Electronic Supplementary Information

pH clock instructed transient supramolecular peptide amphiphile and its vesicular assembly

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Experimental Section

General Information and Materials:
2-Naphthaldehyde, 4,4'-Bipyridyl, Phosphorus Tribromide (PBr₃), Triethylsilane (TES), 1,6-Diphenyl-1,3,5-hexatriene (DPH), decylamine, dodecylamine, tetradecylamine and Gluconolactone (GdL) were purchased from Sigma-Aldrich (USA). Imidazole, Sodium Hydride, Methyl Iodide, Trifluoroacetic acid (TFA) and Urea were obtained from Spectrochem (India). Methyl Bromoacetate and Terephthalaldehyde were acquired from TCI Chemicals (India). Sodium Borohydride, Nile red dye and Urease from Canavalia ensiformis (Jack bean; Activity=345 u/mg) were procured from SRL (India). Cucurbit[8]uril (CB[8]) was synthesized following a previously published protocol and characterized accordingly.¹ Rink amide MBHA resin, protected amino acids and coupling reagents were purchased from Novabiochem. HPLC-grade dimethylformamide (DMF), dichloromethane (DCM) and acetonitrile (ACN) were procured from Spectrochem (India) and Fisher Scientific (India). Solvents were dried whenever required according to the reported procedures. Milli-Q water with a conductivity of less than 2 μS cm⁻¹ was used for all sample preparations. 60-120 mesh silica gel (SRL) was used for column chromatography. Chromatographic purifications were performed on a Luna 5 μm (C18) column (Phenomenex) using a Dionex Ultimate 3000 HPLC. ¹H NMR and ¹³C NMR spectra were recorded using a Bruker Ascend 600 MHz (Bruker, Coventry, UK) spectrometer and referenced to deuterated solvents. Coupling constants (J values) are reported in hertz, and chemical shifts are reported in parts per million (ppm). Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broadened). Electrospray ionization mass spectrometry (ESI-MS) were performed with a Q-Tof-Micro Quadrupole mass spectrometer (Micromass) and data were analyzed using the built-in software. MALDI analysis were performed with Bruker Daltonics - autoflex™ speed MALDI-TOF instrument.
Synthesis:

MV-CHO and Nap-P were synthesized following Scheme S1.

**Scheme S1.** Synthestic route for MV-CHO and Nap-P. (i) NaBH₄, Ethanol/THF, 0°C, 6 h; (ii) HBr, Toluene, Reflux, 3 h; (iii) MeI, Dry DCM, Reflux, Overnight; (iv) Dry Acetonitrile, 80°C, Overnight; (v) NaBH₄, Dry Methanol, RT, 2 h; (vi) PBr₃, Dry DCM, 0°C to RT, 4 h; (vii) Imidazole, NaH, Dry THF, RT (1 h) to Reflux (Overnight); (viii) Methyl bromoacetate, Toluene, 100°C, Overnight; (ix) 1M HCl, 105 °C, 3 h; (x) Solid Phase Peptide Synthesis (SPPS).
4-(Hydroxymethyl)benzaldehyde (A):
NaBH₄ (70.5 mg, 1.86 mmol, 0.25 eqv.) was slowly added to a solution of terephthalaldehyde (1 g, 7.45 mmol, 1 eqv.) in a mixture of ethanol and THF (1:1.5; 10 mL) at 0°C in an ice bath over a period of 30 minutes with constant stirring. The solution was then stirred at 0°C for 6 hours. The reaction mixture was acidified to pH 5 using 2 M HCl and the solvent was evaporated using a rotary evaporator. Water was added to the residue and the product was extracted using ethyl acetate. The organic fractions were collected, dried over anhydrous Na₂SO₄, filtered and evaporated. The crude product was purified using column chromatography (50% Ethyl Acetate/Hexane) to give the product as a white solid. Yield = 74%. ¹H NMR (600 MHz, CDCl₃) δ (ppm) 9.99 (s, 1H), 7.86 (d, J = 8.1 Hz, 2H), 7.52 (d, J = 7.9 Hz, 2H), 4.79 (s, 2H). ¹³C NMR (150 MHz, CDCl₃) δ (ppm) 192.30, 148.09, 130.12, 130.10, 127.80, 127.08, 127.04, 64.51.

4-(Bromomethyl)-benzaldehyde (B):
1.2 mL of 48% aqueous HBr was added to a solution of Compound A (447 mg, 3.28 mmol) in toluene (4 mL) and refluxed for 3 hours. The reaction mixture was cooled to room temperature, DCM was added to it and the organic phase was washed with saturated NaHCO₃ until neutral. The organic phase was dried over anhydrous Na₂SO₄, filtered and evaporated to give the crude product as a yellow solid. Upon further purification using column chromatography (3% Ethyl acetate/Hexane), the product was obtained as a white crystalline solid. Yield= 73%. ¹H NMR (600 MHz, CDCl₃) δ (ppm) 10.01 (s, 1H), 7.86 (d, J = 8.2 Hz, 2H), 7.56 (d, J = 8.1 Hz, 2H), 4.51 (s, 2H). ¹³C NMR (150 MHz, CDCl₃) δ (ppm) 191.67, 144.39, 136.26, 130.32, 129.83, 32.11.

1-methyl-[4,4'-bipyridin]-1-ium iodide (C):
Methyl Iodide (585 μL, 3.85 mmol, 1.2 eqv.) was added to a solution of 4 4'-bipyridyl (500 mg, 3.2 mmol, 1 eqv.) in dry DCM and refluxed overnight. The precipitate so obtained was washed with copious amounts of ether to give the product as a yellow solid. Yield=715.8 mg, 75%. ¹H NMR (600 MHz, D₂O) δ (ppm) 8.86 (d, J = 6.2 Hz, 2H), 8.72 (d, J = 4.2 Hz, 2H), 8.34 (d, J = 6.2 Hz, 2H), 7.86 (d, J = 4.5 Hz, 1H), 4.40 (s, 3H). ¹³C NMR (150 MHz, D₂O) δ (ppm) 153.50, 149.89, 145.55, 142.59, 125.71, 122.42, 47.77. Mass (ESI-MS): m/z calcd. for C₁₁H₁₁N₂⁺ [M]⁺: 171.092, found 171.093.

1-(4-formylbenzyl)-1'-methyl-[4,4'-bipyridine]-1,1'-diium (MV-CHO):
Compound B (674 mg, 2.26 mmol, 1 eqv.) and C (450 mg, 2.26 mmol, 1 eqv.) were dissolved in dry acetonitrile and refluxed overnight under an inert atmosphere of argon. The resultant precipitate was filtered and washed multiple times with ether to give the product as an orange colored solid. Yield = 97%. ¹H NMR (400 MHz, D₂O) δ (ppm) 10.00 (s, 1H), 9.22 (d, J = 6.7 Hz, 2H), 9.07 (d, J = 6.3 Hz, 2H), 8.60 (d, J = 6.4
Hz, 2H), 8.54 (d, J = 6.4 Hz, 2H), 8.06 (d, J = 8.0 Hz, 2H), 7.71 (d, J = 8.0 Hz, 2H), 6.08 (s, 2H), 4.51 (s, 3H). $^{13}$C NMR (100 MHz, D$_2$O) δ (ppm) 195.62, 150.80, 149.61, 146.36, 145.85, 138.97, 136.63, 130.97, 129.70, 127.33, 126.77, 64.19, 48.42. Mass (ESI-MS): m/z calcd. for C$_{19}$H$_{18}$BrIN$_2$O$_2$^[M]$: 495.965, for C$_{19}$H$_{17}$N$_2$O^[M-Br-I-H]$^+$: 289.134, found [M-Br-I-H]$^+$: 289.135.

**Naphthalen-2-ylmethanol (D):**
2-Naphthaldehyde (1 g, 6.4 mmol, 1 eqv.) was dissolved in dry MeOH and was cooled to 0 °C. To this cooled solution, sodium borohyde (266.4 mg, 7.04 mmol, 1.1 eqv.) was added in one portion, and the reaction mixture was allowed to come to room temperature and then stirred at room temperature for 2 hours. After completion of the reaction (confirmed by TLC), the solvent was removed under reduced pressure. To the resulting reaction mixture saturated NaHCO$_3$ was added and it was extracted with DCM. The organic phase was dried over anhydrous Na$_2$SO$_4$, filtered and concentrated under reduced pressure to give the desired material as a white solid. Yield = 88 %. $^1$H NMR (600 MHz, CDCl$_3$) δ (ppm) 7.87 – 7.76 (m, 4H), 7.52 - 7.43 (m, 3H), 4.83 (s, 2H). $^{13}$C NMR (150 MHz, CDCl$_3$) δ (ppm) 138.43, 133.50, 133.06, 128.42, 128.00, 127.82, 126.28, 126.00, 125.54, 125.28, 65.50.

**2-(bromomethyl)naphthalene (E):**
PBr$_3$ (457.5 μL, 4.87 mmol, 1.1 eqv.) was added dropwise to the cooled DCM solution of Compound-D (700 mg, 4.42 mmol, 1 eqv.). Then the reaction mixture was warmed up to room temperature and stirred for 4 hours. Sat. NaHCO$_3$ was added to the reaction mixture and the solution was extracted with DCM. The organic phase was collected, dried over anhydrous Na$_2$SO$_4$, filtered and evaporated with the help of rotary evaporator. The residue was then purified by silica gel chromatography with DCM to yield the desired product as a greyish solid. Yield = 802.2 mg, 82%. $^1$H NMR (600 MHz, CDCl$_3$) δ (ppm) 10.01 (s, 1H), 7.86 (d, J = 8.2 Hz, 2H), 7.56 (d, J = 8.1 Hz, 2H), 4.51 (s, 2H). $^{13}$C NMR (150 MHz, CDCl$_3$) δ (ppm) 191.67, 144.39, 136.26, 130.32, 129.83, 32.11.

**1-(naphthalen-2-ylmethyl)-1H-imidazole (F):**
Imidazole (585.6 mg; 8.6 mmol; 3 eqv.) was taken in dry THF and the solution was cooled to 0°C in an ice bath. Pre-dried NaH (206.46 mg; 8.6 mmol; 3 eqv.) was added to the solution at 0°C with constant stirring. The ice bath was then removed and the solution was allowed to stir at room temperature for 1 hour. Compound E (634 mg; 2.87 mmol; 1 eqv.) dissolved in dry THF was then added to the solution drop wise using a dropping funnel with constant stirring. The solution was allowed to stir for 3 hours at room temperature and then refluxed at 70°C overnight. THF was evaporated from the pale yellow suspension so obtained and ethyl acetate was added to the reaction mixture. The organic phase was washed multiple times with water followed by brine, dried over anh. Na$_2$SO$_4$, filtered and the organic phase was evaporated to give the product as a white solid. The product was taken to the next step without further purification. Yield
= 89%. \(^1\)H NMR (600 MHz, CDCl\(_3\)) \(\delta\) (ppm) 7.86 – 7.75 (m, 3H), 7.58 (d, \(J = 10.4\) Hz, 2H), 7.53 – 7.45 (m, 2H), 7.24 (dd, \(J = 8.5, 1.8\) Hz, 1H), 7.13 – 7.09 (m, 1H), 6.93 (d, \(J = 1.4\) Hz, 1H), 5.24 (s, 2H). \(^{13}\)C NMR (150 MHz, CDCl\(_3\)) \(\delta\) (ppm) 137.59, 133.62, 133.34, 133.08, 129.93, 129.03, 127.92, 127.83, 126.74, 126.55, 126.37, 124.93, 119.45, 51.03. Mass (ESI-MS): m/z calcd. for C\(_{14}\)H\(_{13}\)N\(_2\)\(^+\) [M+H]\(^+\): 209.107, found 209.109.

3-(2-methoxy-2-oxoethyl)-1-(naphthalen-2-ylmethyl)-1H-imidazol-3-ium (G):

**Compound F** (555.6 mg, 2.67 mmol, 1 equiv.) was dissolved in toluene in a round-bottomed flask and methyl bromoacetate (378.8 \(\mu\)L, 4 mmol, 1.5 equiv.) was added to it. The solution was then stirred at 100°C overnight. The white precipitation so formed was collected and washed multiple times with ether and finally dried under high vacuum to give the product as a white solid. Yield = 98.6%. \(^1\)H NMR (600 MHz, DMSO-d\(_6\)) \(\delta\) (ppm) 9.36 (s, 1H), 8.02 – 7.98 (m, 2H), 7.97 – 7.90 (m, 3H), 7.81 (t, \(J = 1.8\) Hz, 1H), 7.60 – 7.52 (m, 3H), 5.71 (s, 2H), 5.30 (s, 2H), 3.74 (s, 3H). \(^{13}\)C NMR (150 MHz, DMSO-d\(_6\)) \(\delta\) (ppm) 167.28, 137.50, 132.74, 132.68, 132.06, 128.84, 127.84, 127.68, 127.64, 126.79, 126.77, 125.66, 124.19, 122.39, 52.79, 52.20, 49.65. Mass (ESI-MS): m/z calcd. for C\(_{17}\)H\(_{17}\)N\(_2\)O\(_2\)\(^+\) [M]\(^+\): 281.128, found 281.132.

3-(carboxymethyl)-1-(naphthalen-2-ylmethyl)-1H-imidazol-3-ium bromide (H):

**Compound G** (900 mg, 2.49 mmol) was dissolved in 25mL 1M HCl and the solution was refluxed for 3 hours. Water in the reaction mixture was then evaporated on a rotary evaporator and the solid so obtained was washed multiple times with ether and finally dried under high vacuum to give the product as a white solid. Yield = 97.8%. \(^1\)H NMR (600 MHz, D\(_2\)O) \(\delta\) (ppm) 8.77 (s, 1H), 7.85 (dd, \(J = 9.9, 6.3\) Hz, 3H), 7.78 (d, \(J = 1.9\) Hz, 1H), 7.54 – 7.49 (m, 2H), 7.38 (d, \(J = 1.9\) Hz, 2H), 7.34 (dd, \(J = 8.5, 1.9\) Hz, 1H), 5.41 (s, 2H), 4.92 (s, 2H). \(^{13}\)C NMR (150 MHz, D\(_2\)O) \(\delta\) (ppm) 170.18, 136.84, 133.03, 132.92, 130.87, 129.24, 128.10, 128.05, 127.81, 127.25, 127.11, 125.59, 123.94, 122.29, 53.18, 50.40. Mass (ESI-MS): m/z calcd. for C\(_{16}\)H\(_{15}\)N\(_2\)O\(_2\)\(^+\) [M]\(^+\): 267.113, found 267.115.

**General Synthesis of the Peptide (Nap-P):**

The peptide was synthesized on Rink amide MBHA resin using standard Fmoc (9-fluorenylmethoxycarbonyl) solid phase peptide synthesis (SPPS) protocol. In a typical coupling, 3 equiv. of protected amino acid (with respect to the loading of the resin), 3 equiv. of HBTU, and 6 equiv. of DIPEA were taken in 5 mL of DMF (for 0.1 mmol scale with respect to the resin loading) and stirred for 5 minutes prior to addition of the mixture to the swelled deprotected resin. The reaction mixture was shaken for 60 min and the resin was washed several times with DMF. The Fmoc-deprotection was achieved by treatment of the resin thrice with 5 mL of 20% piperidine in DMF for 5 minutes followed by thorough washing of the resin with DMF and DCM. The Fmoc-deprotection and coupling steps were repeated until the designed
peptide sequence was obtained. The resin with the loaded peptide was washed several times with DMF and DCM and dried. The dried resin was then treated with a mixture of freshly prepared mixture of 8.5:1:0.5 (TFA/TEA/H$_2$O) and stirred for 1 h. The resin was finally washed with DCM several times. The cleavage cocktail and the washings combined were concentrated to a minimum volume on a rotary evaporator. The cleaved peptide was then precipitated from cold dry ether, centrifuged and lyophilized to get the crude peptide. Purification was done in a semi-preparative HPLC using a Luna 5 μm (C18) column (Phenomenex) and an eluent of acetonitrile and water starting at 5% ACN in H$_2$O reaching at 100% ACN in 30 mins) to complete the chromatogram in 35 mins. (Yield = 65%)

MALDI-TOF: m/z calcd. for C$_{34}$H$_{45}$N$_{12}$O$_{9}$ $^{+}$ [M]$^{+}$, 765.808; found 765.418.

**UV-Visible and Fluorescence Spectroscopy:** UV-Visible spectra were recorded on a PerkinElmer Lambda 750 spectrometer, while fluorescence measurements were performed on a Cary Eclipse (Agilent) spectrophotometer.

**Isothermal Titration Calorimetry (ITC):** ITC was performed using a Nano-ITC instrument from MicroCal for determining the formation constants and thermodynamic parameters for the inclusion complexes. 1 mM Nap-P solution in buffer (10 mM phosphate, pH 7) was injected in parts (each injection, 1.3 μL) at an interval of 2 mins from a 40 μL micro-syringe into the MV-CHO@CB[8] (1:1) solution (0.05 mM, 200 μL) with constant stirring (300 rpm) at 298K. All the solutions were degassed prior to titration. The ITC thermogram showed a 1:1 binding ratio between MV-CHO@CB[8] and Nap-P, thus indicating the formation of the ternary complex.

**Dynamic Light Scattering (DLS) Studies:** The particle sizes of the assemblies were obtained at 298 K using a 632.8 nm He–Ne laser using Zetasizer Nano-ZS90 (Malvern). The stock solution of assemblies were prepared in water and filtered through appropriate filters to remove dust particles, if present before the measurement. All the measurements were performed in triplicates.

**pH Measurements:** The pH curves were recorded on a Hanna HI 2210 pH meter equipped with HI1131 pH probe from Hanna.

**Field Emission Scanning Electron Microscopy (FESEM):** 5 μL of appropriate sample solution at specific time intervals during the pH cycle were casted on a silicon wafer and immediately freeze-dried to cease the kinetics of the pH cycle. FESEM images were then taken on a Gemini SEM 300 (Sigma Zeiss) instrument.

**Field Emission Transmission Electron Microscopy (FETEM):** 5 μL of the sample solution at specific time intervals during the pH cycle were casted on the carbon coated copper grid (300 mesh Cu grid
with thick carbon film from Pacific Grid Tech, USA) and allowed to air dry for 2 minutes and then the excess sample was bloated with a tissue paper. The grid was then immediately freeze-dried and the FETEM images were taken in JEOL 2100F microscopes.

**Atomic Force Microscopy (AFM):** Sample solution at specific time intervals during the pH cycle were casted on a silicon wafer and freeze-dried before the experiment. AFM images were then taken on Oxford Cypher microscope.

**Confocal Laser Scanning Microscopy (CLSM):** Confocal laser scanning microscopic (CLSM) images and videos were recorded on a TCS SP8 microscope from Leica, Germany.

**Preparation of CB[8] stabilized Host-Guest Ternary Complex:**
To prepare 2 mL of 0.75 mM MV-CHO@CB[8] stock solution, appropriate amount of CB[8] (2.6 mg; the overall molecular weight of the used CB[8] was found to be 1730 from the elemental analysis data) and MV-CHO (0.75mg) were taken in a 2 mL volumetric flask and MilliQ water was added to the mixture. The heterogeneous solution was then sonicated for 1 hour at 298K. An equivalent amount of the peptide (Nap-P) was added to the solution of the binary complex and sonicated for 1 hour at 298K. The solution was then kept undisturbed at 298K for 1 day before utilizing for further experiments. The formation of the CB[8] stabilized charge-transfer ternary complex was confirmed by the appearance of a CT band at 396 nm as well as by ITC (Figure 1).

**NMR Study for the pH dependent reversible formation of the supramolecular peptide amphiphile:**
Stock solutions of the ternary complex (1) (1 mM in D_2O) and DA (100 mM in THF-d_8) were prepared. Solution containing equimolar amounts (0.5 mM) of the ternary complex (1) and DA was prepared in D_2O and its NMR spectra was recorded. A drop (1µL) of NaOD was then added to the above solution to induce the formation of imine bonds and thus the SPA and the NMR spectra was recorded again. Finally, few drops (5 µL) of DCl was added to the above solution to neutralize NaOD and break the imine linkages and the NMR spectra was recorded again. The disappearance of the aldehyde peak and the appearance of the imine peak and vice versa in basic and acidic medium, respectively indicate the formation and breakdown of the supramolecular peptide amphiphile.

**Programming of the transient pH states for establishing a tunable pH Cycle:**
Stock solution of Urease enzyme (2 mg/mL) was prepared in MilliQ water and stored at -20°C for further use. Gluconolactone (GdL) and urea were used in the solid form for all experiments. Urea beads were ground to a fine powder to facilitate easy dissolution in water. The required amount of urease (final
concentration: 0.2 mg/mL) was added to MilliQ water and the pH of the solution was adjusted to ~5 using 0.1 M HCl solution. A solid mixture of GdL and urea in the required ratio was added in one portion to the solution and were immediately dissolved by simple hand shaking. The change in pH was then monitored over time at 298K.

**pH Kinetics Study of transient vesicle formation:**

Stock solutions of the ternary complex \((1)\) (1 mM in water) and \(DA\) (100 mM in THF) were prepared. Equimolar amounts (0.1 mM) of the ternary complex and \(DA\) were taken in water and mixed thoroughly. The requisite amount of urease (final concentration: 0.2mg/mL) was added to the solution and the pH of the solution was adjusted to ~5 using 0.1 M HCl solution. The pH cycle was initiated by addition of a 1:3 mixture of GdL (45 mM) and urea (15mM) in solid state to the solution at 298K. The solids were dissolved in the solution by simple hand shaking and the pH changes were monitored over time.

**Dynamic Light Scattering Kinetics Study of transient vesicle formation:**

The solution containing equimolar amounts (0.1 mM) of the ternary complex and \(DA\) along with the required amount of urease (0.2mg/mL) was acidified to pH 5 using 0.1M HCl solution. A 1:3 mixture of GdL (45 mM) and urea (15 mM) were added to the solution and the solution was sonicated briefly to facilitate dissolution. DLS measurements were performed immediately and the change in the hydrodynamic size distribution with time was monitored at 298K. All measurements were performed with a constant angle of 90° and the results were reported as Number Distribution to reflect the number of aggregates formed. All measurements were performed in triplicates.
**Fluorescence Kinetics Study of the repeatability of the formation of the transient vesicle:**

Stock solution (10 mM) of the hydrophobic dye DPH (Diphenylhexatriene) was prepared in THF. Equimolar amounts (0.1 mM) of the ternary complex (1) and DA were dissolved in MilliQ water containing 0.2mg/mL urease and the pH of the solution was adjusted to 5 using 0.1M HCl solution. 10 µM of the hydrophobic dye DPH (Diphenylhexatriene) was added to the solution and then the pH cycle was started by addition of a 1:3 mixture of GdL (45mM) and Urea (15mM). The solution was excited at 355 nm and the emission intensity was monitored at 438 nm over a period of 250 mins.

**Fluorescence Confocal Microscopy Studies:**

Transient vesicle formation was performed as mentioned earlier but in the presence of Nile Red and the phenomenon was monitored through CLSM. Nile red was used as a hydrophobic dye for fluorescence imaging as it specifically accumulates in the hydrophobic vesicle bilayer and shows fluorescence upon vesicle formation. The sample was excited at 488nm and the fluorescence emission was observed at 549 – 662 nm over a period of 120 min at RT.

**Particle Count Analysis from CLSM:**

The particle count from the CLSM images obtained at different time points were analyzed using ImageJ software. In particular, the images were converted to 8 bit format and visible particles were counted with threshold level set at 20, 25 and 30. The particles having a minimum object dimension of 2, 4, 6, 8 and 10 px² were considered for particle count analysis.
Fig. S1 $^1$H NMR of different combinations of MV-CHO, compound H and CB[8] showing the ternary complexation between them.

NOTE: It is to be noticed that in case of 1:1:1 mixture of these three compounds, all the MV-protons and naphthalene protons showed up-field shift due to ternary complex formation. However, no change in position was observed for the imidazolium-protons. This can only happen for this particular combination when MV and naphthalene groups are in a slipped stacked orientation as shown below.
Fig. S1 Thermogram (top) and binding isotherm (bottom) of MV-CHO@CB[8] with H at 298 K showing 1:1 complexation.

Fig. S3 Representative plot of DLS measurements during the transient formation of vesicles in response to the pH clock.
Fig. S4 A) Time dependent emission at 438 nm ($\lambda_{ex} = 355$ nm) of DPH in presence of 1 and DA in water at room temperature. To trigger the vesicle formation, NaOH was added and then to disrupt the vesicles, HCl was added. B) The emission spectra of the same system at different times as mentioned in (A).

Fig. S5 CLSM image of the vesicles formed by 1 and DA at pH 8.

Particle count analysis

The CLSM images recorded at different times (SV1) has been analyzed using different parameters to extract the particle count from each image. In particular, the background threshold level has been set to 20-25-35 (for 8-bit images) and the minimum object dimension has been set to 2,4,6,8 and 10 pixel$^2$ (px$^2$). The results are reported in the following graphs.

The Y axis represents the number of particles calculated and the X axis shows particles of different sizes. In the Z axis, the time is varied. Three different thresholds are plotted as A (20), B (25) and C (35).
Fig. S6 Statistical analysis of the images obtained by confocal fluorescence microscopy (Fig. 4 of the main manuscript).
Fig. S7 Representative plot showing the change in pH during the transient vesicle formation upon three consecutive cycles.
Fig. S8 Representative plot of DLS measurements during the transient formation of vesicles in response to the pH clock by amines of different chain lengths.
Spectroscopic characterization of the synthesized molecules.

Fig. S9 $^1$H NMR spectrum of Compound A in CDCl$_3$.

Fig. S10 $^{13}$C NMR spectrum of Compound A in CDCl$_3$. 
Fig. S11 $^1$H NMR spectrum of Compound B in CDCl$_3$.

Fig. S12 $^{13}$C NMR spectrum of Compound B in CDCl$_3$. 
Fig. S13 $^1$H NMR spectrum of Compound C in D$_2$O.

Fig. S14 $^{13}$C NMR spectrum of Compound C in D$_2$O.
Fig. S15 $^1$H NMR spectrum of MV-CHO in D₂O.

Fig. S16 $^{13}$C NMR spectrum of MV-CHO in D₂O.
Fig. S17 $^1$H NMR spectrum of Compound D in CDCl$_3$.

Fig. S18 $^{13}$C NMR spectrum of Compound D in CDCl$_3$. 
Fig. S19 $^1$H NMR spectrum of Compound E in CDCl$_3$.

Fig. S20 $^{13}$C NMR spectrum of Compound E in CDCl$_3$. 
Fig. S21 $^1$H NMR spectrum of Compound F in CDCl$_3$.

Fig. S22 $^{13}$C NMR spectrum of Compound F in CDCl$_3$. 
Fig. S23 $^1$H NMR spectrum of Compound G in DMSO-d$_6$.

Fig. S24 $^{13}$C NMR spectrum of Compound G in DMSO-d$_6$. 
Fig. S25 $^1$H NMR spectrum of Compound H in D$_2$O.

Fig. S26 $^{13}$C NMR spectrum of Compound H in D$_2$O.
Fig. S27 Analytical HPLC chromatogram of Nap-P.

Fig. S28 MALDI-TOF of Nap-P.
**Descriptions for SVideo 1 and 2**

**SVideo 1:** Time lapsed video (120 mins) for one cycle of the transient vesicle formation by $1 + \text{DA} + \text{Urease}$ in presence of NR and after addition of urea/Gdl showing a target region of the sample.

**SVideo 2:** Time lapsed video (120 mins) for one cycle of the transient vesicle formation by $1 + \text{DA} + \text{Urease}$ in presence of NR and after addition of urea/Gdl showing one vesicle.

**References**