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RESEARCH ARTICLE

The Use of Nano Polymeric Self-Assemblies Based on Novel Amphiphilic Polymers for Oral Hydrophobic Drug Delivery

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ABSTRACT

Purpose To investigate the use of nano self-assemblies formed by polyallylamine (PAA) modified with 5 or 10% mole fluorenylmethoxy carbonyl ($Fmoc_5/_{10}$), dimethylamino-1-naphthalenesulfonyl ($Dansyl_5/_{10}$) and 5% mole cholesteryl group (Ch_5) for oral hydrophobic drug delivery.

Methods Propofol, griseofulvin and prednisolone were loaded into amphiphilic PAAs. Particle size and morphology of drug-loaded self-assemblies were determined using photon correlation spectroscopy and transmission electron microscopy. Solubilising capacity, *in vitro* drug release and formulation stability were analysed by HPLC, and *in vitro* biocompatibility studies (haemolysis and cytotoxicity) were carried out on bovine erythrocytes and Caco-2 cells, respectively. $Dansyl_{10}$ and Ch_5 griseofulvin formulations were administered intra-gastrically to rats, and drug plasma levels were analysed by HPLC.

Results Drug-encapsulated self-assemblies typically have hydrodynamic size of 300–400 nm. $Dansyl_{10}$ exhibited universal drug solubiliser property and had significantly improved prednisolone, griseofulvin and propofol solubility by 145, 557 and 224-fold, respectively. Fmoc polymers resulted in modest drug solubility improvement. These polymers were non-haemolytic, did not enhance cytotoxicity compared to unmodified PAA, and demonstrated significant increase in griseofulvin plasma concentration compared to griseofulvin in water after oral administration.

Conclusions Ch_5 and $Dansyl_{10}$ showed promising potential as nano-carriers for oral hydrophobic drug delivery.

KEY WORDS amphiphilic polymer · hydrophobic drug · nano polymeric self-assemblies · oral delivery · solubiliser

INTRODUCTION

Polymeric self-assemblies have been widely studied for their potential as hydrophobic drug solubilizing agents since they were first reported in 1984 (1). They are commonly formed from amphiphilic polymers where these polymers consist of hydrophilic and hydrophobic segments within the same macromolecules. In aqueous environment, polymeric self-assemblies with core–shell structures are formed upon the aggregation of hydrophobic moieties. The most common type of self-assemblies are spherical polymeric micelles (2) with other less common assemblies such as nanoparticles (3), disc-like structures (4), filamentous structures (5) or vesicles(6) have also been reported. Hydrophobic drugs can physically be encapsulated inside the lipophilic core of these self-assemblies mainly attributed to hydrophobic interaction (6,7). Today, polymeric self-assemblies are widely developed for intravenous administration in particular for cancer therapy (2,7) but their use in other routes of administration such as oral delivery is much less reported (7,8). Recently a few research groups have investigated the use of polymeric micelles in the oral delivery of hydrophobic drugs such as risperidone (9), cyclosporine (10), paclitaxel (11), lodamin (12), griseofulvin (13) and doxorubicin (2). It is thought that apart from the solubilization effect and depending on the type of the amphiphilic polymers, they exhibited other unique properties such as mucoadhesive properties (14), protection against enzymatic degradation (15), inhibition of P-glycoprotein pump (16) or enhancement of

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cellular uptake by CaCo2 cells (17), which showed great potential in oral delivery.

The most common amphiphilic polymer architecture investigated in oral delivery is block copolymers (9), however recently diverse structures such as hydrophobically modified polymers (10,15,18) and dendrimers (19) have also been reported. The hydrophobic pendant groups in hydrophobically modified polymers are traditionally composed of hydrocarbon chains of different lengths such as alkyl, acyl (20) or sterol-like moieties (21). Unlike block copolymer, investigation into the effect of these hydrophobic pendant groups on drug solubilization is seldom investigated. Based on the observations reported for block copolymers, it has been well established that apart from the drug physicochemical properties, the degree of compatibility or interaction between the hydrophobic core-forming polymer and the drug can influence the colloidal stability, encapsulation efficiency and drug release kinetics (2). Rekatas and colleagues reported that block copolymers consisting of polystyrene oxide as the core-forming polymer were able to encapsulate a higher level of drugs with aromatic rings than aliphatic hydrophobic polymers (22).

However, despite most hydrophobic drugs consist of aromatic or cyclic ring systems, to our best knowledge, the attachment of aromatic groups to a pre-formed water soluble polymer backbone, where the aromatic groups serve as the only hydrophobic moiety have not yet been explored for oral hydrophobic drug delivery. Here we investigate the ability of novel poly(allylamine) (PAA) modified with different types and levels of aromatic pendant groups (Fluorenylmethoxy carbonyl (fmoc) and dimethylamino-1-naphthalenesulfonyl (dansyl) on the enhancement of hydrophobic drug solubility and oral absorption (Fig. 1). They will be compared to cholesteryl grafted PAA (Ch), that was recently demonstrated as a potential cancer therapy for parenteral delivery (23) (Fig. 1). Cross-linked PAA has been used clinically as an oral phosphate binder (24) while thiolated PAAs had been investigated as intestinal permeation enhancer (25) but amphiphilic PAA for drug delivery application is seldom reported. Three hydrophobic drugs containing aromatic or cyclic ring structures, propofol (Mw = 178 $gmol^{-1}$, logP = 4.16), prednisolone (Mw = 360 $gmol^{-1}$, logP = 1.8) and griseofulvin (Mw = 353 $gmol^{-1}$, logP = 2.2) will be used as model drugs (Fig. 1). Their physicochemical properties, *in vitro* drug release, formulation stability and *in vitro* biocompatibility will be elucidated and finally their potential in oral delivery of griseofulvin will be investigated *in vivo*.

MATERIALS AND METHODS

15 kDa poly(allylamine) hydrochloride (PAA), propofol, prednisolone, griseofulvin, etoposide, orthophosphoric acid,

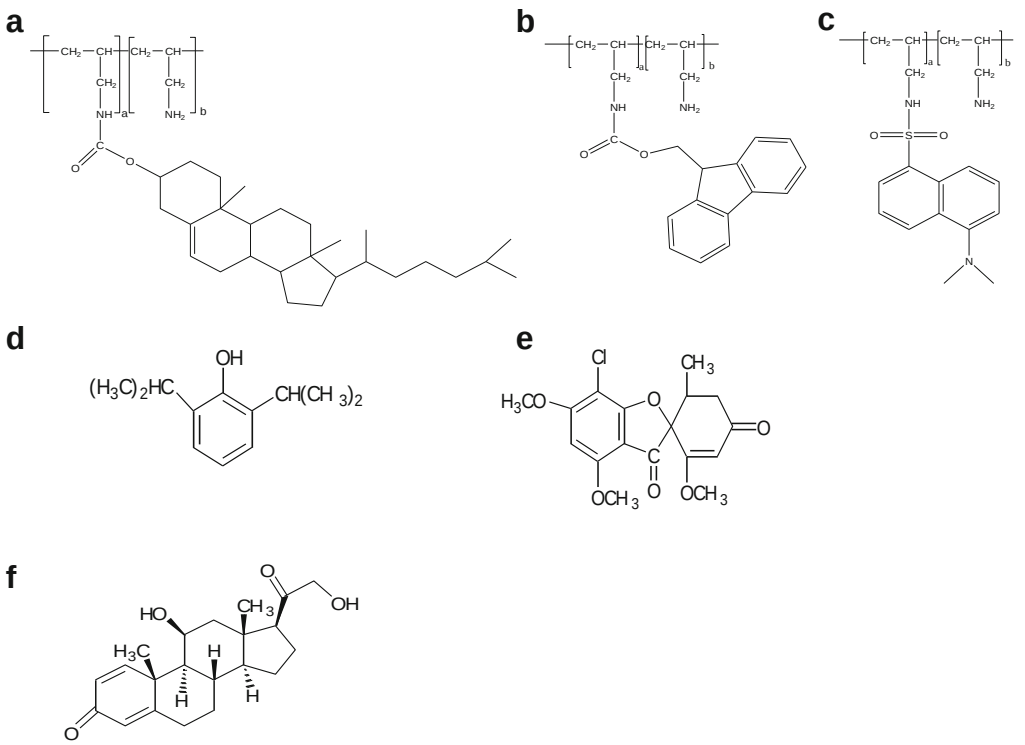

Fig. 1 Chemical structure of (**a**) cholesteryl-PAA; (**b**) Fmoc-PAA; (**c**) Dansyl- PAA; (**d**) propofol; (**e**) griseofulvin; (**f**) prednisolone.

potassium dihydrogen phosphate, octane sulfonic acid, anhydrous sodium acetate, Minimal Essential Media (MEM), Dulbecco's minimal essential media (DMEM), L-Glutamine, Non essential amino acids, Glycerol, Triton-X, 3-(4,5-dimethyl thiazol-2-yl)-2,5diphenyl tetrazolium bromide (MTT) and L-Glycine were purchased from Sigma-Aldrich Co. (UK). HPLC grade solvents, phosphate buffered saline (PBS), Foetal Bovine Serum (FBS), Trypsin EDTA and penicillin streptomycin were purchased from Fisher Scientific (UK). 0.45 μm GDX PVDF syringe filters were from Whatman (UK).

Polymer Synthesis and Characterisation

PAA was reacted with cholesteryl chloroformate, 9 fluorenylmethoxy carbonyl chloride (fmoc-chloride) and 5-Dimethylamino-1-naphthalenesulfonyl chloride (dansyl chloride) based on molar feeds of 20:1 and 10:1 (PAA monomer: hydrophobic group) to yield PAA modified with cholesteryl, fmoc and dansyl pendant groups (Ch, Fmoc and Dansyl, respectively). The novel amphiphilic polymers were characterised by elemental analysis and ^{1}H NMR and the results confirmed 4.7, 4.3 and 7.1% mole modification for Ch_5, $Fmoc_5$ and $Dansyl_5$ respectively and 9.3% for both $Fmoc_{10}$ and $Dansyl_{10}$ (18).The numerals of the polymer abbreviation indicate the% expected mole modification based on the initial molar feeds. 10% mole modification of Ch resulted in an insoluble product and hence no further work was pursued with this polymer.

Drug Loading

Polymer in deionised water (1, 3 and 6 $mgmL^{-1}$) was probe sonicated for 10 min. The hydrophobic drug was added at 1:1, 5:1 and 10:1 initial drug: polymer weight ratios and the drug-polymer solutions were probe sonicated for a further 10 min. All drugs were added in powder form except for propofol which was an oily viscous liquid. After cooling to room temperature, the solutions were filtered using 0.45 μm syringe filters (with pre-filters) to remove any excess drugs.

Quantification of Propofol

Propofol in the self-assemblies was determined using high performance liquid chromatography (HPLC) (Shimadzu prominence UFLC, UK), as previously reported by Qu and colleagues (26). A RP Zorbax ODS 250 mm × 46 mm × 5 μm HPLC column (Hichrom, UK) was used with the flow rate of 1 $mLmin^{-1}$ (80:20 v/v methanol:water) in an isocratic mode. The samples were diluted with mobile phase and 20 μL was injected onto the column. The resultant peak at 7 min was analysed at 229 nm (Shimadzu prominence UFLC, UK). Propofol in the samples were determined using a calibration graph constructed from propofol standards dissolved in methanol (4 μgmL^{-1}–250 μgmL^{-1}), $R^2 = 0.999$.

Quantification of Prednisolone

HPLC consisted of a RP Phenomenex C_{18} 150 mm × 4.6 mm × 3.5 μm column with the mobile phase (36:64 (v/v) acetonitrile:water) and a flow rate of 1 $mLmin^{-1}$. The prednisolone peak eluted at 3 min and detected at λ_{max} 243 nm. Standards were prepared in the mobile phase (6 μgmL^{-1}–25 μgmL^{-1}) and a calibration was constructed, ($R^2 = 0.999$) to determine the concentration of prednisolone in the formulation.

Quantification of Griseofulvin

This method was an adaptation of Trimaille's method (27). In brief the samples were passed through a RP Phenomenex C_{18} 250 mm × 46 mm × 5 μm HPLC column and the peak (9.5 min) was detected at λmax 293 nm . The mobile phase (45:55 v/v) acetonitrile:45 mM potassium dihydrogen phosphate buffer (adjusted to pH 3 with orthophosphoric acid) was at 1 $mLmin^{-1}$ and 20 μL of sample diluted with the mobile phase was injected onto the column. The concentration of griseofulvin in the samples was determined from a calibration graph of griseofulvin standards (0.6 μgmL^{-1}–10 μgmL^{-1}), $R^2 = 0.999$.

For all formulations, the% drug loading capacity (LC) and% drug encapsulation efficiency (EE) was calculated based on the equations below:

$$\% \text{ LC} = \text{drug determined by HPLC} / \text{polymer concentration} \times 100\% \quad (1)$$

$$\% \text{ EE} = \text{drug determined by HPLC} / \text{original drug concentration} \times 100\% \quad (2)$$

Sizing of Nano-aggregates

Hydrodynamic sizes of the drug formulations (in deionised water) were determined using a photon correlation spectroscopy (PCS) (Zetasizer Nano-ZS, Malvern Instruments, UK). All measurements were conducted in triplicate at 25°C and an average value was determined.

Transmission Electron Microscopy (TEM)

Formvar/carbon-coated 200 mesh nickel grids were glow discharged and one drop of the formulations prepared as described above, was dried onto the hydrophilic support film. 1% aqueous methylamine vanadate (20 μL) (Nanovan;

Nanoprobes, Stony Brook, NY, USA) stain solution was applied and the mixture dried down immediately with filter paper to remove excess liquid. The dried samples were imaged with a LEO 912 energy filtering transmission electron microscope at 120 kV. Contrast enhanced, zero-loss energy filtered digital images were recorded with a 14-bit/2 K Proscan CCD camera.

In Vitro Drug Release

The method used was an adaptation of Lee and colleagues (28). The optimum Ch_5 and $Dansyl_{10}$ formulations with the initial polymer: drug weight loading of 1:10,and polymer concentration at 6 $mgmL^{-1}$ were prepared as described previously. The formulation (2 mL) was pipetted in a dialysis tubing (MW cut-off = 12–14 kDa) and dialysed against PBS in sink condition (200 mL, 0.2 M) at 37°C with stirring. At various time points 1 mL of PBS was extracted and replaced with 1 mL of fresh PBS. The amount of drug in the collected PBS was determined using HPLC as described above.

Stability Testing of Formulations

The formulations were prepared as previously described in either solution or freeze dried forms and were stored in air tight desiccators (55% humidity) at room temperature and in the dark. At specific time points, the drug content in the filtered, freeze-dried and reconstituted formulations as well as the formulations in solutions were analysed using HPLC as described above.

Biological Characterisation

Haemolysis Assay

Fresh bovine blood (approximately 50 mL) was washed with copious amount of phosphate buffered saline (PBS buffer) (0.1 M) and centrifuged (2500 rpm) for 10 min at 4°C. The supernatant was discarded. This process was repeated until the supernatant was clear. The red blood cell (RBC) was weighed and fresh PBS was added to achieve 3% (w/v). The red blood cell suspended in PBS (80 μL) was then pipetted into a 96-well round bottom plate. 10 $mgmL^{-1}$ polymer stock solution was prepared in water adjusted to pH7.4. A range of polymer concentrations (0.05–1 $mgmL^{-1}$) were prepared from the polymer stock solution using PBS as the diluents and added (80 μL) to RBC. The plates (160 μL/well) were incubated at 37°C for 4 h before centrifuged at 2500 rpm for 10 min at 4°C. The supernatant (100 μL) was transferred to a flat bottomed 96-well plate and the absorbance was read at 570 nm (microplate reader, Ascend Lab-Systems, UK). PBS and Triton X (80 μL each) were used as the negative and positive controls respectively. The results expressed as percentage haemolysis assuming Triton X gave 100% haemolysis and PBS gave 0% haemolysis. The RBC pellets were viewed under the light microscope (Leica DM3000B, Leica UK) and images were captured.

Cytotoxicity Assay

Caco-2 cells (EDACC, passage number 10) were cultured in minimum essential medium (MEM) containing 10% foetal bovine serum (FBS), 1% L- glutamine and 1% non essential amino acids (NEAA). A range of polymer concentrations in media ($0.2–1\times10^{-4}$ $mgmL^{-1}$) were prepared from stock solution (0.5 $mgmL^{-1}$ in 1:20 water: media). Caco-2 cells (200 μL, 10000 cells/well) in exponential growth were seeded in a 96-well plate and incubated for 24 h at 37°C with 5% CO_2. The media was then removed via aspiration and replaced with the aforementioned polymer solutions (200 μL). After 24 h, the polymer solutions were removed and replaced with fresh media and incubated for a further 24 h. The media was then replaced with fresh media and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (50 μL, 5 $mgmL^{-1}$) was added to the wells and incubated in the dark for 4 h. MTT solution was removed and the purple formazan complexes formed, were dissolved in DMSO (200 μL) and L-glycine buffer (20 μL) (3.75 g glycine and 2.93 g NaCl in 500 mL water and adjusted to pH 10.5).The absorbance was read at 570 nm using a microplate reader (Ascend Lab-Systems, UK) and the percentage cell viability was calculated relative to the positive (Triton X, 1:5 v/v PBS) and negative (media) controls.

In Vivo Oral Absorption Study

Formulation Preparation

Ch_5 and $Dansyl_{10}$ (6 $mgmL^{-1}$), griseofulvin formulations were prepared as described previously using polymer: drug weight ratio of 1:10. Based on HPLC quantification, Dansyl formulation was further diluted with water (1:5) to achieve similar final griseofulvin concentration as Ch_5 (1.2 $mgmL^{-1}$). Griseofulvin (1.2 $mgmL^{-1}$) in distilled water was prepared in a similar manner as described above in the absence of polymer. Polymer solutions were used as controls.

Intragastric Administration and Evaluation of Griseofulvin Absorption

Eighteen male Sprague Dawley rats (280 g, Charles River, UK) were randomly distributed in 5 groups ($n=4$ or $n=3$

for controls) and fasted over night (18 h) with free access to water at all times. The rats were orally dosed with a griseofulvin suspension in water and polymer, griseofulvin formulations prepared above (11.8 $mgKg^{-1}$) via oral gavage (2 mL). Blood samples (approximately 100 μL) were collected using 300 μL microvettes (Microvette®CB300, Vet Tech Solutions, UK) at various time points via tail vein venesection. After the first time point (1 h) food was given to the rats. Blood samples were centrifuged at 2000 rpm for 10 min and the plasma was frozen for further analysis. Griseofulvin was extracted from the plasma by diluting 100 μL plasma with 250 μL acetonitrile. The mixture was vortexed for 30 s and then centrifuged at 3000 rpm for 10 min. The supernatant (50 μL) was injected into a HPLC system consisting of a RP Zorbax ODS column 250 mm× 46 mm×5 μm (Hichrom, UK) with the mobile phase flowing at 2 $mLmin^{-1}$ (50:50 v/v acetonitrile:water). The resultant peak at 3 min was analysed at 260 nm (excitation) and 389 nm (emission) using a fluorescent detector (Varian LC, Varian UK). Griseofulvin present in the samples was determined from a standard calibration curve carried out previously with griseofulvin spiked blank plasma samples (1.9 μgmL^{-1}–10 μgmL^{-1}), $R^2=0.992$. The statistical significance of the results was assessed using two-way analysis on variance ANOVA and Dunnett multiple comparison *t*-test *via* SPSS 13.0 for Windows.

RESULTS

Drug-Loaded Polymeric Self-Assemblies

Ch_5 and $Dansyl_{10}$ formulations were able to improve the solubility of 3 hydrophobic drugs and the level and type of hydrophobic pendant groups had significant impact on maximum drug solubilization (Fig. 2). 10% mole modification improved drug aqueous solubility compared to 5% mole counterparts, which is consistent with the trend reported by others (29). Fmoc pendant groups were less effective in solubilizing the drugs compared to Ch_5 and $Dansyl_{10}$ which exhibited the highest drug encapsulation (Fig. 2). With Ch_5 and $Dansyl_{10}$, increasing polymer concentrations from 1 $mgmL^{-1}$ to 6 $mgmL^{-1}$ increased drug encapsulation regardless of the drug. In contrast no consistent trend was observed with Fmoc and $Dansyl_5$ polymers (data not shown). Optimum solubilization was achieved with Ch_5 and $Dansyl_{10}$ polymers at 6 $mgmL^{-1}$ concentration and polymer weight ratios of 10:1 (Fig. 2). $Dansyl_{10}$ exhibited the highest improvement in drug aqueous solubility demonstrating 145-fold for prednisolone, 224-fold for propofol and 557-fold for griseofulvin respectively (Table I). Unlike most of the reported self-assembled polymers which often demonstrated low drug loading (LC), typically between 5% to less than 20% (13,30), these PAA

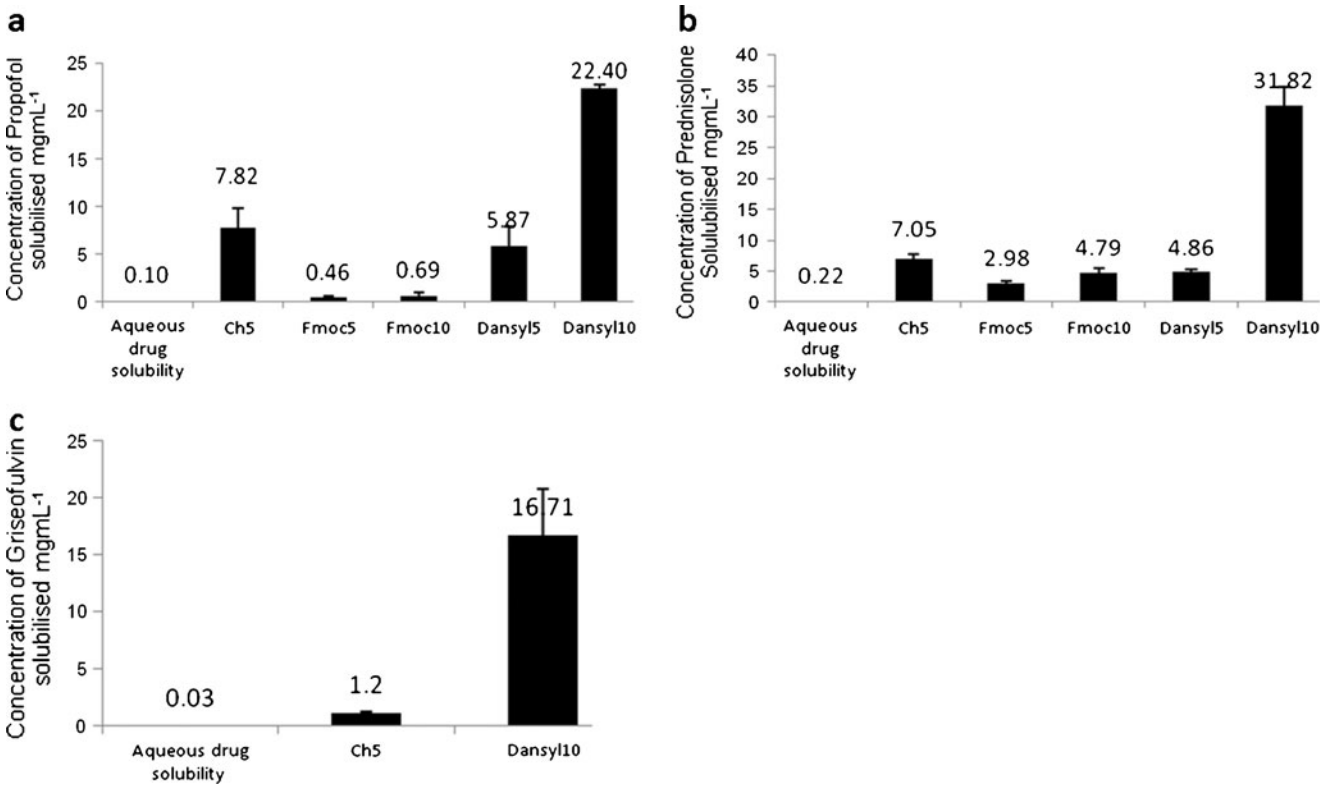

Fig. 2 Maximum drug concentration solubilised by PAA amphiphilies: (**a**) propofol, (**b**) prednisolone and (**c**) griseoufulvin.

Table I Properties of PAA Amphiphiles and Their Drug-Loaded Polymeric Self-Assemblies with Highest Drug Encapsulation

[a]Polymer	$CaCo_2$ IC_{50} (μgmL^{-1})	%LC			%EE			Increment from drug aqueous solubility (−fold)			Size (nm)			PDI					
		P	Pred	Gri	P	Pred	Gri	P	Pred	Gri	unloaded	P	Pred	Gri	unloaded	P	Pred	Gri		
Ch5	37.4 (3.7)	130	117	20	13	12	2	78	32	40	183 (2)	666 (79)	304 (4)	284 (6)	0.167 (0.101)	0.285 (0.004)	0.318 (0.014)	0.209 (0.012)		
Fmoc5	22.9 (3.61)	8	99.2	–	8	20	–	5	14	–	199 (2)	310 (21)	359 (5)	–	0.140 (0.053)	0.125 (0.010)	0.144 (0.001)	–		
Fmoc10	27.9 (9.62)	12	80	–	12	8	–	7	22	–	128 (3)	325 (15)	379 (2)	–	0.163 (0.015)	0.321 (0.110)	0.150 (0.050)	–		
Dansyl5	17.4 (3.5)	98	162	–	20	16	–	59	22	–	120 (6)	402 (12)	431 (21)	–	0.285 (0.025)	0.111 (0.004)	0.125 (0.004)	–		
Dansyl10	24.6 (0.9)	373	530	279	37	53	28	224	145	557	99 (29)	608 (49)	350 (4)	305 (7)	0.367 (0.153)	0.385 (0.009)	0.369 (0.007)	0.254 (0.012)		

Data are presented as mean ± (s.d.), n = 3. [a] The numerals of the polymer abbreviation indicate the%expected mole modification based on initial molar feeds. P = propofol; Pred = prednisolone; Gri = griseofulvin

based amphiphilic polymers have substantial higher LC especially with $Dansyl_{10}$ demonstrating up to 530% LC (Table I). $Dansyl_{10}$ exhibited the highest EE among the polymers ranging from 28% to 53%.

As a whole, the size of drug loaded polymeric self-assemblies increased compared to the unloaded self-assemblies (Table I), and is in agreement with previous reports (30). For Ch_5 and $Dansyl_{10}$, encapsulation of propofol resulted in a significant increase in size (~600 nm) while the rest of the formulations typically have hydrodynamic size of 300–400 nm. An increase in polydispersity index (PDI) is observed in drug loaded Ch_5 after drug encapsulation while this was not evident in $Dansyl_{10}$. It is possible that $Dansyl_{10}$ are more efficient solubilizers than Ch_5 and thus the drug loaded particles are less heterogeneous. TEM images showed that all drug loaded nanoparticles were spherical in shape, however they were smaller than those obtained from PCS measurement (Fig. 3b1-3). This could be due to the fact that PCS measures the hydrodynamic radius of a particle that is generally slightly larger than the actual geometrical radius of a sphere due to solvation of the particle.

In Vitro Drug Release

The *in vitro* drug release of the three drugs from the two best amphiphilic polymers, Ch_5 and $Dansyl_{10}$ were assessed in PBS under sink condition (Fig. 4). Apart from griseofulvin, generally Ch_5 resulted in rapid release where most drugs (between 50 and 60%) had been released in the first 7 h while the release of drugs from $Dansyl_{10}$ formulations were slower with only approximately 20% of the drugs being released after 7 h. The release profile of $Dansyl_{10}$ formulations seems to be independent of the encapsulated drug. It is possible that good compatibility between $Dansyl_{10}$ and the three drugs resulted in a slower release of drug from the self-assemblies, which corresponds well with the high drug loading capacity (31). For most formulations, 100% drug release was achieved between 3 and 4 days (data not shown).

Formulation Stability

Figure 5 shows the amount of drug lost analysed by HPLC over a four-week period. It was found that the freeze-dried propofol formulations following reconstitution did not contain any drug at week 0 indicating the lost of drug in the freeze-drying process, perhaps due to the volatile nature of this drug. Therefore the stability of propofol formulations was subsequently determined using liquid formulations. Over a four-week period, Ch_5, propofol liquid formulations experienced gradual drug lost from 0 to 30% while $Dansyl_{10}$, propofol liquid formulation was able

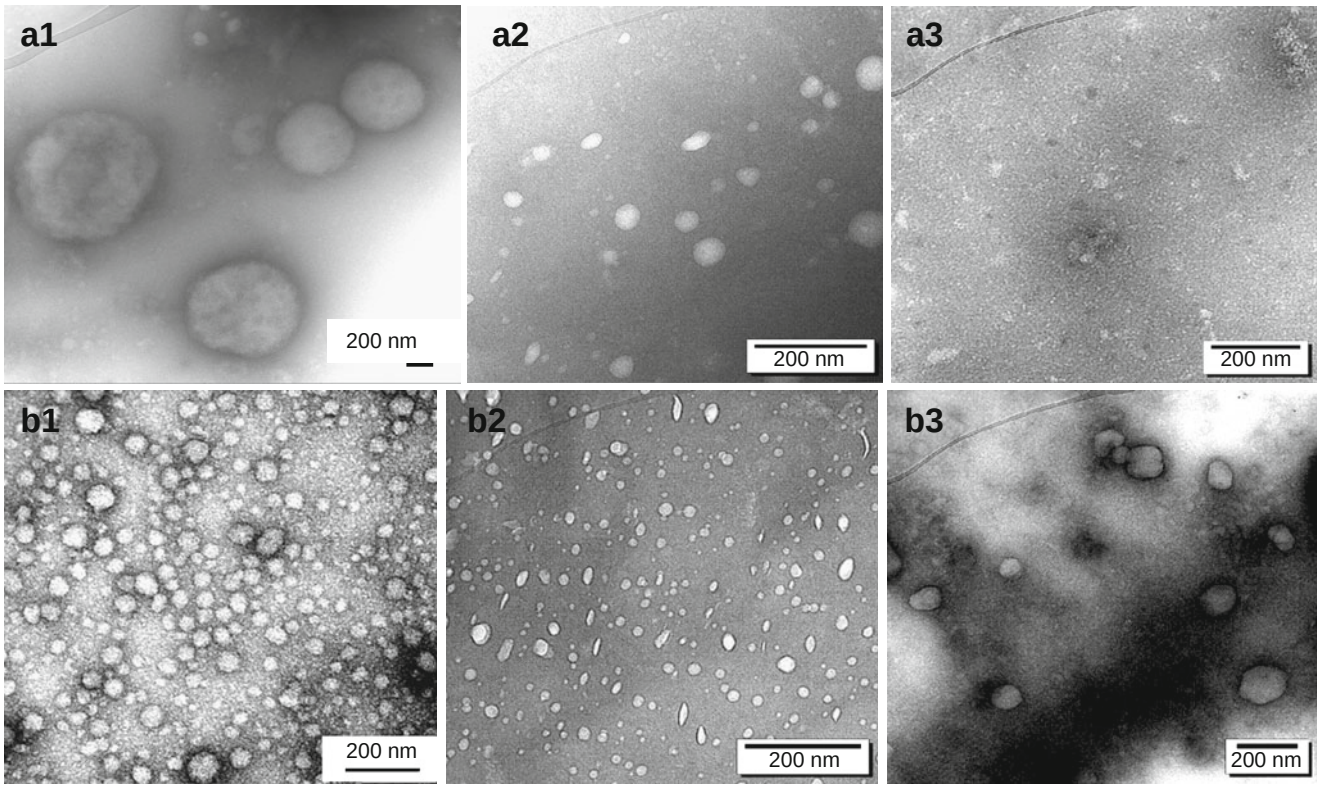

Fig. 3 Negative-stained TEM of (**a**) Ch_5 formulations with 1) propofol, 2) prednisolone and 3) griseofulvin. (**b**) $Dansyl_{10}$, 1) propofol, 2) prednisolone and 3) griseofulvin. All the formulations consisted of 6 $mgmL^{-1}$ polymer and 10:1 initial drug: polymer mass ratio. Bar = 200 nm.

to retain up to 85% of the drug at the end of the 4 week period. This result is consistent with the hydrodynamic size data. The size of propofol encapsulated Ch_5 self-assemblies reduced from 666 nm to 239 nm at the end of the study indicating drug lost while the $Dansyl_{10}$, propofol formulation retained the same size at 677 nm as in week 0 (Table I). The initial drug lost (10–15%) at week 0 from both Ch_5 and $Dansyl_{10}$ freeze-dried prednisolone and griseofulvin formulations was perhaps due to the freeze drying process (Fig. 5). Interestingly, Ch_5 freeze-dried formulations were more stable than $Dansyl_{10}$ formulations as no further notable loss was apparent over the 4 week

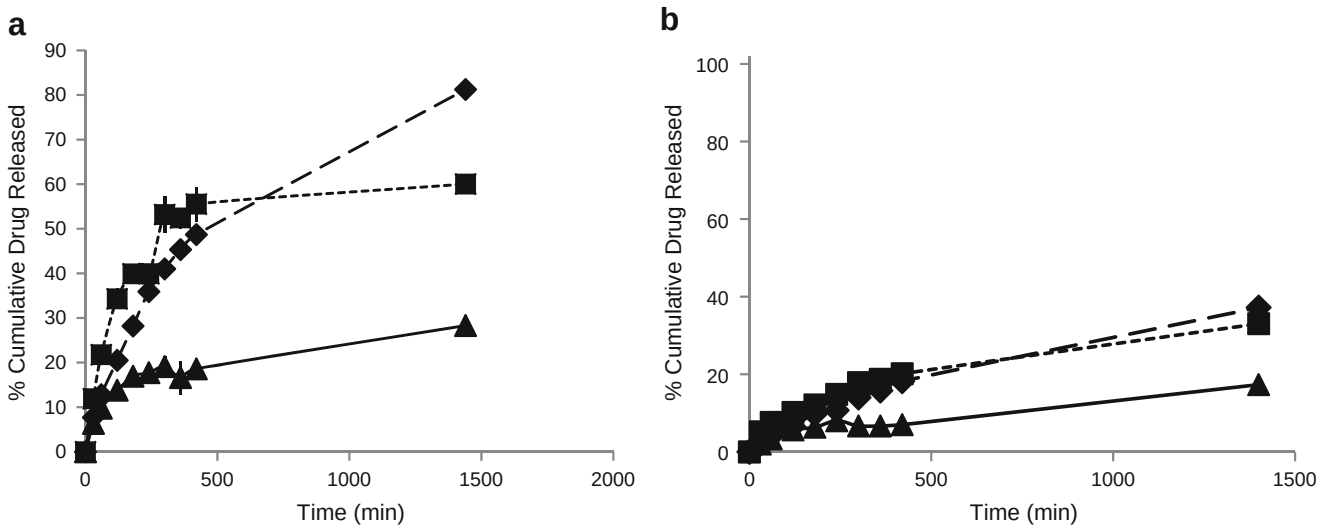

Fig. 4 *In vitro* drug release of hydrophobic drugs from (**a**) Ch5 and (**b**) Dansyl10 formulations carried out in sink conditions. ♦ propofol; ■ prednisolone ▲ griseofulvin. Data presented as $n = 3$, ave ± s.d.

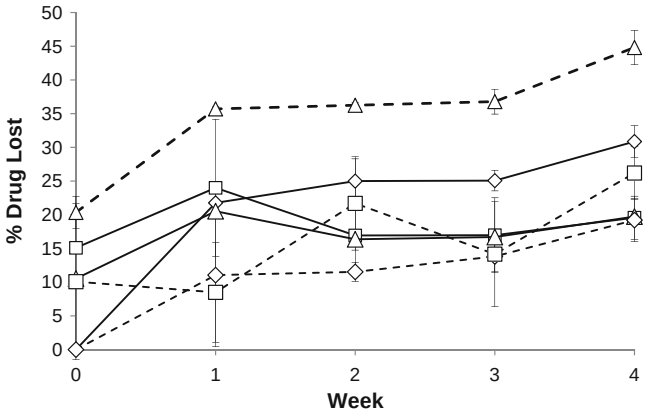

Fig. 5 Percentage drug lost from Ch_5 (*solid line*) and $Dansyl_{10}$ (*dashed line*) formulations over 4 weeks stored in 55% humidity, at room temperature and protected from light. Propofol stored in solution, prednisolone and griseofulvin formulations stored as freeze dried 'cakes'. ◊ propofol; □ prednisolone; Δ griseofulvin. Data presented as $n=3$, ave ± s.d.

however $Dansyl_{10}$, griseofulvin formulation experienced significant drug (40%) lost at the end of the study together with an increase in aggregation size to 1 μm.

Haemocompatibility

Figure 6a shows that apart from $Dansyl_5$, all aromatic grafted PAA polymers were non-haemolytic (<10%) within the concentration range tested, which is similar to the PAA parent polymer. The deviation of $Dansyl_5$ from this trend is not well understood. Unlike other alkyl chain grafted amphiphilic polymers, these aromatic grafted PAA showed better haemocompatibility (10). It has been reported that grafting of hydrophobic alkyl pendant groups tend to increase haemolytic activity due to the anchoring of pendant groups into the red cell (RBC) membrane (32). Our result suggests the inability of aromatic groups to insert into the red blood cell membrane as readily as hydrocarbon chains. Ch_5 polymers at higher concentrations precipitated when in contact with the suspension of RBC in PBS and hence we only tested the haemolytic effect up to 0.1 $mgmL^{-1}$, which showed no haemolytic activity (<0.5%). The RBCs upon incubation with $Dansyl_{10}$ at highest concentration have similar biconcave, spherical shape as RBC in PBS indicating that the polymer did not cause cell lysis or changed its morphology (Fig. 6b).

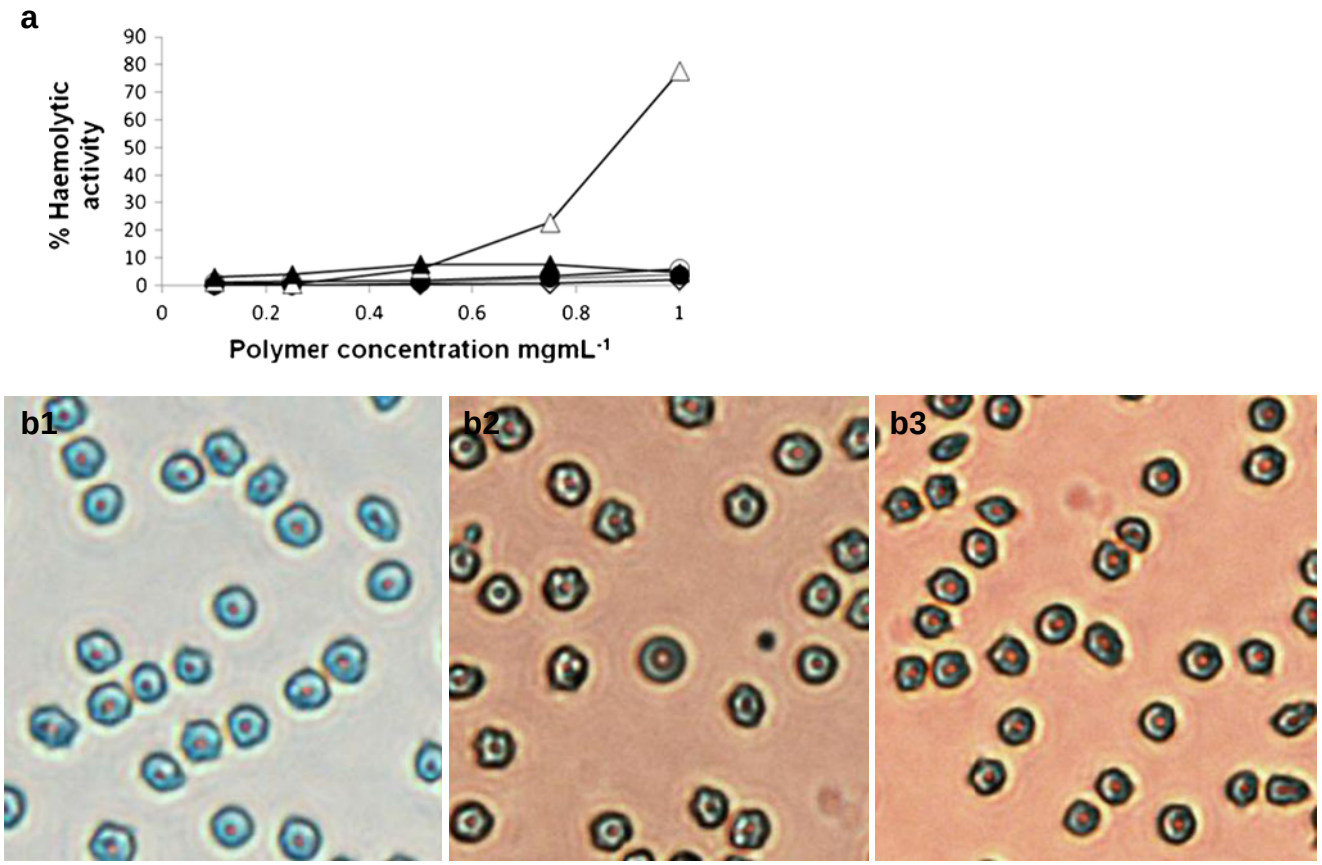

Fig. 6 Effect of PAA and its amphiphilic PAA polymers on bovine red blood cells. (**a**) % Haemolysis of ◊PAA; Δ$Dansyl_5$; ▲$Dansyl_{10}$; ○$Fmoc_5$; ● $Fmoc_{10}$. Data presented as $n=3$, ave ± s.d. (**b**) Morphology of red blood cells upon 4 h incubation with 1) PBS control; 2) $Dansyl_5$; 3) $Dansyl_{10}$ (1 mg/mL^{-1}) (100x magnification).

Cytotoxicity

MTT assay was conducted using CaCo-2 cells to determine the polymer concentration required to kill 50% of the cells and the results are shown in Table I. Higher IC_{50} value indicates the polymer is less cytotoxic. The unmodified PAA has an IC_{50} value of 23.3 ± 20.1 μgmL^{-1}. Modification with the aromatic or cholesteryl moieties did not result in notable differences between the IC_{50} of the modified polymers and the unmodified PAA (Table I). A slight increase in IC_{50} was observed when% of hydrophobic modification for $Dansyl_5$ is increased to $Dansyl_{10}$. It is known that primary amines are cytotoxic (33). It is possible that the reduction of primary amines on the polymer backbone upon higher level of Dansyl modification leads to better biocompatibility.

Intragastric Administration of Griseofulvin Formulations in Rats

Griseofulvin in water and two polymer formulations were administered to rats via oral gavage. No gross acute toxicity was observed in all formulation and control groups. At all time points, the polymer, griseofulvin formulations have significantly higher plasma drug levels than griseofulvin in water ($p < 0.0001$) indicating the ability of these polymers to improve the oral absorption of griseofulvin (Fig. 7). Ch_5 has higher plasma drug concentration when compared to $Dansyl_{10}$ at all time points ($p < 0.001$). The lower absorption observed in $Dansyl_{10}$ formulation could be due to higher critical association concentration (CAC) for $Dansyl_{10}$ (0.25 $mgmL^{-1}$) compared to Ch_5 (0.093 $mgmL^{-1}$) (18). In addition, both polymers also showed different absorption profiles. For Ch_5, the maximum plasma concentration was found at 4 h time point while $Dansyl_{10}$ formulation achieved highest plasma drug concentration at 1 h. This suggests that griseofulvin absorption occurred in the small intestine for Ch_5 while $Dansyl_{10}$ occurred in the stomach.

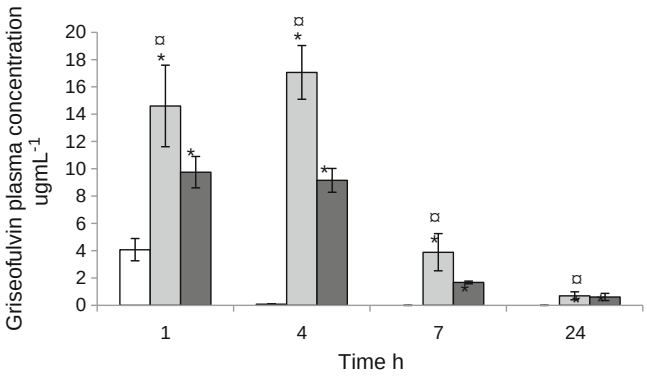

Fig. 7 Mean plasma griseofulvin concentration (μgmL^{-1}) following administration of griseofulvin by oral gavage in rats. ☐ Griseofulvin in water; ▒ Ch5, griseofulvin and ■ $Dansyl_{10}$, griseofulvin. Data presented as n = 4, ave ± s.d. * $p < 0.0001$ polymer formulations *vs.* griseofulvin in water, ¤ $p < 0.001$ $Dansyl_{10}$, *vs.* Ch_5.

DISCUSSION

In this work, we have synthesised four novel aromatic modified PAAs ($Fmoc_5$, $Fmoc_{10}$, $Dansyl_5$ and $Dansyl_{10}$) and sterol modified PAA (Ch_5). Using three hydrophobic drugs with solubility ranging from 0.1 $mgmL^{-1}$ (propofol), 0.22 $mgmL^{-1}$ (prednisolone) and 0.03 $mgmL^{-1}$ (griseofulvin) respectively, we have shown that all modified PAAs described in this work were able to encapsulate these drugs within their hydrophobic core and increased the water solubility. Many studies on amphiphilic block copolymers have shown that increasing the hydrophobic monomer content would result in higher lipophilic content and thus causing stronger interaction with the drug molecules, leading to higher drug encapsulation (34). This result is no exception to the trend where we observed that our novel aromatic modified PAAs with 10% mole modification significantly enhanced drug solubility when compared with their 5% mole counterparts.

Comparison among the aromatic grafted PAAs, reveals the poor solubilizing capacity of Fmoc with low LC and EE compared to Dansyl. We have shown previously that Fmoc modified PAA polymers formed excimers at higher polymer concentrations (18). The flat stereochemistry of aromatic structures allow π-π stacking and hence forming excimers, a known phenomenon supported by others (35). This limits the expansion of the core to accommodate more drugs at higher concentrations (Fig. 8). The trend agrees well with smaller increase in the hydrodynamic size of the loaded self-assemblies compared to Dansyl formulations (Table I). In contrast, the presence of the N,N-dimethylamino side chain in the Dansyl moiety gives rise to a 3D structure, that hinders any stacking interactions of the aromatic rings (18). As a result this allows the self-assemblies to enlarge its core to accommodate larger amount of drug molecules, which is in agreement with an increase of the hydrodynamic size when compared with their unloaded self-assemblies and high LC and EE (Table I; Fig. 8).

Interestingly, $Dansyl_{10}$ self-assemblies seem to have universal drug solubilizing capacity, demonstrating very low excipient to drug ratio across three drugs, i.e. 0.13 (propofol), 0.34 (griseofulvin) and 0.19 (prednisolone). This is significantly lower than traditional drug solubilizers such as low molecular weight surfactants, cyclodextrins or co-solvents systems which typically have excipient to drug ratio ranging from 15:1 to as high at 1000:1 (10) In addition, these novel amphiphilic grafted PAA solubilizers also showed much higher LC (>100%) compared to most of

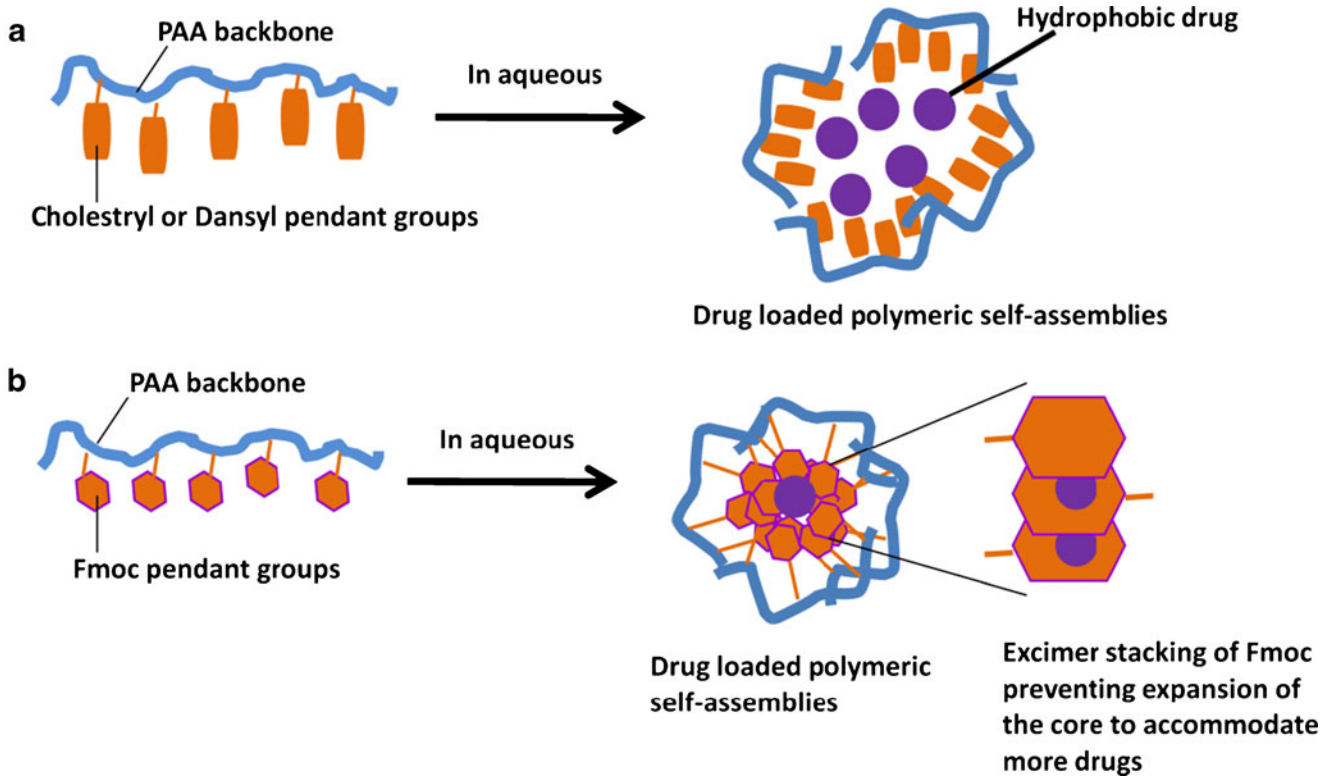

Fig. 8 Proposed drug loaded polymeric self-assemblies structures in aqueous environment (**a**) Ch and Dansyl-PAA and (**b**) Fmoc-PAA.

the reported block amphiphilic polymers (<20%) (8,13,29) and other alkyl or aryl chain grafted amphiphilic polymers based on polyethylenimine (10) or chitosan (36). To the best of our knowledge, preformed water-soluble polymer backbone grafted with aromatic pendant groups which exhibited high LC has not been previously reported. This may be due to better compatibility between the aromatic dansyl pendant groups and the cyclic/aromatic drugs, although more work, i.e. solubility parameters, X-ray diffraction, FTIR data are required to confirm this hypothesis. Another possible explanation could be due to these Dansyl pendant groups acted as hydrotropic agents. Park and colleagues have published extensively on the use of N,N-diethylnicotinamide (DENA) as hydrotropes to increase the solubility of poorly soluble drugs such as paclitaxel (37). They also showed that block amphiphilic polymer consists of polyethylene glycol –b-poly(2-(4-vinylbenzloxy)-N,N-diethylnicotinamide) (PEG-b-PCVBODENA) was able to enhance paclitaxel solubility significantly compared to plain PEG-b-poly(D,L-lactide) (PEG-b-PLA) (18). The DENA group has similarities to the Dansyl pendant groups where both have aromatic structures with a side chain. The authors also reported that DENA enhanced the stability of paclitaxel loaded PEG-b-PCVBODENA polymeric micelles. Freeze-dried prednisolone and liquid propofol $Dansyl_{10}$ formulations also exhibited reasonably good stability over one month period although it would appear that with Ch_5, griseofulvin formulation was more stable than the $Dansyl_{10}$ formulations.

Although Ch_5 did not significantly enhance drug solubility when compared with $Dansyl_{10}$, overall it has superior drug loading capacity to other block amphiphilic polymers. It is expected that cholesteryl pendant group would solubilize prednisolone better due to 'like-dissolves-like' concept. However, this trend is not observed in our study. Instead, Ch_5 increased propofol solubility by 78-fold compared to prednisolone (32-fold) and griseofulvin (40-fold). This may be due to the core forming sterol moieties being rigid and hence restrict entry to larger drug molecules, prednisolone (Mw = 360 $gmol^{-1}$) and griseofulvin (Mw = 353 $gmol^{-1}$) while they are able to accommodate smaller drug molecules like propofol (Mw = 178 $gmol^{-1}$). Previously we showed that Ch_5 core had highest microviscosity compared to cetyl or palmitoyl grafted PAA which may explain the phenomenon observed in this study (3).

Both $Dansyl_{10}$ and Ch_5 consistently achieved optimum drug to polymer initial feed ratios of 10:1 at polymer concentration of 6 $mgmL^{-1}$. For example, at 5:1 drug to polymer initial feed ratio, $Dansyl_{10}$ improved prednisolone solubility by 20-fold but was able to enhance the solubility of prednisolone by 147-fold at 10:1 ratio. For $Dansyl_{10}$ and Ch_5 increased initial drug feed ratios encouraged the

uptake of drugs into the hydrophobic core resulting in lower EE which might be an issue if the drugs are expensive (Table I)

Liu and colleagues had shown that compatibility between drug and the hydrophobic segments forming the hydrophobic core of the block amphiphilic polymers will determine the drug solubilizing capacity as well as the drug release profile (31). They demonstrated that the release rate of ellipticine, a model hydrophobic drug from polymeric micelles was in the order of the compatibility between the hydrophobic segment and the drug where the better the compatibility, the lower the release rate (31). This is similar to the trend observed where $Dansyl_{10}$ exhibited higher LC than Ch_5 for all 3 drugs and slower release profile, presumable due to better compatibility as described by Liu and colleagues although more experimental data such as FTIR and X-ray diffraction are required to confirm this hypothesis

It is understood that biocompatibility of a novel drug solubilizer is equally important as its solubilizing capacity. Alkyl and acyl chains are known to anchor into bilayers of cell membranes creating pores or to form mixed micelles with phospholipid bilayers which will lead to an increase in haemolytic activity and cytotoxicity. In contrast, the presence of cyclic or branching groups decreased haemolytic activity (38). Grafting of either Fmoc or Dansyl aromatic groups did not increase haemolytic activity compared to unmodified PAA. Similar to the cyclic structure, it is possible that the inflexible aromatic structure was not able to anchor into the bilayer as readily as alkyl chains. Interestingly, $Fmoc_5$ appears to deviate from this trend and this is not well understood. The cytotoxicity assay also indicates the addition of aromatic or cholesteryl pendant groups did not enhance the cytotoxicity of PAA.

To elucidate the ability of these PAA amphiphilic polymers in delivering hydrophobic drug orally, griseofulvin was used as a model drug. According to Biopharmaceutics Classification System (BCS), griseofulvin is a class II drug which exhibits poor solubility but high permeability (39). The rate determining step for griseofulvin is the dissolution process. Using similar dose as the clinical dose (11.8 $mgkg^{-1}$), we were able to demonstrate that both $Dansyl_{10}$ and Ch_5 formulations showed significantly higher plasma drug level compared to griseofulvin in water. This could be due to the rate determining step has been eliminated since griseofulvin encapsulated in the self-assemblies would not require dissolution step before absorption. This was the mechanism proposed by Kano and colleagues when they reported the use of block amphiphilic polymer, poly(2-methacryloyloxyethyl phosphorylcholine-co-n-butyl methacrylate) (PMB) for enhancing the oral absorption of griseofulvin (40). They compared extensively the griseofulvin pharmacokinetic data of a range of delivery systems such as niosomes, liposome, self-emulsifying drug delivery systems and spray dried microparticles in rats using data published in the literature. They concluded that PMB have similar C_{max}/Dose ratios with most of the formulations which range from 0.02 to 0.19. Interestingly our result showed a much higher C_{max}/Dose ratios of 1.44 (Ch_5) and 0.85 ($Dansyl_{10}$). Although direct comparison is not applicable, however the high plasma drug concentrations achieved in both PAA formulations and the differences observed between these formulations perhaps indicate there are other contributing factors at play apart from solubilization mechanism.

Although $Dansyl_{10}$ exhibits higher solubilization, however the *in vivo* result demonstrates Ch_5 formulation had significantly higher drug plasma concentrations at all time points with the maximum plasma drug concentration achieved at 4 h. Since it is thought that oral drug absorption using self-assembled nanoparticles is much more complex and hence we cannot assume higher solubilization implies better delivery. To date, there are limited *in vivo* studies on the use of amphiphilic polymers for improving bioavailability of hydrophobic drugs. Pierri and colleagues attempted to use Poly(lactide)-poly(ethylene glycol) micelles as oral carriers for griseofulvin but did not able to proceed to *in vivo* study due to the extremely poor drug loading capacity (4% w/w) (13). The trend we observed could be due to Ch_5 has a much lower CAC (0.0093 $mgmL^{-1}$) compared to $Dansyl_{10}$ (0.25 $mgmL^{-1}$) and hence it did not lose the hydrophobic payload upon dilution in the gastrointestinal tract (18). Another possible explanation could be the polymer architecture affects the interaction between drug loaded self-assemblies with the gut enterocytes. In our previous work, we showed that quaternised palmitoyl modified PAAs were able to promote insulin uptake into cytoplasm of CaCo-2 cells via an active transport while non-quaternised palmitoyl modified PAA did not (17). Therefore, further work is still required to elucidate the interaction between the drug loaded self-assemblies with the intestinal cells and subsequent absorption. In addition, the effect of food, the stomach acidity, the presence of bile salts and other physiological factors might affect the formulations and these issues should also be addressed when using these novel solubilizers for oral delivery.

CONCLUSION

This study demonstrated for the first time attachment of 10% mole aromatic pendant groups (Dansyl) to a pre-formed water soluble polymer backbone poly(allylamine) exhibited superior solubilizing capacity for all three hydrophobic drugs compared to Fmoc PAA or cholesteryl grafted PAA. Its ability to expand its hydrophobic core and possibly better compatibility with the cyclic or aromatic

drugs resulted in slower drug release profile and high drug loading capacity. The *in vivo* study also revealed that Ch_5 and $Dansyl_{10}$ were able to significantly improve the oral bioavailability of griseofulvin, a class II drug suggesting their potential as novel solubilizers for oral delivery.

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