paco}} = C_{\text{HEWL}} = 10 \mu\text{M}$) in H_2O .

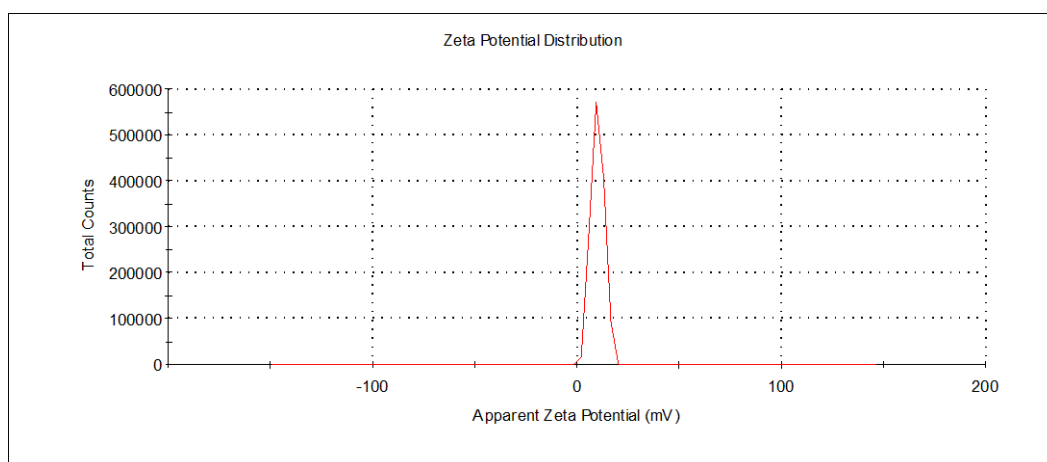


Fig. S37. Zeta potential distribution of the particles for TOPA-alt / HEWL in equimolar ratio ($C_{\text{TOPA-alt}} = C_{\text{HEWL}} = 10 \mu\text{M}$) in H_2O .

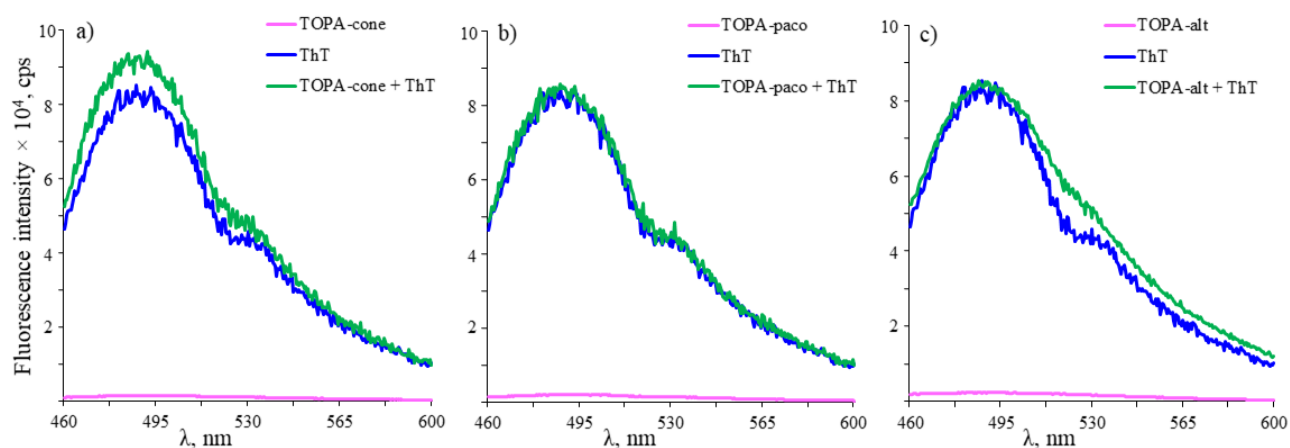


Fig. S38. Fluorescence spectra of: a) TOPA-cone with ThT; b) TOPA-paco with ThT; c) TOPA-alt with ThT in 20mM PBS ($C_{\text{macrocycle}} = C_{\text{ThT}} = 10 \mu\text{M}$).

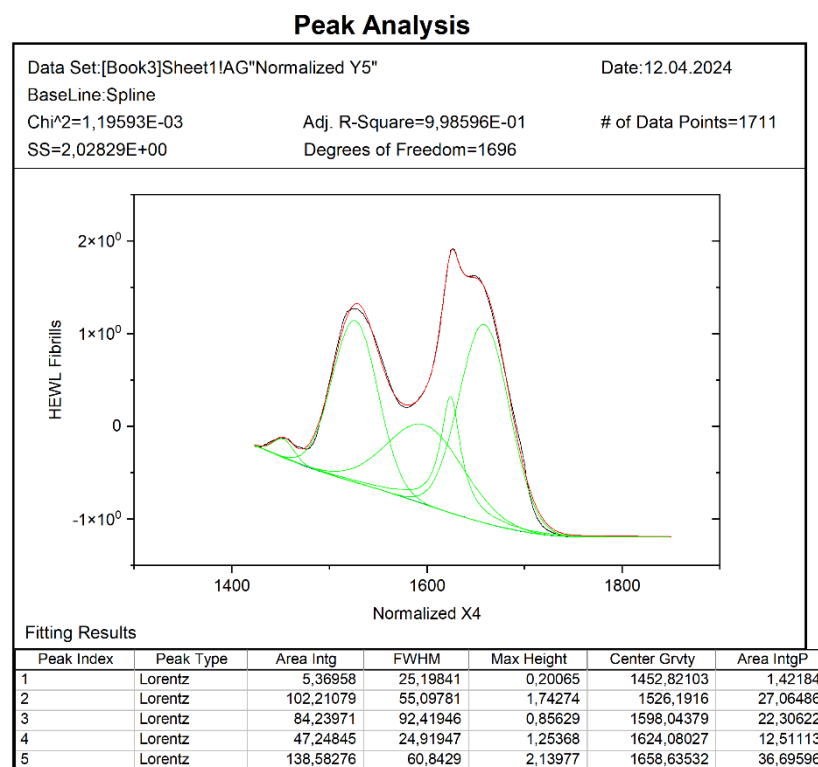


Fig. S39. Fitting parameters in FTIR spectra of α -helix and β -sheet peak in HEWL fibrils. $IntArea \frac{\alpha\text{-helices (peak 5)}}{\beta\text{-sheet (peak 4)}} = 2.92$

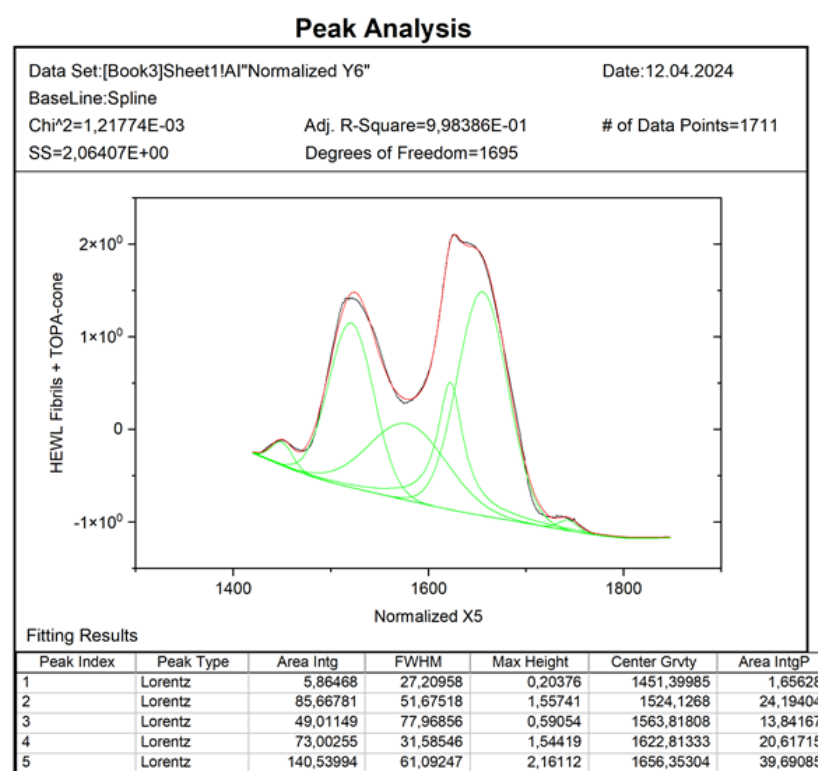


Fig. S40. Fitting parameters in FTIR spectra of α -helix and β -sheet peak in HEWL fibrils with TOPA-cone. $IntArea \frac{\alpha\text{-helices (peak 5)}}{\beta\text{-sheet (peak 4)}} = 1.93$

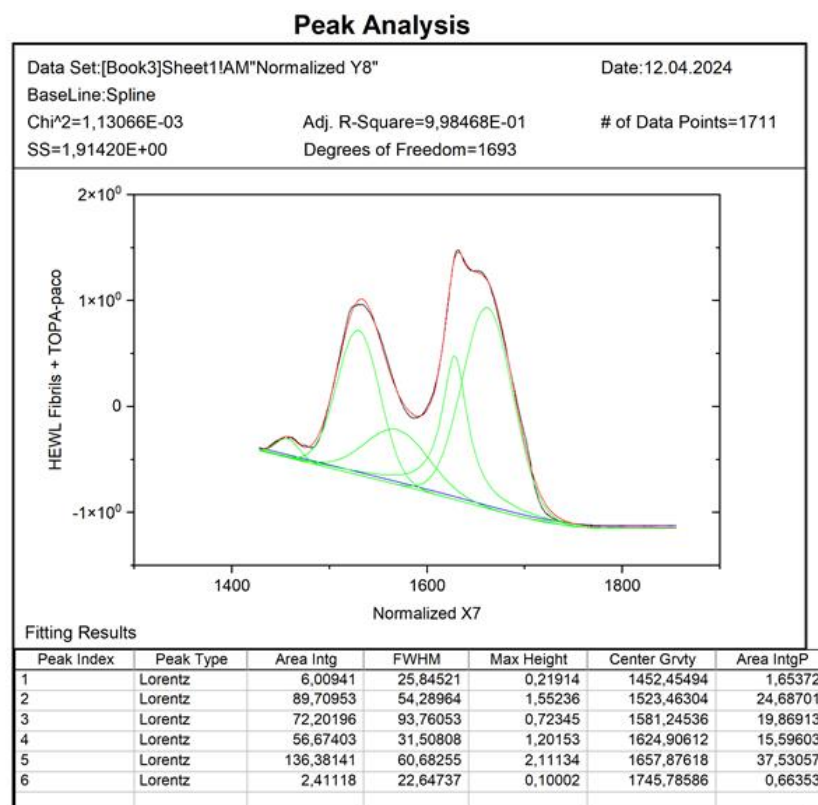


Fig. S41. Fitting parameters in FTIR spectra of α -helix and β -sheet peak in HEWL fibrils with TOPA-paco. $IntArea \frac{\alpha\text{-helices (peak 5)}}{\beta\text{-sheet (peak 4)}} = 2.40$

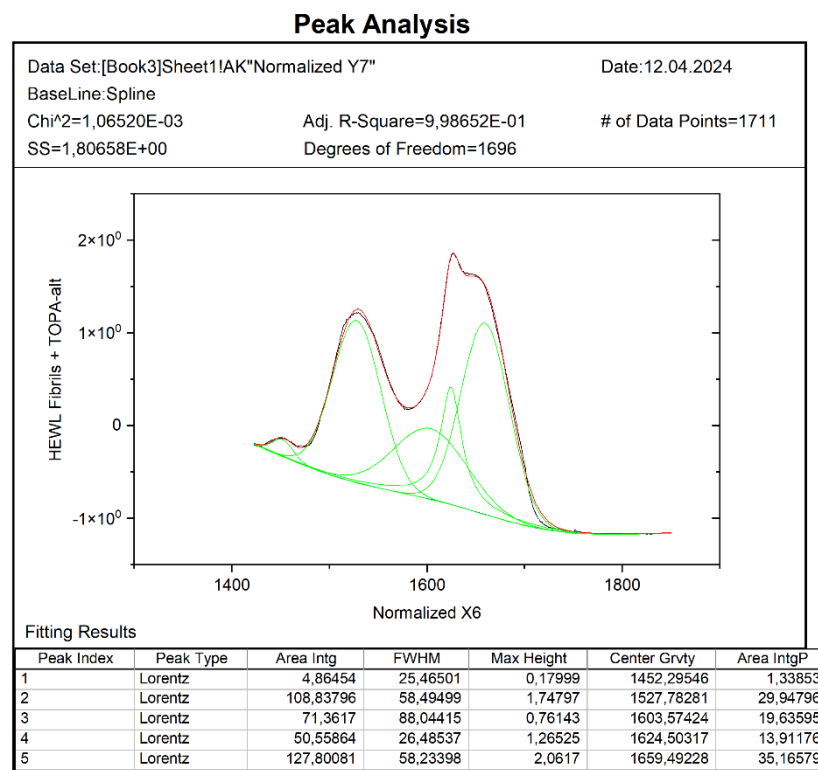


Fig. S42. Fitting parameters in FTIR spectra of α -helix and β -sheet peak in HEWL fibrils with TOPA-alt. $IntArea \frac{\alpha\text{-helices (peak 5)}}{\beta\text{-sheet (peak 4)}} = 2.57$

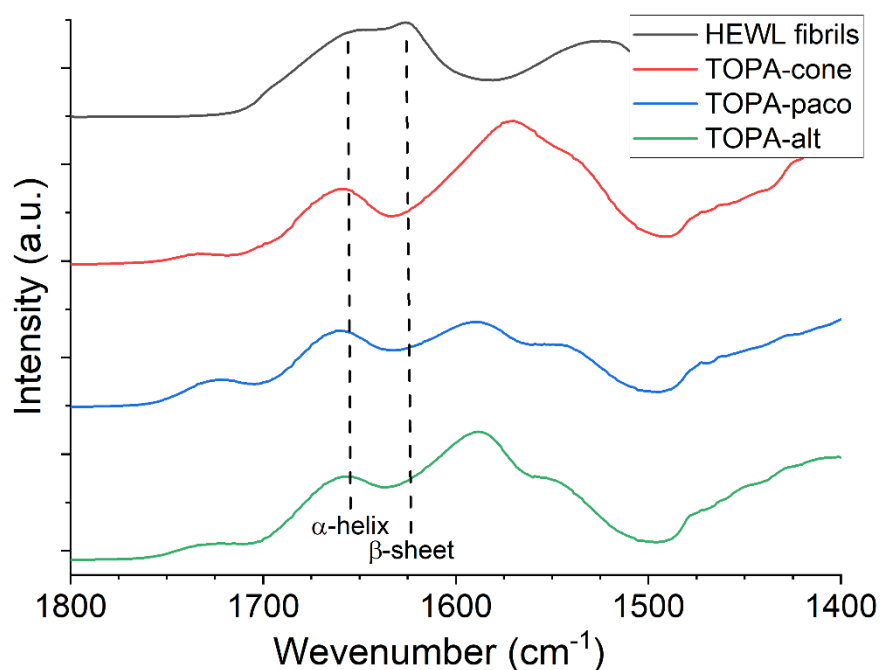


Fig. S43. FTIR spectra of thin films of HEWL fibrils and TOPA macrocycles.

References

- [S1] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J. Immunol. Methods.* 65 (1983) 55–63. [https://doi.org/10.1016/0022-1759\(83\)90303-4](https://doi.org/10.1016/0022-1759(83)90303-4)
- [S2] C.N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, How to measure and predict the molar absorption coefficient of a protein, *Protein Sci.* 4 (1995) 2411–2423. <https://doi.org/10.1002/pro.5560041120>