

CLARE, H., KONG THOO LIN, P., TETLEY, L. and CHENG, W.P. 2012. The use of nano polymeric self-assemblies based on novel amphiphilic polymers for oral hydrophobic drug delivery. *Pharmaceutical research* [online], 29(3), pages 782-794. Available from: <https://doi.org/10.1007/s11095-011-0602-7>

The use of nano polymeric self-assemblies based on novel amphiphilic polymers for oral hydrophobic drug delivery.

CLARE, H., KONG THOO LIN, P., TETLEY, L. and CHENG, W.P.

2012

This version of the article has been accepted for publication, after peer review (when applicable) and is subject to Springer Nature's [AM terms of use](#), but is not the Version of Record and does not reflect post-acceptance improvements, or any corrections. The Version of Record is available online at: <https://doi.org/10.1007/s11095-011-0602-7>

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

1. Introduction

Polymeric self-assemblies have been widely studied for their potential as hydrophobic drug solubilising 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 solubilisation 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 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 [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 solubilisation 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 ($M_w = 178 \text{ gmol}^{-1}$, $\log P = 4.16$), prednisolone ($M_w = 360 \text{ gmol}^{-1}$, $\log P = 1.8$) and griseofulvin ($M_w = 353 \text{ gmol}^{-1}$, $\log P = 2.2$) will be used as model drugs (Fig1). 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*.

2. Materials and methods

15kDa poly(allylamine) hydrochloride (PAA), propofol, prednisolone, griseofulvin, etoposide, orthophosphoric acid, 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). 0.45µm GDX PVDF syringe filters were from Whatman (UK).

2.1 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 ¹H NMR and the results confirmed 4.7, 4.3 and 7.1% mole modification for Ch₅, Fmoc₅ and Dansyl₅ respectively and 9.3% for both Fmoc₁₀ and Dansyl₁₀ [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.

2.2 Drug Loading

Polymer in deionised water (1, 3 and 6mgmL⁻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.

2.2.1 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 x 46 mm x 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}), $R^2 = 0.999$.

2.2.2 Quantification of prednisolone

HPLC consisted of a RP Phenomenex C_{18} 150 mm x 4.6 mm x 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 3min and detected at λ_{max} 243nm. 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.

2.2.3 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 x 46 mm x 5 μm HPLC column and the peak (9.5min) was detected at λ_{max} 293 nm . The mobile phase (45:55 v/v) acetonitrile:45 mM potassium dihydrogen phosphate buffer (adjusted to pH3 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 $\mu\text{g mL}^{-1}$ – 10 $\mu\text{g mL}^{-1}$), $R^2 = 0.999$.

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

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

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

2.3 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.

2.4 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.

2.5 *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 6mg mL^{-1} were prepared as described in section 2.2. 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.

2.6 Stability testing of Formulations

The formulations were prepared as previously described (section 2.2) 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.

2.7 Biological characterisation

2.7.1 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. 10mgmL⁻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 (160uL/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.

2.7.2 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 ($0.2 - 1 \times 10^{-4} \text{ mgmL}^{-1}$) were prepared from stock solution (0.5 mgmL^{-1} in 1:20 water: media). Caco-2 cells ($200 \mu\text{L}$, 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 ($200 \mu\text{L}$). After 24h, the polymer solutions were removed and replaced with fresh media and incubated for a further 24h. The media was then replaced with fresh media and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT) ($50 \mu\text{L}$, 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 ($200 \mu\text{L}$) and L-glycine buffer ($20 \mu\text{L}$) (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.

2.8 *In vivo* oral absorption study

2.8.1 *Formulation preparation*

Ch_5 and Dansyl $_{10}$ (6 mgmL^{-1}), griseofulvin formulations were prepared as described in section 2.2 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.

2.8.2 *Intragastric administration and evaluation of griseofulvin absorption*

18 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 (2mL). 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 30s 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 x 46 mm x 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 ($1.9 \mu\text{g mL}^{-1} - 10 \mu\text{g mL}^{-1}$), $R^2 = 0.992$. 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.

3 Results

3.1 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 solubilisation (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 solubilising 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 mg mL^{-1} to 6 mg mL^{-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 solubilisation was achieved with Ch_5 and Dansyl_{10} polymers at 6 mg mL^{-1} concentration and polymer weight ratios of

10:1 (Fig 2). Dansyl₁₀ exhibited the highest improvement in drug aqueous solubility demonstrating 145-fold for prednisolone, 224-fold for propofol and 557-fold for griseofulvin respectively (Table 1). 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 based amphiphilic polymers have substantial higher LC especially with Dansyl₁₀ demonstrating up to 530 % LC (Table 1). Dansyl₁₀ 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 1), and is in agreement with previous reports [30]. For Ch₅ and Dansyl₁₀, encapsulation of propofol resulted in a significant increase in size (~600nm) while the rest of the formulations typically have hydrodynamic size of 300-400nm. An increase in polydispersity index (PDI) is observed in drug loaded Ch₅ after drug encapsulation while this was not evident in Dansyl₁₀. It is possible that Dansyl₁₀ are more efficient solubilisers than Ch₅ 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.

3.2 *In vitro* drug release

The *in vitro* drug release of the 3 drugs from the two best amphiphilic polymers, Ch₅ and Dansyl₁₀ were assessed in PBS under sink condition (Fig. 4). Apart from griseofulvin, generally Ch₅ resulted in rapid release where most drugs (between 50-60%) had been released in the first 7h while the release of drugs from Dansyl₁₀ formulations were slower with only approximately 20% of the drugs being released after 7h. The release profile of Dansyl₁₀ formulations seems to be independent of the encapsulated drug. It is possible that good compatibility between Dansyl₁₀ and the 3 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 to 4 days (data not shown).

3.3 Formulation stability

Fig 5 shows the amount of drug lost analysed by HPLC over a 4 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 4 week period, Ch₅, propofol liquid formulations experienced gradual drug lost from 0 to 30% while Dansyl₁₀, propofol liquid formulation was able 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 Ch₅ self-assemblies reduced from 666nm to 239nm at the end of the study indicating drug lost while the Dansyl₁₀, propofol formulation retained the same size at 677nm as in week 0 (Table 1). The initial drug lost (10-15%) at week 0 from both Ch₅ and Dansyl₁₀ freeze-dried prednisolone and griseofulvin formulations was perhaps due to the freeze drying process (Fig 5). Interestingly, Ch₅ freeze-dried formulations were more stable than Dansyl₁₀ formulations as no further notable loss was apparent over the 4 week however Dansyl₁₀, griseofulvin formulation experienced significant drug (40%) lost at the end of the study together with an increase in aggregation size to 1µm.

3.4 Haemocompatibility

Fig 6A shows that apart from Dansyl₅, 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 Dansyl₅ 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. Ch₅ 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 0.1mgmL⁻¹, which showed no haemolytic activity (<0.5%). The RBCs upon incubation with Dansyl₁₀ 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).

3.5 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 1. Higher IC₅₀ value indicates the polymer is less cytotoxic. The unmodified PAA has an IC₅₀ value of 23.3± 20.1µgmL⁻¹. Modification with the aromatic or cholesteryl moieties did not result in notable differences between the IC₅₀ of the modified polymers and the unmodified PAA (Table 1). A slight increase in IC₅₀ was observed when % of hydrophobic modification for Dansyl₅ is increased to Dansyl₁₀. 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.

3.6 Intra-gastric 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). Ch₅ has higher plasma drug concentration when compared to Dansyl₁₀ at all

time points ($p < 0.001$). The lower absorption observed in Dansyl₁₀ formulation could be due to higher critical association concentration (CAC) for Dansyl₁₀ (0.25 mg mL^{-1}) compared to Ch₅ (0.093 mg mL^{-1}) [18]. In addition, both polymers also showed different absorption profiles. For Ch₅, the maximum plasma concentration was found at 4h time point while Dansyl₁₀ formulation achieved highest plasma drug concentration at 1h. This suggests that griseofulvin absorption occurred in the small intestine for Ch₅ while Dansyl₁₀ occurred in the stomach.

4 Discussion

In this work, we have synthesised four novel aromatic modified PAAs (Fmoc₅, Fmoc₁₀, Dansyl₅ and Dansyl₁₀) and sterol modified PAA (Ch₅). Using three hydrophobic drugs with solubility ranging from 0.1 mg mL^{-1} (propofol), 0.22 mg mL^{-1} (prednisolone) and 0.03 mg mL^{-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 solubilising 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 8a). The trend agrees well with smaller increase in the hydrodynamic size of the loaded self-assemblies compared to Dansyl formulations (Table 1). 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 1) (Fig 8b).

Interestingly, Dansyl₁₀ self-assemblies seem to have universal drug solubilising 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 solubilisers 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 as 1000:1 [10]. In addition, these novel amphiphilic grafted PAA solubilisers also showed much higher LC (>100%) compared to most of 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 acting 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 Dansyl₁₀ formulations also exhibited reasonably good stability over one month period although it would appear that with Ch₅, griseofulvin formulation was more stable than the Dansyl₁₀ formulations.

Although Ch₅ did not significantly enhance drug solubility when compared with Dansyl₁₀, overall it has superior drug loading capacity to other block amphiphilic polymers. It is expected that cholesteryl pendant group would solubilise prednisolone better due to 'like-dissolves-like' concept. However, this trend is not observed in our study. Instead, Ch₅ 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= 360gmol⁻¹) and griseofulvin (Mw=353gmol⁻¹) while they are able to accommodate smaller drug molecules like propofol (Mw=178gmol⁻¹). Previously we showed that Ch₅ core had highest microviscosity compared to cetyl or palmitoyl grafted PAA which may explain the phenomenon observed in this study [3].

Both Dansyl₁₀ and Ch₅ consistently achieved optimum drug to polymer initial feed ratios of 10:1 at polymer concentration of 6mgmL⁻¹. For example, at 5:1 drug to polymer initial feed ratio, Dansyl₁₀ improved prednisolone solubility by 20-fold but was able to enhance the solubility of prednisolone by 147-fold at 10:1 ratio. For Dansyl₁₀ and Ch₅ 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 1)

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 solubilising 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 Dansyl₁₀ exhibited higher LC

than Ch₅ for all 3 drugs and slower release profile, presumably 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 solubiliser is equally important as its solubilising 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, Fmoc₅ 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 (11.8mgkg⁻¹), we were able to demonstrate that both Dansyl₁₀ and Ch₅ 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 solubilisation mechanism.

Although Dansyl_{10} exhibits higher solubilisation, 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 4h. Since it is thought that oral drug absorption using self-assembled nanoparticles is much more complex and hence we cannot assume higher solubilisation 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 (0.0093mgmL^{-1}) compared to Dansyl_{10} (0.25mgmL^{-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 solubilisers for oral delivery.

5. Conclusion

This study demonstrated for the first time attachment of 10% mole aromatic pendant groups (Dansyl) to a preformed water soluble polymer backbone poly(allylamine) exhibited superior solubilising 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 Ch₅ and Dansyl₁₀ were able to significantly improve the oral bioavailability of griseofulvin, a class II drug suggesting their potential as novel solubilisers for oral delivery.

Acknowledgement

Clare Hoskins was funded by the Research Development Initiative at the Robert Gordon University.

References

1. Bader H, Ringsdorf H, Schmidt B. Water soluble polymers in medicine. *Angew Chem Int Ed Engl.* 1984;123/124:457-85.
2. Kwon G, Kataoka K. Block Copolymer micelles as long-circulating drug vehicles. *Adv Drug Deliver Rev.* 1995;16:295-309.
3. Thompson C, Ding C, Qu X, Yang Z, Uchegbu IF, Tetley L, Cheng W-P. The effect of polymer architecture on the nano self-assemblies based on novel comb-shaped amphiphilic poly(allylamine). *Colloid Polymer Sci.* 2008;286:1511-1526.
4. Anokhin DV, Gearba RI, Godovsky YK, Magonov SN, Makarova NN, Ivanov RI, Bras W, Ivanov DA. Structure and phase behaviour of a disk-necklace polymer: Cyclolinear polymethylsiloxane. *Polymer.* 2007;48:4837-4848.

5. Liao S-C, Lai C-S, Rahman MH, Hsu C-S, Chen H-L, Chen L-A. Supramolecular structures of an amphiphilic hairy-rod conjugated copolymer bearing poly(ethylene glycol) side chain. *Reactive Funct Polym.* 2009;69:498-506.
6. Uchegbu IF. Pharmaceutical nanotechnology: polymer vesicles for drug and gene delivery. *Expert Opin Drug Deliver.* 2006;3:629-640.
7. Bromberg L. Polymeric micelles in oral chemotherapy. *J Control Release.* 2008;128:99-112.
8. Gaucher G, Satturwar P., Jones M-C, Furtos A, Leroux J-C. Polymeric micelles for oral drug delivery. *Eur J Pharm Biopharm.* 2010;76:147-156.
9. Ould-Ouali L, Noppe M, Langlois X, Willems B, Riele PT, Timmerman P, Brewster ME, Ariën A, Pr at V. Self-assembling PEG-p(CL-co-TMC) copolymers for oral delivery of poorly water-soluble drugs: a case study with risperidone. *J Control Release.* 2005;102:657-668.
10. Cheng W-P, Gray AI, Tetley L, Hang TLB, Sch atzlein AG, Uchegbu IF. Polyelectrolyte Nanoparticles with High Drug Loading Enhance the Oral Uptake of Hydrophobic Compounds. *Biomacromolecules.* 2006;7:1509-1520.
11. Lee SC, Huh KM, Lee YT, Cho YW, Galinsky RE, Park K. Hydrotropic polymeric micelles for enhanced paclitaxel solubility: in vitro and in vivo characterisation. *Biomacromolecules.* 2007;8:202-208.
12. Benny O, Fainaru A, Adini F, Cassiola F, Bazinet L, Adini I, PravdaE, Nahmias Y, Koirala S, Corfas G, D'Amato RJ, Folkman J. An orally delivered small-molecule formulation with antiangiogenic and anticancer activity. *Nature Biotechnol.* 2008;26:799-807.
13. Pierri E, Avgoustakis K. Poly(lactide)-poly(ethylene glycol) micelles as a carrier for griseofulvin. *J. Biomed. Mater. Res. A.* 2005;75:639-647.

14. Fefelova NA, Nurkeeva ZS, Mun GA, Khutoryanskiy VV. Mucoadhesive interactions of amphiphilic cationic copolymers based on [2-(methacryloyloxy)ethyl]trimethylammonium chloride. *Int J Pharm.* 2007;339:25-32.
15. Cheng W-P, Thompson C, Ryan SM, Aguirre T, Tetley L, Brayden DJ. In vitro and in vivo characterisation of a novel peptide delivery system: Amphiphilic polyelectrolyte-salmon calcitonin nanocomplexes. *J Control Release.* 2010;147:289-297.
16. Iqbal J, Hombach J, Matuszczak B, Bernkop-Schnürch A. Design and in vitro evaluation of a novel polymeric P-glycoprotein (P-gp) inhibitor. *J Control Release.* 2010;147:62-69.
17. Thompson C, Cheng W-P, Gadad P, Skene K, Smith M, Smith G, McKinnon A, Knott R. Uptake and transport of novel amphiphilic polyelectrolyte-insulin nanocomplexes by Caco-2 cells-towards oral insulin. *Pharm Res.* 2011;28:886-896.
18. Hoskins C, Kong Thoo Lin P, Tetley L, Cheng W-P. Novel fluorescent amphiphilic poly(allylamine) and their supramacromolecular self-assemblies in aqueous media, *Polymers for Advanced Technologies.* 2011;22 in press DOI: 10.1002/pat.1962
19. Sweet DM, Kolhatkar RB, Ray A, Swaan P, Ghandehari H. Transepithelial transport of PEGylated anionic poly(amidoamine) dendrimers: Implication for oral drug delivery. *J Control Release.* 2009;138:78-85.
20. Kato K, Uchida E, Kang EN, Uyama Y, Ikada K. Polymer surface with graft chains. *Prog Polym Sci.* 2003;28:209-259.
21. Yinsong W, Lingrong L, Jian W, Zhang Q. Preparation and characterization of self-aggregated nanoparticles of cholesterol-modified o-carboxymethyl chitosan conjugates. *Carbohydrate Polym.* 2007;69:597-606.
22. Rekasas CJ, Mai SM, Crothers M, Quinn M, Collett JH, Attwood D, Heatley F, Martinin L, Booth C. The effect of hydrophobe chemical structure and chain length on the

- solubilization of griseofulvin in aqueous micellar solutions of block copoly(oxyalkylene)s. *Physical Chemistry Chemical Physics* 2001;21:4769-4773.
23. Hoskins C, Ouaiissi M, Lima S, Cheng W-P, Loureiro I, Mas E, Lombardo D, Cordeiro A, Ouaiissi A, Kong Thoo Lin P. In vitro and In vivo anticancer activity of a novel nano-sized formulation based on self-assembling polymers against pancreatic cancer. *Pharmaceut Res.* 2010;27:2694-2703.
24. Albaaj F, Hutchison AJ. Hyperphosphataemia in Renal Failure: Causes, Consequences and Current Management. *Drugs.* 2003;63:577-596.
25. Vigl C, Leithner K, Albrecht K, Bernkop-Schnurch A. The efflux pump inhibitory properties of (thiolated) polyallylamines. *Journal of Drug delivery Science and Technology.* 2009;19:405-411.
26. Qu X, Khutoryanskiy VV, Stewart A, Rahman S, Papahadjopoulos-Sternberg B, Dufres C, McCarthy D, Wilson CG, Lyons R, Carter KC, Schätzlein A, Uchegbu IF. Carbohydrate – Based Micelle Clusters Which Enhance Hydrophobic Drug Bioavailability by up to 1 Order of Magnitude. *Biomacromolecules.* 2006;7:3452–3459.
27. Trimaille T, Mondon K, Gurny R, Möller M. Novel polymeric micelles for hydrophobic drug delivery based on biodegradable poly(hexyl-substituted lactides). *Int J Pharm.* 2006;139:147-154.
28. Lee J, Cho EC, Cho K. Incorporation and Release behaviour of hydrophobic drug in functionalized poly(D,L-lactide)-block-poly_ethylene oxide) micelles. *J Control Release.* 2004;94:323-332.
29. Qiu LY, Bae YH. Polymer Architecture and Drug Delivery. *Pharm Res.* 2006;23:1-30.
30. Satturwar P, Eddine MN, Ravenelle F, Leroux J-C. pH responsive polymeric micelles of poly(ethylene glycol)-b-poly(alkyl(meth)acrylate-co-methacrylic acid): Influence of the

- copolymer composition on self-assembling properties and release of candesartan cilexetil. *Eur J Pharm BioPharm.* 2007;65:379-387.
31. Liu JB, Xiao YH, Allen C. Polymer-drug compatibility: A guide to the development of delivery systems for the anticancer agent, Ellipticine. *J Pharm Sci.* 2004;93:132–143.
 32. Venkataraman S, Zhang Y, Liu L, Yang YY. Design, Syntheses and Evaluation of Hemocompatible Pegylated-Antimicrobial Polymers with Well-Controlled Molecular Structures. *Biomaterials.* 2010;31:1751-1756.
 33. Aravindan L, Bicknell KA, Brooks G, Khutoryanskiy VV, Williams AC. Effect of acyl chain length on transfection efficiency and toxicity of polyethylenimine. *Int. J. Pharm.* 2009;378:201-210.
 34. Zhang X, Jackson JK, Burt HM. Development of amphiphilic diblock copolymers as micellar carriers of Taxol. *Int J Pharm.* 1996;132:195-206.
 35. Benniston AC, Harriman A, Howell SL, Sams CA, Zhi YG. Intramolecular excimer formation and delayed fluorescence in sterically constrained pyrene dimers. *Chem- Eur J.* 2007;13:4665-4674.
 36. Yuan Y, Lu L-J, Du Y-Z, Hu F-Q. Stearic acid-g-chitosan polymeric micelle for oral drug delivery: in vitro transport and in vivo absorption. *Molecular pharm.* 2010;8:225-238.
 37. Lee J, Lee SC, Acharya G, Chang CJ, Park K. Hydrotropic Solubilization of Paclitaxel: Analysis of Chemical Structures for Hydrotropic Property. *Pharm Res.* 2003;20:1022–1030.
 38. Soderline E, Karlsson L. Haemolytic activity of maltopyranoside surfactants. *Eur J Pharm Biopharm.* 2006;62:254-259.
 39. The Medicines and Healthcare products Regulatory Agency (MHRA), British Pharmacopoeia Volume I, The Stationary Office, 2005.

40. Kano T, Kakinuma C, Wada S, Morimoto K, Ogihara T. Enhancement of drug solubility and absorption by copolymers of e-methacryloyloxyethyl phosphorylcholine and n-Butyl methacrylate. *Drg Metab. Pharmacokinet.* 2011;26:79-86.

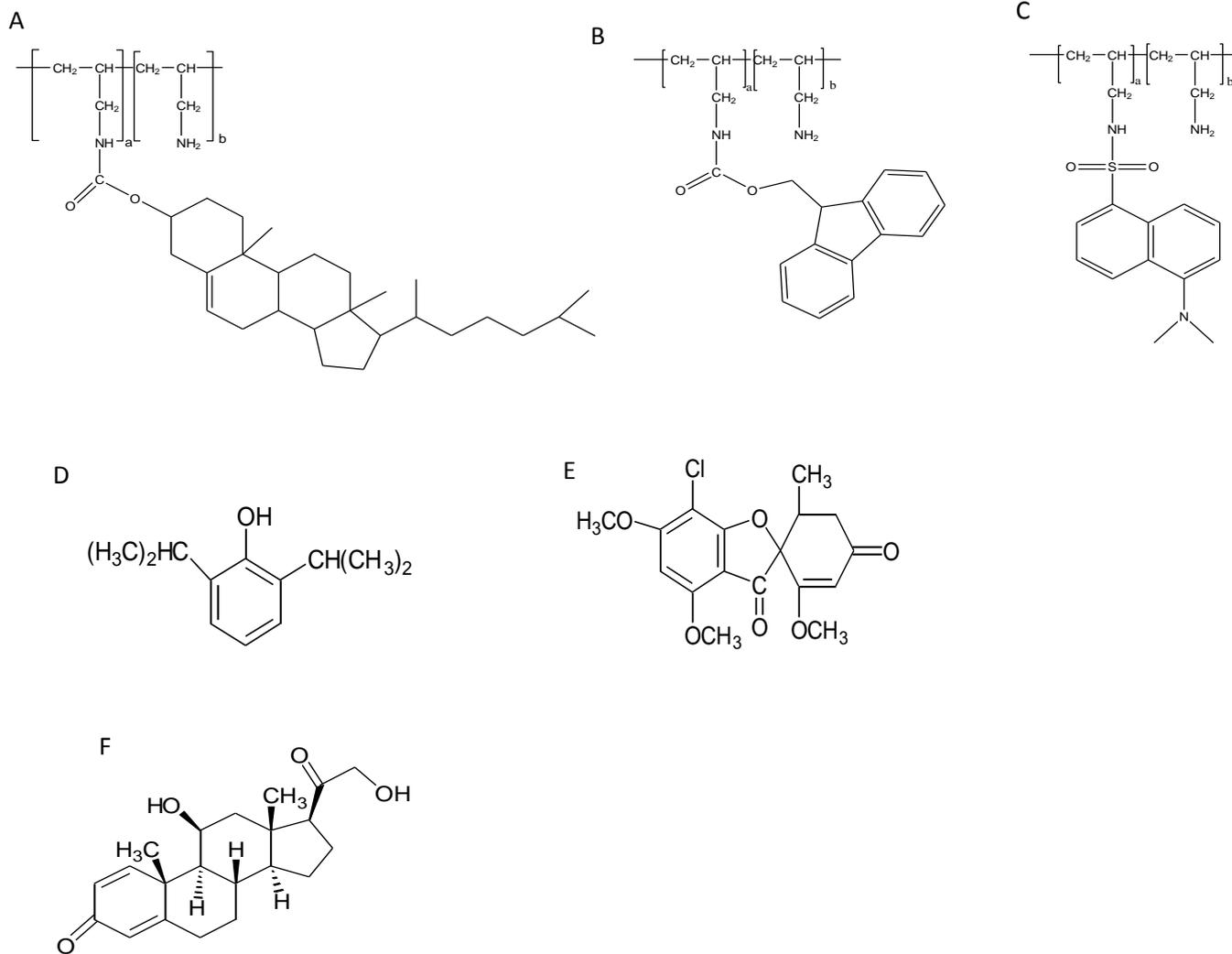


Figure 1: Chemical structure of A) cholesteryl-PAA; B) Fmoc-PAA; C) Dansyl- PAA; D) propofol; E) griseofulvin; F) prednisolone

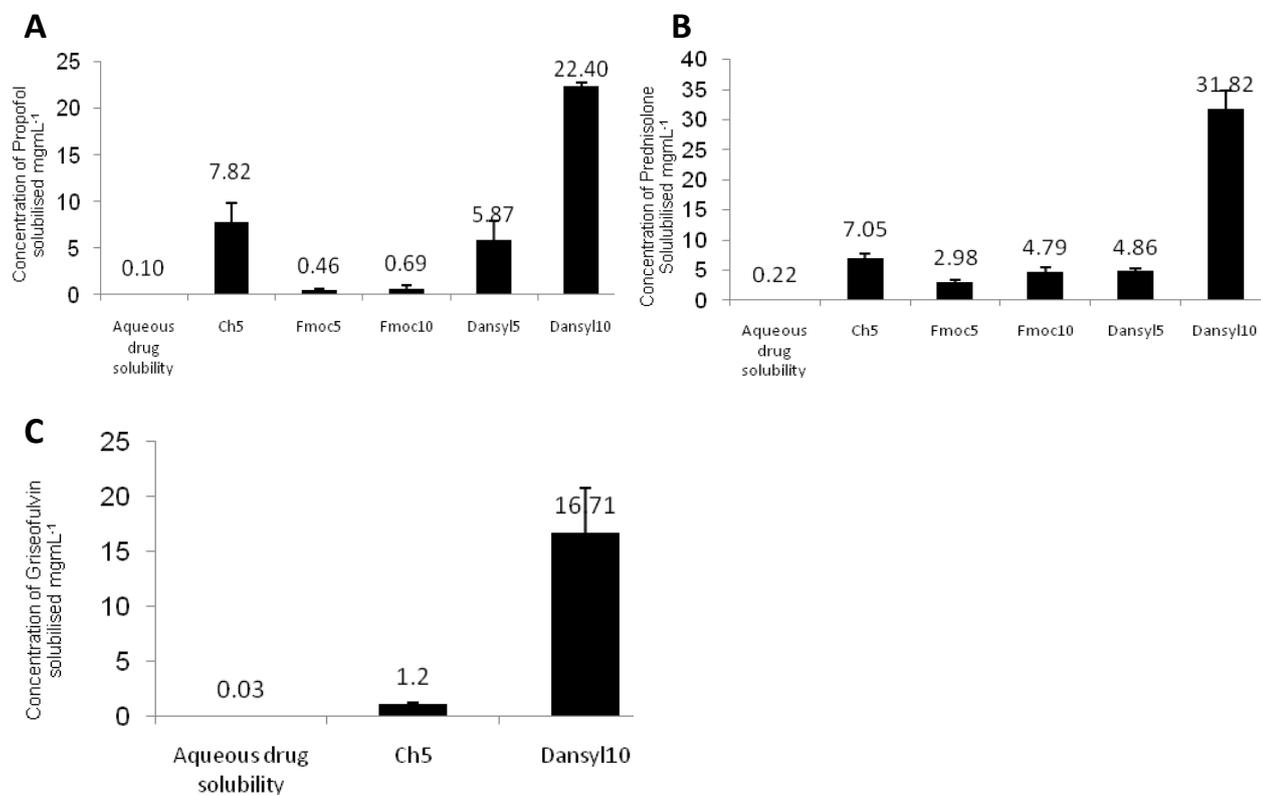


Figure 2. Maximum drug concentration solubilised by PAA amphiphilies. A) propofol, B) prednisolone and C) griseofulvin.

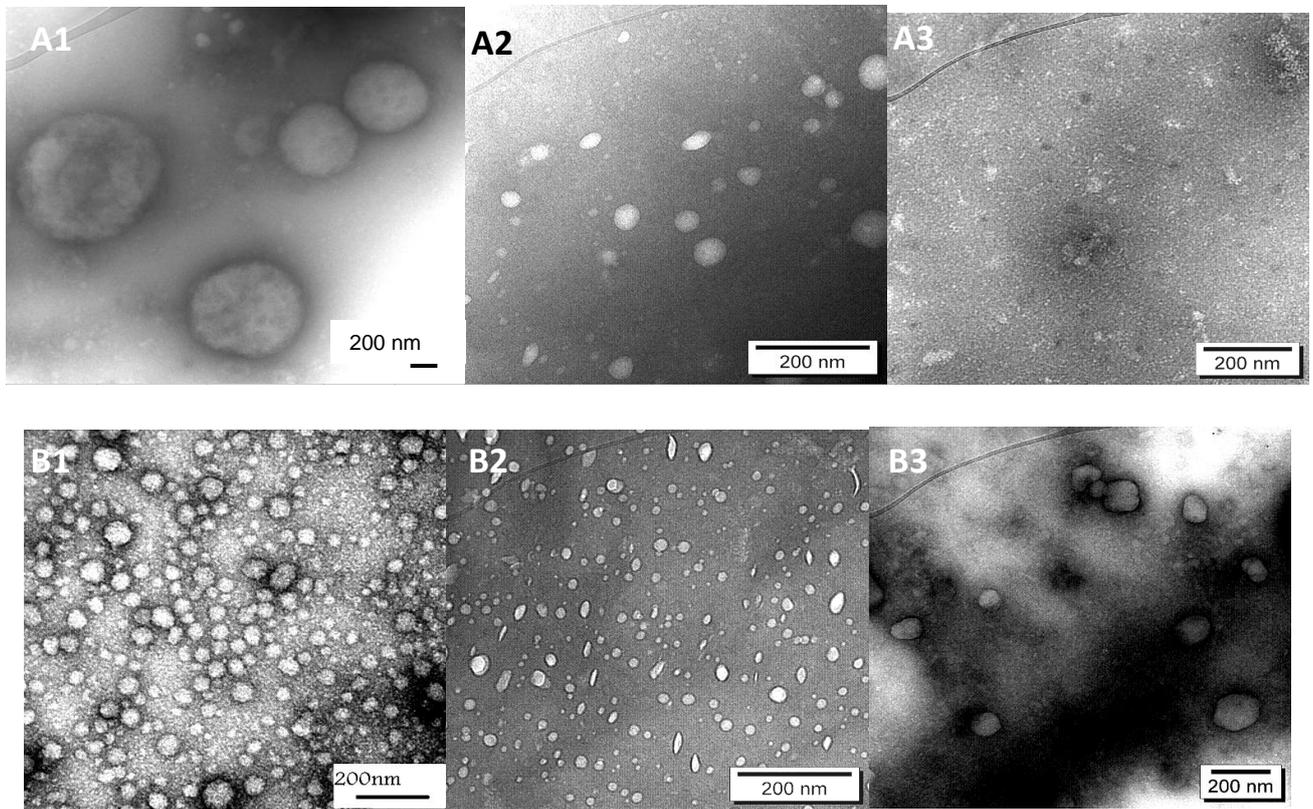


Figure 3. Negative-stained TEM of A) Ch₅ formulations with 1) propofol, 2) prednisolone and 3) griseofulvin. B) Dansyl₁₀, 1) propofol, 2) prednisolone and 3) griseofulvin. All the formulations consisted of 6 mgmL⁻¹ polymer and 10:1 initial drug: polymer mass ratio. Bar=200nm

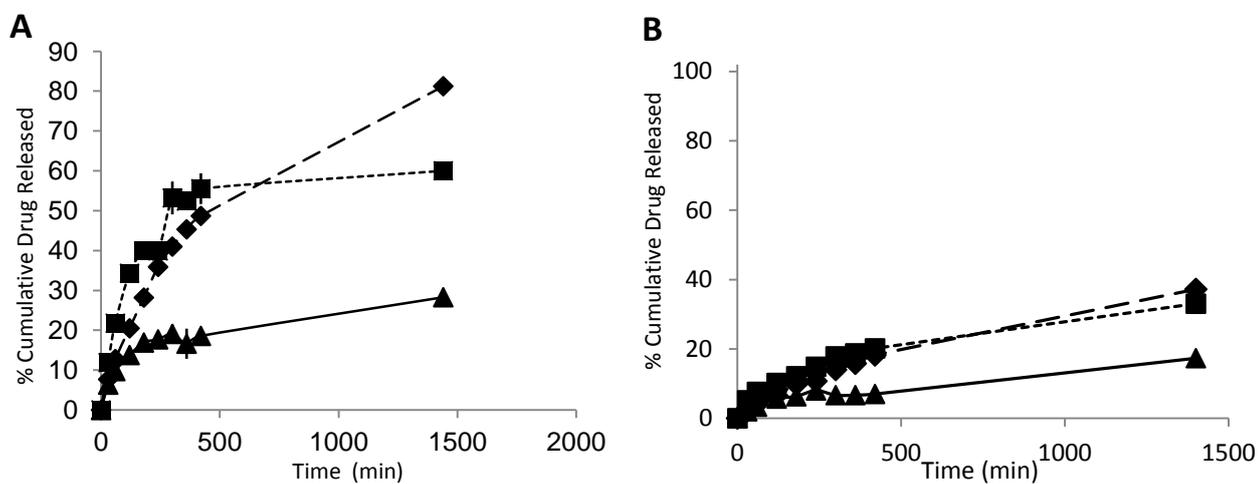


Figure 4. *In vitro* drug release of hydrophobic drugs from A) Ch₅ and B) Dansyl₁₀ formulations carried out in sink conditions. ◆ propofol ; ■ prednisolone ▲ griseofulvin. Data presented as n=3, ave ± s.d.

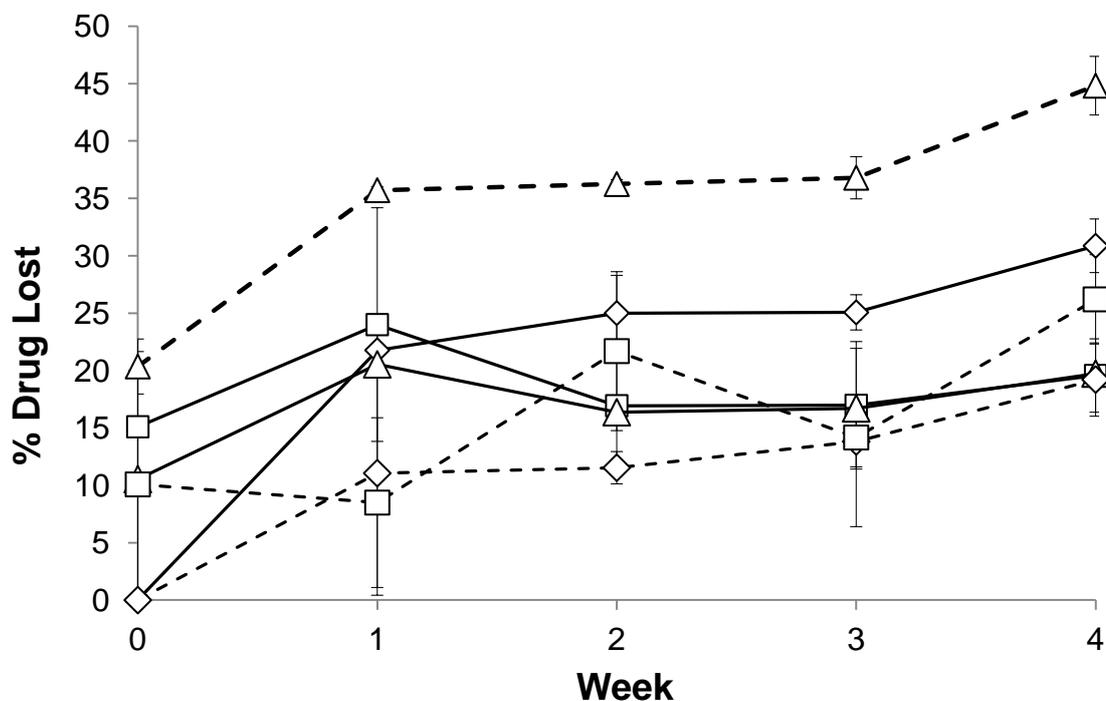


Figure 5. Percentage drug lost from Ch₅ (solid line) and Dansyl₁₀ (dashed line) formulations over 4 weeks stored in 55 % humidity, at room temperature and protected from light. Propofol stored in solution, prednisolone and griseofulvin formulations stored as freeze dried 'cakes'. ◇ propofol ; □ prednisolone; △ griseofulvin. Data presented as n=3, ave ± s.d.

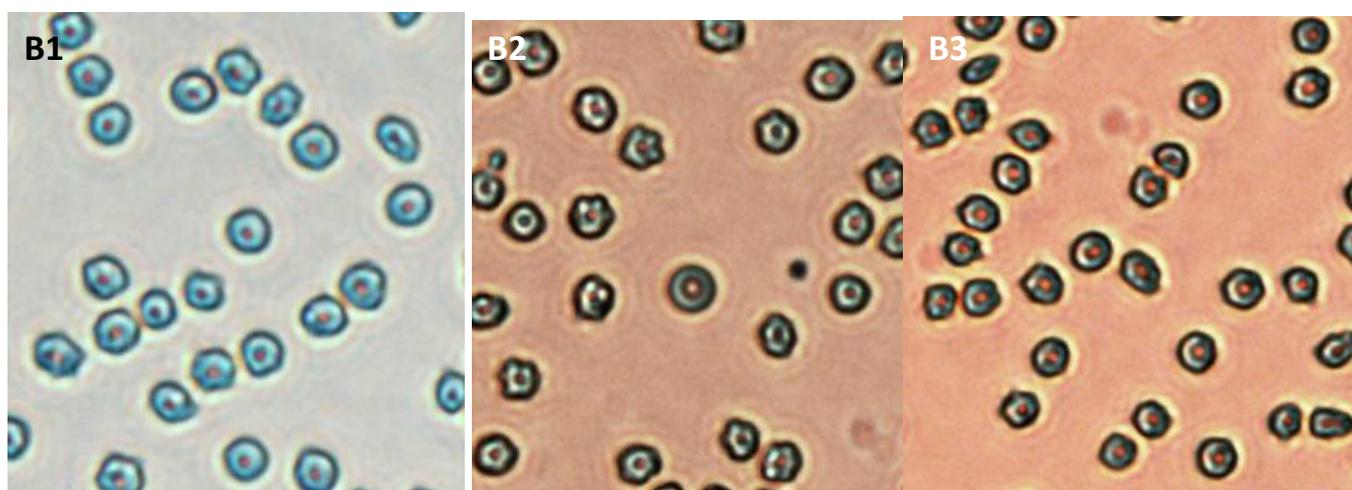
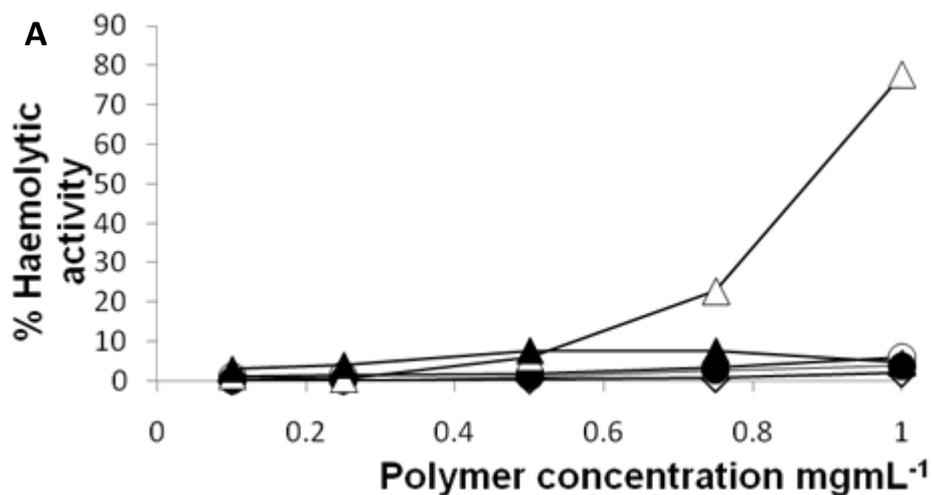


Figure 6. Effect of PAA and its amphiphilic PAA polymers on bovine red blood cells. **A)** % Haemolysis of ◇PAA; △Dansyl₅; ▲Dansyl₁₀; ○Fmoc₅; ● Fmoc₁₀. Data presented as n=3, ave ± s.d. **B)** Morphology of red blood cells upon 4h incubation with 1) PBS control; 2) Dansyl₅; 3) Dansyl₁₀ (1 mgmL⁻¹) (100x magnification)

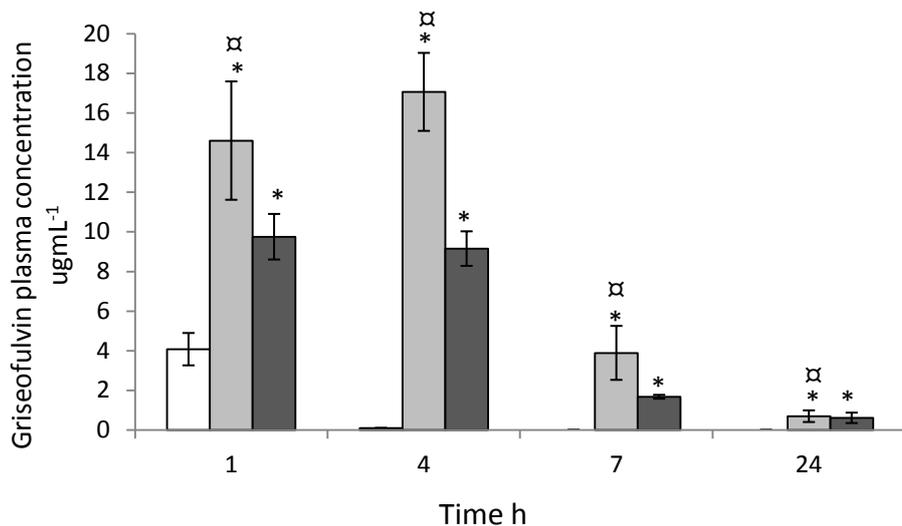


Figure 7. Mean plasma griseofulvin concentration ($\mu\text{g mL}^{-1}$) following administration of griseofulvin by oral gavage in rats. \square Griseofulvin in water; \blacksquare Ch₅, griseofulvin and \blacksquare Dansyl₁₀, griseofulvin. Data presented as $n=4$, $\text{ave} \pm \text{s.d.}$ * $p < 0.0001$ polymer formulations vs. griseofulvin in water, α $p < 0.001$ Dansyl₁₀, vs. Ch₅.

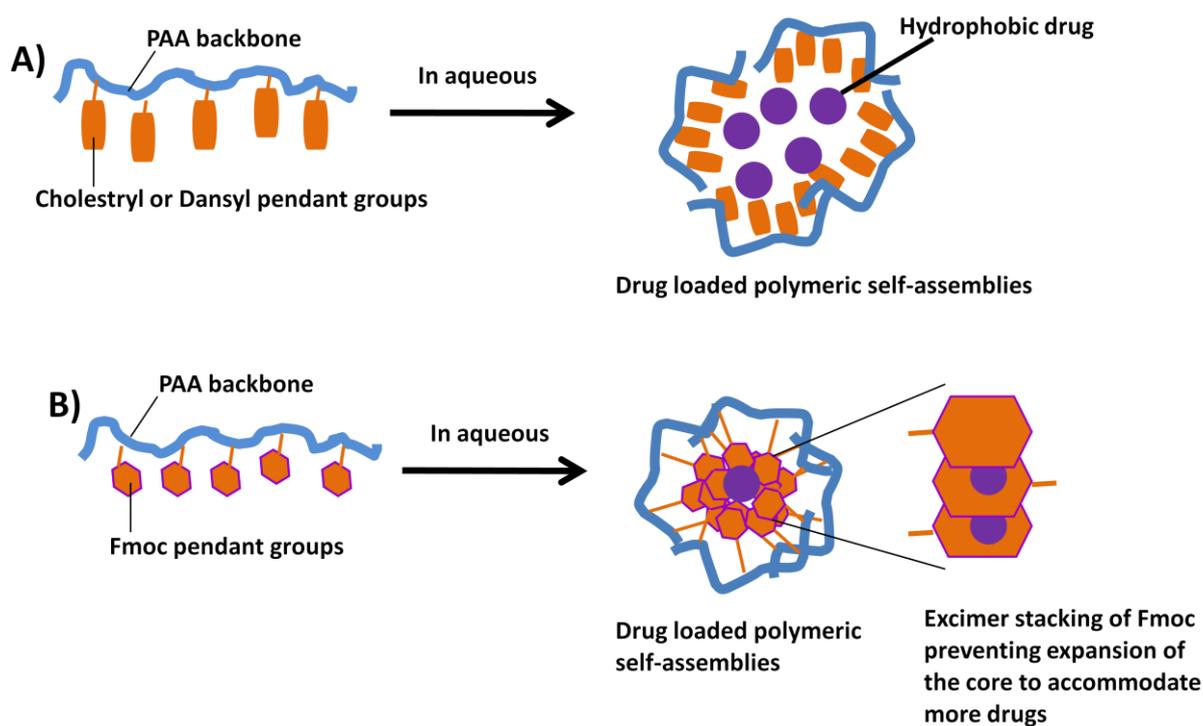


Figure 8. Proposed drug loaded polymeric self-assemblies structures in aqueous environment A) Ch and Dansyl-PAA and B) Fmoc-PAA

Legends to figures

Figure 1. Chemical structure of A) cholesteryl- PAA; B) Fmoc - PAA; C) Dansyl- PAA; D) propofol; E) griseofulvin; F) prednisolone

Figure 2. Maximum drug concentration solubilised by PAA amphiphilicities. A) propofol, B) prednisolone and C) griseofulvin.

Figure 3. Negative-stained TEM of A) Ch₅ formulations with 1) propofol, 2) prednisolone and 3) griseofulvin. B) Dansyl₁₀, 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=200nm

Figure 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.

Figure 5. Percentage drug lost from Ch₅ (solid line) and Dansyl₁₀ (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; △ griseofulvin. Data presented as n=3, ave ± s.d.

Figure 6. Effect of PAA and its amphiphilic PAA polymers on bovine red blood cells. **A)** % Haemolysis of ◇PAA; △Dansyl₅; ▲Dansyl₁₀; ○Fmoc₅; ● Fmoc₁₀. Data presented as n=3, ave ± s.d. **B)** Morphology of red blood cells upon 4h incubation with 1) PBS control; 2) Dansyl₅; 3) Dansyl₁₀ (6 mgmL⁻¹) (100x magnification)

Figure 7. Mean plasma griseofulvin concentration (µgmL⁻¹) following administration of griseofulvin by oral gavage in rats. Griseofulvin in water □ Ch₅, griseofulvin and ■ Dansyl₁₀, griseofulvin. Data presented as n=4, ave± s.d. * p<0.0001 polymer formulations vs. griseofulvin in water, † p < 0.001 Dansyl₁₀, vs. Ch₅

Figure 8. Proposed drug loaded polymeric self-assemblies structures in aqueous environment A) Ch and Dansyl-PAA and B) Fmoc-PAA

Table 1: The properties of PAA amphiphiles and their drug loaded polymeric self-assemblies with highest drug encapsulation. The data presented as mean \pm (s.d.), n=3. ^aThe numerals of the polymer abbreviation indicate the %expected mole modification based on initial molar feeds. P= propofol; Pred= prednisolone; Gri= griseofulvin

| ^a Polymer | CaCo ₂ IC ₅₀ ($\mu\text{g mL}^{-1}$) | %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) |