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Evaluation of liposomes coated with a pH responsive polymer

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Abstract

Liposomes have been coated with the pH responsive polymer, Eudragit S100, and the formulation's potential for lower GI targeting following oral administration assessed. Cationic liposomes were coated with the anionic polymer through simple mixing. The evolution of a polymer coat was studied using zeta potential measurements and laser diffraction size analysis. Further evidence of an association between polymer and liposome was obtained using light and cryo electron microscopy. Drug release studies were carried out at pH 1.4, pH 6.3 and pH 7.8, representing the pH conditions of the stomach, small intestine and ileocaecal junction, respectively.

The polymer significantly reduced liposomal drug release at pH 1.4 and pH 6.3 but drug release was equivalent to the uncoated control at pH 7.8, indicating that the formulation displayed appropriate pH responsive release characteristics. While the coating layer was not able to withstand the additional challenge of bile salts this reinforces the importance of evaluating these types of formulations in more complex media.

Keywords: colonic drug delivery, liposomes, oral drug delivery, targeted drug delivery

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1.0 Introduction

Liposomes have been widely explored as drug delivery vehicles for several decades, offering temporal control of drug release and/or site specific drug delivery for a wide range of drugs with different physiochemical properties. To date they have found clinical utility primarily for the treatment of severe systemic infections and cancer (Cattel *et al*., 2004), for which their parenteral delivery is necessary and appropriate. To further exploit the advantages associated with liposomes (e.g. their ability to interact with cells (Voskuhl and Ravoo, 2008), the relative ease in which they can be produced in a wide range of structural and compositional configurations (Lasic, 1998), their potential in gene transfection (Montier *et al*., 2008) and capacity to carry a vast array of chemical and biopharmaceutical drugs (Lasic, 1998) it is beneficial to explore formulations with potential for non-parenteral delivery. Indeed, a formulation suitable for oral drug delivery (widely accepted as the most practical, efficient and cost effective route for drug administration) could broaden the portfolio of applications for liposomes and open up several new avenues for treatment.

Of growing interest generally in the world of oral drug delivery is colon-targeted delivery for treatment of both local and systemic conditions. It is recognised that this region of the gastrointestinal (GI) tract offers advantages over the stomach and small intestine, e.g. milder pH, lower enzymatic activity, lower bile salt concentrations, longer residence time and slower turnover of the mucus layer. For biopharmaceutical delivery, it also appears to offer the benefit of allowing greater functioning of absorption enhancers, thus allowing reasonable bioavailability of drugs such as peptides which would normally be poorly absorbed from the GI tract (Haupt and Rubinstein, 2002; Sinha *et al*., 2007).

Several researchers have already recognised the potential of combining the advantages of liposomes and colonic drug delivery. Rubenstein's group (Tirosh *et al*., 2009 and Jubeh *et al*., 2004) have investigated liposomal adhesion to healthy and inflamed colonic mucosa *in vitro.* Their work lays important foundations for understanding how liposomes may interact with colonic tissue. D'Argenio *et al*. (2006) have considered liposomes as vehicles for delivery of carnitine for the reversal of colitis. Kesisoglou *et al.* (2005) used liposomes for encapsulating 5-aminosalicylate and 6-mercaptupurine against inflammatory bowel disease. Although for colonic action, administration of the liposomes in all of these studies was either intraluminal or *in vitro* to excised tissue; delivery via oral administration was not considered.

One study that has considered liposomes in the context of oral administration to the colon is that of Xing *et al.* (2003) who describe a multicomponent drug delivery vehicle comprising drug loaded liposomes within Eudragit-coated alginate beads. Although both *in vitro* and *in vivo* results were promising, drug release was controlled by the alginate and not the liposomes and it was not clear whether the liposomes were released to allow them to undergo the advantageous interactions with colonic mucosa that are described above. A further potential drawback of the formulation was the complexity of its preparation (particularly the multiple process steps), potentially limiting economically viable commercial manufacture.

In the present study the emphasis is therefore on simplicity of preparation, with the liposomes retaining dominance as the drug delivery vehicle. Taking the lead from the successful development of commercially available tablet formulations for colonic drug delivery (Baumgart and Sandborn, 2007), the methacrylic acid copolymer Eudragit S100 ® has been used as the coating material. This polymer, with its anionic carboxylic acid side groups, has a solubility threshold of pH 7, remaining insoluble at lower pH values. On the journey through

the gastrointestinal tract, it is generally accepted that pH 7 is not normally reached until at least the distal small bowel/ileocaecal region; thus drug release from formulations coated with Eudragit S100 is likely to commence at the junction between the small intestine and colon, continuing into the colon.

2.0 Materials and methods

2.1 Materials

Liposomal membrane components included egg phosphatidylcholine (EPC) (a gift from Lipoid, Ludwigshafen, Germany, minimum 98 % purity), cholesterol (CH) (Sigma Aldrich, Dorset, UK, and stearylamine (SA) (Sigma Aldrich). SA was incorporated to give the liposomes a positive charge, facilitating electrostatic interaction with the anionic polymer. Vitamin B_{12} (Sigma Aldrich) was chosen as a model drug due to its high solubility in all of the release media used (thus ensuring drug release would not be limited by solubility). Eudragit S100, the pH responsive polymer used for the coating of the liposomes, was a gift from Evonik (Essen, Germany). For the drug release studies 0.1 M hydrochloric acid (HCl), Hanks' balanced salt solution (99.015 mol % water, 0.95 % Hanks' balanced salt and 0.035 % sodium bicarbonate adjusted to pH 6.3 using 0.1 M HCl) and phosphate buffered saline (PBS, increased to pH 7.8 using tribasic sodium phosphate) were used to simulate the pH conditions of the stomach (Sinha and Kumaria, 2003 and Ibekwe *et al*., 2006), small intestine (Ibekwe *et al*., 2006) and ileocaecal junction (Khan *et al.*, 1999), respectively. All components for the release media were purchased from Sigma Aldrich (Dorset, UK). All other chemicals and solvents used were of an analytical grade and used as received.

2.2 Preparation of liposomes and their formulation with Eudragit S100

Liposomes were prepared using EPC and CH in the molar ratio 1:1, with SA comprising 5% of the total lipid. This level of SA (5 mol%) was chosen after an initial screening study showed that it increased the zeta potential of liposomes at pH 7.4 from -12 mV (without SA) to +63 mV. Higher levels of SA were not found to significantly increase zeta potential. The conventional thin film hydration method (Bangham *et al*., 1965) was used to produce multilamellar vesicles (MLVs) for the study. Briefly, the lipids were dissolved in 5 ml chloroform in a 50 ml round bottom flask. The chloroform was then removed using a rotary evaporator, leaving a thin lipid film on the side of the flask which was then dried under nitrogen for 2 hours to remove trace chloroform. The film was then hydrated with an aqueous solution containing 10 mg/ml of vitamin B_{12} in PBS (pH 7.4). During hydration the flask was agitated using a vortex mixer. Excess drug was removed through three cycles of centrifugation and replacement of supernatant with PBS. The final pellet was then re-suspended in 10 ml of PBS.

To prepare the coated liposomes equal volumes of liposomal suspension and aqueous solution of Eudragit S100 of various concentrations (0.0125, 0.025, 0.05 and 0.1 % w/v in PBS) were combined and hand-shaken for 2 minutes.

2.3 Characterisation of liposomes

2.3.1 Zeta potential

Changes in dispersion zeta potential as a function of Eudragit S100 concentration were determined through electrophoretic mobility measurements (Zetamaster, Malvern Instruments, UK) at pH conditions in which the polymer was insoluble. Briefly, 500 µl of the liposome/polymer suspensions (from section 2.2.) were diluted with 20 ml of distilled water

(pH<7) before introducing to the electrophoresis cell. Ten measurements were taken at 25˚C on three independent samples of each preparation.

2.3.2 Light Microscopy

Light microscopy was conducted using an Olympus BX50 light microscope interfaced with a Leica Q500IW computer, with images taken using Ph 3 (phase plate) under the phase contrast setting. A small drop of liposome sample was placed on a pre-cleaned microscope slide before covering with a cover slip. Images were taken at 1000× magnification.

2.3.3. Cryo-electron microscopy (cryo-EM)

Drops of liposomal samples were dispersed into sample wells. The sample holder was then quenched in liquid nitrogen under vacuum conditions. Fracturing of the samples was conducted within the preparation chamber through the use of a fine blade. Samples were fractured using a Polaron Polar Preparation 2000 attached to a Phillips XL 30 Environmental Scanning Electron Microscope (ESEM). The samples were then coated with gold to increase conductivity and transferred into the SEM chamber. Images were taken at a maximum voltage of 3.0 kV to reduce temperature fluctuations associated with higher voltages, with the instrument maintained at -180°C by the periodic addition of liquid nitrogen to the cooling chamber.

2.3.3 Size distribution

Vesicle size and size distribution, as a function of Eudragit S100 concentration, were measured using wet laser diffraction particle sizing (Mastersizer 2000 connected to a Hydro SM small volume sample dispersion unit, Malvern Instruments, UK). Measurements were

carried out in distilled water in which the polymer was not soluble. Three independent formulations of each preparation were each measured 5 times.

2.4 Drug release studies

Drug release studies with uncoated liposomes and liposomes + polymer were conducted in each of the different pH media described in section 2.1. For each release experiment, 1 ml of liposomal suspension was added to 40 ml of preheated (37˚C) release medium and well-agitated in an incubator maintained at 37°C. Sink conditions were maintained throughout each experiment. Aliquots of 1ml were removed at 0, 0.5, 1, 2, 4, 6, 10, 20, 30, 45, 70 and 120 hours and centrifuged to precipitate the liposomes. The concentration of released vitamin B_{12} in the supernatant was determined using UV spectrophotometry against a standard curve obtained at λ=361 nm. All measurements were taken against reference samples of the appropriate dissolution medium. For each formulation, the initial amount of drug (mg drug/ mg phospholipid) prior to release was determined by lysing the liposomes with ethanol and measuring the resulting drug concentration using UV spectroscopy, allowing drug release to be reported as a percentage of the total encapsulated.

Further drug release trials with uncoated and coated liposomes were completed in the presence of bile salts at a concentration representative of that found in the small intestine (10 mM sodium taurocholate in pH 6.3 Hanks' solution). These trials aimed to test the liposomal formulations beyond response to pH alone. Over a period of 4 hours (representative of small intestine transit time) samples were removed and analysed spectrophotometrically at λ=361nm against a reference sample of the release medium.

3.0 Results

The results presented in this section are discussed in section 4.

Table 1 shows the vesicle zeta potential as a function of polymer concentration, where the polymer concentration shown is that of original solution that was mixed with the liposomes. As no further decrease in zeta potential was seen by increasing the polymer concentration beyond 0.05 % this was assumed to be the concentration necessary to cover the surface of the liposomes and was that used in all further studies. Vesicle size (Table 1) was seen to increase with increasing polymer concentration until 0.05 % at which point there was a plateau similar to that seen for the zeta potential results.

Evidence of an association between the polymer and liposomes was also seen using light microscopy. Figure 1A shows the uncoated liposomes at pH 6.3. Typically for MLVs, the size of the vesicles was originally around 5 - 10 μm. On addition of polymer to a system at pH 7.8 no increase in size was observed (Figure 1B), consistent with the fact that the polymer was in solution at these conditions. At pH 6.3 the polymer was seen to precipitate around the vesicles forming larger agglomerates (Figure 1C). A control experiment (results not shown) in which liposomes were excluded showed that polymer 'particles' resulting from precipitation at pH 6.3 were considerably smaller (approximately 200 nm) than the liposomes used in this study. In this way, the agglomerates seen in Figure 1C were assumed to be liposomes + polymer and not precipitated polymer alone.

In Figure 2 typical images from cryo-EM are shown. In Figure 2A the lamellae and central aqueous core of liposomes are clearly visible. In the presence of polymer a crust was

observed around and across the liposomes and the lamellae were no longer visible (Figure 2B).

In Figure 3 drug release profiles for liposomes with and without polymer are shown in the different release media. At pH 1.4 and 6.3 (Figures 4A and B) the amount of drug released was significantly lower at all time points on addition of polymer (Mann Whitney U Test (chosen level of significance α=0.05). For example at pH 1.4, over a 20 hour period, only 10 % of the drug was released, which is in contrast to the 40 % release over the same time period for the uncoated formulation. Over a time period more representative of gastric residence time (boxed graph in Figure 4A) only 2.5 % was released from the coated formulation compared to 10 % for the uncoated. However it can clearly be seen that although drug release was significantly reduced it was not abolished.

Addition of bile salts to the release media significantly increased the drug release rate for both uncoated and coated liposomes. Interestingly there was no statistically significant difference between coated and uncoated formulations in the presence of bile salts indicating that both the structural integrity of the vesicles and the polymer barrier were affected by the bile salts.

4.0 Discussion

The formulation of liposomes into a preparation suitable for colon-targeted oral drug delivery could open up a range of new applications and indications extending the utility of liposomes. However, production and quality control of liposomal preparations can be difficult, hence the need to keep additional process steps and production methods as simple possible. Here we have therefore evaluated a conceptually simple idea of bringing together cationic liposomes and anionic polymer with the intention of creating a pH responsive coat around the liposomes

which would protect the vesicles en route through the stomach and the small intestine. This general route to coating has been previously explored when anionic liposomes were coated with the cationic polymer chitosan (Guo *et al*., 2003; Takeuchi *et al*., 1996, 2005), but no similar work has been completed using a pH responsive polymer for coating. The polymer Eudragit S100 was chosen as the coating material as it is widely used in both commercially available and experimental formulations for colonic targeting e.g. tablets (Khan *et al*., 1999 and 2000), microspheres (Paharia *et al*., 2007) and capsules (Kraeling and Ritschel, 1992).

The use of pH responsive materials for targeted oral delivery is not a perfect science and is not without its drawbacks. For example, substantial inter-patient differences in pH can lead to unpredictable targeting and release (Ibekwe *et al*., 2008). In the case of Eudragit S100, the likelihood of inappropriately early release upstream of the colon can also be increased when partial neutralisation of the polymer's acidic function groups is carried out to facilitate creation of an 'aqueous dispersion' for coating purposes (Ibekwe *et al*., 2006b). Hence although the coating method explored here was one involving only aqueous solutions, unmodified Eudragit S100, albeit at low concentration, has been used to reduce the risk of drug release in the small intestine.

Zeta potential measurements were used to monitor the evolution of the coat. This strategy has previously been used in the development of polymer-coated cationic and anionic liposomal formulations, where the point at which the zeta potential plateaus is taken to indicate saturation of the vesicle surface with polymer (Guo *et al*., 2003; Davidsen *et al*., 2001; Takeuchi *et al*., 2005). Results from our other studies (sizing, cryo-EM and drug release) indicate that such an assumption should be made with caution or that certainly further experimentation should always be carried out to provide information on the physical

characteristics and functionality of the coat. In Table 1, the plateau of the size increase beyond 0.05% indicates that the coat was not building up evenly – instead perhaps developing preferentially on some vesicles before others. Light microscopy images in Figure 1 point to a heterogeneous distribution of polymer and in Figure 2 a discontinuous 'crust' around the liposomes rather than a homogenous coat is observed.

Despite these observations, the polymer was able to substantially slow down drug release at pH 1.4 and 6.3, presumably acting as a diffusional barrier. However, it was unable to protect against bile salts which indicates that premature drug release and liposomal degradation could be expected *in vivo*. This is an interesting finding as it reinforces the importance of going beyond evaluation of liposomal formulations for site specific delivery in the GI tract on the basis of pH shifts alone. The addition of bile salts, while adopted by some researchers in examining *in vitro* liposomal release for oral delivery (e.g. Lee *et al*., 2005) has not been pursued by others (e.g. Guo *et al*., 2003; Filipović-Grčić *et al*., 2001).

Drug release results in Figure 4 indicated that both the liposomes and the coat were disrupted by the bile salts. It was hypothesised that damage to the coat could be due to either the bile salts interacting directly with the polymer, facilitating its dispersion, or a secondary effect of liposomal degradation i.e. once the liposomes were 'digested' the coat dispersed due to the lack of a vesicle core holding it in place. To explore which of these was more likely, we carried out an additional experiment in which Eudragit S100 powder (as received from the manufacturer) was dispersed in either Hanks' solution or Hanks' solution + sodium taurocholate and analysed using wet laser diffraction particle sizing over 2 hours. All material concentrations were equivalent to those of the drug release studies. The resulting polymer particle size distributions were identical in both dispersion media, indicating that the bile salts

did not facilitate polymer dispersion or dissolution. Additionally, infra red spectra of aqueous pastes containing polymer, bile salt and their mixture were recorded using a Fourier transform infra red (FT-IR) spectrometer (FT-IR-6300, Jasco, Great Dunmow, UK) with an attenuated total reflection (ATR) infrared optical unit (golden gate^{TM}, part number 10586, Specac Ltd., Orpington, UK). The purpose of this analysis was to test for the presence of any chemical interaction between the paste components. Any interactions between the Eudragit and the bile salt would result in a shift in the peak positions (e.g. ester vibrations at 1150 cm^{-1} and 1250 cm^{-1}, and C=O vibrations of the carboxylic acid groups at 1705 cm^{-1}) associated with the functional groups involved in the interaction. Examination of the spectra revealed no variation in peak position; in fact, the spectra could be superimposed. It therefore seems likely that disruption to the coat was due to the loss of liposome structure. While liposomes can be designed to increase their resistance to bile salts (Andrieux *et al*., 2009), it would also be necessary to improve the integrity of the coat to prevent bile salt ingress and strategies for encapsulating liposomes within microparticles are therefore being explored.

5.0 Conclusion

Eudragit S100 can be associated with cationic liposomes through a simple mixing strategy creating a barrier that significantly reduces liposomal drug release at pH conditions representative of the stomach and small intestine. The importance of evaluating coated liposomes for oral drug delivery beyond pH shift studies has been demonstrated with the addition of bile salts.

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Table 1. The effect of Eudragit S100 addition upon the particle size (d_{50}), size distribution (span*) and zeta potential of liposomes. Each value represents the overall mean of three independent experiments ± the standard error of the mean. *Span = $\frac{d90-d10}{d50}$

Concentration of polymer coating solution (%w/v)	$d_{(50)}$ (µm)	Span	Zeta potential (mV)
0	7.7 ± 0.1	1.2 ± 0.1	63 ± 2.4
0.01	13.1 ± 2.1	2.3 ± 0.2	45 ± 2.4
0.025	22.0 ± 2.8	1.9 ± 0.4	28 ± 1.9
0.05	22.0 ± 3.4	2.4 ± 0.3	-28 ± 1.3
0.1	20.0 ± 1.7	2.0 ± 0.2	-30 ± 0.5

Figure captions

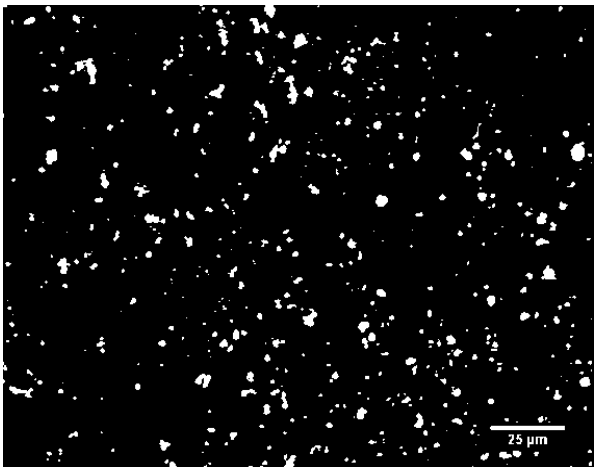
Figure 1. Light microscopy images showing liposomes: (A) without polymer, and in the presence of Eudragit S100 at (B) pH 7.8 and (C) pH 6.3.

Figure 2. Cryo-SEM images of (A) uncoated liposomes in pH 6.3 and (B) liposomes in the presence of Eudragit S100.

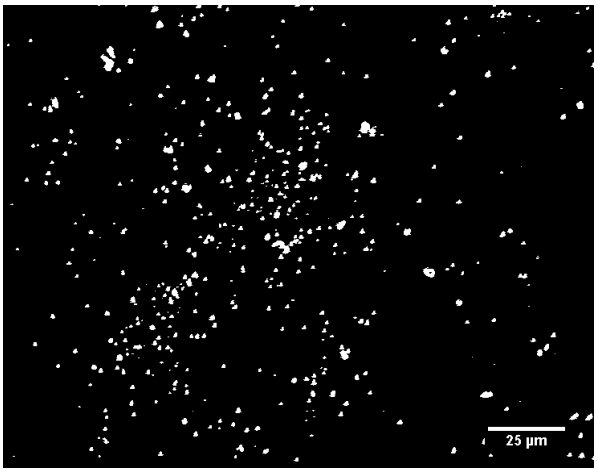
Figure 3. Drug release profiles for liposome formulations with (■) and without (◊) Eudragit S100 at (A) pH 1.4, (B) pH 6.3 and (C) pH 7.8. In Figure 4 (A) drug release over 2 hours is additionally highlighted, corresponding to the typical residence time in the stomach. Each data point represents the overall mean of three independent experiments ± the standard error of the mean.

Figure 4. Drug release profiles for liposome formulations with (●) and without (▲) Eudragit S100 at pH 6.3 in the presence of 10mM sodium taurocholate. Release data from Figure 4 (B) (no bile salts) are shown for comparison with (■) and without (◊) Eudragit S100. Each value represents the overall mean of three independent experiments ± the standard error of the mean.

(A)



(B)



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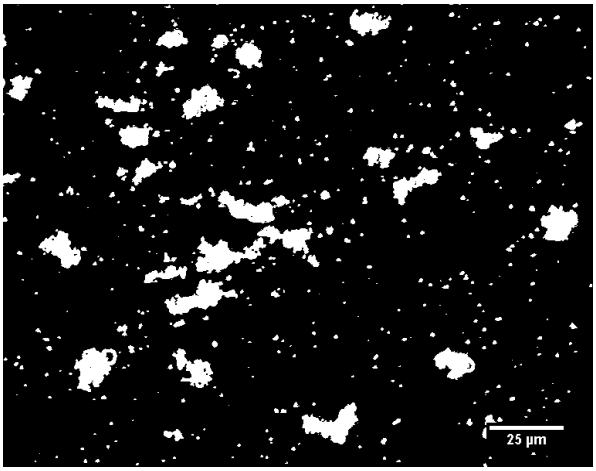
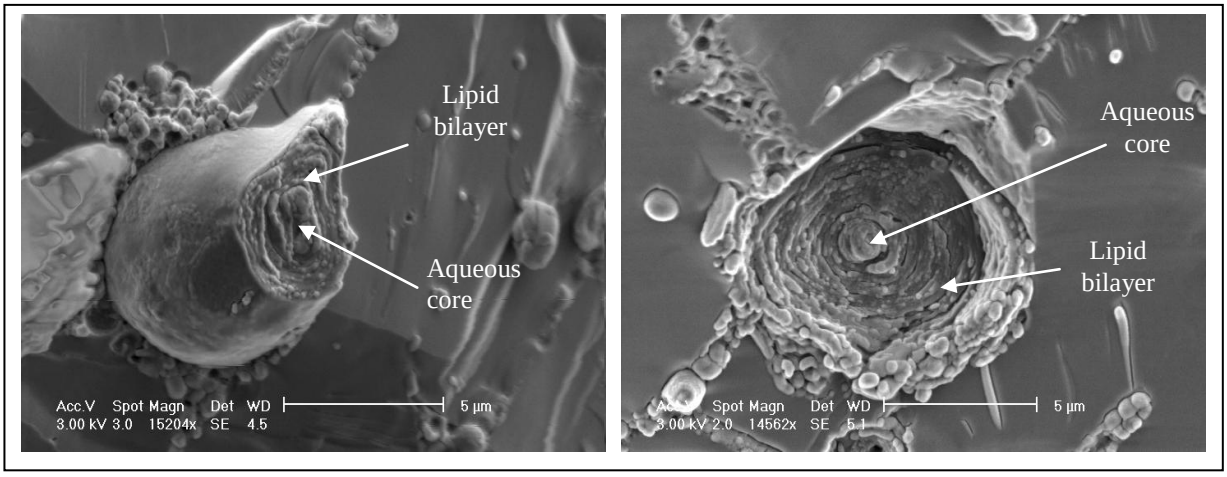
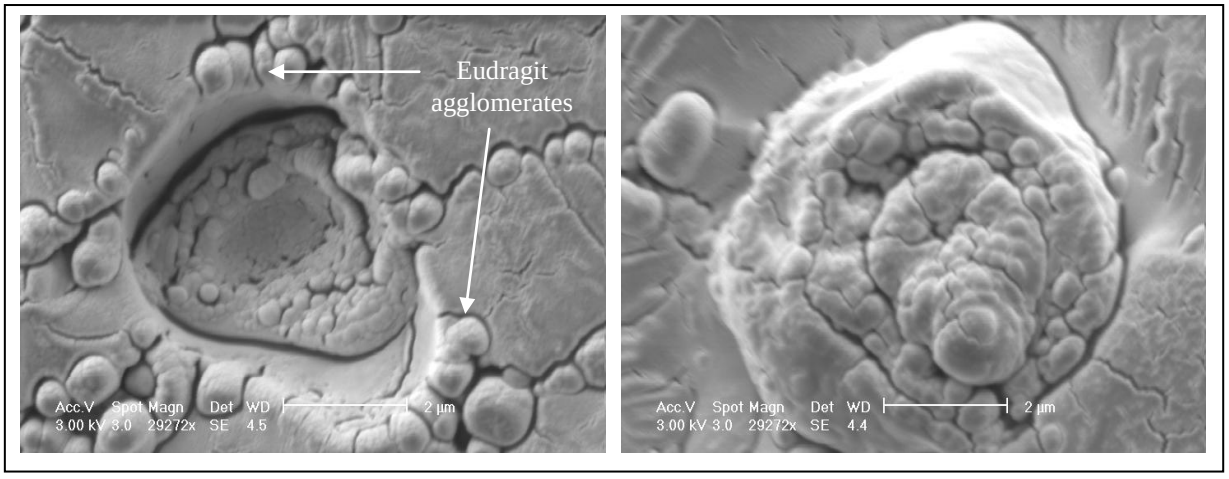


Figure 1.

(A)


Lipid bilayer
Aqueous core
Acc.V Spot Magn Det WD
3.00 kV 3.0 15204x SE 4.5
5 µm
Aqueous core
Lipid bilayer
Acc.V Spot Magn Det WD
3.00 kV 2.0 14562x SE 5.1
5 µm

(B)


Eudragit agglomerates
Acc.V Spot Magn Det WD
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2 µm
Acc.V Spot Magn Det WD
3.00 kV 3.0 29272x SE 4.4
2 µm

Figure 2.

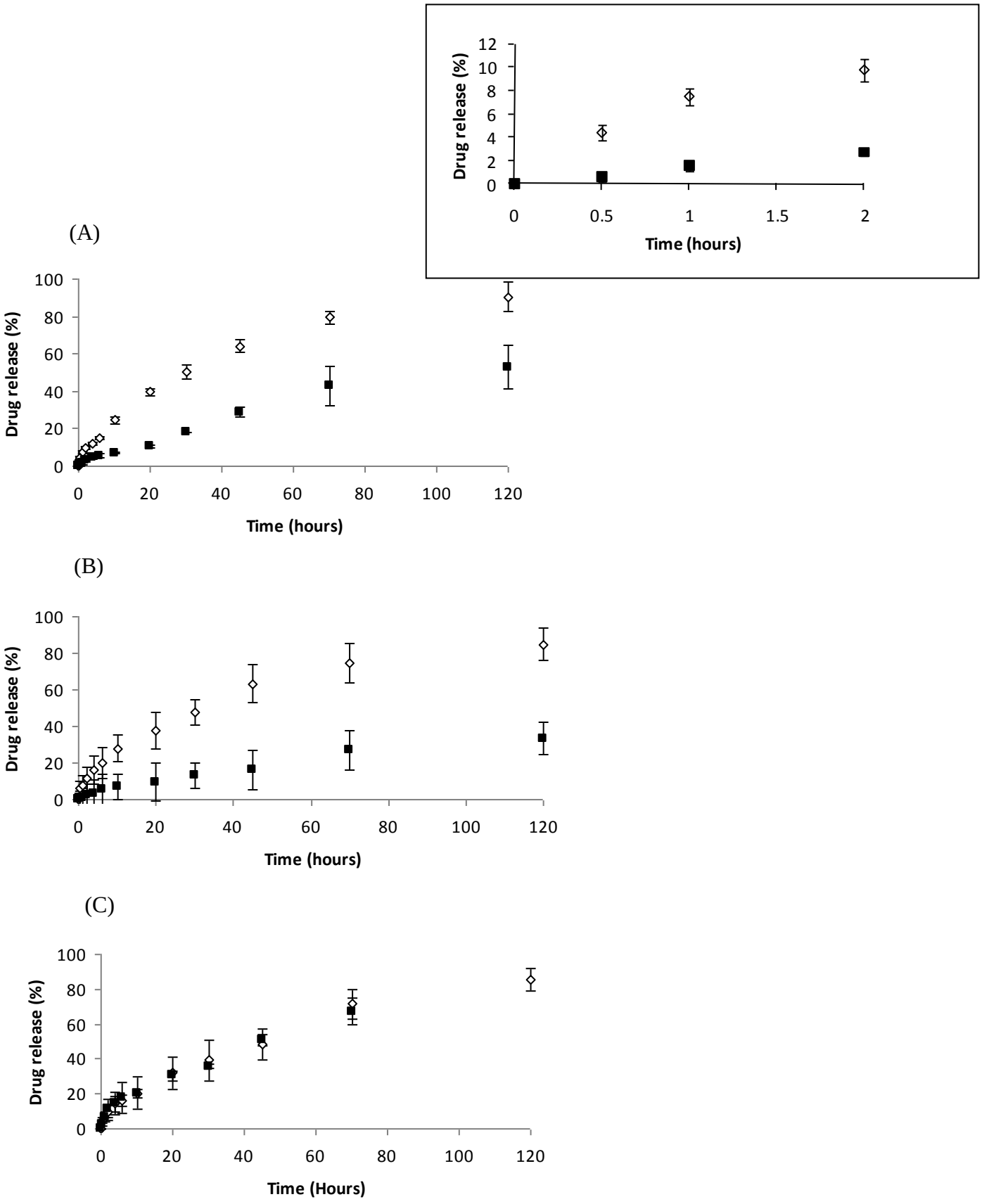
(A)
Drug release (%)
Time (hours)
(B)
Drug release (%)
Time (hours)
(C)
Drug release (%)
Time (Hours)

Figure 3.

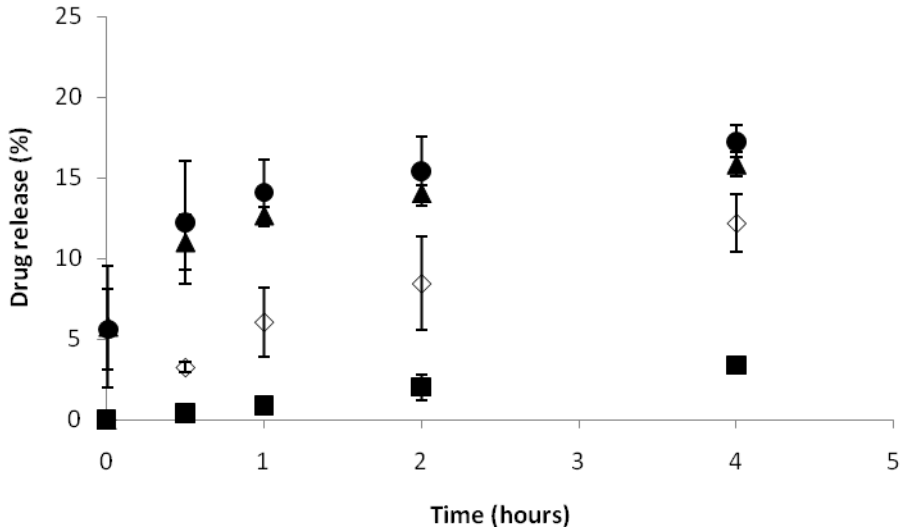

Figure 4.