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Supporting Information

Self-assembled Amphoterecin-B Loaded Self-assembled Nanostructured Lipid Carrier from Cardanol for Delivery and Enhanced Antifungal Activity

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1. Experimental

1.1. Materials

Technical standard Cashew nut shell liquid was purchased from Satya cashew industry (India). Ergosterol, Chitin, Aniline, sodium nitrite, sodium hydroxide and hydrogen peroxide were purched from Sigma-Aldrich (USA). Amphoterecin-B was purchased from high media, India. All solvents were of analytical grade from Merck (India).

1.2. Instrumentation

1.2.1. Ultraviolet-Visible Spectroscopy (UV-Vis). The UV-vis absorbance spectra were recorded on a Shimadzu UV-1601 absorption spectrophotometer against solvent blank reference in the wavelength range of 300−600 nm. Experiments are performed by keeping concentration 0.02 mM samples. In all experiments, solutions were taken in quartz cuvette of 1-cm path length.

1.2.2. Fourier Transform Infrared (FTIR). For IR experiment, samples were dissolved in chloroform and placed onto a KBr pellet and dried. The dried specimen was recorded on Shimadzu 8400 FT-IR spectrophotometer. Absorbance spectra were obtained from 4000 to 400 cm^{-1} with a 4 cm^{-1} resolution, Background spectra were also collected and subtracted.

1.2.3. Nuclear magnetic Resonance (NMR). The ^{1}H and ^{13}C spectra were taken on a Bruker DPX200 (500 MHz for ^{13}C and 200 MHz for ^{1}H) in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard. Both cases 2 mg/ml concentrated solutions were prepared. All signals were referenced to TMS to within ± 0.1 ppm.

1.2.4. Circular dichroism (CD). The Circular dichroism (CD) spectrum was recorded at room temparature using a Jasco J-815 spectropolarimeter equipped with a Jasco PTC-423 S Peltier temperature controller. The scanning rate was 50 nm. min^{-1} with a response time of 2s. Spectrum was recorded at standard sensitivity (100 mdeg) with a data-pitch of 0.5 nmin continuous mode. The scanning range was 260-190 nm and spectrum was the average of two consequent accumulations. The baseline was corrected by subtracting the corresponding buffer blank.

1.2.5. Transmission electron microscopy (TEM). The TEM was performed in JEOL JEM 2100 instrument. A small portion of self assembled samples were drop cast on a Cu-grid. After drying the grid at ambient temperature, the samples were directly imaged under TEM.

1.2.6. Dynamic light scattering (DLS) measurements

The dynamic light scattering experiment was performed with an argon ion laser system (DLS, Malvern Instruments, Series 4700) at 25 °C. Two mg/ml concentration in cyclohexane of all self-assembled compound was filtered with 200 nm syringe filter and dispersion was used for analysis. Scattering angle of 90° was used for the DLS measurements of particle size, and the results are reported as the average and standard deviation from more than twenty DLS measurements over a time period sufficient to reach equilibrium.

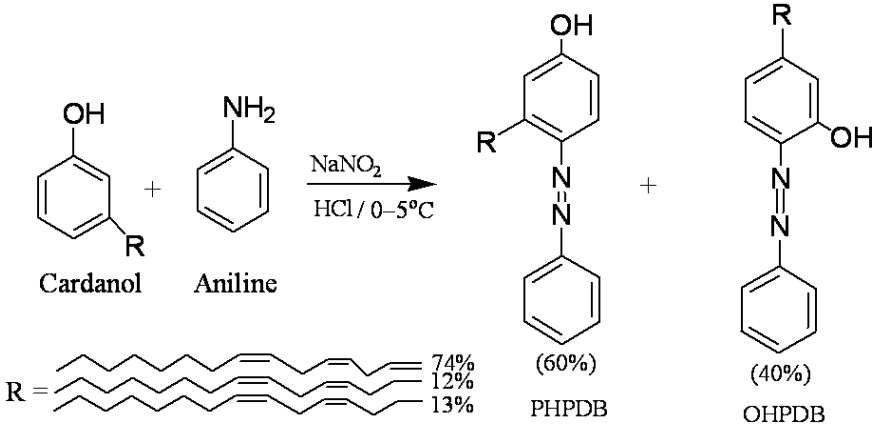
1.2.7. Surface tension measurements

The surface tension values of self-assembled AmpB-PHPDB complex was measured with pendant method (ramé-hart Model 250 Standard Goniometer/ Tensiometer) at 25 °C where liquid phase is cyclohexane and liquid pendant drop is suspended in air as gaseous external phase. Each liquid droplet volume employed in this study was typically 1.8±0.1 μL. All the measurements were repeated 10 times and their average value taken for all calculations.

1.2.8. Isothermal titration calorimetry (ITC). The titration was performed using a ITC_{200} Systems (GE Healthcare, USA) coupled with non-reactive Hastelloy® cells for chemical resistance. The titrations were carried out 0.25×10^{-3} molar solution with 0.1×10^{-3} molar chitin in dimethyl sulphoxide (DMSO) and 0.25×10^{-3}amphoterecin-B with 0.1×10^{-3} molar ergosterol in methanol. All solutions were degassed right before the experimental runs in same condition at 25 °C and at 180 sec intervals utilizing a stir speed of 310 rpm. Blank ITC experiments were done to correct heat of dilution effects. Origin 7.0 (OriginLab Corp., MA) was used to analyze the ITC data to determine the binding constant (K) and enthalpy of binding (ΔH) directly from the binding thermograms.

1.3. General synthesis procedure

Synthesis of three different Cardanol diazonium compounds was carried out according to the modified procedure of Bhunia *et al*[1]. via electrophilic substitution reaction (Scheme.1). Aniline (2.325 gm, 25 mmol) was dissolved in 50 ml 1(N) HCl solution and diazotized with sodium nitrite (1.7 gm, 25 mmol in 10 ml of water) solution at 0°C with constant stirring separately. The solution was diluted with chilled methanol. Cardanol (7.4 gm, 25 mmol) was dissolved in a chilled 5% methanolic potassium hydroxide solution and added dropwise to the diazonium salt solution. The whole system was kept in an ice bath within the range 0–10°C. The red dye formed was stirred for a further period of 6 h and poured into dilute HCl solution with stirring. The red dye was separated, washed thoroughly with water, and dried. The mixture of products (OHPDB and PHPDB) was purified by column chromatography on silica gel (60–120 mesh) using petroleum ether and ethyl acetate (5:1 and 10:1, respectively) as eluent. The purity was confirmed by using U.V, FTIR, NMR and MALDI-MS analysis.



Scheme S1. Synthesis of 4- [(4'-Hydroxy-2-pentadecenylphenyl)diazenyl] benzene (PHPDB) and 4- [(2'-Hydroxy-2-pentadecenylphenyl)diazenyl] phenol (OHPDB).

(i) (4- [(4'-Hydroxy-2-pentadecenylphenyl)diazenyl] benzene (PHPDB): Red viscous liquid; yield: 6.18 gm; U.V (DMSO, nm): 364 (-N=N-); IR (KBr, cm^{-1}): 3438 (Ar–OH), 2909, 2851 (–CH2–), 1593,1520 (Ar, C=C), 3002, 859 (cis –CH=CH–), 1463 (-N=N-), 1297 (symmetric C=C), 1165 (asymmetric C=C); ^{1}HNMR (500 MHz, $CDCl_3$, δ ppm): 7.8 (d, 2H), 7.6 (d, 1H), 7.3(m, 1H), 6.79 (d, 2H), 6.73 (d, 1H), 6.70 (d, 1H), 5.99-4.89 (m, 4H), 3.06 (t, 2H), 2.71(m, 4H), 2.0-0.84 (m, 15 H); ^{13} CNMR ($CDCl_3$, δ ppm): 14.23, 22.79, 25.71, 27.36, 29.11, 29.90, 31.53,32.03, 113.9,114.06, 116.64, 117.55, 122.71, 129.94, 130.52, 131.44, 143.55, 144.99, 156.36.

(ii) 4- [(2'-Hydroxy-2-pentadecenylphenyl)diazenyl] phenol (OHPDB): Red viscous liquid; yield: 4.08 gm; U.V (DMSO, nm): 355 (-N=N-); IR (KBr, cm^{-1}): 3358 (Ar–OH), 2919, 2853 (–CH2–), 1593, 1503 (Ar, C=C), 3009, 815 (cis –CH=CH–),1463 (-N=N-), 1296 (symmetric C=C), 1150 (asymmetric C=C); ^{1}HNMR (500 MHz, $CDCl_3$, δ ppm): 7.91 (d, 2H), 7.62 (d, 1H), 7.46 (m, 1H), 7.17 (d, 2H), 6.8 (d, 1H), 6.73 (d, 1H), 5.99-4.89 (m, 6H), 3.06 (t, 2H), 2.71(m, 4H), 2.0-0.84 (m, 21H); ^{13} CNMR ($CDCl_3$, δ ppm): 14.23, 22.79, 25.71, 27.36, 29.11, 29.90, 31.53,32.03,114.23, 116.75, 123.28, 124.90, 128.23, 130.45, 136.94, 144.96, 148.28, 156.52, 159.87.

1.4. Estimation of drug entrapment efficiency and drug loading content

Self-assembled AmpB was prepared in choloroform solution after overnight rotation at 1000 rpm. The methanolic solution of PHPDB was added to self-assembled AmpB and mixed well. Then mixture solution was added drop wise in cyclohexane solvent at room temperature with 1000 rpm. After 2 h, organic layer was separated from the mixture and vortexed for overnight. The residual drug in methanol layer was estimated by UV-vis spectrophotometically at 384 nm. The percentage of drug entrapment efficiency and loading content were calculated following the equation 1 and 2.

$$\text{Entrapment efficiency (\%)} = \frac{A_{\text{Total drug}} - A_{\text{Unencapsulated drug}}}{A_{\text{Total drug}}} .100 \qquad (1)$$

$$\text{Drug loading content (\%)} = \frac{\text{Encapsulated drug}}{\text{Amount of PHPDB}} .100 \qquad (2)$$

Where, $A_{\text{Total drug}}$ is the optical density of drug added in methanol and $A_{\text{unencapsulated}}$ drug is the optical density of residual drug in methanol phase.

1.5. In vitro AmpB release.

The release of AmpB from the PHPDB-AmpB complex was investigated in water phase at three different pH as 7, 5.5 and 3 at room temperature. For each experiment, 5 ml of PHPDB-AmpB complex in cyclohexane solution (2 mg/mL) was suspended in 5 mL aqueous medium with stirring 100 r.p.m. The amount of released free AmpB in the aqueous phase was analyzed by UV-spectrophotometrically at 384 nm. The cumulative drug release percent (Er) was calculated by the following equation:

$$E_r\,(\%) = \frac{V_e \sum_{1}^{n-1} C_i + V_0 C_n}{m_{drug}} .100 \qquad (3)$$

Where, m_{drugs} represents the initial amount of loaded drugs, V_0 is the whole volume of the release media (V_0= 5 mL), Ve is the volume of the replace media (Ve=3 mL) and C_n represents the concentration of drugs in the nth sample. Three groups of replicate measurements were carried out for each time point.

1.6. Antifungal assay

The minimum inhibitory concentration (MIC) of all synthesized compounds against planktonic cells of *Candida albicans* SJ11 (hospital isolate) and *Candida tropicalis* NCIM 3110 strains were determined according to the guidelines of the Clinical and Laboratory Standards Institute [CLSI (M27-A2 document, NCCLS] [National Committee for Clinical Laboratory Standards, document M27-A, National Committee for Clinical Laboratory Standards, Wayne, Pa]. A 200 µL of total volume in each well was made by containing the growth medium (RPMI 1640) with different concentrations of individual compounds (500 to 0.48 µg.mL^{-1}) and fungal inocula load was 3.5 ×10^6 CFU/mL. Two wells of the plate used as growth (without antifungal) and sterility (without inocula) controls. The plates were incubated at 30°C for 18h. Experiments were carried out in 96-well plate in triplicate on three different sets following Mandal *et al*[2].

1.7. Biofilm eradication test

The formation of *Candida albicans* biofilm on polystyrene plat was carried out according to Mandal et al. [2012]. Each well is containing 200 µL of RPMI 1640 medium with inoculums dose 3.5 X 10^6 CFU/ mL and incubated for 72 h at 30ºC. To determine the effect of only AmpB and self-assembled AmpB loaded PHPDB complex on biofilm eradication, complexes were added to mature biofilm, after 1h incubation with MIC dose, wells are washed gently and visualized in scanning electron microscopy. The remaining biofilm metabolic activity was quantified by the XTT-reduction assay as described earlier[4].

1.8. Haemolytic assay/ Evaluation of red blood cell lysis

Haemocompatibility study was performed using standard protocol following Mandal et *al.*[3]. In brief, blood was collected from 6-week-old male BALB/c mice in a heparinized tube and red blood cells (RBC) were obtained by centrifugation at1500×g for 5 min in 4°C.The collected RBC pellet was diluted in 20mM HEPES saline buffer (pH 7.4) to make a 5% (v/v) solution. The RBC suspension was added to HEPES-buffered saline (−ve control), 1.0% Triton X-100 (+ve control) and incubated with different compounds at a fixed concentration of 16 μg mL^{-1} for 60 min at 37°C. After centrifugation at 12,000rpm at 4°C, the supernatants were transferred to a 96-well plate. Hemolytic activity was determined by measuring the absorption at 570nm (Biorad Microplate reader 5804R). Control samples of 0% lysis (in HEPES buffer) and100% lysis (in1%TritonX-100) were employed in the experiment. All assays were performed intriplicate. Hemolytic effect of each treatment was expressed as percent of cell lysis relative to the +ve control cells (% control) using the following formula: [(Abs_{570} of samples)/ (Abs_{570} of (+) ve control cells)]×100, where absorbance is abbreviated to Abs.

1.9. Cytotoxicity assay

MTT [(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)], assay was performed to determine cell cytotoxicity following the method described earlier by Mandal et *al.*[4]. Human Embryonic Kidney 293 (HEK cells (2.0×10^3) were seeded in 100 μL complete DMEM medium per well in 96 well plates. Plates were incubated at 37^0C in 5% CO_2 for 24 hours for cell attachment. Cells were treated with individual compounds with variable concentration from 5-100 μg/mL and incubated at 37^0C in 5% CO_2 for 48h. Three wells were used in the 96 well plates for each derivative and repeated three times. For the MTT assay, thiazolyl blue tetrazolium bromide solution (100 μL; 1 mg/ mL) in incomplete medium was added and this mixture incubated for 4 hours. After that, 100 μL of dimethylsulphoxide (DMSO) was added and the plates were rotate for 5 minutes. Optical density was recorded at 550 nm with DMSO as the blank. Percentage of cell viability was plotted against concentrations of derivatives. Cells treated without any compound used as control.

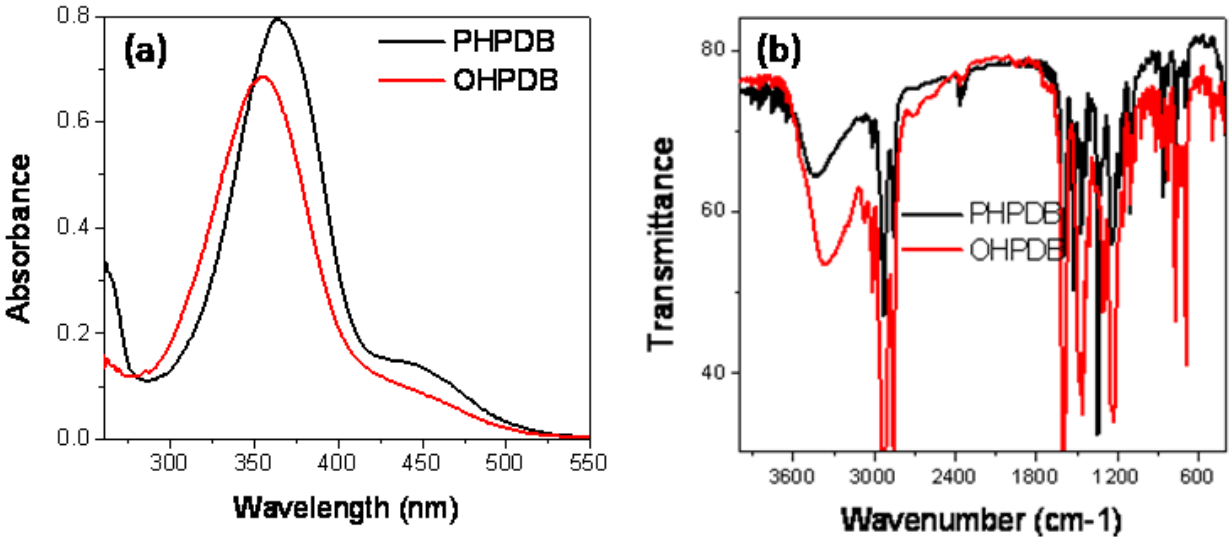


Fig. S1. (a) UV spectrum of (i) PHPDB (ii) OHPDB; (b) FT-IR spectrum of (i) PHPDB (ii) OHPDB.

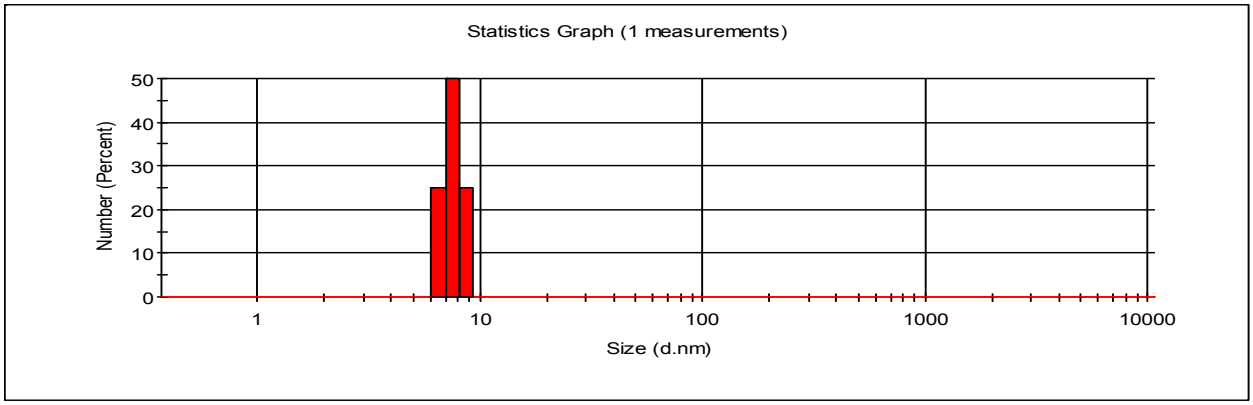


Fig. S2 (a). Particle size distribution from DLS measurment of self-assembled Amp B.

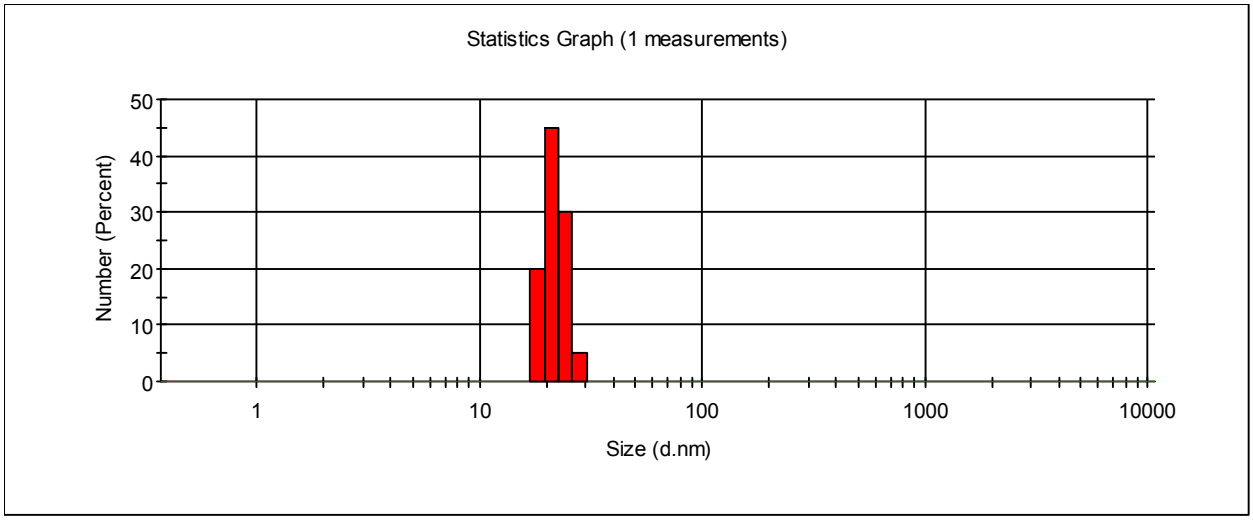


Fig. S2 (b). Particle size distribution from DLS measurment of self-assembled PHPDB.

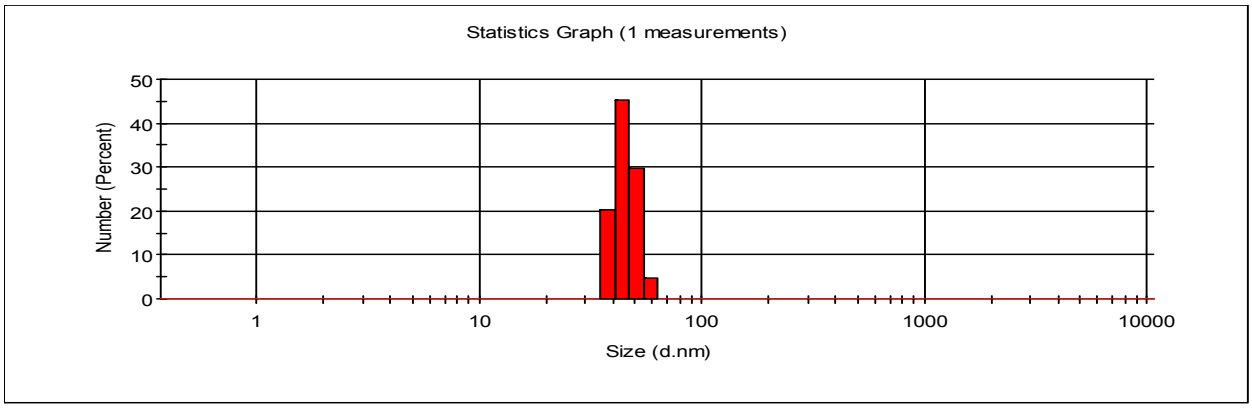


Fig. S2 (c). Particle size distribution from DLS measurment of self-assembled Amp B loaded PHPDB complex.

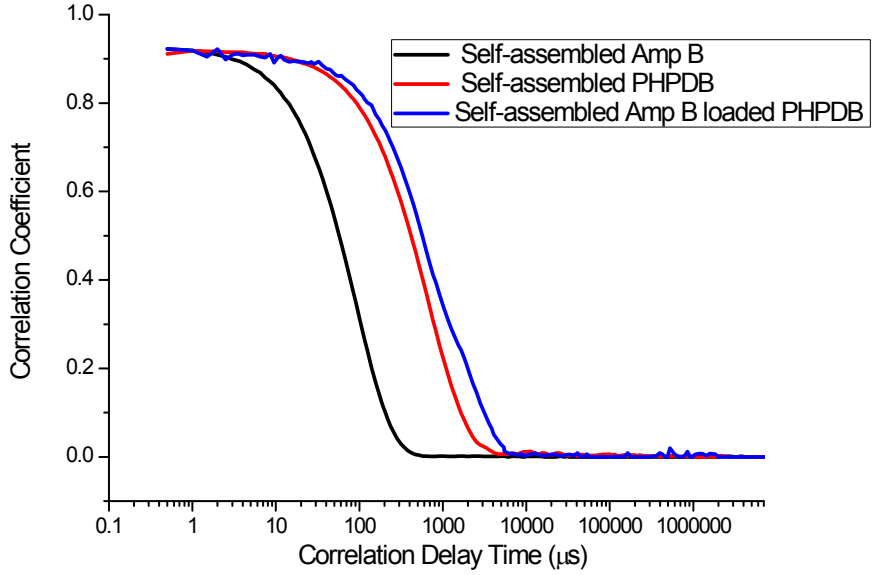


Fig. S2 (d). Correlogram representation for correlation function of self-assembled AmpB (black colour), PHPDB (red) and AmpB loaded PHPDB complex (blue). The obtained correlogram represents the poor correlation function and higher poly dispersive index (0.747) of self-assembled Amp B loaded PHPDB complex. This is might be due to the presence of two different nanostructures (PHPDB and AmpB) in the same environment and their continuous gregation. Therefore, z-average diameter from cumulants was higher than average particle size diameter.

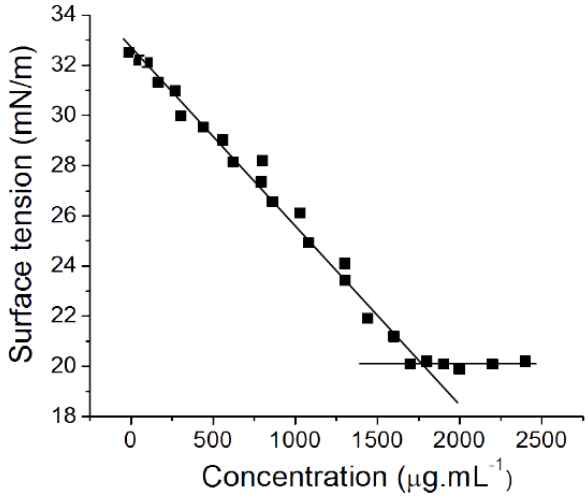


Fig. S3. Determination of particle association concentration. Surface tension was measured with variation of different concentration of self-assembled Amp-PHPDB complex.

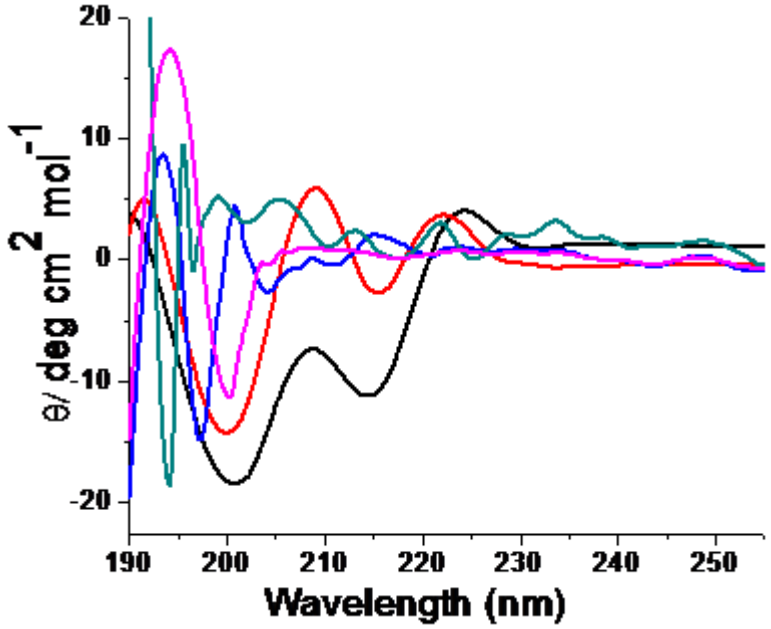
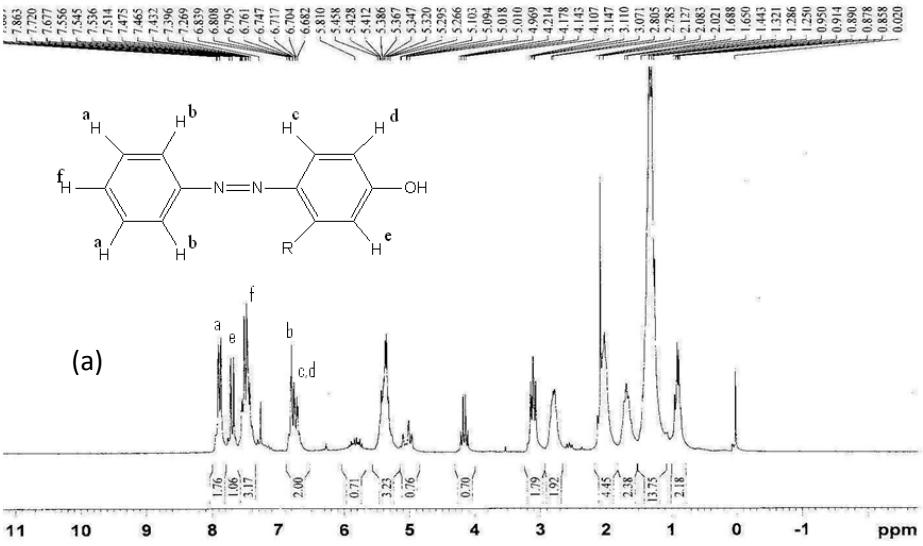


Fig. S4. Circular dichroism spectra of pure and self-assembled compounds. (i) Pure PHPDB (black colour), (ii) self-assembled PHPDB (red colour), (iii) pure amphoterecin-B (green colour), (iv) self-assembled amphoterecin-B (pink color), (v) self-assembled amphoterecin-B loaded PHPDB (blue colour).


a
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N=N
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(a)
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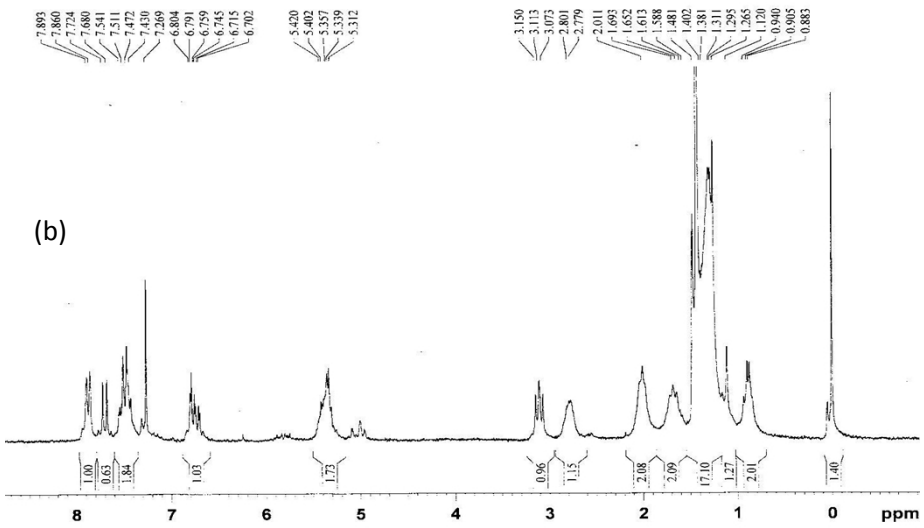
(b)
ppm

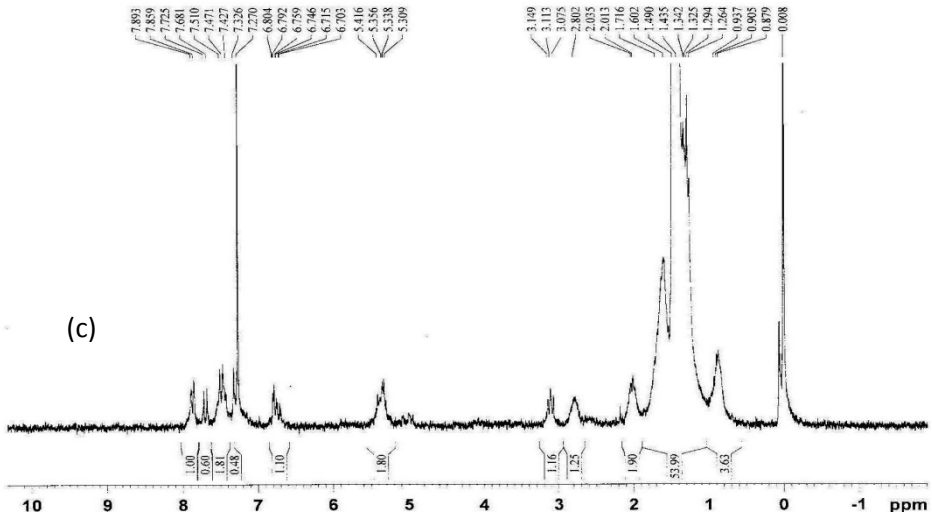

Fig. S5: The ^{1}HNMR spectra of PHPDB. Spectra obtained from pure PHPDB (a),self-assembled PHPDB (b), and self-assembled AmpB loaded PHPDB complex (c).

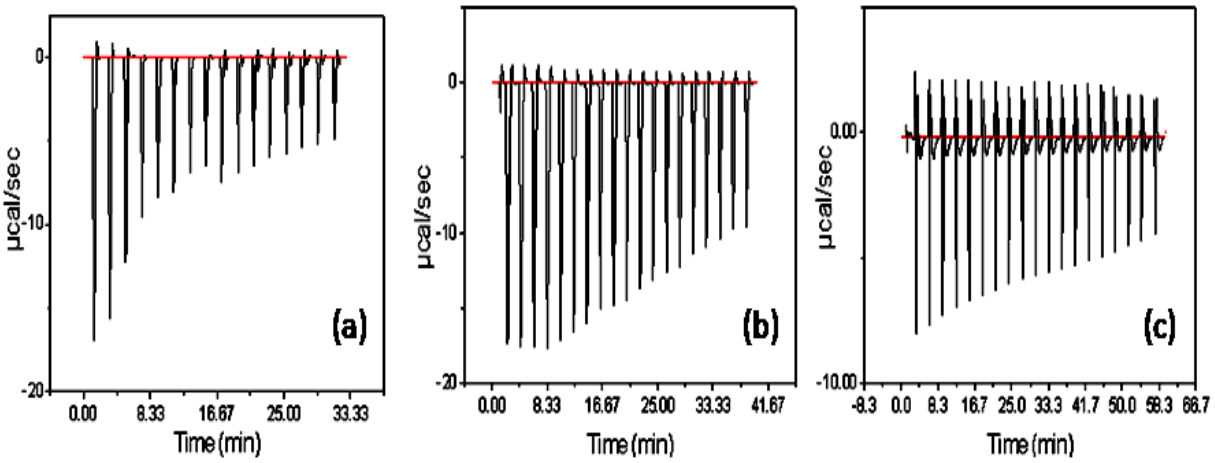

Fig. S6. ITC based binding thermogram plot of Amphoterecin-B with Ergosterol and PHPDB with chitin. Binding thermogram obtained from interaction between ergosterol with pure AmpB (a) and with self-assembled AmpB (b); chitin with self-assembled PHPDB (c).

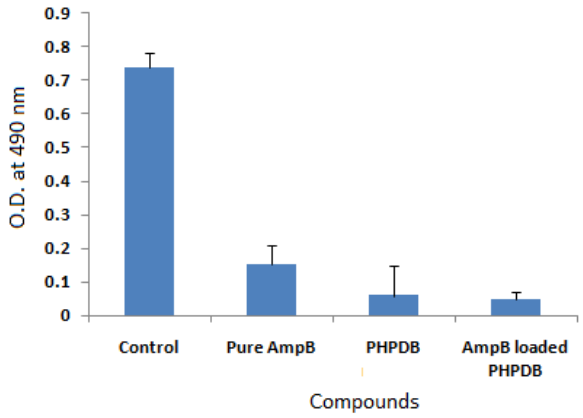

Fig. S7. XTT-assay quantification data are the mean of triplicates

Table S1: Determination of drug entrapment efficiency and loading content in variable amount of drugs.

PHPDB ($mg.mL^{-1}$)	Self-assembled AmB ($\mu g.mL^{-1}$)	Drug entrapment efficiency (%)	Drug loading content (%)
2	100	35.15±3.45	1.75±0.22
2	200	47.33±2.76	4.7±0.54
2	300	51.24±2.11	7.9±1.08

Table S2: Data derived after fitting the raw heat associated data with nonlinear regression.

Parameters	Pure Amphoterecin-B	Self assembled Amphoterecin-B	Self assembled PHPDB
K	1.29± 2.53 E^6 M^{-1}	1.181± 6.2 E^5M^{-1}	1.44 ± 2.65 E^6 M^{-1}
ΔH	-4.348 E^5 cal/mol	- 1.292 E^7 cal/mol	-4.039 E^4 cal/mol
ΔS	-1.253 E^3 cal/mol/deg	-4.341 E^4 cal/mol/deg	-112 cal/mol/deg
R^2	0.97626	0.99728	0.99750

Table S3. IC_{50} determination of synthesized compounds through MTT assay in HEK 293 cell lines. Data are the mean of triplicates (n=3) and bars, represent standard deviation (±SD).

Sl. No	Compound(s) name	IC50 value ($\mu g.mL^{-1}$)
1	Cardanol	15.86±0.31
2	AmphotericinB (AmpB)	30.48±1.21
3	Self-assembled AmpB	42.37±2.12
4	PHPDB	21.15±0.86
5	Self-assembled PHPDB	32.92±1.28
6	Self-assembled AmpB loaded PHPDB	35.42±1.48

References

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