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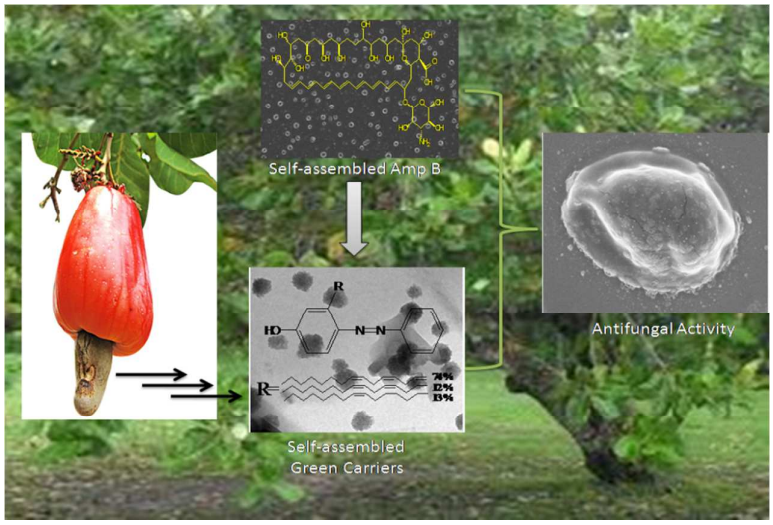
Graphical abstract



Benzenediazonium functionalized cardanol self-assembled loaded with self-assembled AmpB for sustained release. Approach is exerting dual role as self-assembled AmpB binds to ergosterol whereas PHPDB soft green carriers binds to chitin and enhanced the antifungal activity which helps to control fungal infections caused by biofilm forming MDR pathogens.

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Self-assembled Amphoterecin-B Loaded into Self-assembled Cardanol Derivative as Soft Green Carrier for Delivery and Enhanced Antifungal Activity

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Self-assembled strategies are used for soft green carrier(SGC) preparation from cardanol after modification with benzenediazonium functional moieties(PHPDB). A spherical core-shell like self-assembled structure of AmphotericinB(AmpB) loaded into PHPDB and released in aqueous medium. AmpB and PHPDB show ergosterol and chitin binding affinity respectively, sustain enhanced activity with proficient biofilm eradication.

The emergence of MDR pathogens has become a great concern to public health. WHO has recognized multi-drug resistant pathogens as one of the top three threats to human health [1] and appeal for developing innovative approaches to control MDR pathogens. Including numerous approaches, combination therapy or sustained release of antibiotics with appropriate vehicles are successful in application[2-3]. The increased incidences of fungal infections are often serious with an associated mortality rate of 40%.[4-5] The choice of antifungal drugs are limited due to their low number of availability in market in comparison to antibacterial drugs. AmpB is a broad spectrum, lipophilic polyene antifungal drug used for the treatment of systemic fungal diseases. It has selective activity towards fungal cells by binding to ergosterol, the major sterol of fungal cells.[6-7] Continuous application and abuse of AmpB over last 30 years, assist the pathogens to become resistant. In this context, combination therapy of AmpB along with sustained release should be more advantageous to control the high biofilm forming fungal MDR pathogens.[8]

There are systematic barriers to overcome in developing the biologically active compounds into clinically useful agents. Several biologically active compounds never become clinically useful because of their toxicity, low permeability and bioavailability. AmpB is toxic and especially causing renal toxicity.[9-11] It also causes general malaise,[12] anaemia,[13] and phlebitis.[14] Another biologically active molecule is cardanol, a yellow to brown coloured phenolic lipid carrying a C-15 side chain at meta-position known as m-pentadecenylphenol.[15] Cardanol is the major component of technical CNSL of *A. occidentale*, also having cytotoxic activity. The unsaturated lipid chain of cardanol increased the permeability of liposomal bilayer membrane resulting leakage of potassium ions from cell.[16-18] Therefore, optimization of the physiochemical characteristics (charge, lipophilicity, hydrogen bonding, size) or modification of a drug is a general strategy to facilitate the safe application. Self-assembled diazonium cardanol enhance antifungal activity with chitin binding ability, has been reported in our previous paper[19]. Hence, attempts to develop the self-assembled structures of both AmpB and cardanol are interesting to reduce the toxicity. Cardanol was synthesized to PHPDB and OHPDB for the preparation of self-assemble based nanostructure in hydrophobic environment (Fig. 1). The main purpose is to develop the SGC for delivery of AmpB, where PHPDB also have antifungal activity which helps to kill the resistant pathogens using dual activity from drug itself and PHPDB carriers, mostly useful to completely remove the biofilm from infected area.

Cardanol is a bio-based amphiphilic viscous phenolic lipid purified from technical CNSL using reverse phase-HPLC and characterized by FTIR, NMR and MS analysis.[19] Diazonium compound of cardanol were synthesized by electrophilic substitution reaction with aniline in presence of $NaNO_2$ and dil. HCl (Scheme S1).[20] A semi-viscous liquid diazonium compound was developed, washed gently with water, and dried with Na_2SO_4 for overnight. Ortho- coupling (OHPDB) and para-coupling (PHPDB) products (Fig. 1) were separated and purified by column chromatography on silica gel (60–120 mesh) using

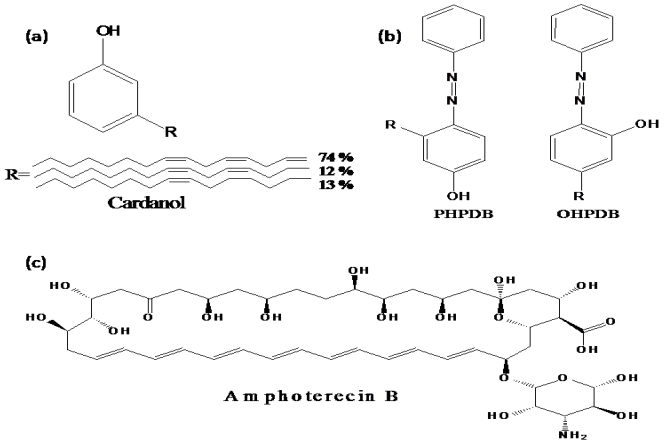

Fig. 1. Chemical structure of compounds. Structure of known cardanol (a) and amphotericin B (c) and proposed diazonium derivatives, PHPDB and OHPDB (b).

petroleum ether and ethyl acetate (5:1) and (10:1), respectively. OHPDB and PHPDB are characterized through UV-vis, FTIR and ^{1}HNMR analysis (Fig. S1 and S5,a). Self-assembled structure of 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 at room temperature with 1000 rpm. After 2 h, organic layer was separated from the mixture and vortexed for overnight. Further, only PHPDB without AmpB was processed as same way for the preparation of self-assembled structure. A colloidal dispersion was prepared and observed under TEM, the images revealed that AmpB adapts a spherical core shell like structure with a diameter 10 nm (Fig. 2a).

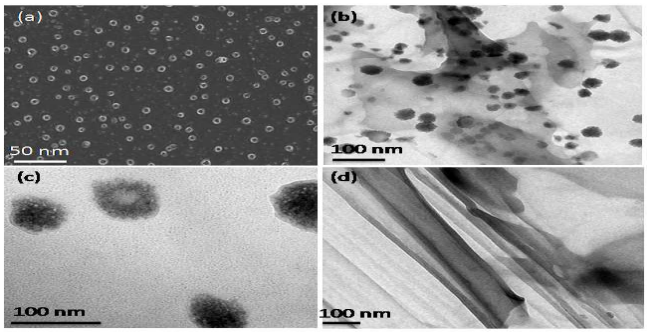

Fig. 2. TEM images of prepared self-assembled compounds. Self-assembled Amphoterecin-B (a), self-assembled PHPDB (b), self-assembled Amphoterecin-B loaded PHPDB (c) and self-assembled OHPDB (d).

Interestingly, PHPDB formed spherical morphology but not OHPDB, as well as AmpB was successfully loaded on the hydrophobic surface of PHPDB (Fig. 2b-2d). The DLS analyses revealed the presence of 7-10 nm self-assembled AmpB, 30-40 nm self-assembled PHPDB nanosphere and 50-70 nm self-assembled AmpB loaded PHPDB complex in solution (Fig. S2). The poly dispersive index and zeta potential values are 0.747 and 13.2±2.14 mV, respectively of the ampB-PHPDB complex. The particle association concentration values were observed 1700 µg.mL^{-1} for self-assembled AmpB-PHPDB complex (Fig. S3). In OHPDB, the C15 hydrophobic side chain lies at the para position of benzenediazonium which aggregates in linear fashion by the influence of hydrophobic interaction and forms sheet like structure (Fig. 2d).

The molecular interactions involved in the AmpB loaded PHPDB, have been investigated using CD and UV-vis and ^{1}HNMR analysis. CD spectra of self-assembled PHPDB show a broad negative band at 200 nm along with a new positive band at 192 nm for aggregation. Self-assembled AmpB not only gives a pronounced negative band at 200 nm but also a sharp positive peak near 194 nm is typical for core shell structure. Whereas, the shift of π-π* transition in 200 to 203 nm region along with a new positive band at 201 nm suggests that formation of PHPDB-AmpB complex (Fig. S4). The absorbance spectrum of AmpB shows three intense bands at 408, 385 and 365 nm with one small band at 330 nm in methanolic solution. Moreover, it also shows a small solder at 222 nm due to π-π* transition of polyene funtionality. However, PHPDB exhibit a strong absorption at 348 nm along with a weak band at 225 nm due to diazonium chromophoric (-N=N-) group and π-π* transition of unsaturated side chain, respectively. The red shift of absorption peak from 225 to 231 nm is indicating the appearance of the self-assembled form of PHPDB (Fig. 3a). Whereas, a strong broad red shift at 242 nm gives good indication for the formation of PHPDB-AmpB complexs which is aggregated by π-π stacking interaction between polyene of AmpB and unsaturated chain of PHPDB. Moreover, ^{1}HNMR analysis revealed that the major change in line width broadening between 1.26 to 1.716 ppm in AmpB loaded PHPDB complex due to their aggregation through hydrophobic interaction (Fig. S5,c).

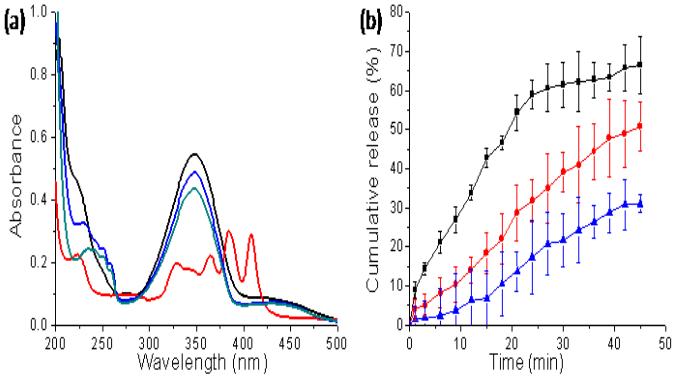

Fig. 3. Drug loading and release behaviour study. UV-visible spectrum of pure PHPDB (blue color), pure AmpB (black color), self-assembled PHPDB (green color), self-assembled Amp-B loaded PHPDB (red color) (a). Drug release kinetics represent in aqueous phase at pH 3.0 (blue color); pH 5.5 (red color) and pH 7.0 (black color). Release kinetics were performed each 3 minutes interval (b). Data are the mean of triplicates ± S.E.

The loading and release of AmpB from the PHPDB-AmpB complex was investigated by solvent diffusion method[20] (Fig. 3). The percentage of drug entrapment efficiency increased with increasing the drug concentration and optimized loading efficiency was observed at 200 µg. mL^{-1} (Table S1). AmpB was extracted in aqueous phase from complex at pH 3 in room temperature. The amount of released AmpB was analyzed

spectrophotometrically at 384 nm with time (min) and maximum release observed at 30 min onwards (Fig. 3b).

In general, AmpB is highly soluble in water at pH 2-3[21] and therefore selected pH 3.0 to 5.5 was best for sustained release without disturbing the SGC which remains in solvent phase. All the synthesized compounds with their self-assembled structures were tested for antifungal activity against *Candida albicans* SJ11 (hospital isolate) and *Candida tropicalis* NCIM 3110.[22] It was observed that AmpB loaded PHPDB complex having highest antifungal activity in comparison to AmpB itself (Table 1). The result suggests that PHPDB is a good candidate for antifungal agent itself, as well as in combination with AmpB. AmpB directly binds to ergosterol in cellular lipid bilayer membranes, the major sterol of fungal cells and forming a transmembrane channel helps to leak out the monovalent cations causing fungal death.

Table 1. Minimum inhibitory concentration ($\mu g.mL^{-1}$) of all synthesized compounds against *C. albicans.*

Compounds	*C. albicans* SJ11	*C. tropicalis* NCIM3110
Cardanol	62.5	31.2
PHPDB	62.5	31.2
AmpB	31.2	1.9
Self-assembled PHPDB	31.2	15.6
Self-assembled AmpB	31.2	1.9
Self-assembled PHPDB with Self-assembled AmpB complex	7.8	1.0

The ITC experiment was performed to evaluate the ergosterol binding activity of both AmpB and self-assembled AmpB. The binding isotherm with ergosterol revealed that affinity was more with pure AmpB than self-assembled form due to the aggregation of free molecule by reducing the binding site.

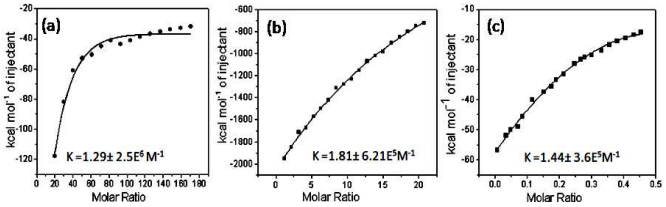

Fig. 4. ITC based binding isotherm plot of Amphoterecin-B with Ergosterol and PHPDB with chitin. Binding isotherm obtained from interaction between ergosterol with pure AmpB (a) and with self-assembled AmpB (b); chitin with self-assembled PHPDB (c).

In aqueous medium at pH 3, the self-assembled AmpB become unwinds slowly, increases the solubility and the activity of AmpB is prolonged. Simultaneously, self-assembled PHPDB was evaluated for ergosterol binding ability and no significant change have been observed in binding thermogram or isotherm was observed (data not shown), whereas affinity was prominent with chitin molecule, the cell wall composition of C. albicans, ergosterol lipid and chitin makes a rigid layer surrounding the fungal membrane. The binding isotherm and thermogram for ergosterol with AmpB and chitin with PHPDB are shown in Fig. 4 and S6, respectively. The binding constant (K), entropy (ΔS), and enthalpy (ΔH) of their reactions (Table S2) clearly indicates that the interactions of pure and self-assembled AmpB with ergosterol are based on enthalpically derived because the values of ΔS and ΔH are negative (where $\Delta H > \Delta S$). Moreover, interactions parameters of PHPDB with chitin also showed the same trend, suggesting that all experimental results are in good agreement for the exothermic nature. Therefore, it is obvious from overall interaction that AmpB loaded PHPDB binds exothermically with cell wall membrane components.

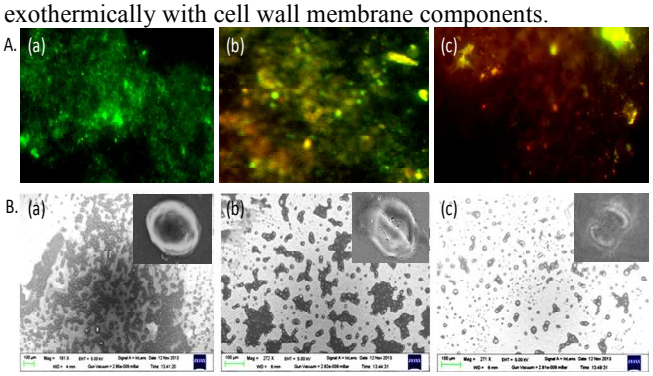

Fig. 5. Images of biofilm eradication ability of compounds in *C. albicans* SJ11. The stain was used BacLight Live/Dead dye under fluorescence microscope where green and red fluorescence indicating live and dead cells, respectively. Images were captured from 48h old biofilm grown over cover slip (A.a); biofilm containing cove slip treated with AmpB (A.b) and biofilm containing cove slip treated with AmpB loaded PHPDB (A.c). The cover slip was treated with drugs in RPMI medium for 2h, gently washed and again grown in fresh RPMI medium for 24 h. AmpB loaded PHPDB showed better activity than only pure AmpB which significantly reduced live cells and most of the cells were dead against C. albicans SJ11. SEM images of old biofilm containing cover slip after treatment with only PBS buffer (B.a); treated with AmpB (B.b) and treated with AmpB loaded PHPDB (B.c). Pictures are inset representing the SEM images of individual cells before and after treatment, revealed drastic damage occurred after treatment with AmpB loaded PHPDB complex. Biofilms were treated for 2h and drugs were prepared in PBS (1X) buffer, SEM images were captured immediately after treatment

The microtiter plate assay[22] was used to evaluate the feasibility of AmpB loaded PHPDB complex over old biofilm formed by *C. albicans* SJ11. Figure 5 shows the inhibition of fungal growth in biofilm were about 81% and 99% for pure AmpB and AmpB loaded PHPDB complex, respectively. The result suggests that PHPDB in combination with AmpB effectively kill the bacteria in established biofilms by drastically reducing the metabolic activity (Fig. S7). Thus, both vehicle and drug are representing the same activity with different action which could be used in future for the treatment of old fungal infections where biofilm is also a major concern. Haemolytic

assay[23] was also performed to examine the compatibility of the compounds with red blood cells and their efficacy in biological systems. The results show that upto 1 h incubation of compounds with RBC, the self-assembled complexes are slightly less toxic than original pure compounds (Fig. 6). MTT assay was also performed against human embryonic kidney 293 cells to check the toxicity. It was observed that cardanol having highest toxicity followed by PHPDB among them and toxicity was reduced for self-assembled structures in comparison to pure compounds (Table S3). During self-assembled structure formation, all the functional moieties interact with each other and decreasing the surface reacting group helps to reduce the toxicity.

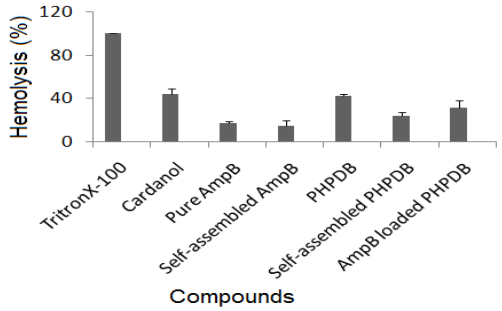

Fig. 6. Hemolytic assay of all synthesized compounds. All the compounds were used at a fixed concentration of 16 µg. mL-1 for 60 min at 37°C. The treatment with triton X-100 (1.0%) was considered as 100% of haemolysis. Data are the mean of triplicates ± S. D.

Conclusions

The system is exerting dual role as AmpB strongly binds to ergosterol as well as PHPDB binds to chitin and enhanced the antifungal activity. Thus, the strategy could be used for the prevention and treatment of biofilm-related old fungal infections caused by MDR pathogens. the successes of self-assembly based complexes preparation using biologically active natural molecules might have an attractive prospect in drug delivery**.**

Notes and references

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Electronic Supplementary Information (ESI) available: Experimental protocols, characterization of compounds, supporting figures, and tables are available in supplementary section.

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ABBREVIATIONS

AmpB, Amphotericin B; CNSL, cashew nut shell liquid; CD, circular dichroism; DLS, Dynamic light scattering measurement; FTIR, fourier transform infra red spectrophotometer; HPLC, high performance liquid chromatography; ITC, isothermal titration calorimetry; MDR, multi-drug resistance; SGC, soft green carriers; NMR, nuclear magnetic resonance spectrometer; OHPDB, 4- [(2'-Hydroxy-2-pentadecenylphenyl)diazenyl] benzene; PHPDB, 4- [(4'-hydroxy-2-pentadecenylphenyl) diazenyl] benzene; RBC, red blood cell; TEM, transmission electron microscope; UV-vis, ultraviolet-visible spectroscopy; WHO, World Health Organization.