Supporting Information

A Transient Vesicular Glue for Amplification and Temporal Regulation of Biocatalytic Reaction Networks

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A. Materials and methods

Materials

All commercially available reagents were used as received. Adenosine 5'-triphosphate disodium salt hydrate (ATP), Adenosine 5'-diphosphate sodium salt (ADP), Adenosine 5'-monophosphate disodium (AMP), Guanosine 5'-triphosphate sodium salt hydrate (GTP), Amberlite IRA-400, 1,6-diphenyl-1,3,5- hexatriene (DPH), Cytochrome C (CytC) from equine heart, Glucose Oxidase (GOx) from Aspergillus niger, Apyrase from potatoes (PA), Laccase from Trametes versicolor, D-Glucose anhydrous, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Triton X-100 and hydrogen peroxide (30 %, w/v solution) were purchased from Sigma-Aldrich. Particularly, PA was dissolved in 1.0 mL of Milli-Q water making a concentration of 100 U/mL and preserved at -20 °C for further use. Hexadecyl amine, sodium tripolyphosphate anhydrous, nile red, 3,3',5,5'-tetramethylbenzidine (TMB), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Calcein reagent and peroxidase ex. horseradish (HRP) were procured from SRL, India. S-methylisothiouronium sulfate and dodecyl amine were purchased from Alfa Aesar. Tetradecyl amine, stearylamine and fluorescein-5-isothiocyanate (FITC) were obtained from TCI Chemicals. Sodium metal and p-Toluenesulfonic acid monohydrate were purchased from SpectroChem. All the stock solutions were prepared by weighing.

Methods

NMR Spectroscopy. NMR spectra were recorded using a Bruker AV400 NMR spectrometer operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. ^{31}P NMR was recorded in 400 MHz Bruker spectrometer with 5 mm BBO probe. Multiplicity is given as follow: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad peak.

ESI-MS. ESI-MS experiments were conducted on MicroMass QTOF mass spectrometer.

UV-Vis and Fluorescence Spectroscopy. UV-visible spectra were measured at 25 ºC on PerkinElmer Lambda750 Spectrometer and fluorescence emission on a Hitachi F-7000 spectrophotometer using quartz cuvette with 1 cm pathlength. Kinetics measurements were performed on a Molecular Devices SpectraMax M5^{e} microplate spectrophotometer. Measurements were performed in a non-tissue culture treated 96-well plate (Corning, flat bottom, non-binding surface). Turbidity measurements and some fluorescence emission studies were also performed on the SpectraMax M5^{e} microplate instrument.

Isothermal Titration Calorimetry (ITC). Thermodynamics of non-covalent bindings was performed on MicroCal VP- ITC instrument from Malvern.

Nanoparticle Tracking Analysis (NTA). Particle counts were analyzed using Malvern Nanosight LM14C Nanoparticle Tracking Analyser.

Dynamic light scattering (DLS) and Zeta potential. DLS and zeta potential measurements were performed on a Malvern Zetasizer Nano ZS instrument.

Electron Microscopy. Transmission Electron Microscopy (TEM) images were recorded on ThermoFischer Scientific Talos L120C electron microscope. The Field Emission Scanning Electron Microscopy (FESEM) analysis was carried out using the Ultra55 FESEM Karl Zeiss MonoCL instrument with 3 kV and 5 kV emission voltage.

Confocal Laser Scanning Microscopy (CLSM). Confocal images were taken using a Confocal Zeiss LSM880 (Airyscan) microscope.

Small Angle X-ray Scattering (SAXS). SAXS was recorded on Small Angle X-ray Scattering (SAXS) Low Angle instrument from Nanostar.

Circular Dichroism (CD). CD experiments were performed at 25 °C using JASCO, J-815 CD spectrometer. All the spectra are baseline corrected and θ (ellipticity) values were recorded in mdeg units.

Fluorescence lifetime. The measurements were done using a time-correlated single photon counting (TCSPC) spectrometer (Edinburgh FLS980).

B. Design of the ATP-driven self-assembled system

Synthesis of the ligands

1-Hexadecylguanidinium chloride (C_{16}Gua) was synthesized according to a reported protocol (Figure S1).[1] First, a methanolic solution of methylisothiourea sulphate was prepared, and then sodium metal (dissolved in methanol) was gently added to it at 0 °C. The resulting mixture was stirred for 1 h at 0 °C followed by the addition of hexadecyl amine. The mixture was stirred at 25 °C for 2 h and then at 40 °C for 38 h resulting in the formation of 1-Hexadecylguanidinium sulphate. Sulphate anion was exchanged to tosylate anion by adding p-Toluenesulfonic acid monohydrate (p-TsOH.H_2O) to the solution. The solution was heated at 60 °C for 2 h and the resulting precipitate was filtered off. The filtrate was kept at -20°C to give colourless crystals. Recrystallization from acetonitrile gave 1-hexadecylguanidinium p-toluene sulfonate salt as fine colourless crystals. The p-toluene sulfonate salt was converted to chloride ion via ion exchange on Amberlite IRA-400 chloride form. The desired product formation was confirmed by ^{1}H, ^{13}C and ESI-MS (Figure S2a-c).

Following the same protocol, dodecylguanidinium chloride (C_{12}Gua), tetradecylguanidinium chloride (C_{14}Gua) and octadecylguanidinium chloride (C_{18}Gua) were synthesized and product formation was successfully confirmed by mass spectrometry (Figure S2d-f).

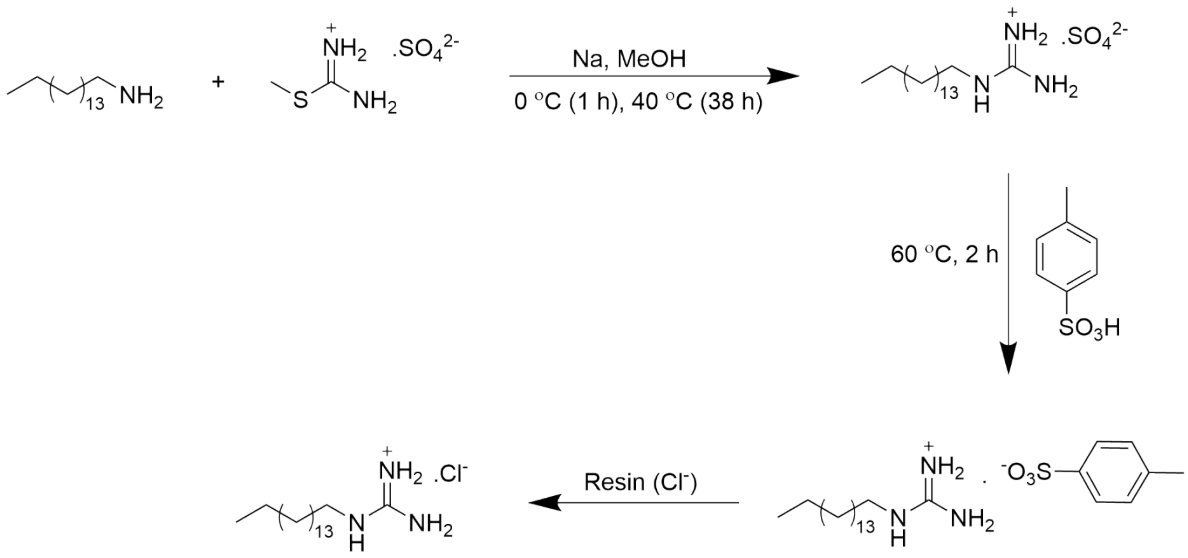


Figure S1. Synthesis of 1-Hexadecylguanidinium chloride.

Characterization of the ligands

1-Hexadecylguanidinium chloride (C_{16}Gua):

^{1}H NMR (δ ppm, DMSO-d_6, 298 K, 400 MHz): 0.86 (t, 3H, CH_3), 1.24 (m, 26H, 13 CH_2), 1.45 (m, 2H, $NHCH_2CH_2$), 3.08 (m, 2H, $NHCH_2$), 6.91, 7.32 and 7.68 (br and m, respectively, 5H, guanidinium).

^{13}C NMR (δ ppm, DMSO-d_6, 298 K, 100 MHz): 157.24, 41.12, 31.67, 29.07, 28.94, 28.51, 26.32, 22.33, 13.04.

The ^{1}H and ^{13}C NMR Spectra of C_{16}Gua are shown below in Figure S2a and S2b.

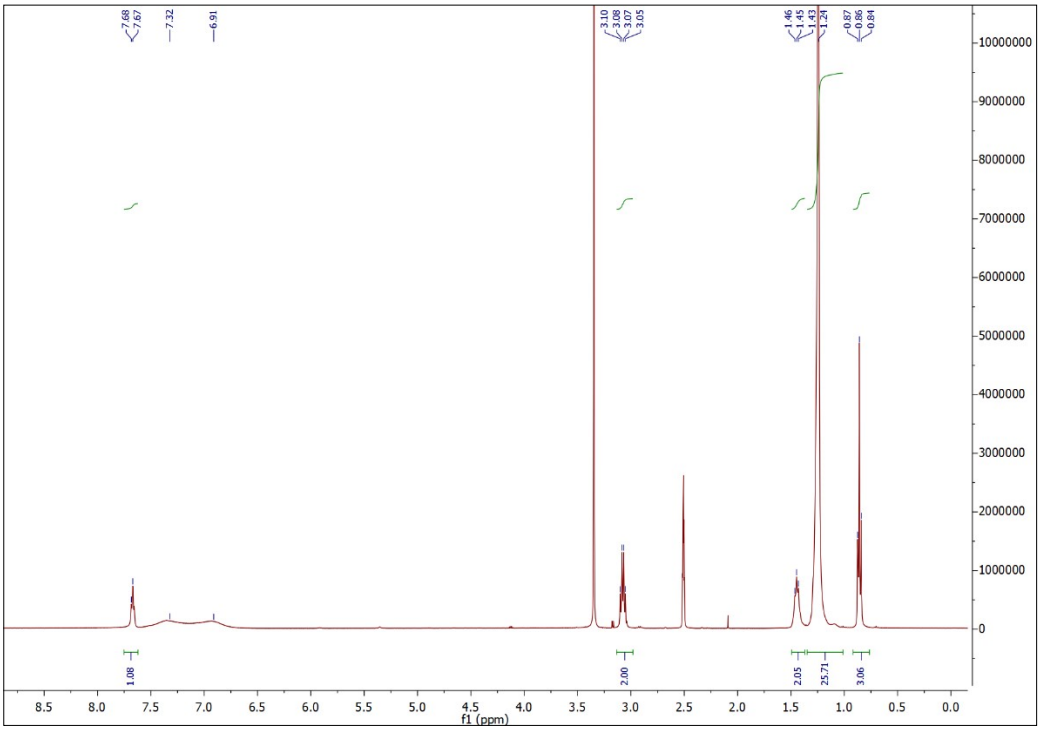


Figure S2a. ^{1}H NMR spectrum of C_{16}Gua.

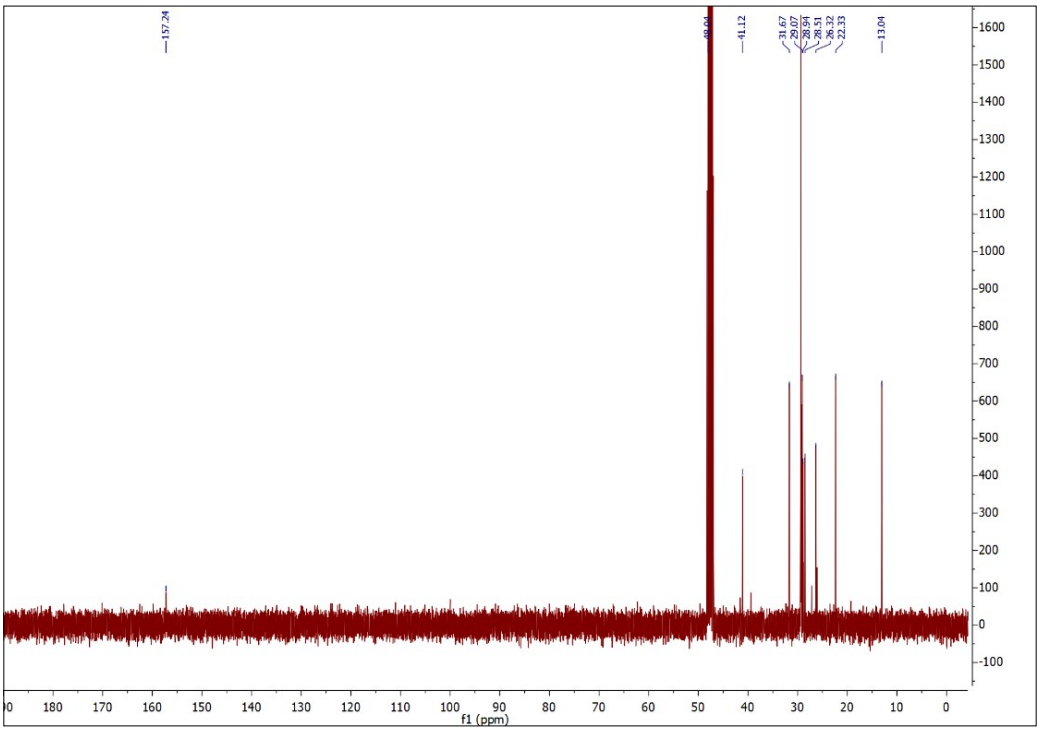


Figure S2b. ^{13}C NMR Spectrum of C_{16}Gua.

Further, molecular weight was confirmed by ESI-MS (ESI$^+$). [M+H] $^+$: m/z calculated for $C_{17}H_{37}N_3$ 284.5040, Found: 284.3066, as can be seen in Figure S2c.

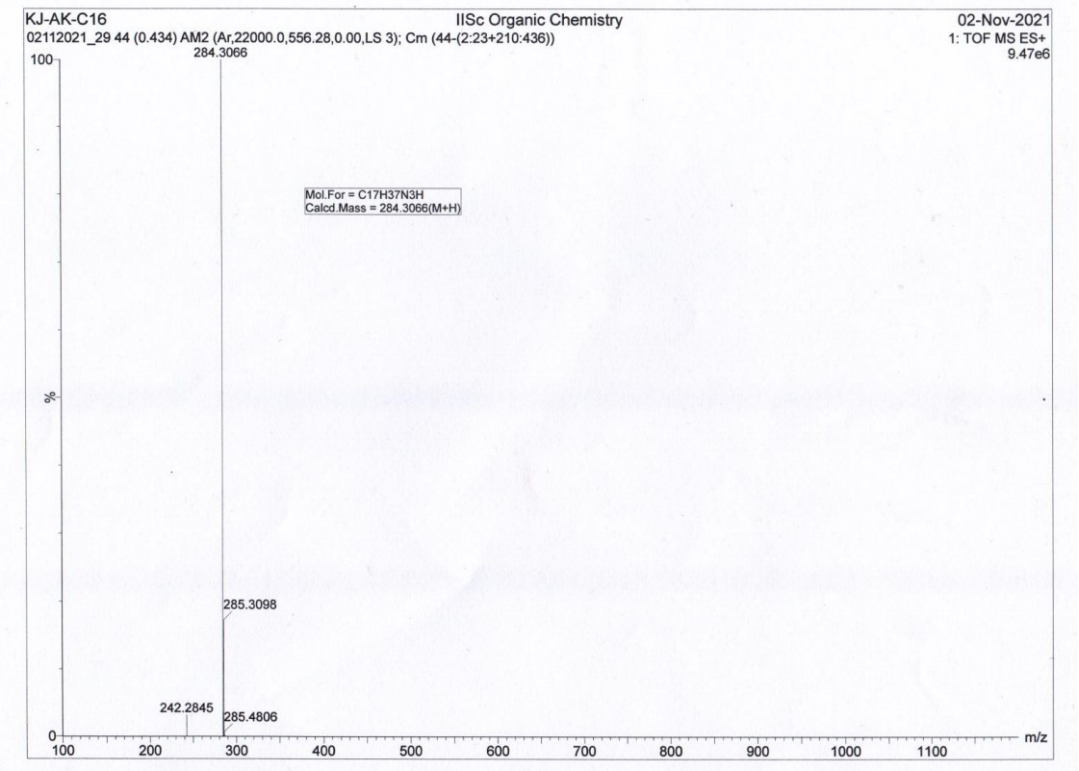


Figure S2c. ESI-MS spectrum of C_{16}Gua.

C_{12}Gua, C_{14}Gua and C_{18}Gua were also characterized by mass spectrometry. Figure S2d, S2e and S2f shows the mass spectrum with expected m/z peak, indicating the successful formation of C_{12}Gua, C_{14}Gua and C_{18}Gua, respectively.

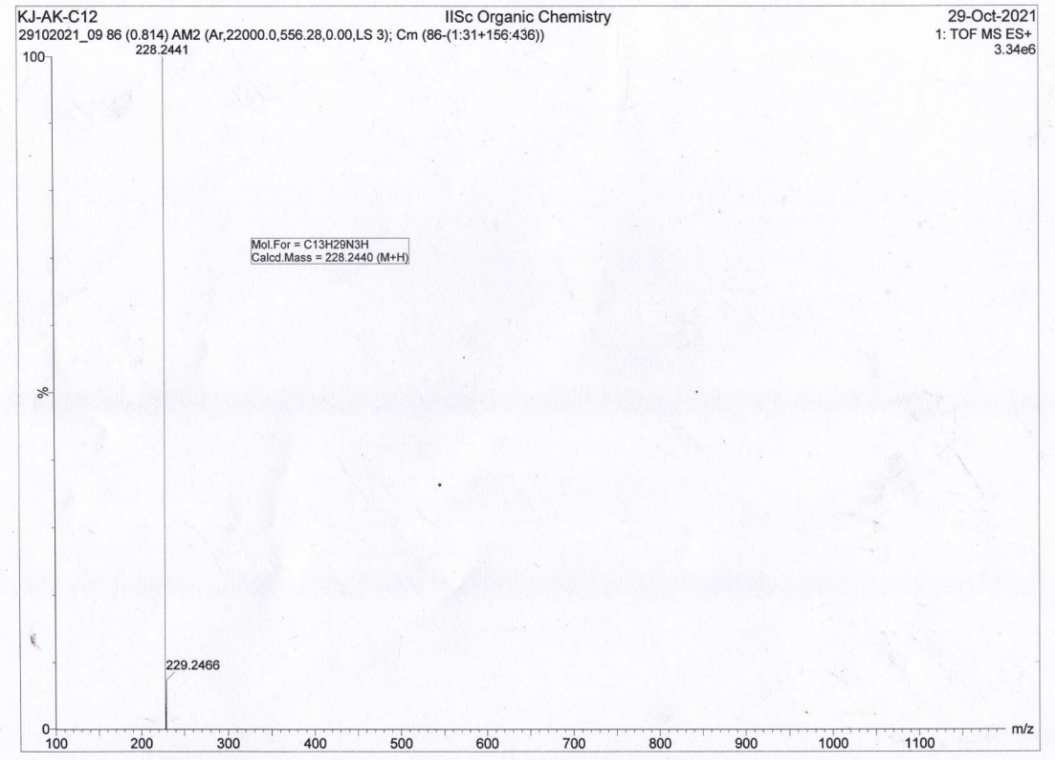


Figure S2d. ESI-MS spectrum of C_{12}Gua.

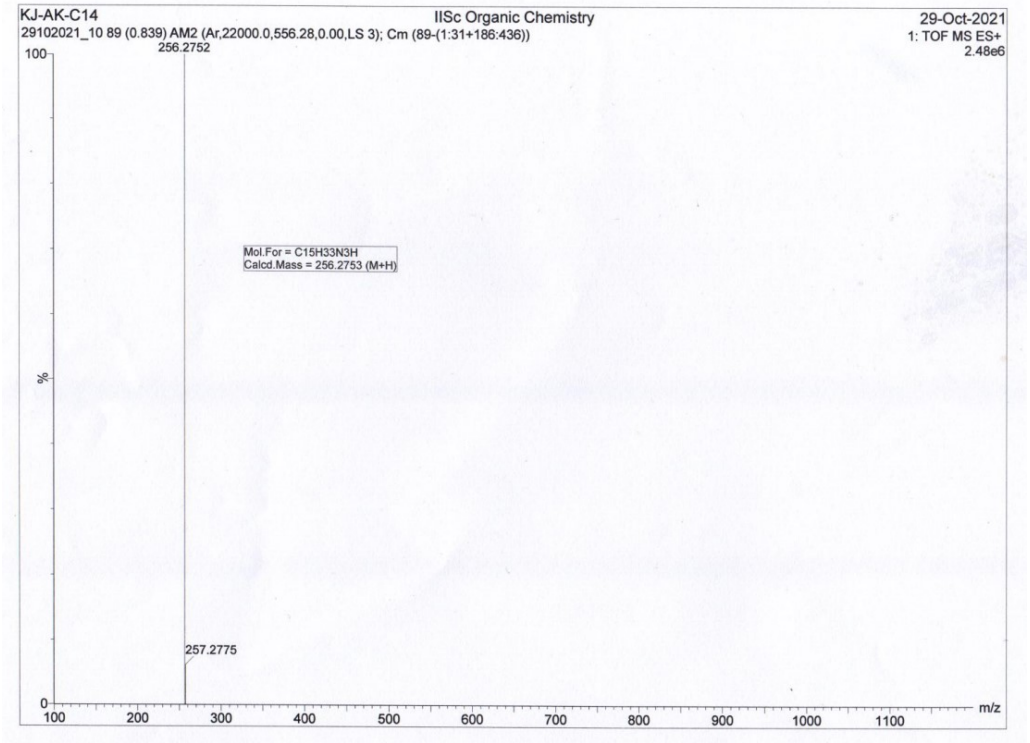


Figure S2e. ESI-MS spectrum of C_{14}Gua.

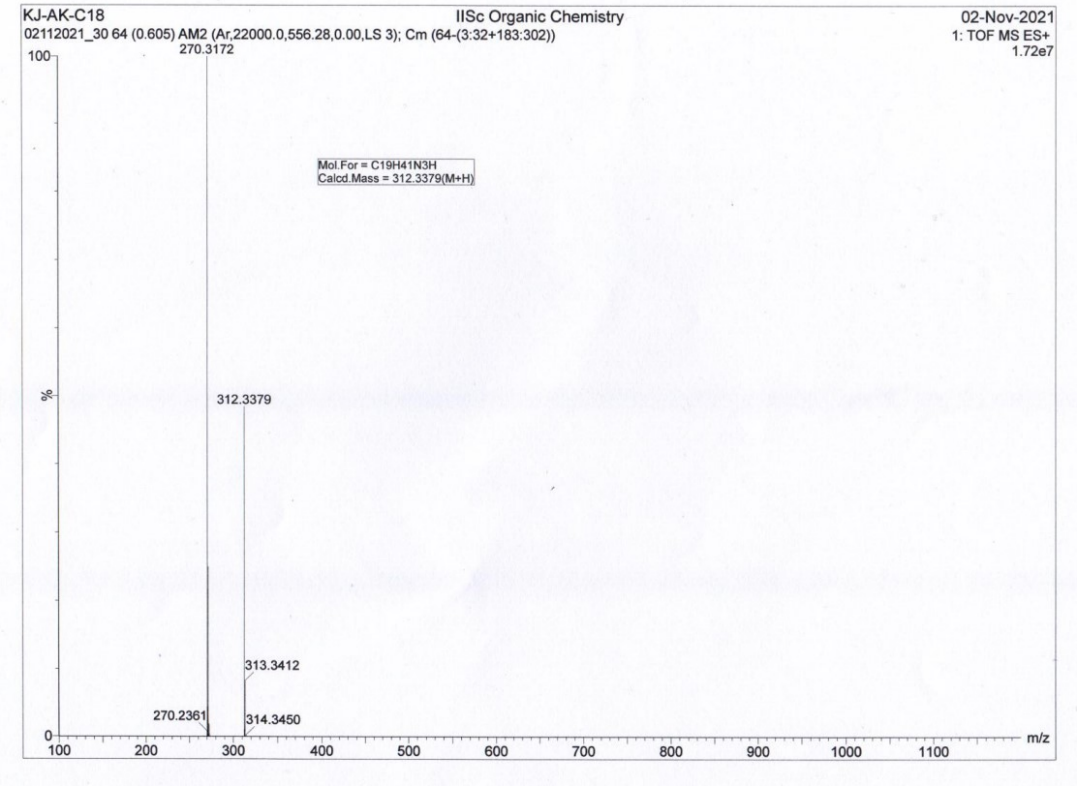


Figure S2f. ESI-MS spectrum of C_{18}Gua.

Effect on ^{31}P NMR of ATP with the addition of C_{16}Gua

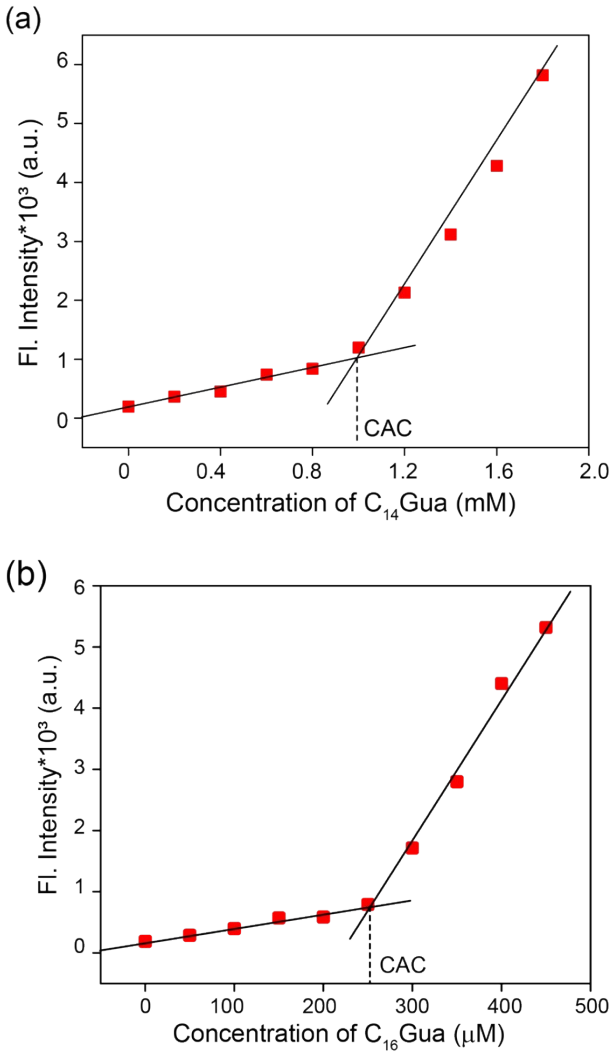
To study the interaction of C_{16}Gua with ATP, the impact on electronic environment around phosphates of ATP was studied. ATP was titrated with increasing concentration of C_{16}Gua, and the ^{31}P-NMR spectra were recorded. To the solution of 20 mg of ATP in 0.5 mL D_2O and 0.25 mL of DMSO-d_6, 0.25 equivalents of C_{16}Gua (dissolved in same volume ratio of solvents) was added each time and spectra were recorded successively after each addition. Guanidinium-phosphate interactions *via* electrostatic and H-bonding affects the ^{31}P NMR signals of ATP.

C. ATP-driven self-assembled vesicles

1. Spectroscopic studies

Critical aggregation concentration (CAC) of the surfactants C_nGua (n = 12, 14, 16, 18) using DPH dye

Fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH) is almost non-fluorescent in aqueous medium but upon incorporation in hydrophobic core, exhibits strong fluorescence. The fluorophore has a characteristic emission maximum at 428 nm upon excitation at 355 nm. Stock solution (1 mM) of DPH dye was prepared in THF solvent for all the studies. Fluorescence intensity at 428 nm as a function of the amount of the surfactant added to an aqueous buffer solution containing DPH (2.5 µM) was measured. CAC was determined via intersection point of the linear parts of the titration curves. The same protocol was followed to determine the CAC of the surfactants with varying 'C' chain length. CAC of C_{14}Gua, C_{16}Gua and C_{18}Gua was found to be ~995 µM, ~260 µM, ~88 µM, respectively. C_{12}Gua having a small 'C' chain length is expected to have CAC much higher. The graphs can be seen in Figure S3.



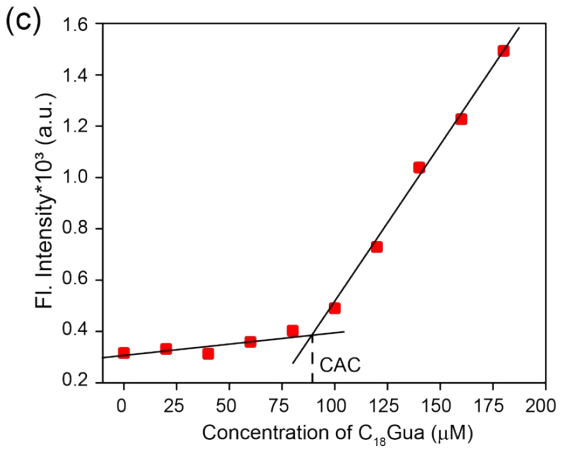


Figure S3. CAC of **a.** C_{14}Gua, **b.** C_{16}Gua and **c.** C_{18}Gua. Experimental conditions: [HEPES] = 1 mM, pH 6.0, [DPH] = 2.5 μM, T = 25 °C, Excitation wavelength = 355 nm and Emission wavelength = 428 nm. au, arbitrary units.

Fluorometric study of DPH intercalation in assembly

The stabilizing effect of ATP on aggregate formation was studied by titrating increasing amounts of ATP to a constant concentration of C_{16}Gua. DPH was used as an apolar probe that gets intercalated in the hydrophobic layers of **C_{16}VesiGlue** and thus shows enhanced fluorescence as a result of restricted rotation.[2] An immediate increase in the fluorescence intensity originating from DPH was observed on increasing addition of ATP to the buffered solution (HEPES, 1 mM, pH 6.0) containing 100 μM C_{16}Gua and 2.5 μM DPH dye, which got plateaued at ~33 μM of ATP. From the results, the stoichiometric molar ratio of ~1:3 between ATP and surfactants in the aggregates was determined.

Absorbance study of DPH intercalation

DPH dye, as a hydrophobic probe absorbs at 355 nm. An experiment was performed where absorbance at 355 nm was studied with increasing concentration of ATP in the solution containing the C_{16}Gua and DPH dye. It was observed that absorbance increases with increasing amount of ATP owing to π-π stacking of the DPH molecules resulting from the intercalation in the hydrophobic region of the **C_{16}VesiGlue**. At C_{16}Gua concentration of 100 μM, increase in absorbance was observed until ATP concentration of 33 μM was reached (Figure S4).

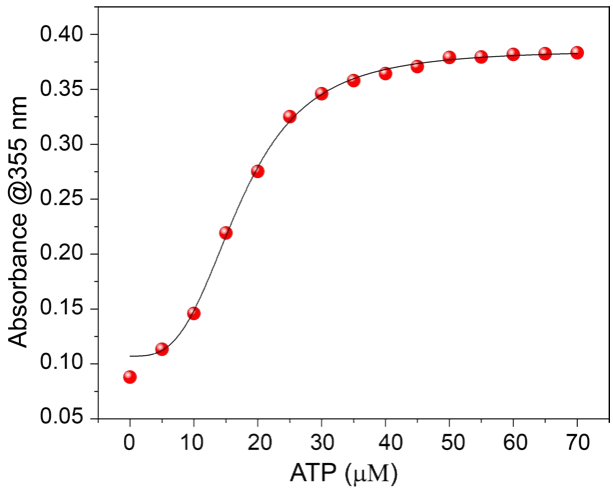


Figure S4. Absorbance of DPH at 355 nm by titrating 100 μM C_{16}Gua with increasing concentration of ATP. The black line represents non-linear fitting to guide the eye.

Fluorometric study above and below CAC

Titrating increasing amounts of ATP to a constant concentration of C_{16}Gua, a molar ratio of ~1:3 between ATP and surfactants in the aggregates was determined. An immediate increase in the fluorescence intensity originating from DPH was observed upon the addition of ATP to C_{16}Gua, independent of the fact whether the surfactant concentration was below (Figure S5a) or above (Figure S5b) the CAC (50 μM and 450 μM respectively). In all cases, an increase was observed only until a molar ratio of ATP to surfactant of ~1:3 was attained. At surfactant concentrations above the CAC (at 450 μM of the surfactant), an increase in fluorescence intensity was observed until ~150 μM of ATP. In this case, a significant fluorescence intensity was also observed in the absence of ATP, because above the CAC the surfactant is already aggregated in micelles that can solubilize DPH.

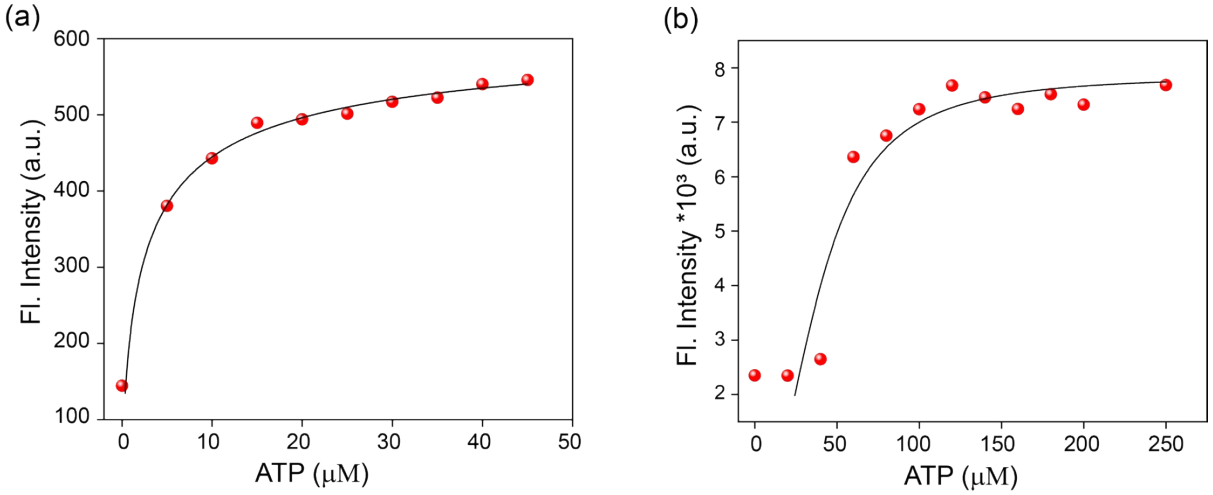


Figure S5. Fluorescence intensity at 428 nm (a.u.) as a function of the increasing ATP amount added to aqueous buffer solutions of C_{16}Gua **a.** below its CAC i.e., 50 μM and **b.** above CAC i.e., 450 μM,

containing DPH (2.5 µM). Experimental conditions: [HEPES] = 1 mM, pH 6.0, T = 25 °C, λ_{ex}= 355 nm. The black line represents non-linear fitting to guide the eye.

Fluorescence spectra of DPH

To 100 µM of C_{16}Gua solution, 33 µM of ATP was added in presence of 2.5 µM DPH and fluorescence spectra were recorded in the range of 380- 550 nm using an excitation wavelength of 355 nm (Figure S6).

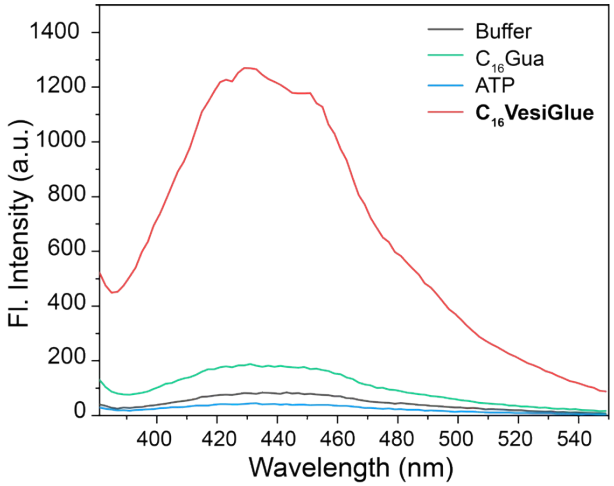


Figure S6. The fluorescence spectra of DPH at 1:3 ATP:C_{16}Gua forming **C_{16}VesiGlue**. Experimental conditions: [HEPES] = 1 mM, pH 6.0, T = 25 °C.

CAC of 1: 3 ATP:C_{16}Gua

The increasing concentration of 1:3 ATP:C_{16}Gua was added to the buffered solution of 2.5 µM DPH and fluorescence intensity was measured at 428 nm. The intersection point of the linear parts of the titration curves was considered to be the CAC of the system, which was found to be ~50 µM (Figure 2b).

Lifetime of DPH in C_{16}VesiGlue

Fluorescence lifetime was determined using a time-correlated single photon counting (TCSPC) spectrometer (Edinburgh FLS980). The lifetime of DPH in a solution (buffer) was compared with when it gets encapsulated inside the hydrophobic region of the **C_{16}VesiGlue**. DPH was excited with a laser of 375 nm followed by the measurement of lifetime of DPH intercalated in the hydrophobic bilayer of **VesiGlue**. For data fitting of the decay curves, bi-exponential fitting model was used. The average lifetime was calculated using the equation:

$$\tau(avg) = \frac{A_1(\tau_1)^2 + A_2(\tau_2)^2}{A_1\tau_1 + A_2\tau_2}$$

The data reveals an increase in lifetime of DPH from 3.934 ns to 5.832 ns upon addition of ATP (33 μM) to the solution containing C_{16}Gua (100 μM) and DPH (3 μM) in buffer. The result demonstrates that the probe is localized in a more hydrophobic environment which is consistent with the ATP assisted **VesiGlue** formation.

Turbidity of C_{16}Gua with different phosphate analogues

The turbidity measurements were carried out by measuring absorbance at 500 nm, as none of the components of the system absorb light at 500 nm. Turbidity was defined as (100-%T), where %T is percentage transmittance observed due to scattering by the assembly. Turbidity measurements were carried out by varying the phosphate analogue concentration with respect to a fixed concentration of C_{16}Gua (100 μM). Different analogues that were used for assembly formation are ATP, sodium triphosphate, GTP, ADP and AMP. This wavelength was chosen as none of the ligand and the phosphate component absorbed at this region. The increase in absorbance directly corelates the increased turbidity of the solution.

Kinetics of C_{16}**VesiGlue** *formation*

The kinetics of **VesiGlue** formation was studied by measuring the increase in DPH fluorescence intensity at 428 nm with time upon the addition of 8.3 μM of ATP to an aqueous buffered solution of surfactant C_{16}Gua (25 μM) and DPH (2.5 μM). From the kinetics measurements, constant value of fluorescence intensity was observed within 15 min. (Figure S7).

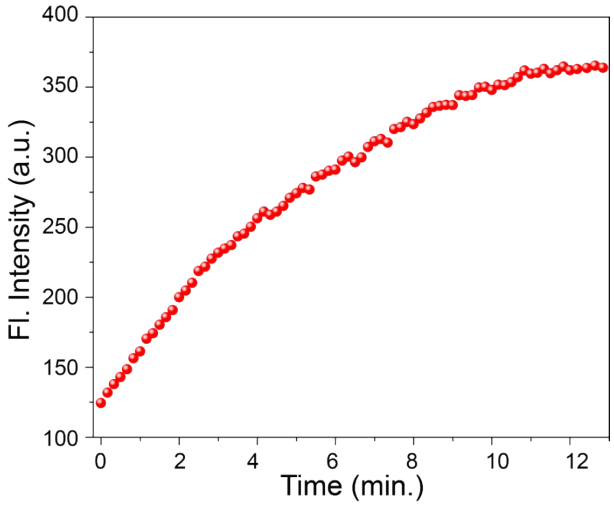


Figure S7. Fluorescence intensity at 428 nm (a.u.) as a function of time upon the addition of ATP (8.3 μM) to an aqueous buffered solution of surfactant C_{16}Gua (25 μM) containing DPH (2.5 μM). Experimental conditions: HEPES (1 mM, pH 6.0).

2. Isothermal Titration Calorimetry (ITC)

Isothermal Titration Calorimetry was employed for determining the thermodynamics of **VesiGlue** formation by the C_{16}Gua and ATP. The heat flow as result of the binding process was recorded using a high-sensitivity MicroCal VP-ITC instrument from Malvern. All solutions were prepared in 1 mM HEPES buffer pH 6.0. The reaction cell containing 100 μM C_{16}Gua was titrated by injecting 2 μL each from 0.75 mM ATP contained in the titration syringe. Data acquisitions were done through the computer software provided by MicroCal VP-ITC. The integration of heat flow tracing determined the heat of reaction produced after each injection. The data was fitted using one set of sites binding model. The thermodynamic parameters of binding were used to analyze and understand the nature of the interactions involved in the process of **VesiGlue** formation and the process was found to be exothermic in nature.

3. Nanoparticle Tracking Analysis (NTA)

The analysis of size distribution of the particles was done using NanoSight instrument (Malvern, UK) considering its robustness compared to the traditional DLS technique. The particle size distribution of the **VesiGlue** formed by the 50 μM C_{16}Gua and 16.6 μM ATP prepared in 1 mM HEPES (pH 6.0) was measured after incubating the sample for 20 min. The sample was injected onto the stage using 1 mL syringe. Camera level was adjusted until all the particles were discreetly visible, autofocus was utilized accordingly and ideal particle per frame value was set at 20-80 particles/frame. Five 1-min videos were captured for each measurement and the videos were analysed using the in-built NanoSight software. The resulting size was observed to be in the range of 100-250 nm, with maximum particles of size 150 nm and few particles of larger size. The particle count was found to be in the range of 10^9 particles per mL of solution.

4. Dynamic Light Scattering (DLS)

The hydrodynamic diameter of the **VesiGlue** formed by the 50 μM C_nGua, (n= 12, 14, 16 and 18) and 16.6 μM ATP prepared in 1 mM HEPES (pH 6.0) was measured after incubating the sample for 20 min and then placing the solution in disposable low volume cuvette. A minimum of 3 measurements were made. The hydrodynamic diameter found is depicted in Figure S8.

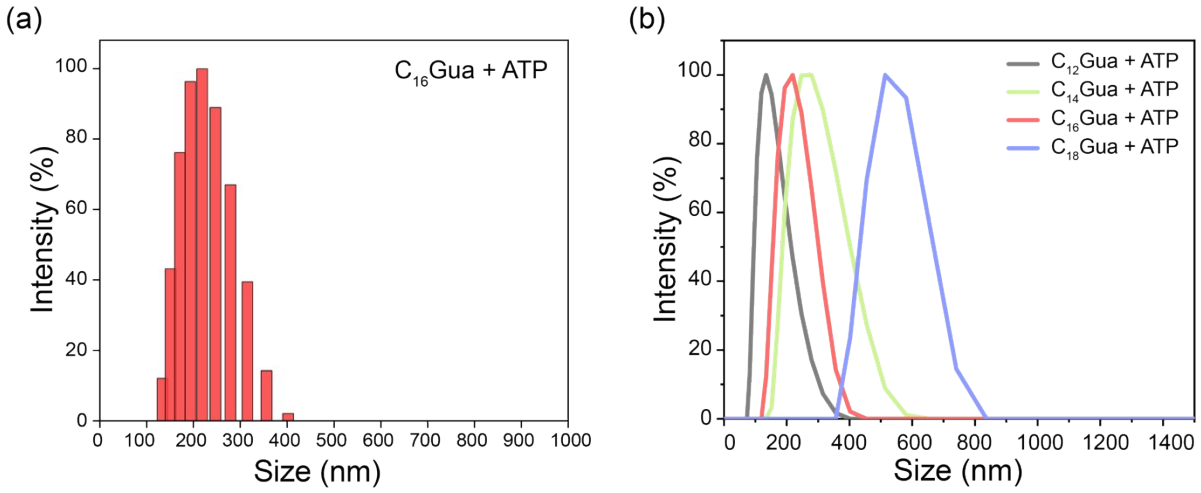


Figure S8. a. Hydrodynamic diameter of 50 µM C_{16}Gua + 16.6 µM ATP (**C_{16}VesiGlue**) in aqueous buffered solution. **b.** Hydrodynamic diameter of **VesiGlue** formed by different chain length ligand (C_nGua) and ATP.

5. Stability of C_{16}VesiGlue formed with time

Turbidity

The high stability of **C_{16}VesiGlue** with 1:3 ratio of ATP: C_{16}Gua emerged from the observation of near constant turbidity values over prolonged periods (>24 hours) (Figure S9). The turbidity (absorbance at 500 nm) was measured over a period for the buffered solution containing 100 µM C_{16}Gua, to which 33 µM of ATP was added.

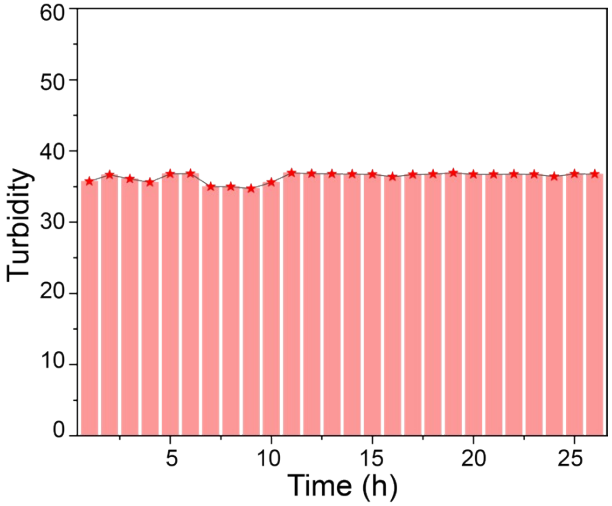


Figure S9. Turbidity of **C_{16}VesiGlue** formed by 100 µM C_{16}Gua and 33 µM ATP with increasing time.

DLS

Further, to study the stability of the **C_{16}VesiGlue**, DLS was recorded after one day of incubation. The observed data is shown in figure S10.

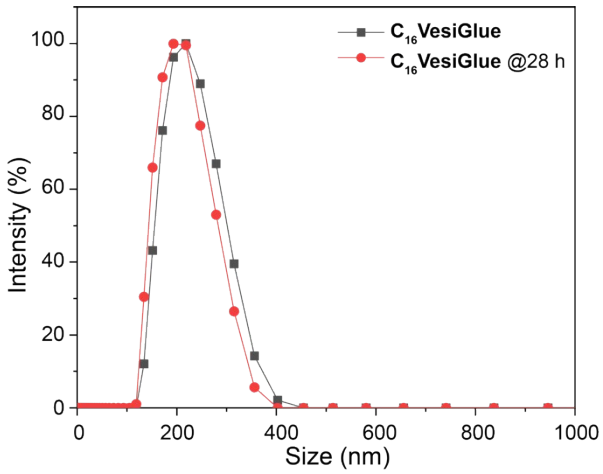


Figure S10. Hydrodynamic diameter of 50 µM C_{16}Gua + 16.6 µM ATP in aqueous buffered solution after 28 h.

C_{16}VesiGlue formation in high salt concentration

The formation of **C_{16}VesiGlue** was performed in presence of high ionic strength solution (150 mM NaCl). Turbidity was measured at 500 nm with increasing concentration of ATP to a fixed concentration of C_{16}Gua (100 µM) in salt solution (Figure S11). It was found that there is not much effect of salt on the system and the assembly is still formed and stable suggesting the high strength of the multivalent H-bonding and electrostatics interactions involved between phosphates and guanidinium.

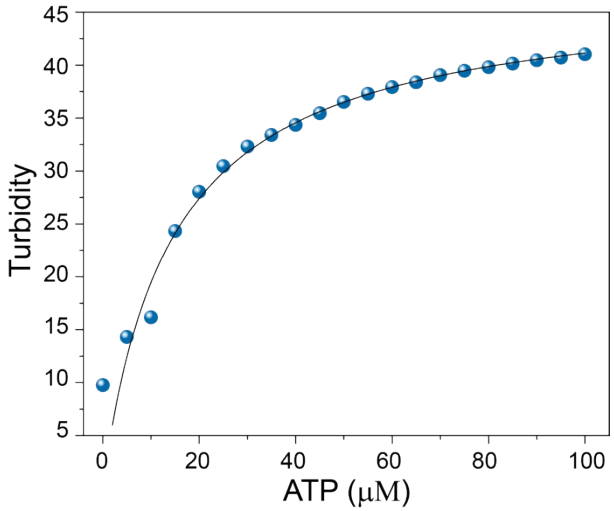


Figure S11. Turbidity of **C_{16}VesiGlue** formed by C_{16}Gua (100 µM) with increasing ATP in 150 mM NaCl solution.

6. GTP-driven self-assembled vesicles

Fluorometric study of DPH intercalation

The stabilising impact of GTP on aggregate formation was investigated by titrating increasing amounts of GTP to a fixed concentration of C_{16}Gua. DPH, an apolar probe gets intercalated in the hydrophobic layers of the assembly resulting enhanced fluorescence due to restricted rotation. An immediate increase in the fluorescence intensity originating from DPH was observed on increasing addition of GTP to the buffered solution (HEPES, 1 mM, pH 6.0) containing 100 μM C_{16}Gua and 2.5 μM DPH dye, which got saturated at ~33 μM of GTP. The findings revealed a stoichiometric molar ratio of 1:3 between GTP and surfactants in aggregates.

Nanoparticle Tracking Analysis (NTA)

The analysis of size distribution of the particles was done using NanoSight instrument. Particle size distribution of the assembly formed by the 50 μM C_{16}Gua and 16.6 μM GTP prepared in 1 mM HEPES (pH 6.0) was measured after incubating the sample for 20 min. The sample was injected onto the stage using 1 mL syringe. Camera level was adjusted until all the particles were discreetly visible, autofocus was utilized accordingly and ideal particle per frame value was set at 20-80 particles/frame. Five 1-min videos were captured for each measurement and the videos were analysed using the in-built NanoSight software. The resulting size was observed to be in the range of 150-300 nm, with maximum particles of size 190 nm. The particle count was found to be in the range of 10^8 particles per mL of solution.

Small Angle X-ray Scattering (SAXS)

The powder sample of assembly was prepared via freeze drying the solution of 100 μM of C_{16}Gua with 33 μM GTP. The freeze-dried sample was then transferred to the sample holder and the data was measured by Nanostar Small Angle X-ray Scattering (SAXS) Low Angle instrument.

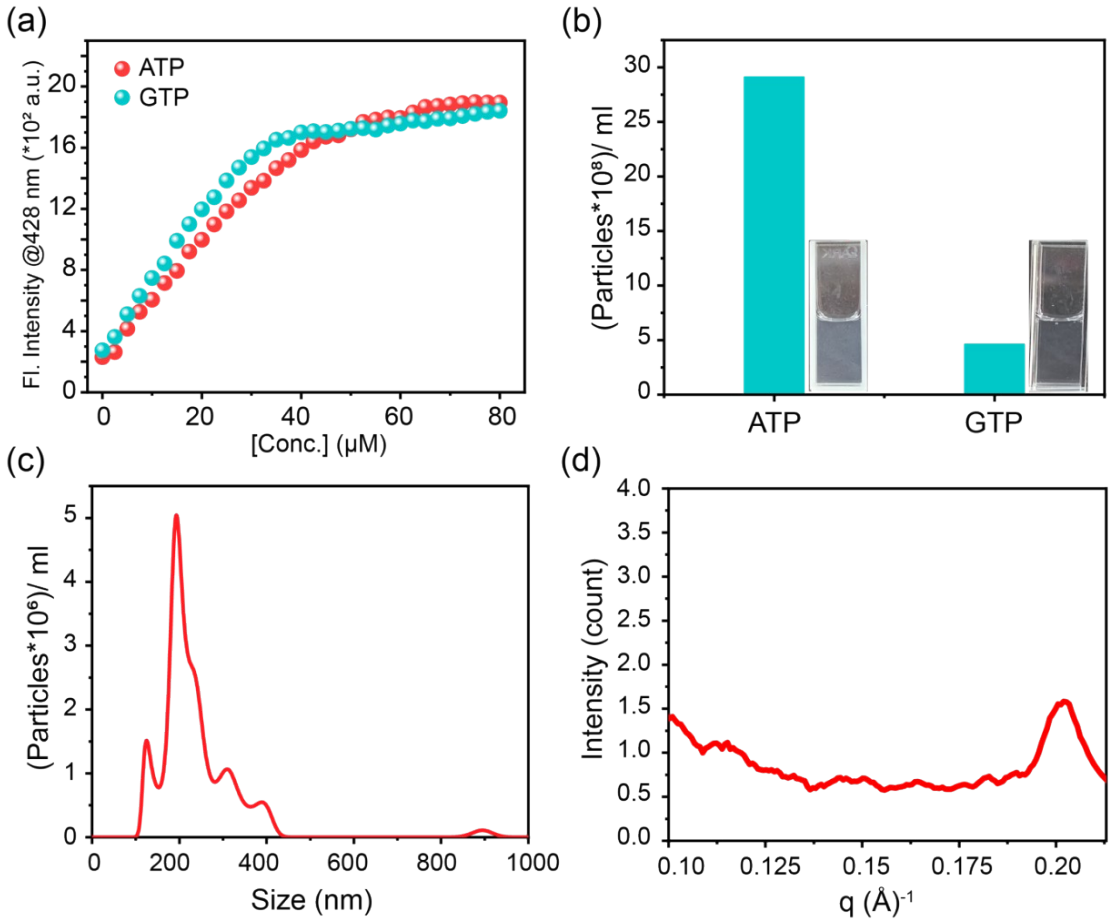


Figure S12. (a) Fluorescence intensity at 428 nm with increasing concentration of ATP (Red) and GTP (Cyan) in an aqueous buffered solution containing 100 μM of C_{16}Gua and 2.5 μM of DPH, suggesting the binding ratio of ATP:C_{16}Gua as well as 1:3 GTP:C_{16}Gua. (b) Number of particles per ml in assembly formed via 50 μM of C_{16}Gua and 16 μM of ATP vs GTP, measured using Nanoparticle tracking analysis (NTA). (c) NTA of 1:3 GTP:C_{16}Gua assembly in buffer showing the particle size distribution (d) SAXS pattern of vesicle formed via 1:3 GTP:C_{16}Gua.

7. Zeta potential

All the samples were prepared in 1 mM HEPES buffer pH 6.0. ATP (33 μM) was added to 100 μM C_{16}Gua and the ζ-potential was recorded in a Zetasizer disposable cuvette. A minimum of 3 measurements were made for each sample.

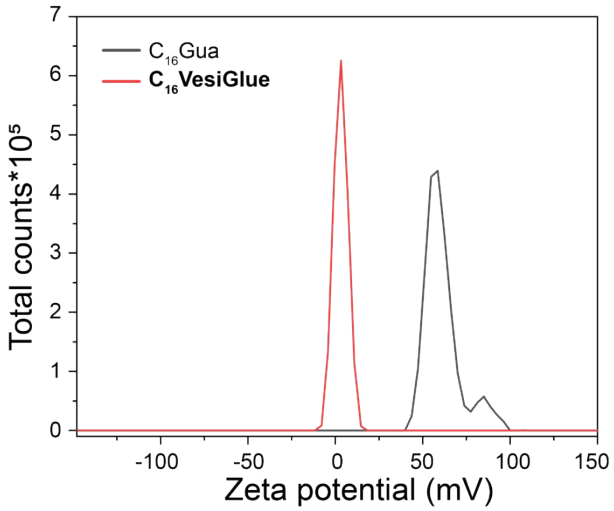


Figure S13. Zeta potential (mV) of **C_{16}VesiGlue.**

8. Electron Microscopic studies

Transmission Electron Microscopy (TEM)

TEM images of the **C_{16}VesiGlue** formed by the ligand and ATP were recorded on Talos L120C electron microscope. Samples for TEM imaging was prepared by premixing the 50 µM C_{16}Gua and 16 µM ATP in MQ water. 5 µL of the sample was drop casted on a carbon-coated copper grid and incubated for 1 min. For staining purposes, it was then placed on a drop of uranyl acetate (2%) for 30 s. The sample was initially air dried followed by the complete solvent evaporation under vacuum before the stained grid was imaged. TEM images showed the vesicles of varying size from 150 nm to 300 nm, with few particles of larger size.

Field emission Scanning Electron Microscopy (FESEM)

Sample for SEM imaging was prepared by mixing C_{16}Gua (25 µM) and ATP (9 µM) in MQ water and incubating for 2 h followed by drop casting 2 µL of the sample on a silicon wafer. The control sample was prepared the same way, in absence of ATP. The samples were allowed to initially air dry and later under reduced pressure. The samples were then sputter coated with approximately 5 nm layer of Au before studying under SEM. The size of the spherical assembly (**C_{16}VesiGlue**) varied in the range of ~150-300 nm. FESEM micrograph of the **C_{16}VesiGlue** is shown in Figure S14 along with the control of only C_{16}Gua, where no assembly formation take place.

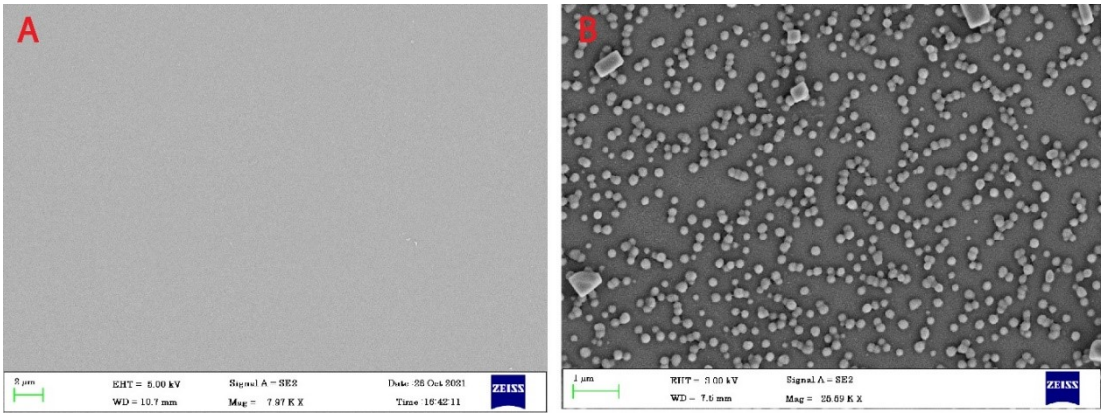


Figure S14. SEM Image of **a.** C_{16}Gua alone and **b.** ATP assisted **C_{16}VesiGlue.**

9. Assembly of C_{16}Gua above CAC

DLS

The hydrodynamic diameter of the 300 μM of C_{16}Gua prepared in 1 mM HEPES (pH 6.0) was measured by placing the solution in disposable low volume cuvette.

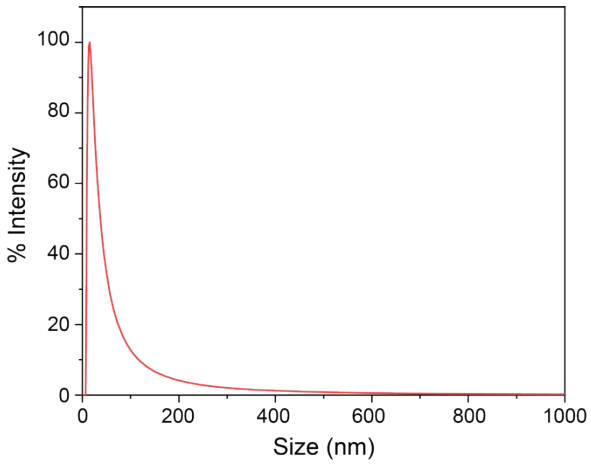


Figure S15. DLS measurements of C_{16}Gua above CAC. The Z-average of particles calculated from the DLS data is 29.3 nm with PDI (polydispersity index) value of 1.604.

TEM

Talos L120C electron microscope was used to take TEM images. To prepare the TEM sample, micelle solution was dropped on carbon-coated copper grid and then dried under ambient environment. Next, uranyl acetate solution (2%) was dropped on the hydrophobic film (parafilm), and then the grids were laid upside down on the top of the uranyl acetate solution for 30 s.

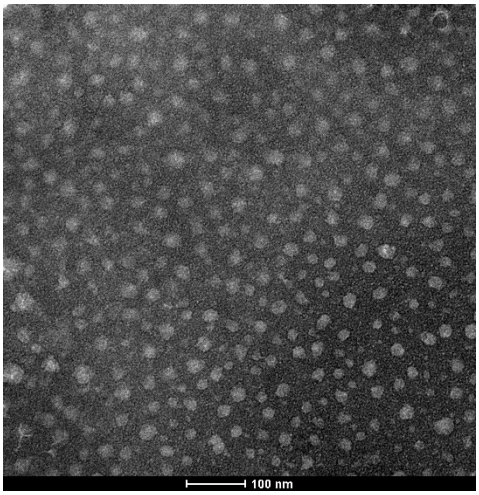


Figure S16. Representative negatively stained TEM image of C_{16}Gua above CAC.

10. Small Angle X-ray Scattering (SAXS)

To prepare the powder sample, aqueous solution of **VesiGlue** (100 µM C_{16}Gua + 33 µM ATP) and **VesiGlue** containing CytC (0.5 µM) was freeze-dried. The freeze-dried sample was then transferred to the sample holder and the data was measured by Nanostar Small Angle X-ray Scattering (SAXS) Low Angle instrument.

11. Confocal Laser Scanning Microscopy (CLSM)

To the aqueous buffer containing 5 µM of Nile red, was added 50 µM C_{16}Gua and 16 µM of ATP. The solution was incubated for 15 min. and the confocal images were recorded using a Confocal Zeiss LSM880 (Airyscan) microscope. The excitation wavelength was chosen to be 561 nm.

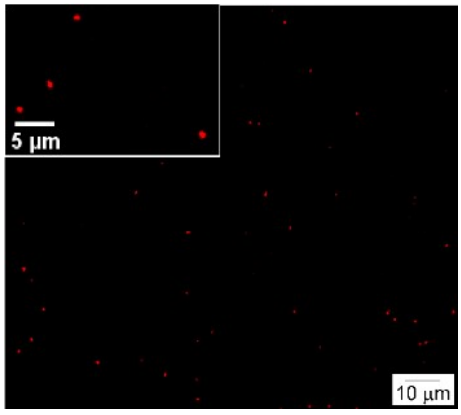


Figure S17. Confocal image of **C_{16}VesiGlue** stained with Nile red (red fluorescence, Ex: 561 nm, Em: 630 nm), Scale bar 10 µm and 5 µm.

12. Calcein dye release assay

10 µM of calcein was loaded to **VesiGlue** (100 µM C_{16}Gua and 33 µM ATP). The fluorescence of 10 µM of free calcein in buffer was considered to be 100 % release and the fluorescence intensity of calcein in presence of **VesiGlue** was taken as 0 % release. Lysis of **VesiGlue** was understood in terms of the percentage leakage of calcein from the trapped **VesiGlue**, which was monitored by recording the increasing fluorescence intensity at 515 nm (excit. = 490 nm) upon increasing Triton X-100. Complete vesicle disruption corresponding to 100 % leakage was achieved by addition of 350 µM Triton X-100 as shown in figure S18.

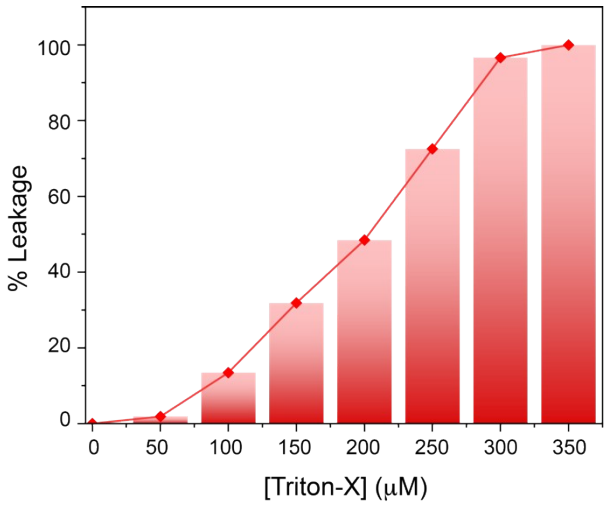


Figure S18. Percentage of calcein dye leakage with increasing concentration of Triton X. Experimental conditions: [HEPES] = 1 mM, pH 7.4, [Calcein] = 20 µM, T = 25 °C, Excitation wavelength = 490 nm and Emission wavelength = 515 nm.

13. Effect of salt on assembly formed via different phosphate analogues

The fluorescence of the DPH Dye was measured when intercalated in the assembly formed via C_{16}Gua (100 µM) and different phosphate analogues (33 µM ATP/GTP/TP) in 96 well plate. To the solutions, was added different concentration of salt (25 mM and 50 mM NaCl) and fluorescence of DPH was again recorded (Figure S19). The resulting increased fluorescence suggests further entrapment of the DPH dye in the hydrophobic bilayer owing to the increased rigidity of the vesicle bilayers.

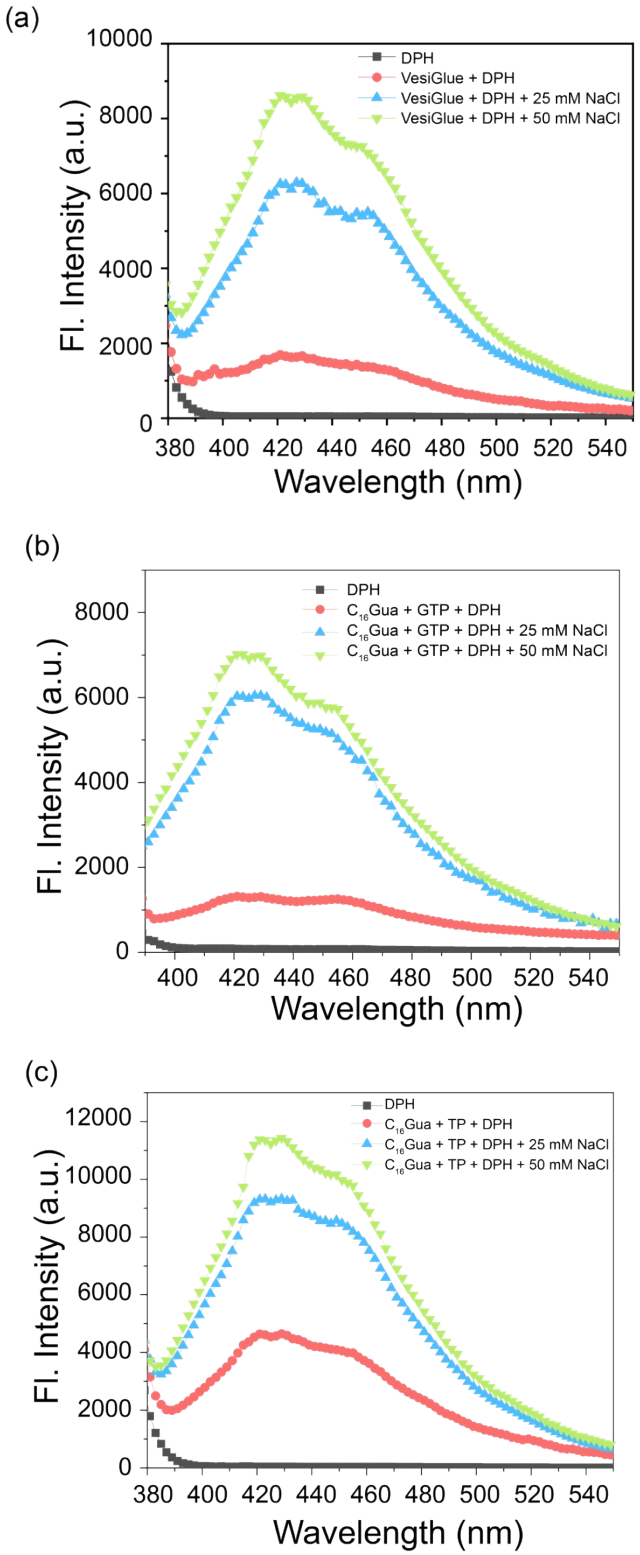


Figure S19. Fluorescence spectra of DPH dye upon addition of salt concentration to assembly formed via C_{16}Gua and **a.** ATP, **b.** GTP and **c.** TP.

"Vesicular glue" for molecular recognition of proteins

1. CLSM

CytC was tagged with a fluorophore fluorescein isothiocyanate (FITC) following the reported protocol.[3] Briefly, 5 mg of CytC was added to 2.5 mg of FITC in 10 mM HEPES buffer of pH 7.2. The mixture was stirred at room temperature for 30 min. followed by insoluble FITC removal through centrifugation. The supernatant was dialysed at 4 °C and desalted through an Amicon Ultra-2mL centrifugal filter unit (COMW 3000; Merck Millipore). Fluorescence confocal images were captured for a buffered solution of the **VesiGlue** formed via C_{16}Gua (50 µM) and ATP (16.6 µM) in presence of Nile red (2.5 µM). To visualize the co-localization of CytC on the **C_{16}VesiGlue** surface, FITC-labelled CytC (5 µM) was added to the Nile red entrapped **VesiGlue**. Nile Red stained the **VesiGlue** by its entrapment inside the hydrophobic bilayer while FITC-labelled CytC was visualized to be anchored on the **VesiGlue** resulting in the overlap of two fluorescent signals.

2. Fluorescence of ATP

The Fluorescence emission spectra of ATP were recorded in Hitachi F-7000 spectrophotometer in presence of ligand and CytC. The result demonstrates the interaction of C_{16}Gua with ATP and further CytC with **C_{16}VesiGlue** surface exposed ATP. Experimental conditions: HEPES (1 mM, pH 6.0), [ATP] = 33 µM, [C_{16}Gua] = 100 µM, [CytC] = 5 µM.

3. DLS

The hydrodynamic diameter of the **C_{16}VesiGlue** was measured in presence of CytC. The increased diameter of the overall **VesiGlue** indicates the coverage of protein around the **C_{16}VesiGlue**. The samples were prepared in HEPES buffer pH 6. Measurements were done after incubating the sample for 10 min and placing the solution in disposable low volume cuvette. The observation is as shown in Figure S20.

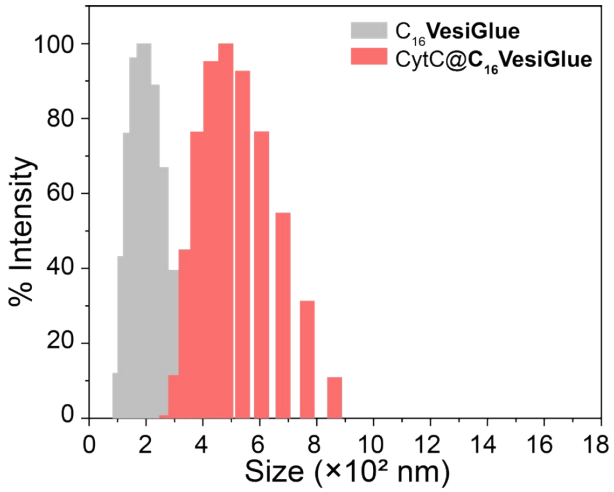


Figure S20. Size measured by DLS of protein (CytC) anchored **C_{16}VesiGlue**.

4. Zeta Potential

All the samples were prepared in 1mM HEPES buffer pH 6. 100 μM of C_{16}Gua was assembled using 1/3rd concentration of different phosphate analogues and 0.5 μM of CytC was added to it to study the change in surface charge. The ζ-potential was recorded in a Zetasizer disposable cuvette. A minimum of 3 measurements were made for each sample.

5. DPH dye leakage experiment

To study the stability of **C_{16}VesiGlue** on addition of CytC, an experiment was performed where DPH was encapsulated inside the **VesiGlue**. To the 1 mM HEPES pH 6 buffered solution containing 2.5 μM DPH Dye, **VesiGlue** was formed by addition of 100 μM C_{16}Gua and 33 μM ATP. To the resulting dye entrapped **VesiGlue**, was added 0.5 μM of CytC and the fluorescence spectra was recorded after 30 min. incubation. No significant change in the fluorescence of DPH upon CytC addition suggests that the **C_{16}VesiGlue** are intact when CytC is anchored on its surface. The study was performed with all the necessary controls.

6. ITC

Isothermal Titration Calorimetry was employed to determine the binding affinity of CytC with **VesiGlue**. All solutions were prepared in 1 mM HEPES buffer pH 6. The reaction cell containing **VesiGlue** formed by 400 μM C_{16}Gua and 133 μM of ATP was titrated by injecting 5 μL each of 1 mM CytC contained in titration syringe. Data acquisitions were done through the computer software developed by MicroCal VP-ITC. The integration of heat flow tracing determined the heat of reaction produced after each injection. Similar conditions were used for the control study of CytC titration with assembly formed by C_{16}Gua and triphosphate. Also, the binding affinity of CytC with assembly of C_{16}Gua and GTP was determined using the same protocol. The results are shown in Figure S21.

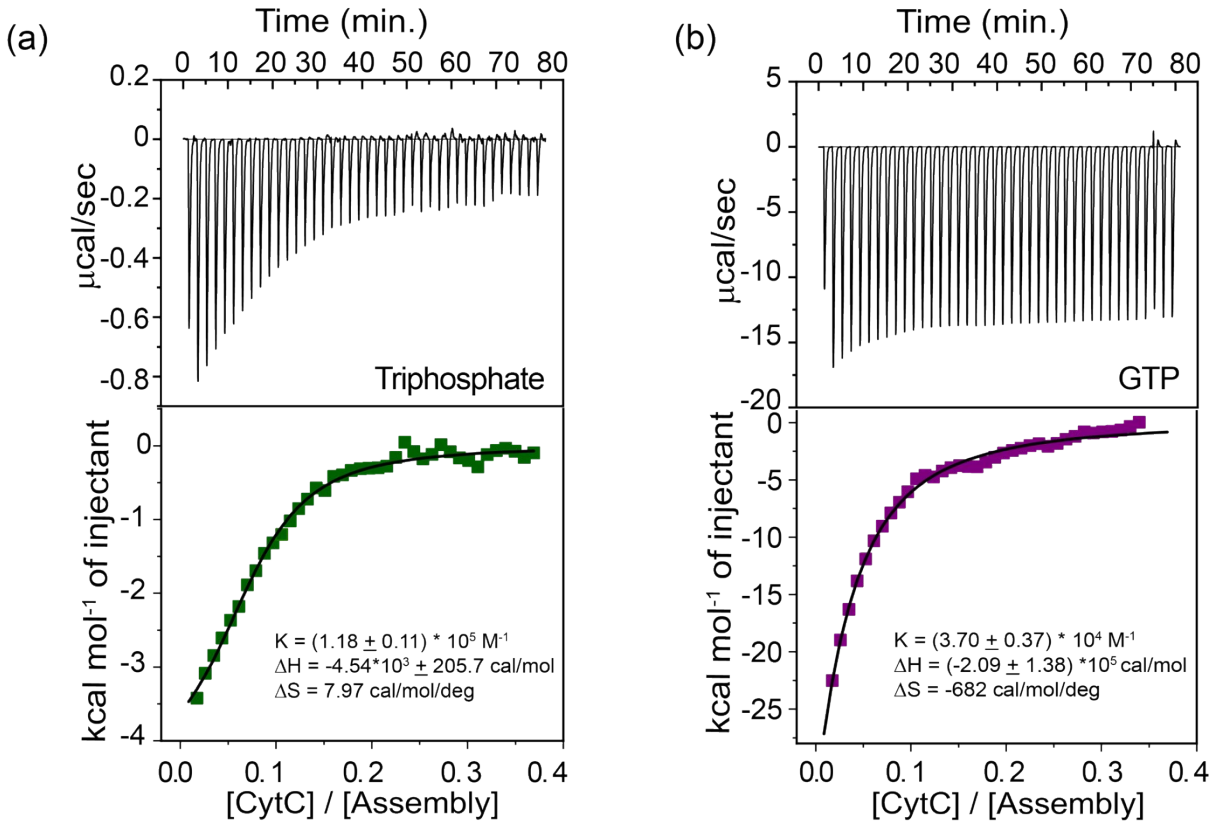


Figure S21. ITC titration of increasing concentration of CytC (syringe) to the assembled structures formed via C_{16}Gua and **a.** Sodium triphosphate, **b.** GTP.

ITC experiment was performed to determine the binding affinity of CytC with just the ATP. All solutions were prepared in 1 mM HEPES buffer pH 6. The reaction cell containing 133 µM of ATP was titrated by injecting 5 µL each of 1 mM CytC contained in titration syringe. Data acquisitions were done through the computer software developed by MicroCal VP-ITC. The integration of heat flow tracing determined the heat of reaction produced after each injection. The observed result can be seen in Figure S22.

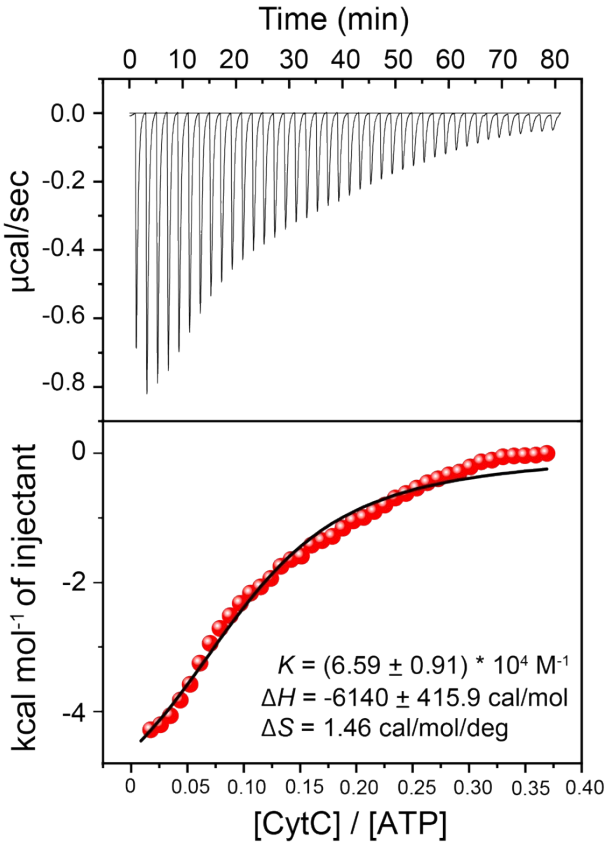


Figure S22. Integrated heat flow signals as a function of [CytC]/[ATP] molar ratio. The best fit was obtained by single-site non-competitive binding model.

ITC in presence of 100 mM NaCl

ITC was employed to determine the binding affinity of CytC with assembly formed by C_{16}Gua and GTP/ TP in salt. All solutions were prepared in 1 mM HEPES buffer pH 6. The reaction cell containing assembly formed by 400 µM C_{16}Gua and 133 µM of GTP/ TP was titrated by injecting 5 µL each of 1 mM CytC contained in titration syringe. Data acquisitions were done through the computer software developed by MicroCal VP-ITC. The integration of heat flow tracing determined the heat of reaction produced after each injection. The results are shown in Figure S23.

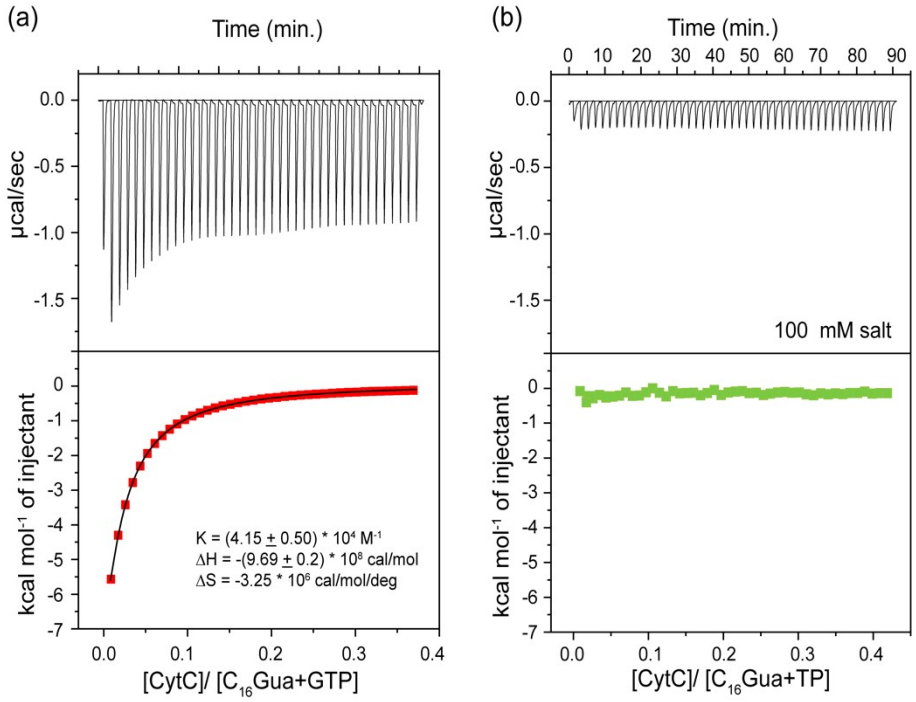


Figure S23. Integrated heat flow signals as a function of (a) [CytC]/[C_{16}Gua+GTP] and (b) [CytC]/[C_{16}Gua+TP] molar ratio in presence of 100 mM NaCl. The best fit was obtained by single-site non-competitive binding model.

Intactness of assembly in presence of NaCl

The turbidity of the assembly formed via C_{16}Gua (100 µM) and different phosphate analogues (33 µM ATP/GTP/TP) in buffer (1 mM HEPES, pH 6) were measured with addition of increasing NaCl concentration (0-150 mM) (Figure S24).

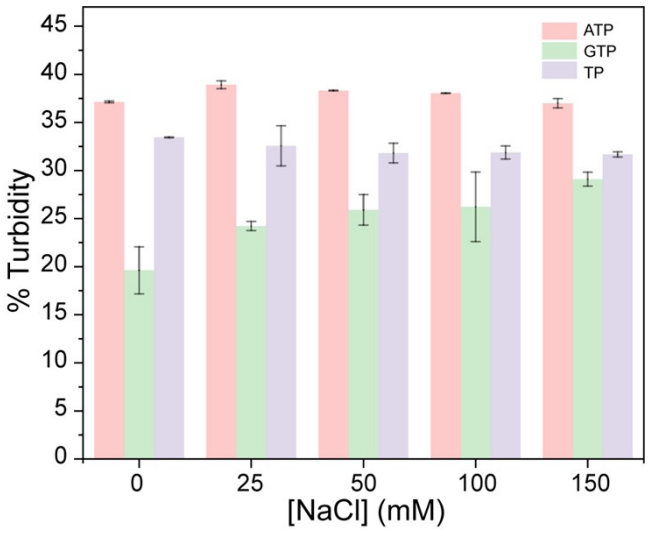


Figure S24. Turbidity of **C_{16}VesiGlue** and assembly formed by C_{16}Gua and GTP/ TP upon addition of increasing salt (NaCl).

7. UV-Vis spectra of CytC

Effect of **C_{16}VesiGlue** (formed via 100 µM C_{16}Gua and 33 µM ATP) on native structure of 5 µM CytC was investigated *via* UV-Visible spectroscopy studies. The characteristic Soret peak of CytC at 414 nm was found to be retained when bound to the **C_{16}VesiGlue** concluding the withholding of the native structure of the protein (Experimental condition: 1 mM HEPES pH 6).

8. Circular Dichroism (CD) spectra of CytC

To investigate the structural stability of CytC, the effect of **C_{16}VesiGlue** on the secondary structure of protein was studied using CD spectroscopy. All CD measurements were done at far-UV range using a JASCO CD spectropolarimeter (model J-810) equipped with a peltier temperature-controlled cell holder. A 0.1 cm path length cylindrical quartz cuvette (Hellma, Forest Hills, NY, USA) was used for all the measurements. The solutions of C_{16}Gua, ATP, CytC, and the **C_{16}VesiGlue** were prepared in 1 mM HEPES (pH 6). The CD spectra of CytC (10 µM) alone and along with **C_{16}VesiGlue** of C_{16}Gua (200 µM) and ATP (66 µM) were recorded from 190 nm to 240 nm. Also, all the required controls were measured following the same conditions.

The spectra reveals that the presence of C_{16}Gua, ATP or **C_{16}VesiGlue** did not cause any change to the native conformation of CytC with the retainment of characteristic bands of the protein at 208 nm (π– π* transition) and 222 nm (n– π* transition).

E. Amplified enzyme activity on VesiGlue

1. Activity assays for CytC enzyme

Exploring the origin of activity enhancement

Zeta Potential of VesiGlue in presence of TMB substrate

The samples were prepared in 1 mM HEPES buffer pH 6.0. TMB (100 µM) was added to**C_{16}VesiGlue** and the ζ-potential was recorded in a Zetasizer disposable cuvette. A minimum of 3 measurements were made for each sample.The zeta potential of **VesiGlue** in presence of TMB substrate changed from -0.433 mV to +0.845 mV, suggesting the accumulation of TMB on the surface of the **VesiGlue**.

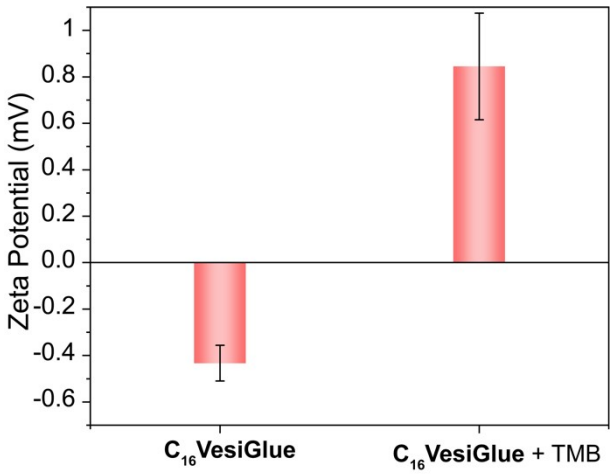


Figure S25. Zeta potential of **C_{16}VesiGlue** in the presence of TMB substrate in buffer (HEPES, 1 mM, pH 6). *n* = 3 technical replicates, mean ± SD for all the independent studies.

Role of adenine and the stability of TMB intermediate

Adenine may act as a distal histidine and help in the conversion of H_2O_2 to OH radical during the peroxidase activity of CytC as demonstrated in figure S26a.

The stability of TMB intermediate was monitored via recording the absorbance at 652 nm with time. All the samples were prepared in 1 mM HEPES pH 6. The stability of the intermediate was much higher for **VesiGlue** than in the other cases, indicated by the lag phase before the decrease in absorbance values. The results are shown in figure S26b.

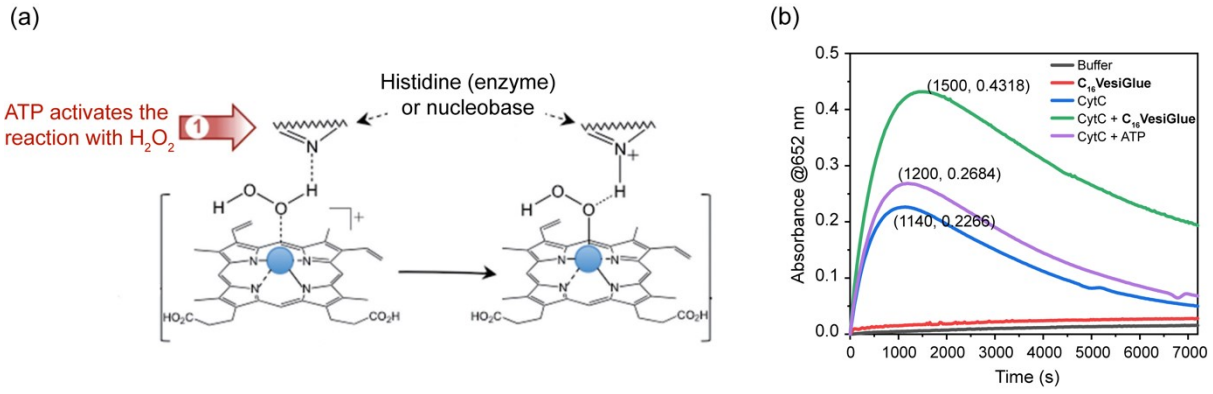


Figure S26. (a) Schematic for the mechanism of adenine promoting the binding of H_2O_2 towards Heme. (b) The oxidation of TMB is monitored via the appearance of the intermediate (652 nm).

Specific Activity of CytC in presence of assembly formed by different capping agents

The activity of the CytC was measured using TMB as a substrate in presence of the C_{16}Gua assembly formed via different phosphate analogues like ATP, ADP, AMP, GTP and sodium triphosphate. We assume from the literature that the role of ATP in enhancing the specific

activity of CytC involves interactions of ATP molecule with various amino acids in CytC. **C_{16}VesiGlue** are able to enhance the activity of CytC by almost 8.4 folds. It was observed from the control study that neither triphosphate nor GTP can increase the specific activity of the protein. This signifies the importance of Adenine unit of ATP in the activity enhancement. Inability of C_{16}Gua to form vesicles in presence of ADP and AMP do not enhance the CytC activity, which further suggests the importance of CytC anchoring for its activity enhancement. Experimental Conditions: [HEPES] = 1 mM (pH 6), [C_{16}Gua] = 240 µM, [ATP] = 80 µM, [ADP] = 80 µM, [AMP] = 80 µM, [Sodium triphosphate] = 80 µM, [GTP] = 80 µM, [CytC] = 0.5 µM, [TMB] = 100 µM, [H_2O_2] = 2 mM. One unit of specific activity of the CytC@**VesiGlue** system is defined as 1.0 µmole of product formed in one minute per mg of the enzyme.

Peroxidase activity of CytC at different concentration of C_{16}VesiGlue

The experiment was performed in 1 mM HEPES pH 6 in 96 well plate maintaining the final volume to be 250 µL. The **C_{16}VesiGlue** was taken in increasing concentration and 0.5 µM of CytC was allowed to incubate with it for 5 min. To the solution was added 100 µM of TMB and the reaction was initiated by the addition of 2 mM of H_2O_2. The activity was measured by monitoring the reaction of TMB oxidation (λ_{max} = 650 nm) with time for 15 min. (ε_{650} nm is 39000 M^{-1} cm^{-1} in water). The fixed concentration of CytC was found to have increased activity with increasing **C_{16}VesiGlue** concentration, where the concentration of **VesiGlue** was represented by the number of particles per mL (calculated from NTA).

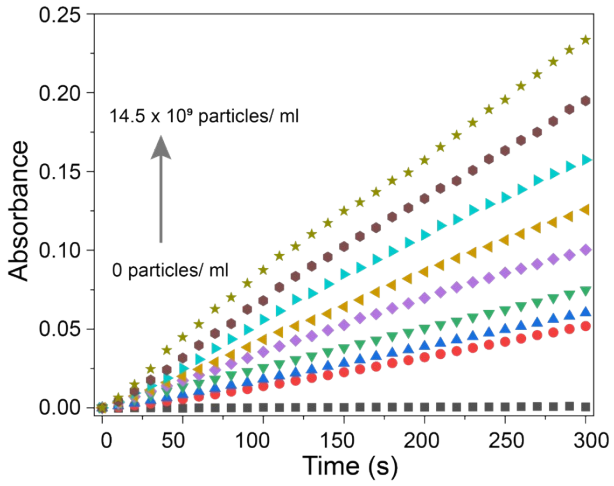


Figure S27. Change in absorbance at 650 nm as a function of time at varying no. of **C_{16}VesiGlue.**

Enzyme Kinetics

The activity was monitored in HEPES buffer by oxidation of TMB. Reactions were carried out with CytC (0.5 µM) in presence of **C_{16}VesiGlue** (100 µM C_{16}Gua + 33 µM ATP), H_2O_2 (2

mM) and varying concentrations of TMB in 1 mM of HEPES buffer of pH 6.0 in a total of 250 µL of solution. Absorbance at 650 nm was recorded immediately after the addition of TMB and H_2O_2 solutions (ε_{650} nm for ox. TMB is 39000 $M^{-1}cm^{-1}$ in water). Activity of CytC alone and in presence of only ATP was determined under the same conditions.

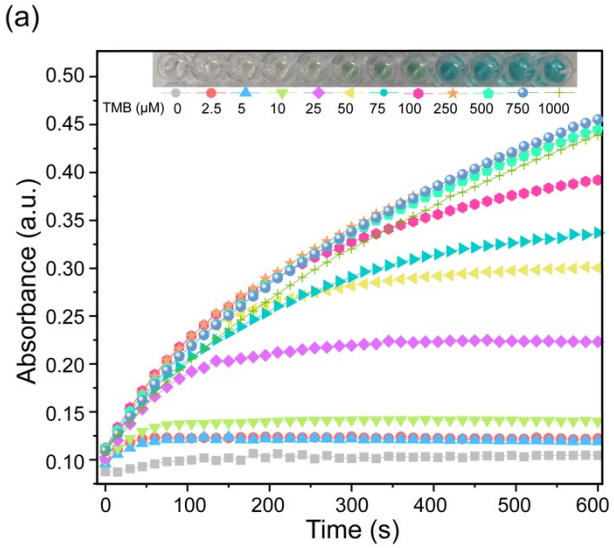


Figure S28 a. Kinetics of product formation (TMB_{ox}) measured by an increase in absorbance at 650 nm in the presence of CytC glued on **C_{16}VesiGlue** with increasing TMB concentration.

Table 1. Kinetic parameters of the catalytic activity of CytC in the different assembled states

System	**K_M (µM)**	**V_{max} (µM/min.)**	**k_{cat} (*10^{-3} $min.^{-1}$)**
CytC	44.16	0.70	1.40
CytC+ATP	40.08	0.95	1.90
CytC+**C_{16}VesiGlue**	33.97	1.58	3.16

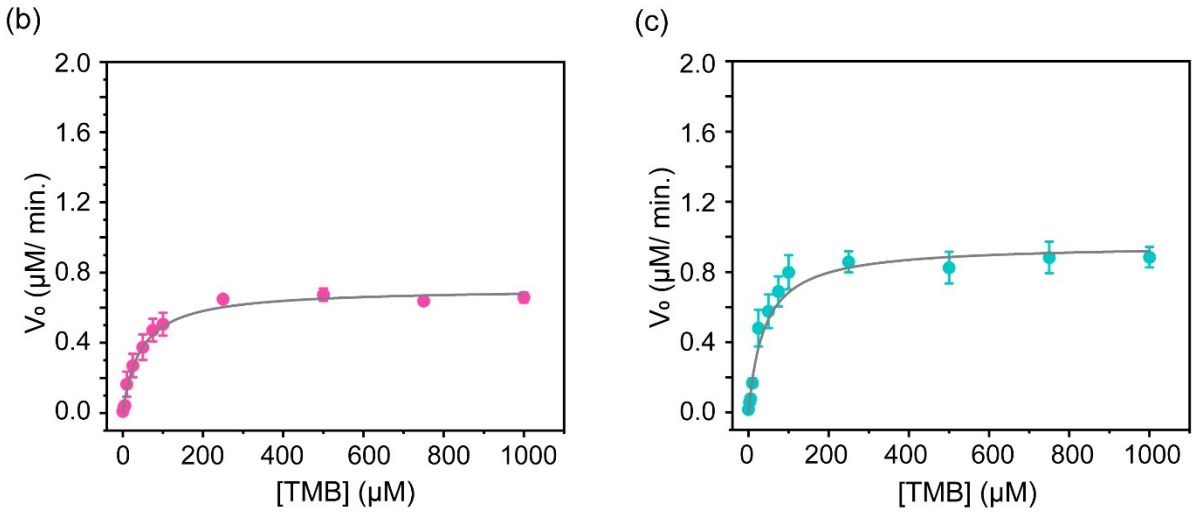


Figure S28. Michaelis-Menten plot of **b.** CytC activity. **c.** in presence of ATP

Specific activity of CytC at different equivalents of ATP to C_{16}Gua

The experiment was performed in 1 mM HEPES pH 6 in 96 well plate maintaining the final volume to be 250 µL. The ATP was added in increasing concentration to the solution containing 0.5 µM of CytC and 100 µM of C_{16}Gua. The mixture was allowed to incubate for 15 min. followed by the addition of 100 µM of TMB and the reaction was initiated by the addition of 2 mM of H_2O_2. The activity was measured by monitoring the reaction of TMB oxidation (λ_{max} = 650 nm) with time for 15 min. (ε_{650} nm is 39000 M^{-1} cm^{-1} in water). The results are shown in figure 4c.

Specific Activity of CytC in presence of CTAC and ATP

To compare our system with another, which is formed by comparatively weak interactions, we choose CTAC as the surfactant to form assembly with ATP. The activity of CytC was again measured by monitoring the change in absorbance of TMB oxidized product at 650 nm for a period of 15 min. Experimental Conditions: [HEPES] = 1 mM (pH 6), [C_{16}Gua] = 100 µM, [CTAC] = 100 µM, [ATP] = 33 µM, [CytC] = 0.5 µM, [TMB] = 100 µM, [H_2O_2] = 2 mM.

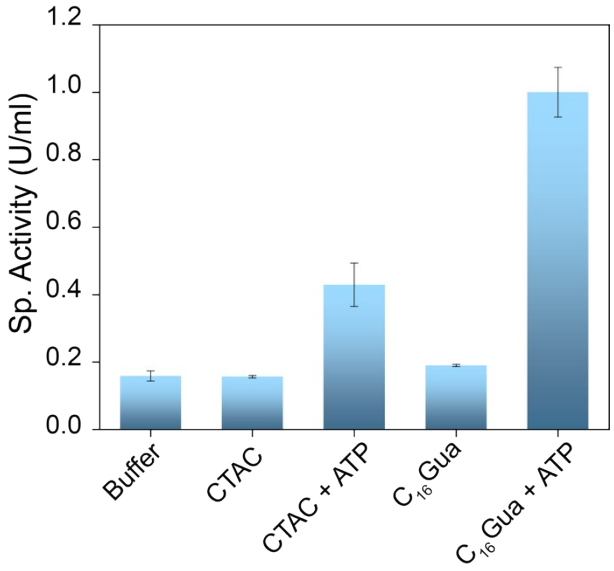


Figure S29. Specific activity of CytC in presence of different surfactants.

2. HRP and Laccase activity

The enzyme activity of HRP was determined in 1mM HEPES buffer of pH 6 using TMB assay for peroxidase. The HRP oxidizes TMB in the presence of hydrogen peroxide. Reactions were carried out with HRP (0.075 nM) and assembled structures of 100 μM C_{16}Gua and 33 μM ATP. TMB (100 μM), H_2O_2 (2 mM) in 1 mM of HEPES buffer in a total of 250 μL of solution. Absorbance at 650 nm was recorded for 15 minutes immediately after the addition of TMB and H_2O_2 solutions.

The laccase enzymatic activity was determined by using the substrate 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS). 1 mM HEPES buffer of pH 6 was used for the assay. Laccase catalyzes ABTS to form an oxidized ABTS having blue colour. Enzymatic activity of laccase was compared in presence and absence of the **C_{16}VesiGlue**. For the enzyme assay of the samples, 1 μg/mL of the laccase were incubated with **C_{16}VesiGlue** (100 μM C_{16}Gua and 33 μM ATP) and reaction rate was monitored by addition of 100 μM ABTS. Activities were calculated by the increase in absorbance at 415 nm.

F. <u>Cascade reactions on the VesiGlue surface</u>

The experiment was performed by incubating various concentration of GOx in 50 μL of HEPES buffer (1 mM, pH 6) with 50 μL of glucose (100 mM) at 37 °C for 45 min. 25 μL of the above solution was added to the 225 μL buffered solution containing CytC (0.5 μM) templated **C_{16}VesiGlue** (formed via 100 μM C_{16}Gua and 33 μM ATP). To the mixture was added 100 μM of TMB and the solution was incubated for 45 min and the oxidized TMB was quantified by recording the absorbance at 650 nm with time.The change in colour was clearly visible by naked eye. The linear increase in absorbance at 650 nm was observed with increasing concentration of GOx. The absorbance spectra were recorded from 500-750 nm with time. Additionally, the effect of increasing concentration of glucose was studied on the cascading rate. 25 μl of 1 mg/mL of GOx was incubated with 175 μL of buffer containing varying concentration of glucose. 25 μL of the above solution was added to the buffered solution containing CytC (0.5 μM) templated **VesiGlue** (formed via 100 μM C_{16}Gua and 33 μM ATP) in presence of 100 μM of TMB in a volume of 225 μl. Again, the change in absorbance at 650 nm was recorded.

1. ITC

Isothermal Titration Calorimetry was employed to determine the binding affinity of GOx with **VesiGlue**. All solutions were prepared in 1 mM HEPES buffer pH 6. The reaction cell containing **VesiGlue** formed by 400 μM C_{16}Gua and 133 μM of ATP was titrated by injecting 5 μL each of 0.5 mM GOx contained in titration syringe. Data acquisitions were done through the computer software developed by MicroCal VP-ITC. The integration of heat flow tracing determined the heat of reaction produced after each injection. The results are shown in Figure S30.

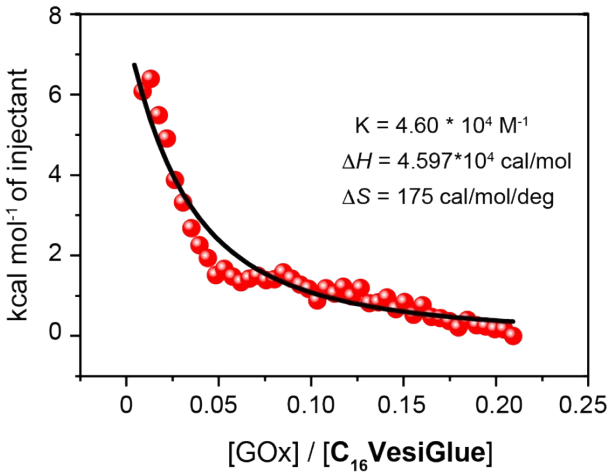


Figure S30. Integrated heat change as a function of GOx/**VesiGlue** molar ratio. The best fit was obtained by single-site non-competitive binding model.

2. Effect of gluconic acid produced during cascade on the pH and stability of C_{16}VesiGlue

Varying concentration of GOx was incubated in 50 µL of HEPES buffer (1 mM, pH 6) with 50 µL of glucose (100 mM) at 37 °C for 45 min. The above solution (25 µL) was added to the 225 µL buffered solution containing **C_{16}VesiGlue** (formed via 100 µM C_{16}Gua and 33 µM ATP) and pH of the solution was measured using pH meter.

The turbidity of the respective solution was measured via recording the absorbance at 500 nm.

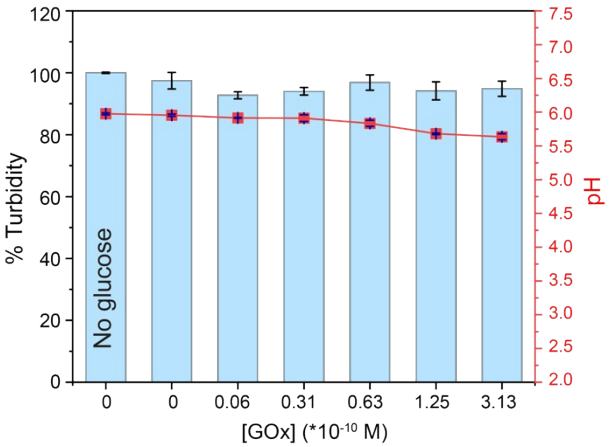


Figure S31. The change in pH (red dots) and turbidity (blue bars) of solution containing **VesiGlue** with increasing gluconic acid production because of increasing GOx concentration.

G. Temporal control of enzyme activity and biocatalytic cascade

1. Formation and disruption study of the C_{16}VesiGlue

Fluorescence of DPH

The disruption of **C_{16}VesiGlue** by the enzyme potato apyrase was confirmed by fluorescence studies using hydrophobic fluorophore DPH. In presence of the enzyme, hydrolysis of ATP takes place which leads to the release of the fluorophore from the entrapped core due to disruption of **C_{16}VesiGlue** accompanied by a decrease in fluorescence intensity at λ = 428 nm with time. Upon addition of increasing PA concentration (0, 0.5, 1 U/mL) to the assembled system formed via 25 μM of C_{16}Gua and 8.3 μM ATP, the fluorescence of DPH was found to decrease.

The reversible **VesiGlue** formation was shown by performing 3 cycles with the same sample, adding new batches of ATP to C_{16}Gua, each time the signal returned to the starting value. Fluorescence intensity at 428 nm was measured upon three repetitive additions of 16 μM ATP to a solution of 50 μM C_{16}Gua and DPH (2.5 μM) in the presence of potato apyrase (1 U/mL).

The controllable lifetime of **CytC@VesiGlue** was shown by performing 3 continuous cycles with repetitive addition of ATP. Fluorescence intensity at 428 nm was measured upon three repetitive additions of 16.6 μM ATP to a solution of 50 μM C_{16}Gua and DPH (2.5 μM) in the presence of potato apyrase (1 U/mL) and CytC (0.5 μM) and compared to that of system without CytC under same conditions (Figure 6b).

NTA

Further, NTA measurements were carried out to measure the change in the number of particles per mL accompanying the degradation of the **C_{16}VesiGlue** due to ATP hydrolysis by the enzyme potato apyrase. Measurements were performed after 30 min. of adding the PA to a solution of **C_{16}VesiGlue**. The quantity of **C_{16}VesiGlue** could be recorded via video before and after the addition of PA. The decrease in particles per mL signifies the breakdown of assembled structure upon hydrolysis of ATP. NTA measurements of solutions of surfactant (50 μM) and ATP (16 μM) in the presence and absence of potato apyrase (1 U/mL) was performed in HEPES 1 mM, pH 6.

CLSM

To study the dynamic nature of the **C_{16}VesiGlue** formed, potato apyrase (2 U/mL) was added to the nile red intercalated assembly formed via C_{16}Gua (50 mM) and ATP (16.6 mM). The sample was incubated for 15 min followed by recording the images on Confocal Zeiss LSM880 (Airyscan) microscope. Interestingly, only few to no particles could be observed proving the destruction of assembly in presence of potato apyrase.

2. Temporal regulation of CytC activity

The investigation of temporal control of specific activity of CytC under dissipative conditions was done by monitoring the peroxidase activity of CytC towards pyrogallol substrate. Because of the interfering effect of TMB Substrate in presence of the enzyme potato apyrase, pyrogallol was chosen. The oxidation of pyrogallol leads to the formation of the product purpurogallin that can be monitored at λ_{max} = 420 nm (ε420 nm is 2640 M^{-1} cm^{-1} in water). Upon addition of increasing amount of PA (0-5 U/mL) to the CytC templated assembled system, the **C_{16}VesiGlue** started disrupting and the activity of CytC was found to decrease.

The upregulation and downregulation of CytC peroxidase activity was indeed evidence of temporal control. The experiment was performed in different wells of a 96 well plate, where the first well contained **C_{16}VesiGlue** while next well contained the enzyme PA in addition to **C_{16}VesiGlue**, followed by wells containing **C_{16}VesiGlue+PA** and sequential addition of ATP at varying intervals of time. For the preparation of CytC decorated **C_{16}VesiGlue**, 100 μM of C_{16}Gua was incubated with 33 μM ATP for 15 min followed by addition of 0.5 μM CytC and incubated for approximately 5 min at room temperature. The peroxidase activity of CytC was checked by monitoring the absorbance of oxidation product of 10 mM of pyrogallol solution and adding 30 mM of H_2O_2 as an initiator of the reaction. The presence of PA disrupts the **VesiGlue** leading to a significant decrease in the peroxidase activity. However, replenishing the system with ATP reforms the template which increases the activity again. The cycle was repeated three times.

The regulation of CytC peroxidase activity was performed in absence of C_{16}Gua, following the same protocol as stated above and results are shown in figure S32.

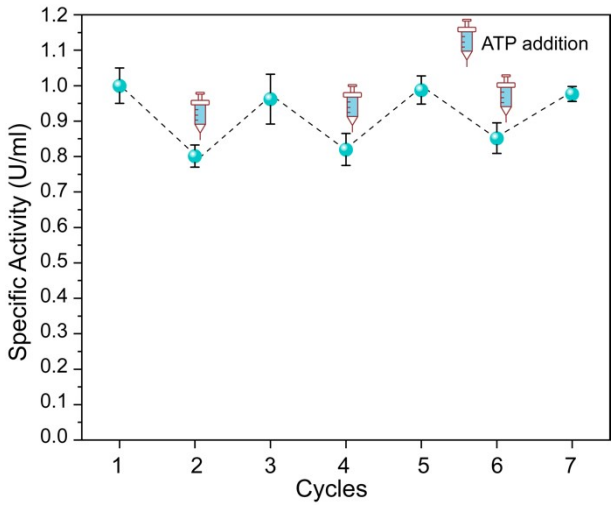


Figure S32. Normalized specific activity of ATP bound CytC measured by the catalyzed oxidation of pyrogallol (*n* = 3 technical replicates, mean ± SD). Temporal regulation of CytC activity was achieved through oscillation of ATP concentration by the presence of PA and addition of ATP when it is hydrolysed at 30 min.

The time dependent CytC activity regulation over **VesiGlue** formation and disruption was performed by monitoring the amount of oxidized product formed upon pyrogallol addition. **VesiGlue** (100 μM of C_{16}Gua + 33 μM ATP) bound CytC (0.5 μM) was monitored for its catalytic activity in presence of PA (3 U/ml) by adding the substrate (10 mM). Once PA disrupts the **VesiGlue** by cleaving the trivalent ATP, the activity was reduced significantly due to the unbound CytC in the solution. Subsequent replenishment of substrate to 10 mM did not attain the catalytic rate same as that of the CytC@**VesiGlue** system. However, reviving the system with ATP resulted in enhanced activity of CytC similar to that of the first cycle. We successfully carried out the activity regulation for three continuous cycles (Figure 6g).

3. Temporal regulation of cascade reaction

Modulation of the biocatalytic cascade reaction was achieved by gaining the temporal control over the **VesiGlue** system using PA. 100 μL of 100 mM glucose was incubated with 100 μL of 1 mg/mL GOx. 50 μL of the solution was added to the 200 μL of the system containing 0.5 μM CytC templated on **VesiGlue** (100 μM C_{16}Gua and 33 μM ATP). The activity of the system was measured using the substrate pyrogallol (30 mM). The two-step cascade reaction has faster rate in presence of **VesiGlue**, however the rate decreases by 90 % upon addition of PA (2 U/mL) with sufficient incubation time. Interestingly, the re-enhancement in the rate of purpurogallin formation could be achieved upon successive addition of 33 μM ATP, thus demonstrating the two reversible cycles of cascade. Additionally, we showed linear dependency between the GOx/CytC/**C_{16}VesiGlue** cascade reaction and the substrate glucose concentration. The experiment was performed by incubating various concentration of glucose in 100 μL of HEPES buffer (1 mM, pH 6) with 100 μL of GOx (1 mg/mL) at 37 °C. 50 μL of the above solution was added to the 200 μL buffered solution containing CytC (0.5 μM) templated **VesiGlue** (formed via 100 μM C_{16}Gua and 33 μM ATP) and CytC templated **VesiGlue** in presence of PA. The activity of the system was measured by recording the absorbance at 420 nm as a function of time.

H. Thermodynamic parameters for ITC studies

System	ΔH (cal/mol)	TΔS (cal/mol)	ΔG (cal/mol)
C_{16}VesiGlue formation	-297600	-289954	-7646

System	ΔH (cal/mol)	TΔS (cal/mol)	ΔG (cal/mol)
C_{16}Gua + ATP + CytC	-8030	-563.22	-7466.78
ATP + CytC	-6140	435.08	-6575.08
C_{16}Gua + GTP +	-209000	-203236	-5764

CytC			
C_{16}Gua + TP + CytC	-4540	2375.06	-6915.06
C_{16}Gua + ATP + NaCl + CytC	-7400	-497.66	-6902.34
C_{16}Gua + ATP + GOx	45970	52150	-6180

I. References

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