Supporting Information

Sound-driven dissipative self-assembly of aromatic biomolecules into functional nanoparticles

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Experimental Section

Materials

Tryptophan, 1-diphenyl-2-picrylhydrazyl (DPPH), Dulbecco's phosphate-buffered saline (D-PBS), bovine serum albumin (BSA), filipin from S. filipinensis, Hydrogen peroxide (30 %) and EIPA (Ethylisopropyl amiloride) were purchased from Sigma Aldrich. Methanol (99.9%) HPLC grade formic acid was purchased from Fisher chemicals. Pitstop 2 was purchased from Abcam (Cambridge, UK). Doxorubicin hydrochloride (~99%) was purchased from Chem Inc. (USA). Triton X-100 was purchased from Chem-Supply. Phalloidin Alexa 480, rabbit anti-EEA-1 monoclonal antibody and rabbit anti-Rab7 monoclonal antibodies were purchased from Cell Signalling Technology. Alamar blue assay kit, Rabbit LAMP-1 and goat anti-rabbit IgG secondary antibody Alexa Fluor 647 were supplied by Invitrogen. Fetal bovine serum (FBS) was purchased from Bovogen. *Dulbecco's Modified Eagle's medium* (DMEM) was purchased from Lonza. All the solutions were prepared in high purity water.

Ultrasonic treatment of Tryptophan

The sonication of 10 ml of 1 mg/ml Tryptophan was carried out at ultrasonic frequency and power of 355 kHz and 2 W/cm² (Power delivered per area of ultrasonic transducer) respectively as reported previously ^{1, 2}. The ELAC Nautik USW 51-052 ultrasonic plate transducer powered by a T&C Power Conversion, Inc. was used for the sonication. The OH radical yield was determined by 'Weissler'' method¹ and was estimated to be 1 mM after 1 h sonication. .

The sonication was performed in the Milli-Q water for 5 hours at temperature of 37 ± 2 °C. The nanoparticles were then separated by centrifugation at 6000 rpm for 10 min and washed several times before the characterisation.

HPLC and Mass spectrometry

Shimadzu SCL-10AVP HPLC equipped with UV detector set at 205 nm was utilised to carry out the further analysis of sonicated product. The Phenomenex "Jupiter 5u C18 300A" column was used. The injection volume was 20 μ L and flow rate was 1 ml/min with methanol: water: formic acid (70:30:0.5) used as an eluent. Electrospray ionisation mass spectrometry was performed by Agilent Accurate-Mass QToF LC/MS in positive ion mode to estimate molecular weight of the sonicated product.

Scanning electron microscopy (SEM)

The morphology and size of the particles was observed by field emission scanning electron microscope (Quanta 200 FEI) at 10 kV. The drop of nanoparticle suspension was placed on the metallic support with carbon tape and was sputter coated with gold prior to imaging.

Molecular Dynamics Simulations

The Molecular Dynamics (MD) simulations were carried out with the GROMACS 4.6.5 software package ³. Six replicas of the compound 4 in the Figure 1a (hereafter dTrp) were randomly inserted in a cubic box of 64 nm³ and hydrated with 2058 pre equilibrated SPC water molecules ⁴. The W2-OH parameters were obtained by starting from those provided by the Prodrg server ⁵ with slight modifications introduced by analogy with the standard gromos 43a1 parameters. In particular, the tryptophan parameters were used as model, except for the hydroxyl group, for which the corresponding tyrosine parameters were used. The torsional dihedral angle connecting the two aromatic rings was described according to previous report ⁶. The charges were assigned by analogy with the gromos 43a1 force field ⁷ considering a neutral pH, as a consequence both the amino and the carboxyl groups were considered charged. The simulation

was carried out according to a protocol previously used, with slight changes ⁸⁻¹⁰. Briefly, the system was energy-minimized and then equilibrated using a 100 ps MD, where the positions of the dTrp atoms were harmonically restrained. Production simulations were performed for 1 µs, at a temperature of 300 K, controlled by means of the V-rescale scheme ¹¹. Pressure coupling was applied using the Berendsen algorithm, with a time constant of 1.0 ps and a reference pressure of 1 bar ¹². Bond lengths were constrained with the LINCS algorithm ¹³. Short-range electrostatic interactions were cut-off at 0.9 nm and long range electrostatic interactions were calculated using the particle mesh Ewald (PME) algorithm ¹⁴. Simulations were run with a 2 fs time step. To evaluate the Solvent Accessible Surface Area (SAS) and the number of hydrogen bonds, the g_sas and g_hbond tools in GROMACS was used with the default settings. The structural figures were produced by using VMD ¹⁵ or Chimera ¹⁶. At each time, a single dTrp molecule was considered part of an aggregate if the minimum distance with any other molecules in the aggregate falls below 0.35 nm. The minimum distances were evaluated with the g_mindist tool in GROMACS between all the pairs of molecules; starting from these distances, a homemade program was used to obtain the number of molecules in the aggregates.

Surface tension measurements

The surface tension of dissolved dimer solutions at different concentration was measured in water/air system using pendant drop method on OCA 15 EC. The slope of the graph of surface tension vs lnC was used to calculate the surface excess of dimers using Gibbs adsorption isotherm equation given as follows.

$$\Gamma = \frac{-1}{RT} \frac{\partial \gamma}{\partial lnC}$$

Where R is Gas constant (8.314 J/ mol K), T is the temperature in K, γ is the surface tension, C is the concentration of the solute, Γ is the surface excess. Then the area occupied by each molecule was calculated using the expression $1/N_A\Gamma$ where N_A is Avogadro's number.

Fluorescence spectroscopy

To study the optical properties of the sonicated product Shimadzu RF-5301PC fluorescence spectrophotometer (Shimadzu) equipped with a xenon lamp and 1.0 cm optical length quartz cell was utilized to obtain the fluorescence excitation and emission spectra. The fluorescence spectra were obtained at different wavelength in PBS (pH=7.4).

Dynamic Light Scattering (DLS), ζ-potential

The zeta potential and hydrodynamic diameter of the particles was determine using ZEN0040, Malvern Instruments. The 100 μ L particles (2mg/ml) were suspended in Milli-Q and zeta potential and hydrodynamic diameter of particles were determined.

Protein Adsorption

The dTrpNPs were incubated with 100 % Fetal bovine serum and the adsorption of protein was determined from the increase in size of particle measured by Malvern Analytical NanoSight NS300. Nanosight combines the principle of light scattering and Brownian motion of particles in liquid suspension and use the Stokes Einstein equation to obtain the hydrodynamic diameter of the particles.

Quantum yield

The quantum yield of the sonicated product with an emission maximum at 690 nm was determined by using Rhodamine B as a reference standard in water. Following equation was used to estimate the quantum yield

$$\frac{\phi_{f1}}{\phi_{f2}} = \frac{(1 - 10^{-A2})n_1^2\alpha_1}{(1 - 10^{-A1})n_2^2\alpha_2}$$

Where ϕ is the quantum yield, A is the Absorbance, n is the refractive index and α is the area under the fluorescence emission spectrum and subscript 1 and 2 illustrated the reference sample and sonicated product, respectively.

Antioxidant activity

The antioxidant activity was monitored using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. To 3 ml DPPH solution (250 μ M in 50:50 ethanol: water), tryptophan with and without ultrasound treatment was added and absorption of DPPH was recorded at 520 nm. Percentage of radicals scavenged can be determined by using following equation:

% Radical scavenging activity = $[(A_{DPPH} - A_{sample})/A_{DPPH}] \times 100$

Where A_{DPPH} and A_{sample} are the absorption values for blank DPPH solution and after the addition of sonicated sample, respectively

Loading of DOX to dTrpNPs

0.5 mg/ml dTrpNPs, obtained after 5 hr of sonication, were incubated with 20 μ l Dox solution giving final Dox concentration of 280 μ g/ml. The particles were incubated overnight with Dox. They were well washed five times with water to remove excess Dox and were refrigerated at 4°C.The amount of Dox loaded was quantified using fluorescence intensity at λ_{ex} =480 nm.

Release of Dox from dTrpNP

dTrpNPs loaded with the Dox were incubated with 0.01 M PBS buffer (pH=7.4) at 37 °C. The release of Dox was studied as a function of incubation time. The mixture was centrifuged, and

supernatant was collected and analysed by fluorescence spectroscopy at an excitation wavelength of 480 nm.

Cellular Uptake of dTrpNP using Flow cytometry

The association and uptake of the dTrpNPs with human breast adenocarcinoma MDAMB-231 breast cancer cell line was evaluated using Flow cytometry. Since the particles were fluorescent, no further functionalization of particles was required. The cells after incubation with the particles at different times (2 h, 4.5 h, 6.5 h, 12 h and 24 h) were detached using trypsin, then washed twice using 1% BSA/PBS and centrifugation at 400 rpm for 5 mins at 4 °C. After this, cells were analysed using BD Accuri[™] C6 flow cytometer under different fluorescence channels and FlowJo was used for data analysis.

Cell Viability

The human breast adenocarcinoma MDA-MB-231 cells were plated in 96 well plate with seeding density of 40,000 cells per well in 100 μ L DMEM media with 10 % FBS. The cells were incubated for 24 hours in 5% CO₂ at 37°C. The particles were added into the cells at different concentrations for 24 and 48 h in triplicates and cell viability of the sonicated tryptophan was assessed by addition of alamar blue assay. Similarly, DOX loaded dTrpNPs and free doxorubicin were at different DOX concentration to compare the cell viability after 24 h and 48 h.

Mechanism of internalization of dTrpNP

To study the mechanism of internalization MDA-MB-231 cells were seeded with seeding density of 50,000 cells per well in 24 well plate and incubated overnight at 37 °C. The cells were incubated with different endocytosis inhibitors namely filipin (5 μ g/ml), pitstop-2 (12 μ g/ml), and Ethylisopropyl amiloride (EIPA) (15 μ g/ml) to inhibit caveoloe-dependent endocytosis, clathrin-dependent endocytosis and micropinocytosis for 30 min and then incubated with

dTrpNP (final concentration 10 μ g/ml) for 2 h. The cells were washed twice with PBS and detached with trypsin. Then cells were washed twice using 1% BSA/PBS and centrifuged at 400 rpm for 5 min at 4 °C. The cells were analysed using Apogee A50-Micro Flow Cytometer and FlowJo was used for data analysis.

Laser Scanning Confocal Microscope Imaging

All samples with cells were imaged using Nikon A1R confocal microscope with a 60×1.4 NA oil immersion objective. For the live cell imaging the MDA-MB-231 cells were seeded with seeding density of 40,000 cells per well in DMEM media with 10 % FBS were incubated with the dTrpNPs at 37 °C for 24 h and Dox loaded dTrpNPs for 2-24 hrs and after refreshing the culture media, the cells were observed under the confocal microscope.

For intercellular trafficking, the particles were removed after 2.5 h and were kept at 37 °C for 2.5 h, 5 h and 21.5 h in fresh media corresponding to observation times of 2.5 h, 5 h, 8.5 h and 24 h. Then for each sample the media was removed after the desired time, cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 10 min. After washing 3 times again the cells were permeabilized with 0.1 % TritonX-100 (in PBS) for 5 min and washed again with PBS and Blocked for 1 hour with 1 % BSA solution in PBS. After this, cells were incubated with rabbit anti-EEA1 monoclonal antibody (2 μ g/ml), rabbit anti-Rab7 monoclonal antibody (2 μ g/ml) and rabbit LAMP-1 (1 μ g/ml) for 1.5 h followed by 3 time washing with PBS and further incubation for 1 h with goat anti-rabbit Alexa Fluor 647 conjugate secondary antibody (2 μ g/ml). The samples were then imaged using confocal microscope. The 647 laser was set at the power where no signal from the dTrpNPs was observed. The Pearson's correlation function was determined with ImageJ software.

Characterisation of dTrpNp



Figure S1: HPLC of the sonicated tryptophan at different sonication times up to 3 hours. The HPLC analysis shows the conversion of tryptophan (retention time 3.2 min) into products. The peak at retention time 4.4 min (Figure 2b) is attributed to the dimers and hydroxylated dimers of tryptophan, whereas the peak at 2.7 min (Figure 2c) is attributed to the hydroxylated tryptophan species. Both peaks increase as a function of sonication time. Visible dTrp aggregates were formed after 3 h sonication.



Figure S2: ESI/MS of sonicated tryptophan showing formation dimer and hydroxylated dimers. The HPLC data was further supported by the ESI-MS, which was performed in positive ion mode. (a) mass spectrometry of sonicated tryptophan confirms the formation of hydroxylated products such as Trp+OH, Trp+2OH and Trp+3OH at m/z of 221, 237 and 253, respectively. The peak at m/z 237 could be also ascribed to the degradation of tryptophan into N'-Formylkynurenine. In addition to these, different dimeric species with different degree of hydroxylation were also obtained (e.g. the peaks at m/z 407, 423, 439), (b) The mass spectroscopy of dTrpNps after dissolution. The peak at m/z 407.50, 429 and 445 correspond to Trp-Trp, Trp-Trp+Na, and sodium adduct of dTrp (Trp-Trp+ONa) respectively. Peak at m/z 370 can be fragmentation of Trp Dimer. Apart from dimer, trimer at m/z 631 was also observed.



Figure S3: (a) EDS analysis of the tryptophan nanoparticles confirming the presence of carbon, oxygen and nitrogen and increase in oxygen percentage. The energy dispersive X-ray spectroscopy of the tryptophan nanoparticles also confirmed the presence of carbon, nitrogen and oxygen with atomic percentages of 68 %, 11 % and 21 % respectively. The atomic percentage of C, N, and O in tryptophan itself is 68.7 %, 14.6 %, 16.7 % respectively. Therefore, there is an increase in the percentage of oxygen with could be attributed to the presence of multiple hydroxyl groups, (b) FTIR spectra of dTrpNP and Trp, in dTrpNP peaks

lost intensity and were broadened as compared to monomeric tryptophan. Peaks at 1695 cm⁻¹ corresponds to C=O stretch, 1596 cm⁻¹ to C=C aromatic stretch, 1324 cm⁻¹ to C=C indole stretch. The peak at 3400 cm⁻¹ in Trp is due to N-H, however, broad additional peak in dTrpNP at 3340 cm⁻¹ corresponds to O-H stretch, (c) NMR spectra of dTrpNP. Peaks at 8 and 13 ppm correspond to O-H proton on different position of the aromatic ring and 10.3 ppm is due to N-H proton in indole. Peaks between 6.7 to 7.5 ppm correspond to aromatic and indole protons.



Figure S4: a) Fluorescence emission spectra of tryptophan solution at different sonication time at λ_{ex} = 310 nm, b) Fluorescence excitation spectra of tryptophan solution at different sonication time at λ_{em} = 370 nm, c) Absorbance spectra of tryptophan solution at different sonication time, and d) Fluorescence emission spectra of tryptophan at different excitation wavelengths.



Figure S5: (a) Fluorescence emission spectra of dTrpNPs at different excitation wavelengths before dissolution, and after dissolution at $\lambda_{ex} = 400$ nm (green) (b) Fluorescence excitation spectra at emission wavelength 680 nm and fluorescence emission spectra at excitation wavelength 575 nm of dTrpNPs before dissolution



Figure S6: (a) The numerical simulation of the change in bubble radius as a function of oscillation time at 355 kHz and 2 W/cm² calculated using bubble dynamic equations and scheme illustrating the changes in the bubble size (at the top) and (b) SEM of dTrp nanoaggregates formed above cac in acidic environment.



Figure S7: Normalised FCS function G(t) for 568 nm in tryptophan sonicated suspension. The fit residual has been shown in the inset. The fast and slow components at correlation times 0.02349 ± 0.0072 ms and 6.50 ± 0.49 ms respectively were obtained, attributed to free dTrp dimers (fast component) and nanoparticles (slow component). The diffusion coefficient of free tryptophan dimers and nanoparticles was found to be $870\pm78 \,\mu\text{m}^2/\text{s}$ and $4.3\pm0.33 \,\mu\text{m}^2/\text{s}$.



Figure S8: Titration curve of the dTrpNPs after dissolution showing changes in pH against number of moles of OH ions



Figure S9: (a) Particle size distribution and (b) SEM image of dTrpNP obtained during cycle 2 where the NP obtained during cycle one were dissolved in alkaline conditions and then ultrasound was applied after adjusting pH at 2.



Figure S10: (a) Photostability study of sonicated tryptophan suspension showing fluorescence intensity as a function of time when irradiated at 401 and 488 nm. (b) Fluorescence intensity of nanoparticles when irradiated at 488 nm as a function of number of storage weeks at pH 5 showing that the intensity didn't decrease, showing the stability of nanoparticles, (c)

fluorescence emission spectra of dTrpNP in PBS and protease when irradiated at 488 nm, (d) pH dependence of fluorescence intensity of dTrpNPs at excitation wavelength of 480 nm. The green emission of dTrpNPs at 555 nm increased 3 folds in alkaline environment This may be ascribed to the deprotonation of the aromatic hydroxyl groups at pH 9 and to the higher quantum yield of the deprotonated species as indicated by the potentiometric titration curve (**Figure S7**).



Figure S11: (a) Cell viability percentage of dTrpNPs against MDA-MB 231 cells after 24 h and 48 h showing the cytocompatibility of nanoparticles, and (b) Flow cytometry of the dTrpNPs under different fluorescence channels such as FITC (blue), TRITC (green), AF 647 (red) illustrating association of particles to cells.

Table S1: Table summarizing the PCC values depicting colocalization of dTrpNP with Early endosome, Late endosome and Lysosome at different incubation times

	2.5 h	5h	8.5 h	24 h
Early endosome	0.563	0.502	0.23	0.23
Late endosome	0.529	0.6385	0.312	0.3
Lysosome	0.323	0.373	0.255	0.21



Figure S12: Schematic showing proposed mechanism for the intracellular trafficking, cellular uptake and endosomal escape of dTrpNPs. The particles are uptaken by endocytosis leading to the formation of early endosome (pH~6.5) (step 1) due to the acidification (activity of ATP dependent proton pump), then can enter late endosome/lysosome (pH~5.5) (step 2). The protonation can mediate the flow of ions and water into the endosome/lysosome compartments and counter ions (Cl-) to maintain electrical neutrality (step 3). The process would lead to the high osmotic pressure (step 4) and ultimately escape of dTrpNPs. Later the particles can undergo dissolution in the cytosol.

Antioxidant properties of dTrpNPs

The antioxidant and radical scavenging properties of dTrpNP nanoparticles were evaluated using the assay based on DPPH radical reduction. Tryptophan itself can act as an antioxidant, since the indole group is a hydrogen donor and it can quench oxygen radicals.

However, the higher degree of hydroxylation significantly enhances the antioxidant properties of dTrpNP compared to Tryptophan, as shown in Figure S13. We have also considered the applicability of dTrpNP as a platform for sustained anticancer drug delivery



Figure S13: DPPH radical scavenging activity of tryptophan with and without ultrasound (USultrasound) after 2 and 10 min of incubation with of DPPH solution



Cell stained with Phalloidin (480 nm)

Fluorescence due to DOX (560 nm)

Merged image showing DOX accumulation Nucleus

Figure S14: Confocal microscopy images of the MDA-MB -231 human breast cancer cells when treated with doxorubicin after 2 h incubation.

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