AN INVESTIGATION INTO LIPOSOMAL FORMULATIONS FOR TARGETED DRUG DELIVERY TO THE COLON

Matthew Ernest John Barea

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SUMMARY

It has been well documented that the oral route is the most cost effective route for drug delivery and encourages patient compliance. Recent studies have shown the numerous advantages associated with specific drug delivery to the colon, highlighting its favourable conditions and long transit time as the main advantages. A number of *in vitro* studies also show that the delivery of liposomes to the colon could provide further advantages due to bonding to the colonic mucosa in both healthy and inflamed regions. Despite these apparent advantages no oral liposomal formulation has been developed for targeted delivery to the colon as yet. This work sets out to develop a formulation which can be taken orally, that will remain intact through the stomach and small intestine to release the liposomes, and subsequently the active ingredient, in to the colon.

Initially, experiments were conducted in which liposomes were directly coated with the pH responsive polymer Eudragit S100. Although the coating was shown to slow drug release in simple pH buffers, it was realised it could not protect the lipid membrane from the model bile salt sodium taurocholate. Development of the formulation moved onto the production of Eudragit S100 microspheres to provide a solid barrier to protect the liposomes. A number of production variables including homogenisation speed and time were investigated to provide a homogenous microsphere population suitable for encapsulating liposomes. Due to the solvents required in the microsphere production it was essential to protect the liposomes, which was done by coating them with the enzyme triggered polymer chitosan. This coating would not only protect the liposomes from the solvents but also provide a trigger release when the liposomes reach the colonic microflora. The final stage involved encapsulating chitosan-coated liposomes within the Eudragit microspheres to produce a novel, colon targeting liposome-in-microsphere (LIM) formulation. Through cryo-SEM chitosan-coated liposomes could be observed within the microsphere core. Subsequent drug release studies showed that the LIM

formulation remained intact through the simulated stomach and small intestine conditions with drug release occurring in the colonic conditions where the model enzyme β-glucosidase could solubilise the chitosan coating.

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Contents

ABBREVIATIONS

5-ASA	5-aminosalicylic acid
CH	cholesterol
DCM	dichloromethane
DOTAP	1,2-Dioleoyl-3-Trimethylammonium-Propane
DOPE	1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine
DSC	differential scanning calorimetry
EPC	egg phosphatidylcholine
FTIC	fluorescein isothiocyanate
FTIR	fourier transform infrared spectroscopy
GI	gastro intetsinal
GPC	gel permeation chromatography
GUV	giant unilamellar vesicle
HCl	hydro-chloric acid
IBD	inflammatory bowel disease
LD_{50}	median lethal dose
LIM	liposome in microsphere
LUV	large unilamellar vesicle
MLV	multilamellar large vesicle
MMC	migrating motor complex
MVV	multivesicular vesicle

OLV	oligolamellar vesicle
PDI	polydispersity index
SA	stearylamine
SEM	scanning electron microscopy
SPC	soy phosphatidylcholine
SUV	small unilamellar vesicle

1.0 Introduction

ABSTRACT

The oral drug delivery route is widely seen as the most practical and cost efficient route of administration. Despite the well documented advantages of colonic drug delivery including long residence time, neutral pH and low enzyme activity, very few oral colonic drug delivery systems have made it to market. Recent investigations have shown that liposomes have a number of advantages for the prolonged release of active ingredients in the colon. Coupled with the advantages associated with specific targeting to the colon for drug delivery it is proposed that liposomal formulations capable of targeted drug release to the colon could be useful for both localised and systemic treatment.

The first part of this chapter investigates the various routes of administration available, reviewing oral delivery in detail with an emphasis to colonic targeting. The complexities of the GI tract as a route for drug delivery are outlined, with pH and transit times being discussed in detail. It introduces the concept of various triggers for site specific drug delivery and summarises the advantages and disadvantages of each method. The second part discusses the production and applications for liposomes and introduces the possibility of coating for site specific colonic drug delivery. The final part of the chapter introduces the overall aims and objectives of this work.

1.1 Introduction

The principal goal of targeted drug delivery is to deliver the drug to a specific area with increased efficiency and reduced adverse effects than less specific drug administration. This can be achieved through the prolonged and localised delivery of the drug to the diseased tissue thus ensuring maximal therapeutic effects are achieved whilst minimising possible side effects through excessive exposure. For site specific drug delivery a number of routes of administration can be adopted, each of which have a number of advantages and disadvantages (Langer 1998) which are discussed in the following section.

1.2. Routes of drug administration

1.2.1. Enteral drug delivery

1.2.1.1. Oral administration

Oral administration is widely regarded as the most practical, efficient and cost effective route for drug dosage and is responsible for over eighty percent of the best selling pharmaceutical products available (Lennernas and Abrahamsson, 2005). It not only allows for complete patient independence and therefore compliance but it is normally cost effective to manufacture and offers a lengthy shelf life which is normally determined by the active drug itself rather than the formulation components (Mathiowitz, 1999). There are however a number of complications associated with oral drug delivery including the varying conditions observed within the GI tract and the large populations of digestive enzymes which will degrade most peptide and protein drugs (Chen and Langer 1998).

1.2.1.2. Rectal administration

The use of rectal drug administration has a number of advantages, including being able to administer drugs to the colon without needing to negotiate the hostile environments of the stomach and small intestine. The use of rectal administration also allows for specific targeting to be achieved as a number of pathways are not required to reach the colon and varying transit times across the ileo-caecal junction would not be an issue. Furthermore, the use of rectal administration allows for drugs to be effectively administered whilst the patient is undertaking bouts of vomiting, nausea or unconscious convulsions (van Hoogdalem *et al*.,

1991). The potential disadvantages of rectal administration include patient compliance and the causes of bowel movements on the administration time of the dosage.

1.2.2. Parenteral drug delivery

Parenteral delivery is the most direct route for drug delivery and therefore has a major advantage over other methods. This means the drug can be administered to the specific site instantaneously, or can be within the circulation shortly after administration, which is essential when the drug is required immediately. The parenteral administration route can be achieved through the intravenous, intramuscular or subcutaneous approach. Despite the obvious advantages of this approach there are a number of disadvantages which limit the use of parenteral administration for all applications. The administration of the drug is required to be conducted by a qualified health care professional and levels of patient compliance are low due to the associated pain and subsequent fears. Some forms of subcutaneous and intramuscular delivery can be self administered but patient compliance and willingness is still low. If parenteral administration is required then a sustained treatment is observed as more favourable as it reduces the pain associated with frequent injections and the need for a healthcare professional to administer it. Myocet® (Enzon Pharmaceuticals) is a liposomal formulation containing the active ingredient doxorubicin. It is used in the treatment of metastatic breast cancer and is a non-pegylated version i.e. it does not have the polyethylene glycol (PEG) coating associated with other liposomal formulations containing doxorubicin (Doxil®, Caelyx®).

1.2.3. Transdermal drug delivery

Transdermal delivery refers to the delivery of drugs through the skin which requires penetration through the two sublayers of the epidermis to reach the microcirculation of the dermis. The advantages associated with transdermal delivery include ease of use/application and the possibility to provide non-invasive sustained release where other routes may not be applicable (oral). A current dermally administered liposomal formulation is LMX-4 (Ferndale Pharmaceuticals Ltd) which contains the local anaesthetic lidocaine and is therefore marketed as a fast and effective pain relief from medical procedures involving injection (taking blood, insertion of cannula etc.).

1.2.4. Pulmonary drug delivery

Pulmonary drug delivery refers to drugs administered through the airways and has a number of advantages and applications. The use of pulmonary drug delivery would not only be advantageous due to the large surface area offered by the alveoli but also effective systemic treatment would be possible as well as localised treatment. Drawbacks associated with pulmonary drug delivery include the treatment can be short-term and the formulation can soon be cleared by the mucociliary escalator.

Due to the numerous advantages associated with the oral drug delivery route the current investigation will go on to explore the possibility of developing an oral formulation and the subsequent issues that may arise.

1.3. Advantages of colonic drug delivery

After briefly reviewing a number of routes of administration it is generally accepted that the oral route is the most cost effective and patient compliant route. With this in mind it is essential to determine the best site for drug delivery within the GI tract and subsequently design a formulation for the application. Over recent years the colon has received a large amount of interest as a possible drug delivery site for the treatment of both localised diseases (bowel cancer, ulcerative colitis and Crohn's disease) (McConnell *et al*., 2008; Ibekwe *et al*., 2008a; Patel *et al*., 2008), but also for the administration of systematically-acting proteins and therapeutic peptides such as insulin (Tiwari *et al*., 2010). Protein and peptide drugs are known to be degraded by digestive enzymes present within the stomach and small intestine. These proteases are lower in concentration in the colon which means it is a more favourable site for delivery (Kumar *et al*., 2011). The reduced proteolytic activity in the colon compared to that of the small intestine mean certain drugs that are enzymatically labile in the small intestine would be more effectively absorbed (Mathiowitz, 1999). The specific targeted delivery to the colon would also allow the dosage to be reduced, therefore minimising any possible systemic side effects that may occur. As an example, the specific treatment of ulcerative colitis is through the administration of the active ingredient 5-aminosalicylic acid (5-ASA; Mesalazine) which is available in tablet, enema and suppository formulations. It has been shown that the oral formulation has a high absorption level within the upper GI tract of approximately 75% whilst only 19% was absorbed in the colon (Segars and Gales, 1992).

Due to the topical nature of the drug administration of 5-ASA it is necessary to get the highest efficiency of delivery to the site of infection which is the large intestine. With this in mind it has been shown that the use of enemas and suppositories have proven the most effective at treating ulcerative colitis, but the problem of patient compliance remains.

The colon offers a number of further advantages for drug delivery including a mild pH and a long residence time (on average 35 hours in total), which are reviewed within this chapter. Few drugs (e.g. nisodipine and dilazep hydrochloride) are known to be preferentially absorbed in the colon; therefore specific formulations are required to target the colon (Fasinu *et al*., 2011).

1.4. Anatomy and physiology of the gastro intestinal (GI) tract

Although oral administration to the colon offers considerable advantages it is also the most complicated route of drug delivery due to the wide variation in pH, enzyme and bacterial levels. It is therefore essential to completely understand the environment that the formulation will be exposed to and any influences they may have. This not only entails understanding the different conditions that are found within the GI tract but also understanding the patient variability that can occur, especially regarding both transit times and chemical differences. It is also important to understand that these conditions can fluctuate through varying illnesses which either need to be taken into account when designing specific formulations, or ideally, producing a formulation with certain flexibility to take into account these changes. This has also led to the concept of absorption windows whereby certain drugs display region specific absorption and therefore drug exposure at the specific site is essential for effective drug dosing (Davis, 2005). To review the GI tract it can be split into the three main sections of the stomach, small intestine and large intestine (Figure 1.1).

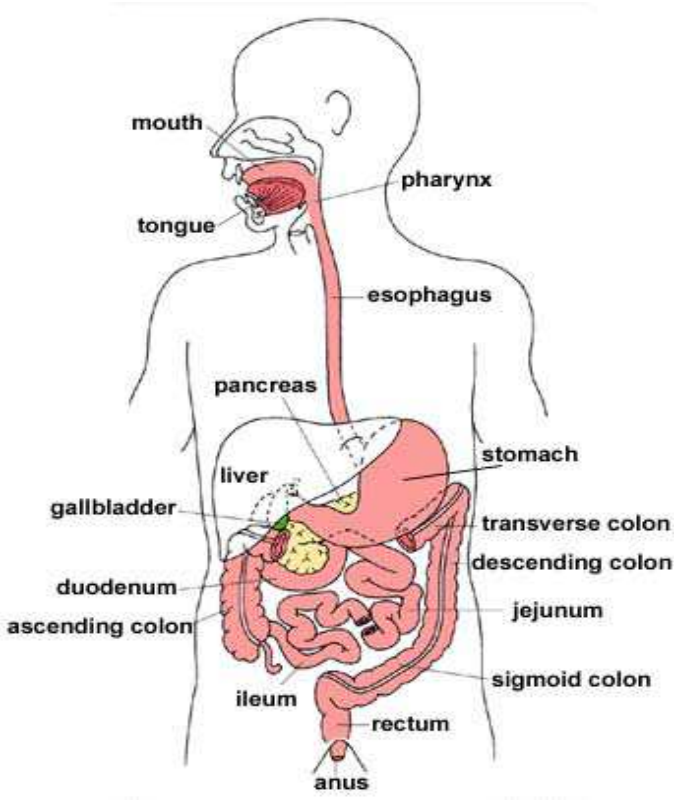


Figure 1.1. Structure of the gastrointestinal tract showing the position of the stomach, small intestine (duodenum, jejunum, ileum) and the colon (http://www.umm.edu/digest/howworks.htm).

1.4.1. Anatomy and physiology of the stomach

After oral ingestion food travels down the oesophagus (20 – 30 seconds transit time) to the stomach, whereby digestion starts through the storage, mixing and production of a chyme to then be processed further in the small intestine. The stomach is a muscular cavity which produces gastric secretions upon the entering of food. The gastric juices that are secreted are made up of water, hydrochloric acid (HCl) (0.15 M), pepsin and mucin. The presence of the hydrochloric acid makes the pH of the stomach approximately 1-3, which is an optimum pH for the protein digesting enzymes present in the gastric juice to inhabit. The low pH coupled with a high intensity of stomach contractions makes for a hostile environment for orally administered formulations to travel. The exit of food from the stomach is controlled by the pyloric sphincter which relaxes to release the stomach contents into the small intestine. This release will only occur for smaller objects within the fed stomach (<10mm) whilst larger objects (>20 mm) will be retained and processed until broken into smaller particles (Davis, 2005).

1.4.1.1. Structure of the stomach

The stomach is a large muscular sac located high in the abdominal cavity just below the diaphragm. The stomach wall is made up of four layers; the mucosa, submucosa, muscularis externa and the serosa. When in the fasted state the mucosa of the stomach lies in folds but can then distend to approximately 5 dm^3 after a large meal (Clegg and Mackean, 1999). The muscularis externa has three separate layers of muscle which through their contraction causes the contents of the stomach to be continually mixed and churned.

1.4.1.2. pH changes in the stomach

The pH changes throughout the GI tract have been well investigated observing a number of variables for subjects including feed status, age, sex and health. The standard method to measure GI tract pH uses a radiotelemetric capsule which is orally taken and can emit a signal to an antenna every second to record the pH (Ibekwe *et al*., 2008a; Ewe *et al*., 1999). Experiments taken place in both the fasted and fed states show that the pH of the stomach is in the region of 1 – 3 (Ibekwe *et al*., 2008a; Dressman *et al*., 1990; Fallingborg *et al*., 1989), which is due to the concentration of HCl. As a result of all the *in vivo* measurements the British Pharmacoepoeia recommends a pH 1-1.5 0.1M HCl to be used for simulated gastric media when assessing delayed release dosage forms (British Pharmacoepoeia 2010, Appendix XII A309).

1.4.1.3. Transit times in the stomach

The transit time within the stomach can vary considerably and is mainly dependent upon the feed status of the subject (Varum *et al.*, 2008). The stomach transit time in the fasted state is normally in the range 1 – 2 hours which is due to the migrating myoelectric complex (MMC). The MMC involves a prolonged period (40 – 60 minutes) of low mechanical activity followed by intense regular contractions of 4 – 6 minutes, before emptying of the stomach into the small intestine (Davis, 2005). The MMC can be interrupted by the presence of food and therefore what is normally a regular transit time becomes irregular and can be in the range of a few seconds through to a number of hours. It has also been shown that the size of the formulation can influence transit time in the fed state with smaller dosage forms having a more reliable transit time but larger formulations being retained in the stomach.

1.4.2. Anatomy and physiology of the small intestine

1.4.2.1. Structure of the small intestine

The small intestine is on average 5 metres long, with a normal range of 3-7 metres and is responsible for the majority of digestion and food absorption that takes place in the human body. As the main role of the small intestine is digestion and absorption, this also makes it the primary site for drug absorption. The small intestine is split into the duodenum, jejunum and ileum, with each section having different characteristics and therefore providing a varying environment for drug dosage formulations. A dosage form in the small intestine will be subjected to a completely different environment compared to that of the stomach due to its relatively high pH and presence of bile salts. The small intestine achieves high levels of absorption through its greatly increased surface area, which is provided by the large population of villi and microvilli lining the epithelial surface (Figure 1.2). The role of digestion is completed by the digestive juices which are present within the small intestine or secreted due to a reflex response or hormone trigger. The two main digestive juices found in the small intestine are bile and pancreatic juice.

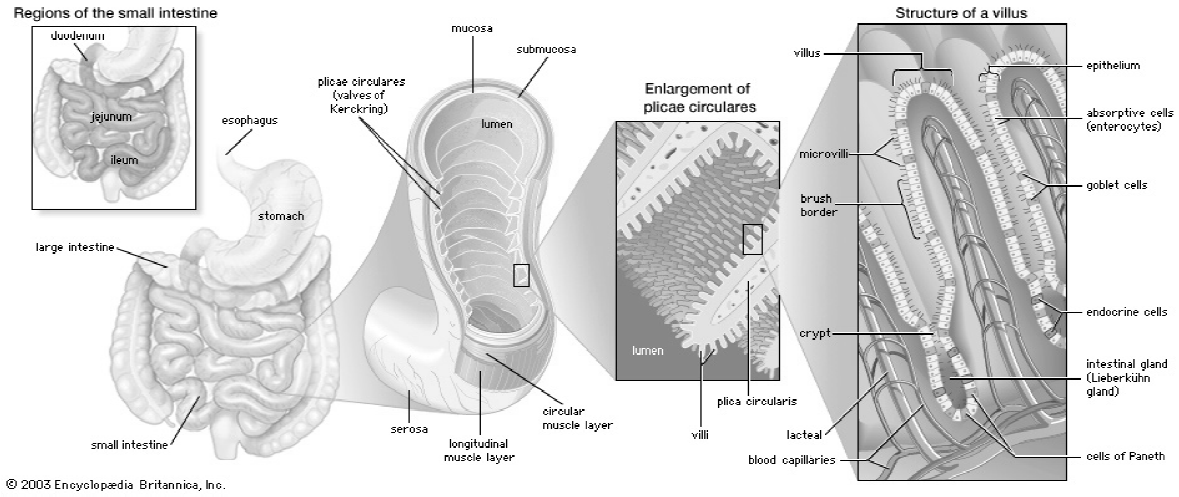


Figure 1.2. Schematic diagram showing the sections of the small intestine and the subsequent wall structure showing the villi and microvilli (http://www.britannica.com/EBchecked/topic/549336/small-intestine).

1.4.2.2. pH changes of the small intestine

Studies have generally showed similar values for the pH within the stomach, but the small intestine shows more of a range of pH values. pH changes within the small intestine can be

observed not only due to individual variability, but also variability has been observed over 7 days for the same subject (Ibekwe *et al*., 2008).

The small intestine has a pH ranging from 5.7 in the pylorus to 7.7 in the ileum. The pH in the duodenum is normally measured within the range of 6.0 to 6.6 in both the fasted and fed states (Ibekwe *et al*., 2008; McConnell *et al*., 2008; Fallingborg *et al*., 1989; Evans *et al*., 1988). The pH then gradually increases down the small intestine with values in the range of pH 6.8 – 7.4 (Ibekwe *et al*., 2008; Evans *et al*., 1988) observed for the jejunum. The pH of the small intestine then peaks at the ileum whereby values recorded have been in the range of 7.2 – 7.7 (Ibekwe *et al*., 2008a; Fallingborg *et al*., 1989; Evans *et al*., 1988). This patient variability in pH through the small intestine can therefore be problematic when designing a pH responsive drug dosage formulation as each patient could have a different point within the GI tract where drug release will be initiated.

1.4.2.3. Transit times in the small intestine

The transit times for the small intestine can range considerably from 1-6 hours depending upon the subject, with the mean being 3 hours (McConnell *et al*., 2008; Davis *et al*., 1986). The transit time for dosage forms of differing size is statistically similar in both the fasted and fed states (Rouge *et al*., 1996). The range in small intestine transit has not only been attributed to subject variability but it has been suggested that the timing of food ingestion may have a considerable influence on the transit time (McConnell *et al*., 2008). It has been suggested that the intestinal motility may be influenced by the MMC and the subsequent points along the GI tract (oesophagus, throughout the small intestine).

1.4.2.4. Digestive juices found within the small intestine

Bile is produced in the liver cells, stored in the gall bladder and released into the duodenum via the bile duct. Bile is made up of bile salts, bile pigments, cholesterol and salts. The main role of bile is to lower the surface tension of fat globules allowing emulsification to take place and therefore aid the subsequent hydrolysis of fat by lipase. The enterohepatic (bile) system is an efficient one with more than 95% of bile salts being reabsorbed in the terminal ileum, with less than 5% (0.2 – 0.6 g) being lost in faeces daily (Redinger, 2003). The reabsorbed bile salts are then actively transported to the gall bladder where newly synthesised bile salts can replenish to the required levels. Bile salts are bile acids that have been

compounded with a cation, usually sodium. Approximately 80% of bile salts in the human body are made up from the salts of taurocholic and glycocolic acids and therefore *in vitro* studies can be completed using the model bile salt sodium taurocholate.

Pancreatic juice includes a number of enzymes including amylase, trypsin, peptidases and lipases which are responsible for a number of roles including the hydrolysis of starch to maltose and the hydrolysis of proteins to polypeptides.

1.4.3. Anatomy and physiology of the large intestine

1.4.3.1. Structure of the large intestine

The large intestine consists of the caecum and appendix, colon and rectum and is approximately 1.5 metres long. The large intestine has a diameter in the range of 6 – 6.3 cm and therefore is approximately twice that of the small intestine. The large intestine is joined to the small intestine at the ileo-caecal junction where the entry of digested food is controlled by the ileo-caecal valve. The large intestine is structurally very similar to the small intestine but without the villi for increased surface area. The colon's two main roles involve absorption, which takes place in the proximal half, and storage, which occurs in the distal half. These two processes result in the colon having a lower water content and fluid mobility than other areas of the GI tract. These conditions mean any drug residing in the colon will have higher residency times due to the reduced capacity of enzymatic biodegradation, which will in turn allow for the maximum possible drug uptake efficiency in patients.

Some of the advantages associated with colon specific drug delivery can be seen as problematic for example the reduction in water content can lead to sluggish and propulsive movements which may lead to size segregation. Furthermore, the variability of transit time for both the stomach and small intestine may influence certain drugs that have a reduced absorption window.

1.4.3.2. pH changes of the large intestine

When previously designing colonic drug delivery systems it was generally accepted that the pH of the GI tract continued to rise through to the colon, therefore allowing for specific pH controlled release to be observed. However, it has been shown that the GI tract pH increases distally to a peak at the ileo-caecal junction (Ibekwe *et al*., 2008a; McConnell *et al*., 2008).

The pH has then been shown to reduce in to the range 5.6 - 6.2, which is a significant decrease in comparison to the distal small intestine (Evans *et al*., 1988; Fallingborg *et al*., 1989; Ibekwe *et al*., 2008a). Once the pH has decreased on arrival into the large intestine very few fluctuations in pH are observed, which is due to the low motility experienced throughout. A limited range in pH is seen through the large intestine of a healthy subject but some variability can be seen due to certain diseased states (McConnell *et al*., 2008). Reduced pH values of 4.7 and 5.3 have been recorded for ulcerative colitis and Crohn's disease respectively. This indicates not only patient variability needs to be taken into account but also the effect the specific disease may have on the targeted area.

1.4.3.3. Transit times of the large intestine

The transit times observed within the large intestine can vary considerably, which provides a number of issues if designing a time-dependant drug delivery system. Before entering the colon it has been shown that dosage forms accumulate in the ileo-caecal junction for a variable period of time up to approximately 3 hours (Ibekwe *et al*., 2008b). Despite this, Ibekwe *et al*. (2008b) observed an average transit time of 74 minutes for the fasted state and 81 minutes in the fed state for the ileocaecal junction, with only one subject from eight showing a transit time of over 100 minutes. The variable transit times are continued through the ascending (up to 14 hours) and transverse colon (up to 48 hours), with transit through the descending colon being relatively fast (Varum *et al*., 2010).

1.4.3.4. Microflora of the large intestine

The stable pH and lengthy transit times found within the large intestine provide the perfect conditions for bacterial growth with over 400 species residing and a range of $10^{11} – 10^{12}$ CFU/g in comparison to the stomach (10^2 CFU/g) and small intestine ($10^4 – 10^7$ CFU/g). The main bacteria found in the large intestine are that of the oxygen intolerant anaerobic nature with the main isolated species being *Bacteroides*, B*ifidobacterium* and *Eubacterium* (Gorbach *et al*., 1967). The energy needs of the bacteria are fulfilled by fermenting various types of substrates (di, tri – polysaccharides, mucopolysaccharides) that have been left undigested by the small intestine. The colonic microflora are able to break down these polysaccharides by producing a large number of reductase enzymes and polysaccharidases (Jain *et al*., 2007).

The previous sections have outlined the varying conditions of the GI tract and the subsequent issues that may arise when designing a targeted drug delivery formulation. Transit times and varying pH values throughout the GI tract have been summarised (Tables 1.5 and 1.6).

Table 1.5. Table summarising the mean varying pH values recorded throughout the GI tract for healthy fasted subjects in a number of studies.

Reference	**Stomach**	**Small intestine**			**Large intestine**		
		Duodenum	Jejunum	Ileum	Proximal colon	Mid colon	Distal colon
Evans *et al*., 1988	1.0 – 2.5	6.6	6.63	7.49	6.37	6.61	7.04
Fallingborg *et al*., 1989	1 – 3	6.4	7.0	7.3	5.6	5.7	6.6
Dressman *et al*., 1990	1.7	6.1					
Pye *et al*., 1990		6.6	7.4	7.5	6.4	6.6	7.0
Russell *et al*., 1993	1.3	6.5					
Ibekwe *et al*., 2008a	1.4	6.5	6.8	7.2	6.5		

Table 1.6. Table summarising the varying transit times (hours) recorded throughout the GI tract for healthy fasted subjects in a number of studies.

Reference	**Stomach**	**Small intestine**			**Large intestine**		
		Duodenum	Jejunum	Ileum	Proximal colon	Mid colon	Distal colon
Davis *et al*., 1986		3 - 4					
Evans *et al*., 1988		5.7					
Fallingborg *et al*., 1989	0.9	8			17.5		
Wilding, 2001	0 - 2		0.5 - 2	0.5 – 2.5	2 - 72		
Rouge *et al*., 1996		3 ± 1			1 - 60		

In summary, the colon offers an advantageous site for drug delivery due to its near neutral pH, long transit time and relatively low proteolytic enzyme activity (Yang *et al*., 2002). Not only is the colon advantageous for the treatment of localised diseases but it also a suitable site for systemic treatments. The main challenges associated with targeting the colon for drug delivery is being able to preserve the formulation through the harsh acidic conditions of the stomach and subsequent rise in pH coupled with the influence of bile salts in the small intestine.

1.6. Strategies for ensuring site-specific targeting and release to the colon

A number of challenges have been presented when attempting to create a formulation for colon-specific drug delivery (Kumar *et al*., 2011):

(i) The primary challenge is that of creating a formulation that can withstand the upper GI tract and lead to subsequent drug release in the near neutral environment of the colon.

(ii) Due to the high viscosity of the colonic fluid it is assumed that drugs need to be in the form of a solution within the colon to allow for successful delivery.

(iii) The effect of the resident microflora needs to be taken into account as it may cause the metabolic degradation of the drug.

(iv) Drug transport across the mucosa and into the systemic circulation can be reduced by the low surface area in the colon.

With these challenges in mind a number of polymers have been investigated to produce formulations suitable for colonic drug delivery. These polymers can be used in a variety of ways from a direct spray coating formulation through to microsphere formulations composed of a number of polymer layers. Each coating polymer can be categorised depending upon their specific trigger within the GI tract i.e. what causes them to solubilise, degrade or swell to cause the drug release.

1.6.1. Time dependent triggers

Time dependent formulations work on the concept of releasing the drug after a specified time e.g. taking into account transit through the stomach and small intestine to subsequently release in the large intestine. Time delayed systems that have been developed (e.g. Pulsincap, Time-Clock) rely on a multi component system which involves the use of an outer enteric coating which is insoluble in the stomach (Philip *et al*., 2010; Hebden *et al*., 1999; Steed *et al*., 1997). This allows for any variety in stomach transit time to be exempt from the calculations for the time controlled component of the formulation i.e. only the small intestine and ileo-caecal junction need to be accounted for. An example of a time dependant formulation is that of the enteric coated time-release press coated (ETP) tablets whereby a multilayer concept is adopted (Philip *et al*., 2010) (Figure 1.3). The formulation consists of a drug containing core tablet with a rapid release function, which is surrounded by a press coated swellable hydrophobic polymer Hydroxypropyl cellulose (HPC). The HPC layer provides a timed released function and is dependent upon the weight or composition of the layer. The final coating is an enteric layer which not only protects the formulation through the stomach but also means the varying transit time of the stomach does not need to be accounted for. Despite this the varying transit times through the GI tract remain an issue with the possibility of varying transit times through the small and large intestine causing either

premature drug release or even reducing drug release if colonic transit is accelerated, as has been observed in patients with IBD (Philip *et al*., 2010)

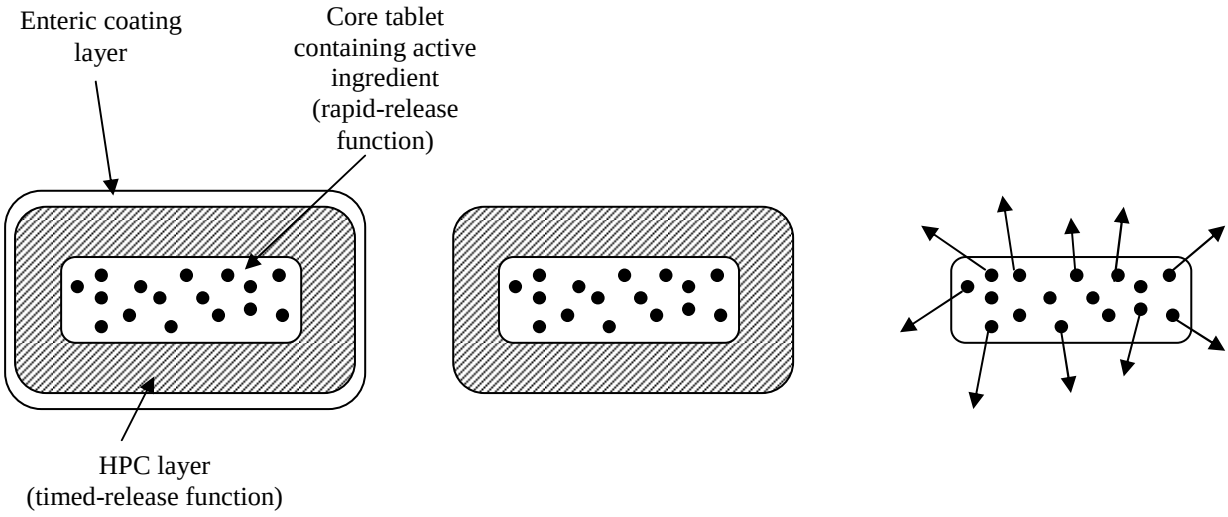


Figure 1.3. Schematic diagram showing the design of the ETP tablet. The enteric coating is rapidly dissolved after gastric emptying, exposing the timed release HPC layer. The HPC layer slowly erodes throughout the small intestine to expose the rapid release core tablet for colonic drug delivery.

1.6.2. pH dependent triggers

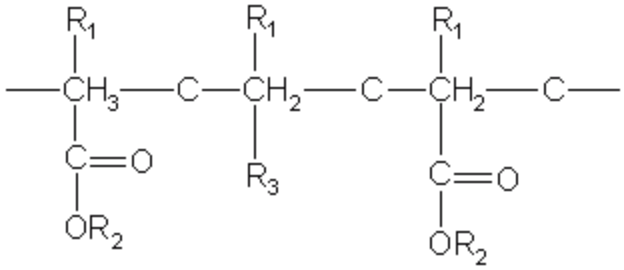
pH dependent formulations attempt to exploit the increase in pH that is observed along the GI tract, from the acidic stomach through to the slightly alkaline ileo-caecal junction. Coating the formulation with a pH sensitive polymer would protect the contents to the point whereby a pH is reached that would solubilise the polymer and therefore release the active component. There is a range of pH responsive polymers that may be utilised in the production of a pH controlled drug dosage form (Table 1.7). From the pH levels through the small intestine (section 1.3.2.2) it is apparent that for ileo-caecal and subsequent colonic drug delivery the polymer would need a pH threshold level of approximately 7 as any polymer below this level would begin to release in the duodenum. Despite the pH of the ileum increasing to above 7 in most cases the nature of the polymer solubilisation would mean that drug delivery would not occur until the large intestine. Despite this due to the nature of the pH targeting a number of researchers refer to it as ileo-caecal junction delivery as opposed to colon specific delivery. With this in mind the most widely used polymers for colonic drug delivery are within the

Polymethacrylic-acid-methylmethacrylate-ester-copolymer, brand name Eudragits (Evonik, Darmstadt, Germany).

Table 1.7. Widely used pH responsive polymers in oral drug delivery.

Brand name	Polymer	Target site	Threshold pH	References
Eudragit L100 (Evonik)	Poly(methacylic acid-co-methyl methacrylate) 1:1	Jejunum	6.0	(Eudragit.evonik.com)
Eudragit S100 (Evonik)	Poly(methacylic acid-co-methyl methacrylate) 1:2	Colon	7.0	(Eudragit.evonik.com)
Eudragit L-30D-55 (Evonik)	Poly(methacylic acid-co-ethyl acrylate) 1:1	Duodenum	5.5	(Eudragit.evonik.com)
Eudragit FS 30D (Evonik)	Poly(methyl acrylate-co-methyl methacrylate-co-methacrylic acid) 7:3:1	Colon	7	(Eudragit.evonik.com)
Opadry, Sureteric (Colorcon Ltd.)	Polyvinyl acetate phthalate (PVAP)		5.0	Liu *et al*., 2011;
CAP	Cellulose acetate phthalate		6.0	Dalmoro *et al*. (2010); Liu *et al*. (2011)

pH responsive Eudragit polymers are acrylate-based pharmaceutical polymers which include methacrylic acid copolymers (gastroresistant) and aminoalkyl methacrylate copolymers (gastrosoluble) (Röhm Pharma Polymers) (Figure 1.4). There are a number of methacrylic acid copolymers in the Eudragit range which all dissolve at different pH conditions and subsequently at a different section of the GI tract (Figure 1.5). For the application of colonic drug delivery, Eudragit L100 and S100 are mostly used, which dissolve within the pH range of 6 - 7 and 7 - 8 respectively. This indicates that the use of Eudragit S100 as an enteric coating would provide colon targeted drug delivery, with dissolution of the coating being initiated through the jejunum/ileum regions where the pH has been seen to increase to a maximum of approximately 7.2 at the ileo-caecal junction (Ibekwe *et al*., 2008a).



$R_1 = CH_3$, H
$R_2 = CH_3$, CH_3CH_2
R_3 = COOH (Eudragit® L and S)
$R_3 = COOCH_2CH_2N$ (CH3) Cl- (Eudragit® RL and RS)

Figure 1.4. Chemical structures of Eudragit S,L, RL and RS (Chourasia and Jain, 2003).

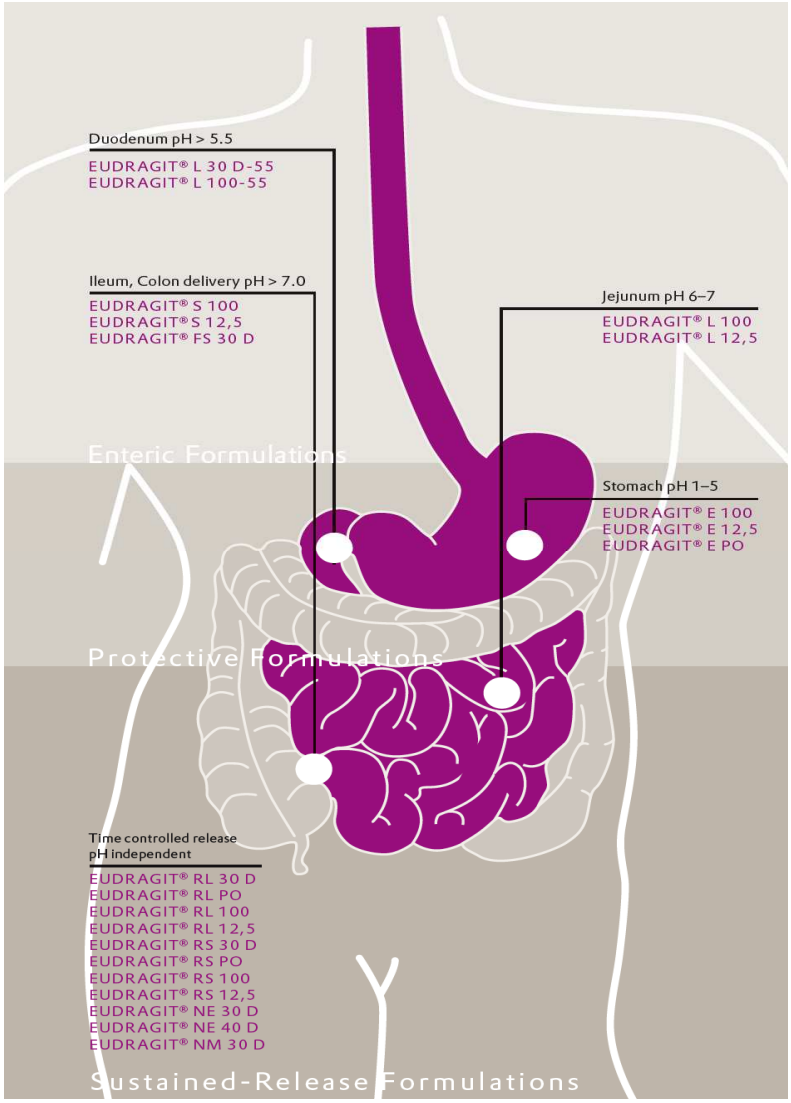


Figure 1.5. Range of pH responsive Eudragit polymers and their anticipated point of solubilisation. (www.evonik.com)

The use of Eudragit S100 and L100 as pH responsive drug formulations is well proven, with a number of products currently on the market including Asacol (Medeva Pharma) and Salofalk (Dr Falk Pharma). Despite these drugs making it to market a number of studies have indicated that in some patients Asacol failed to disintegrate in the GI tract (Schroeder *et al*., 1987; Sinha *et al*., 2003). The inability of Asacol to disintegrate during GI tract transit was attributed to rapid small intestine transit and therefore implying the formulation was not subjected to a pH above 7 for a long enough duration. This theory is reinforced by Ibekwe *et al*. (2008a) where the *in vitro* disintegration of Eudragit S100 coated tablets was compared to the relative pH and transit time throughout the GI tract of the participant. It was shown that in seven out of eight participants tablet disintegration occurred in the ascending colon during the fasted state, with one participant showing disintegration in the ileo-caecal junction. Contrasting this, in both the fasted and fed states it was observed that in three out of the eight subjects the tablet failed to disintegrate. The three tablets that failed to disintegrate could not be attributed to a single limiting factor (pH value, residence time), but was attributed to a number of factors that make up the complexities of pH responsive formulation *in vivo*. The majority of tablets in the fed state were seen to disintegrate in the ileo-caecal junction and therefore Ibekwe *et al*. (2008a) defined the use of Eudragit S100 for ileo-caecal targeting as opposed to the normally defined colonic targeting. The drop in pH observed upon entry to the small intestine would indicate Eudragit S100 would begin to solubilise prior to reaching the colon, but due to the quick transit time through the ileo-caecal junction and the nature of delayed release formulations, it is anticipated that minimal drug release will occur prior to the colonic region.

A number of studies conducted by Khan *et al*. (1999 and 2000) showed that it was possible to modify the polymer characteristics, through the use of a combination of different grades of Eudragit (S100 and L100) as an enteric coating. Khan *et al*., (1999, 2000) showed that the addition of Eudragit L100 to Eudragit S100 in varying ratios altered the pH at which the tablet solubilised. This would enable formulations to be created with high accuracy, facilitating the possibility of tailoring them, depending upon the specific GI tract pH of the patient. Although this work proved useful in indicating that specific formulations can be produced, the inclusion of Eudragit L100 led to polymer solubilisation occurring at a pH less than 7, which would therefore cause drug release in the proximal small intestine, as opposed to the distal region at the ileo-caecal junction. Furthermore, despite the ability to produce a

more specific pH solubilisation point between the range of Eudragit L100 and S100 (pH 6 and 7), it would still be susceptible to the intra and inter patient variables, as discussed previously. With this in mind, it is hypothesised that a system using the pH threshold of Eudragit S100 would enable solubilisation in all patients at the distal section of the small intestine, with a view for continued drug release throughout the large intestine.

1.6.3. Microbially triggered polymers

Microbially triggered polymers aim to utilise the vast quantity of anaerobic bacteria found within the colon (section 1.4.3.4.). The main polymers that have been investigated are non-starch polysaccharides which can only be solubilised by the microflora in the colon and therefore will remain intact throughout the stomach and small intestine. Examples of the biodegradable polysaccharides used in colonic drug delivery include amylase, chitosan dextran, guar gum and pectin (Kumar *et al*., 2011). Each of these polysaccharides are solubilised within the large intestine due to specific enzymes as they are resistant to GI enzymes found within the small intestine. These polysaccharides are also soluble in acidic conditions and therefore an enteric coating would be required to prevent solubilisation in the stomach. Of the polysaccharides investigated a number of studies have managed to coat liposomes with chitosan through a number of techniques (Mady *et al*., 2009; Laye *et al*., 2008; Wei and Bin, 2003; Guo *et al*., 2003; Henriksen *et al*., 1994 and 1997).

1.7. Systems for colon specific drug delivery

A number of approaches have been adopted in creating formulations for drug delivery to the colon, each of which using either one or a combination of the polymers previously introduced. A number of formulations have shown varied degrees of success through both *in vitro* and *in vivo* trials, each of which are outlined in the following section.

1.7.1. Prodrugs

Prodrugs are pharmacologically inactive derivatives of a parent drug molecule that require *in vivo* transformation, either spontaneously or by enzymatic degradation, to form the active drug (Tiwari *et al*., 2010). Prodrugs for colonic drug delivery are hydrolysed by the extensive number of enzymes produced by the colonic microflora including azoreductase, β-galactosidase, β-xylosidase, nitro-reductase and glycosidase deaminase (Sinha and Kumria,

2001). These enzymes have led to a number of conjugates being investigated as possible prodrugs for colonic drug delivery including amino acid, glycoside, glucuronide, azo, dextran, and cyclodextrin. A prodrug for colonic drug delivery is normally successful if it is hydrophilic and bulky to minimise absorption in the upper GI tract, but upon arrival into the colon is converted into a more lipophilic drug molecule which is available for absorption. The advantages of prodrugs include their ability to deliver high percentages of the active ingredient directly to the colon with limited side effects and much simpler production techniques required in comparison to more complex multi-particulate drug carrier systems.

The prodrug approach has a number of disadvantages including the lack of versatility as the formulation depends upon the functional group available on the drug moiety for chemical linkage. A further drawback is the fact that prodrugs are classified as new chemical entities and would therefore require more extensive trials than using an existing active ingredient in a drug delivery formulation. Furthermore to this the possible biological side effects of the carrier molecule are relatively unknown and would therefore require further investigation or prodrugs would need to be limited to the use of natural polysaccharide carriers (dextrans, cyclodextrins) as opposed to the synthetic carriers.

1.7.2. Pressure-controlled colon delivery capsules (PCDCs)

Digestive processes in the colon are controlled by forcible peristaltic movements that are termed mass peristalsis (Patel *et al*., 2008). The mass peristaltic waves only occur three to four times a day and are of a short duration. Despite this, the mass peristalsis temporarily increases the luminal pressure within the colon, which can be exploited for targeted drug delivery. PCDCs were initially developed by Takaya *et al*., (1995) by producing capsular shaped suppositories coated with the water-insoluble polymer ethyl cellulose. Sufficient fluid is present in the stomach and small intestine to prevent drug leakage. Due to the reabsorption of water in the colon, the viscosity of the luminal content increases, causing mass peristalstis. The increased pressure associated with mass peristalsis (reported up to 110 mmHg in healthy subjects with a duration of 14 seconds (Rao *et al.*, 2001)), causes the PCDC to rupture and release the contents into the colon. The variables reported to influence the performance of PCDCs are the thickness of the ethylcellulose membrane, and the capsule size and density.

1.7.3. Azo hydrogels

A number of studies have described the synthesis and characterisation of azo hydrogels for colonic drug delivery (Brondsted and Kopecek, 1991; 1992; Kopecek *et al*., 1992; Akala *et al*., 1998). Colon targeting is achieved by combining pH-sensitive monomers with azo cross-linking agents in the hydrogel structure. During transit through the GI tract the hydrogel continues to swell as the pH increases (to a maximum of approximately 7.4) at the ileo-caecal junction. Due to the swelling throughout the GI tract, upon entering the colon, the hydrogel cross-links are accessible to colonic enzymes and can therefore be degraded through the cleavage of the cross-links.

1.7.4. Novel colon targeted delivery system (CODES^{TM})

CODES^{TM} is a colon specific drug delivery system that uses specific polysaccharides that are only degraded by bacteria available in the colon (Yang *et al*., 2003; 2002; Takemura *et al*., 2000; Watanabe *et al*., 1998). The system usually involves the core tablet consisting of the active polysaccharide plus any other excipients coated by three different polymer layers (Figure 1.6). The three coating layers (from inside to out) include an acid soluble polymer, a hydroxypropyl methylcellulose (HPMC) barrier layer and finally a pH responsive enteric coating. Upon entry into the GI tract the core is protected by the enteric coating until the small intestine at which point the pH rises above 7 and therefore causes the enteric coating and the HPMC barrier to dissolve. The inclusion of the HPMC barrier is necessary to prevent any interaction between the oppositely charged enteric coating and acid soluble polymer. The acid soluble polymer (in many cases Eudragit E100) is soluble at pH<5 and therefore is only slightly permeable/swellable at a higher pH (those found in the small intestine). Upon arrival into the large intestine the bacteria degrade the polysaccharide and subsequently cause a significant decrease in pH in the surrounding area to increase dissolve the acid soluble polymer layer and lead to extensive drug release. In Figure 1.6 the degradation of lactulose generates organic acid therefore leading to a localised decrease in pH to allow for drug release through the dissolution of acid soluble coating layer. Other polysaccharides used within the CODES™ include mannitol, maltose, stachyose and fructooligosaccharide.

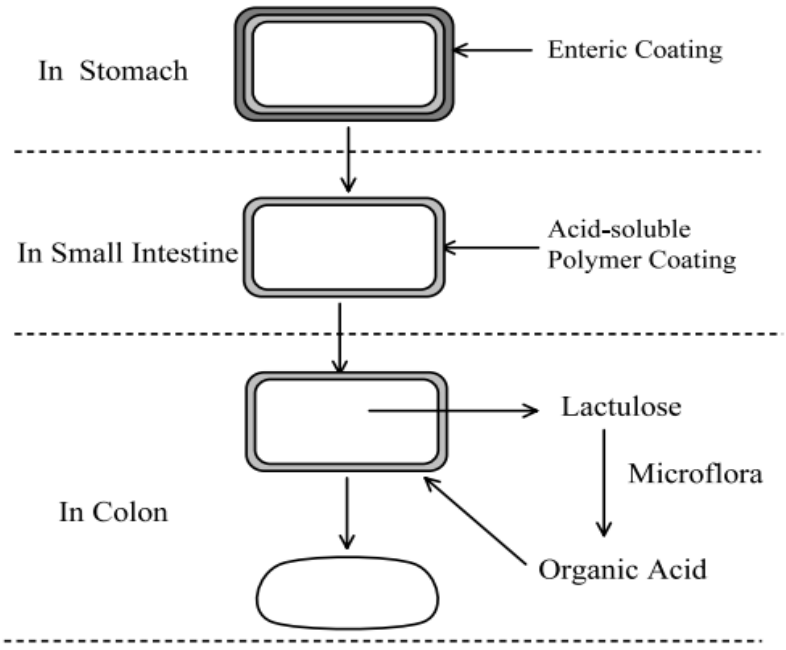


Figure 1.6. Schematic diagram showing the components of CODES^{TM}. The diagram indicates the release of lactulose maintained within a traditional tablet core as the active ingredient within the system (Takemura *et al.*, 2000).

Whilst these formulations have identified and addressed the advantages of colonic drug delivery, little consideration is given for the actual uptake of the drug within the colon. A number of researchers have shown that using liposomes for site specific colonic drug delivery can provide a number of advantages over a standard reservoir system. This is discussed below after a general introduction to liposomes.

1.7.5. Liposomes as drug delivery vesicles

1.7.5.1. Introduction to liposomes

Liposomes are micro-particulate spherical vesicles which spontaneously form when phospholipids are appropriately dispersed in an aqueous medium. The phospholipid molecules contain a hydrophobic, non polar tail (fatty acid chain) attached to a hydrophilic polar head.

When placed in an aqueous environment the lipids align, with the hydrophilic heads facing the aqueous solution and the hydrophobic tails pointing inwards (Figure 1.7). The formation of the bilayer leads to closed phospholipid vesicles forming, which are considered stable due to the significant hydrophobic effect (Tanford, 1980). Each bilayer encapsulates an aqueous

space, with the number of spaces equalling the number of bilayers within the liposome. Classification of liposomes is determined through the number of bilayers and the mean diameter of the vesicle (Table 1.8), which is principally determined by the production method.

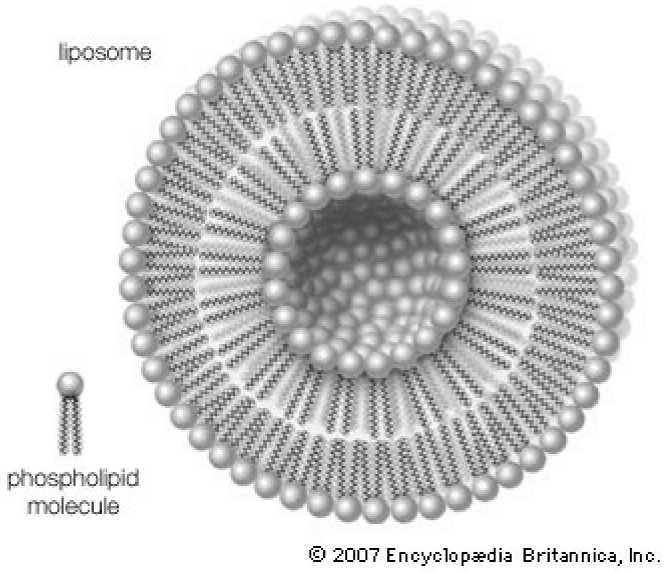


Figure 1.7. Arrangement of phospholipid molecules into a spherical liposome vesicle with an aqueous core (http://www.britannica.com/EBchecked/topic/342808/lipid/257727/Biological-membrane-lipids).

Table 1.8 – Classification of liposomes through their diameter and number of bilayers, Storm and Crommelin (1998)

Liposomal type	Abbreviation	Typical diameter
Multilamellar large vesicle	MLV	>0.5µm
Oligolamellar vesicle	OLV	0.1-1µm
Small unilamellar vesicle	SUV	20-100nm
Large unilamellar vesicle	LUV	>100nm
Giant unilamellar vesicle	GUV	>1µm
Multivesicular vesicle	MVV	>1µm

The membrane transition temperature (T_m) of liposomes is often described as the point at which the bilayer membrane changes from a well ordered solid state to a fluid, more disordered state (Table 1.9). The T_m is not only important for the production of liposomes but also its ability to retain any molecules encapsulated within the aqueous spaces can be greatly affected.

Bilayer properties of liposomes can be modified through both the alteration of the lipid component and/or the addition of further constituents, for example cholesterol (CH). Changes in liposomal mechanical properties can be observed when various types of lipid are used, due to their difference in T_m. Both naturally occurring and synthetic phospholipids are used in the production of liposomes with their characteristics varying considerably. Naturally occurring phosphatidycholine derived from egg yolk or soya bean (EPC or SPC), are used extensively in liposome production due to their biocompatibility, low transition temperature (facilitating

liposome production) and availability. Synthetic phospholipids used in the production of liposomes include dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidic acid (DMPA), which have an increased T_m but offer a more stable membrane. It has been shown that the inclusion of CH can further stabilise the bilayer and therefore decrease their permeability in the liquid state with amounts up to 3 molar % softening the bilayer, and any amounts above 3-4 molar % showing a stiffening effect and therefore a reduction in the rate of drug release (Lemmich *et al*., 1996). Not only can altering the phospholipid and the inclusion of CH add stability to the membrane but it can also help prevent bilayer solubilisation through the attack of bile salts (Rowland and Woodley, 1980).

Table 1.9 – Phospholipids and their corresponding transition temperature, T_m

Phospholipid	Transition temperature, T_m (°C)
Egg Phosphatidycholine (EPC)	-5 to -15
Soya Phosphatidycholine (SPC)	-20 to -30
Dimyristoylphosphatidylcholine (DMPC)	23.5
Dipalmitoylphosphatidylcholine (DPPC)	41.3
Distearoylphosphatidylcholine (DSPC)	55.1

The properties of liposomes make them ideal for use as a drug delivery system due to their ability to carry a variety of substances, structural versatility and non toxicity of their

components. The aqueous spaces within a liposome can be used to store hydrophilic drug molecules whilst hydrophobic drugs can be encapsulated within the bilayer. The advantages of using liposomes as a drug delivery system are those relating to (Storm and Crommelin, 1998):

Structural and physiochemical characteristics – liposomes are a very diverse family of vehicles and can be tailored to suit the application.

Distribution – liposomes can target, either actively or passively, specific areas in the body for treatment (site specific) or can be directed away from sites that are sensitive to the toxic nature of the drug (site –avoidance).

Duration – liposomes act as a reservoir of drug that is slowly released over time leading to increased duration of action with a fewer number of administrations.

Protection – liposomes can protect the drug from certain detrimental factors that may be present in the host e.g. degradative enzymes.

Internalisation – liposomes can interact with target cells allowing the intracellular delivery of drugs that would normally have unfavourable physiochemical characteristics in this respect e.g. DNA molecules.

Amplification – if the drug is an antigen, liposomes can act as immunological adjuvant in vaccine formulations.

Due to the number of advantages of using liposomes for drug delivery, research and development has taken place to produce liposomes suitable to treat a number of conditions. A number of liposomal products have been developed for parenteral administration and are present in marketplace today, e.g. the anticancer products Doxil® (Ortho Biotech), Myocet® (Zeneous) and DaunoXome® (Gilead Sciences) (Zhang *et al*., 2008). The other main field of liposomal drug delivery is in the treatment of systemic fungal infections including Abelcet® (Enzon) and Ambisome® (Gilead Sciences). Other drugs are emerging on the market which use liposomes via the intravenous route including Visudyne® (QLT, Novartis) which is a

liposomal form of the drug verteporfin which is administered for the treatment of age related macular degeneration (Zhang *et al*., 2008). The majority of liposomal drug products within the marketplace are for intravenous administration. To extend their applicability to a wider range of disease states, it would be beneficial to create formulations for oral administration.

1.7.5.1. Advantages of liposomes for colonic site specific delivery

A number of researchers have recognised the potential advantages of producing liposomal formulations that deliver the active ingredient to the colonic region. As mentioned previously specific targeting to the colon provides the ability to deliver active ingredients both topically and systemically, it has been shown that liposomes have the ability to adhere to both healthy and inflamed colonic mucosa *in vitro* (Tirosh *et al.*, 2009*;* Jubeh *et al.*, 2004). Tirosh *et al*. (2009) showed that negatively charged (anionic) liposomes preferentially attached themselves to the inflamed mucosa of the rat colon in low pH conditions characteristic of those found during ulcerative colitis. The lower pH values observed in inflammatory bowel disease relate to the production of mucosal bicarbonate and lactate, bacterial fermentation of carbohydrates and mucosal absorption of short chain fatty acids which could all influence the pH of the colon. In comparison, at a neutral pH no preferential attachment of liposomes was observed and therefore no improved rate of active ingredient uptake (transferrin) was identified. This indicates that the use of anionic liposomes would be preferential when treating ulcerative colitis due to their increased affinity to inflamed colonic mucosa. Similarly, Jubeh *et al*. (2004) reported a two-fold increase in anionic liposomal adhesion to inflamed colonic mucosa in comparison to that of neutral or cationic liposomes. Interestingly, a three-fold increase in adhesion for cationic liposomes was observed compared to neutral and anionic liposomes for healthy colonic mucosa which would indicate that they may be suitable for the systemic delivery of proteins through adhesion to healthy colonic mucosa.

The interaction of liposomes with colonic mucosa has led a number of researchers to explore the possibility of treating both local and systemic diseases through liposomal colonic targeting. D'Argenio *et al*. (2006) 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 explored.

One study that has explored the oral route for colonic liposomal drug delivery is that of Xing *et al*. (2003). The study describes a multicomponent drug delivery system which comprised drug loaded liposomes within Eudragit-coated alginate beads. The resultant formulation was assessed *in vitro* using simple buffer solutions of pH 1.2, 6.8 and 7.4. After two hours in pH 1.2 buffer very little release was shown indicating that the coating mechanism would survive the stomach *in vivo*. Upon exposure to the pH 6.8 phosphate buffer for three hours considerable drug release was shown (approximately 45%) which was attributed to the dissolution of the Eudragit S100 coating to expose the gel beads subsequently causing drug release. This would indicate that drug release would occur prior to the ileum and therefore significant amounts of the active ingredient would not reach the target of the colon. In the final three hours in pH 7.4 phosphate buffer it was shown that drug release up to approximately 90% could be observed. Despite the obvious protection of the formulation through simulated stomach conditions the formulation has a number of drawbacks that could be improved upon. It is indicated that drug release was controlled by the alginate and not the liposomes therefore making it unclear as to whether the liposomes could be subsequently released allowing them to adhere to the colonic mucosa, a major advantage of using liposomes as previously described. Furthermore, the *in vitro* drug release trials showed considerable drug release whilst in a simple pH 6.8 phosphate buffer, indicating that exposure to *in vivo* conditions containing bile salts would cause further drug release and therefore reduce the quantity of drug delivered to the colon.

1.7.5.2 Production of liposomes

As liposomes are produced by the spontaneous interaction between phospholipids and water (with agitation of some form – Lasic 1998), the emphasis when producing liposomes is mainly on the ability to form vesicles of the right size and structure with the highest entrapment efficiency (New, 1990). A wide variety of techniques has been adopted to produce liposomes which include mechanical dispersion techniques, dried-reconstituted vesicles, and solvent dispersion techniques (ethanol/ether injection vesicles and reverse phase evaporation vesicles). Most methods of producing liposomes can be said to involve three stages, which include the drying down of lipids from organic solvents, dispersion of the lipids

in aqueous media and subsequent purification of the resultant liposomes. In this work it was decided that a method based on that of Bangham *et al*. (1965) would be adopted throughout, as the actual production of liposomes is not under investigation, but the possibility of producing a formulation suitable for colonic drug delivery is. This method enabled direct comparison with the numerous studies that have investigated the coating of thin film hydration vesicles (Takeuchi *et al*., 2005; Guo *et al*., 2003; Khan *et al*., 2000).

Thin film hydration

The original method of producing liposomes was established by Bangham *et al*. (1965) and is a simple thin film hydration method, also known as the 'hand shaking' method. The method involves producing a lipid solution in an organic solvent such as chloroform. The lipid solution is then placed in a round bottomed flask, with a rotary evaporator being used to remove the solvent and leave a lipid film deposited on the sides of the flask. Any residual solvent is then removed by drying the film under a stream of nitrogen. The film is then hydrated with an aqueous buffer that is above the T_m of the lipid mixture. The flask is then agitated through hand shaking (sometimes glass beads are used) which displaces the thin film from the walls of the flask causing the production of liposomes. The liposomal suspension produced consists of a heterogeneous MLV suspension with liposomes over 1 µm in size. Subsequent processing (see below) can then be completed to produce a more homogenous formulation with a specific size range (SUVs, LUVs etc.).

Sonicated vesicles

To produce the homogenous SUV liposomal sample it is necessary to use a method which imparts energy at a high level on the lipid suspension (New, 1990). Initially Huang *et al.* (1969) produced SUVs with an approximate diameter of 25 nm by using probe sonication whilst Johnson *et al*. (1969) produced similar SUVs using a ultrasonic bath for a prolonged period of time (1 – 1.5 hours).

Probe sonication can be used when suspensions require high energy in a small volume e.g. high concentration of lipid or the use of a viscous aqueous phase. As a consequence of this process heat is given off and therefore it is essential that the suspension is maintained at a

constant temperature by the use of a cooling bath to prevent any lipid degradation. A further issue that may arise through probe sonication is the possible contamination of the sample through the degradation of the probe therefore suspending Ti particles within the sample. Subsequent centrifugation or gel permeation chromatography can be used to separate any small MLVs remaining from the SUV population.

The ultrasonic bath method is more suitable for dilute lipid concentrations of a higher volume and as there is a lower energy input, there is subsequently a lower risk of lipid degradation due to heat. The risk of contaminants entering the solution is reduced as the formulation can be maintained in a sealed container throughout the sonication process. The main drawbacks of the bath sonication method include the need for prolonged sonication time and the final liposome size may not be homogenous and thus there may be a requirement for centrifugation (Barenholtz *et al*., 1977).

Membrane extrusion

The use of membrane filters to reduce the size of liposomes has been investigated considerably with two main methods having evolved. The first method uses what is described as a tortuous path membrane which consists of a number of fibres criss-crossed over each other, which leads to specific channels which liposomes are forced through. The channel or pore size is controlled by the density of the fibres used in the membrane manufacture. The drawback of this method is that larger liposomes can become stuck within the membrane channels and this therefore blocks the filter. The more widely used method is that of the Nuclepore membrane which consists of uniform pores through a thin sheet of polymer. A range of pore sized membranes can be obtained, ranging from 1 µm to 50 nm. Liposomes which are much greater in size than the membrane will be broken down into smaller vesicles when extruded through the membrane. The liposomes which are only marginally larger than the pore size will be able to change their conformation and squeeze though the pore, which means despite a number of extrusions a small percentage of the liposomes will be larger than the pore size itself.

1.8. Objectives of current work

The overall objective of this work was to develop and evaluate a (polymer-coated) liposomal system that will potentially allow for site specific drug delivery at the colon following oral administration. The liposomal component will be developed to encapsulate a model drug that can be either encapsulated in the aqueous space or the lipid bilayer. It is hypothesised that the development of a formulation containing materials with both a pH and enzyme triggered response would increase the accuracy of the targeting and subsequently reduce the problems associated with inter and intra patient variability.

With the overall objective in mind, the specific aims were to:

Introduce colonic drug delivery outlining the specific advantages and challenges involved. Investigate the characteristics of specific coating polymers used in targeted colonic drug delivery, comparing the difference between enzyme and pH controlled mechanisms.

Produce a coated liposomal formulation for the purpose of site specific drug delivery to the colon. The direct coating of liposomes will be investigated with the possibility of producing a more robust system by encapsulating liposomes within a solid microsphere.

Assess each formulation using a number of standard techniques to characterise formulations indicating any change in particle charge, size and morphology.

Conduct drug release trials *in vitro* simulating the stomach, small intestine and colon to assess the formulation and its suitability for the desired application of colonic drug delivery. *In vitro* studies will include model bile salts and enzymes to produce an accurate representation of the GI tract.

2.0 Material characterisation

ABSTRACT

A number of different polymers are used in the production of site specific drug delivery vesicles. The polymers used can be categorised by the specific triggers that cause their solubilisation and/or degradation whether it be pH, residence time or enzymatic action. The two polymers used throughout this thesis have been introduced in this chapter. Eudragit S100, a pH responsive polymer, and chitosan, an enzyme triggered polysaccharide, have been selected to produce a site-specific drug delivery system to target the colon and subsequently release liposomes containing the active ingredient. Knowledge of the two polymers has been built up through a number of techniques including gel permeation chromatography (GPC), Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM). GPC provided an insight into the specific molecular weight of Eudragit S100 (122,500) and chitosan (237,500) which are shown to have a considerable impact on the dissolution. FTIR provided an insight into the 'as received' nature of the polymers which further enables the prediction of the solubilisation mechanisms/speeds due to the specific chemical structure. SEM offered a visual guide to the polymers with characterisation being essential to observe any changes that may be observed during subsequent processing. In depth discussions of the specific solubility mechanisms for each polymer are included to give an insight into their response throughout the GI tract conditions.

2.1.1. Introduction

In order to investigate the possibility of coating of liposomes with polymers it is essential to understand the polymer and subsequently the coating mechanism that may occur. The overall aim of this chapter is to introduce the polymers to be used throughout this thesis and subsequently provide an insight into their specific properties. A number of techniques will be used including Fourier transform infra-red spectroscopy (FTIR) and gel permeation chromatography (GPC) to identify the differences in the polymers and therefore hypothesise how they may coat liposomal surfaces. These techniques will indicate the specific properties including the specific chemical bonds, molecular weight and morphology of the polymer. Furthermore, imaging techniques will be adopted to visualise the polymers in their as received state and therefore allow comparisons to be made when the liposome coating is undertaken.

2.1.2. Introduction to polymer science

Polymers can simply be described as long chain molecules of high molecular weight (Sperling, 1999). They are termed macromolecules which are produced by covalent bonding of repeat monomer units through a process called polymerisation. Bonding along the chain is strong but intramolecular forces only take place through weaker forces of either van der Waals or hydrogen bonds.

Polymers can adopt two morphologies; amorphous or semi-crystalline. The amorphous state refers to a randomly entangled structure with no long range order. The glass transition temperature (Tg) refers to a range of temperatures whereby the amorphous polymer changes from a brittle solid to a viscous liquid upon heating. Crystallisation within the polymer is prevented due to either the irregular structure or due to rapid quenching of the sample which prevents the formation of ordered crystalline structures. Tg is not only dependent upon the chemical structure of the polymer but is also influenced by the cooling rate adopted through the crystallisation temperature (Tc), therefore the thermal history of a sample can have a considerable effect upon its properties.

The semi-crystalline state of a polymer refers to a highly ordered microstructure that alters from a solid to a liquid at the melting point (Tm). Many polymers are semi-crystalline and have regions of both highly ordered chains and random entanglements therefore, altering in

state over a range of temperatures. Semi-crystalline polymers can be seen to form spherical structures that are termed spherulites (Figure 2.1). It has been shown that the spherulite consists of thin lamellae crystals that have grown radially, which are made of extensively folded polymer chains. The lamellae regions are separated by thin regions of amorphous material. The initial process of crystallisation is termed nucleation, and can either involve the attachment of polymer chains to a nucleating agent (heterogeneous nucleation), such as dust or an inorganic material, or the chains become aggregated within the melt (homogenous nucleation). The spherulite grows by repeated branching, which develops an intermediate wheat sheaf structure, with even further branching creating the spherical structure.

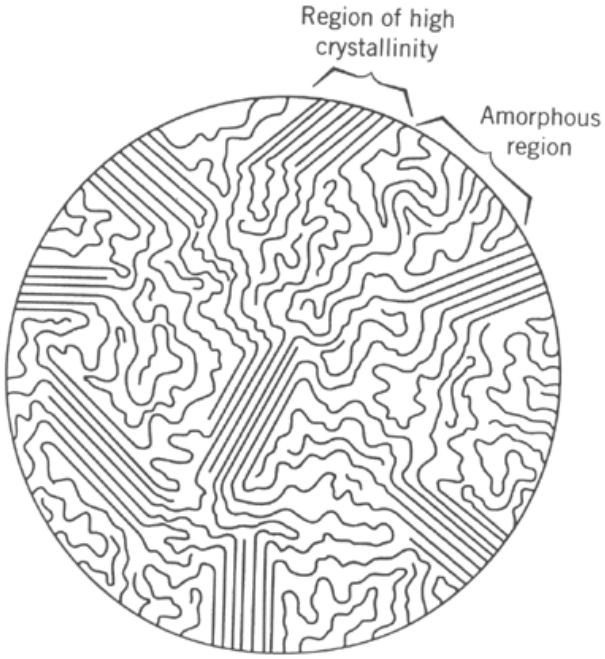


Figure 2.1.Schematic diagram of polymer spherulite with chain folded lamellae (Callister, 2003)

2.2. Polymers for oral colonic drug delivery

2.2.1. Introduction to polymers for oral colonic drug delivery

A number of polymers have been explored for the application of triggered oral colonic drug delivery including pectin, alginate, chitosan, Eudragit and cellulose acetate phthalate (CAP) (Tiwari *et al*., 2010; Sinha and Kumria, 2003). As previously discussed, the main triggers for polymer degradation and subsequent drug release utilise either the changes observed in the

GI tract (pH, enzyme, temperature) or relate to a specific transit time through the tract. A number of studies have reviewed the advantages of each method, with no single trigger release mechanism consistently providing a successful release in all experiments (Yang *et al*., 2002; Patel *et al*., 2008). It has therefore been considered that a trigger system utilising two or more of the mechanisms would be more successful as it would reduce the influence that patient variability may have upon the drug delivery system (Bajpai *et al*., 2008). This strategy has been seen with the success of the CODES^{TM} technology which uses two different polymers (pH and enzyme controlled) to provide drug release in the colonic region. Due to the significant variance that can be observed in transit times through the GI tract within both the fed and fasted states it has been decided to concentrate on the specific triggers of pH and enzyme change throughout this thesis.

To investigate oral colonic drug delivery it has been decided two polymers will be investigated, one being pH dependant and one enzyme controlled. The pH triggered polymer selected is that of Eudragit S100 which has been used in a number of studies for colonic drug delivery and has also shown flexibility as it can be combined with Eudragit L100 to adjust its specific solubility profile (Khan *et al*., 1999a; 1999b). The enzyme triggered polymer to be used is chitosan, which is emerging as very promising for colonic drug delivery due to being solubilised by enzymes only found within the colon (Jain and Jain, 2007). In depth investigations into each of these polymers are required to further understand their properties and solubility mechanisms. A number of studies have been undertaken to characterise the polymers in their 'as received' state and therefore build up an understanding of how they will respond to the changing conditions both experimentally *in vitro* but also what would be observed *in vivo*.

2.2.2. Eudragit S100

2.2.2.1. Introduction

The most commonly used polymers for pH responsive drug delivery are methacrylic acid and methyl methacrylate ester copolymers which are marketed under the brand name of Eudragits (Evonik, Germany). A range of pH responsive Eudragits are available (S100, L100, L30-D55), with each one showing a different solubility trace (Figure 2.2). The Eudragit range of polymers have been used in a number oral delivery systems due to the number of advantages they possess, including:

- Protects active ingredients sensitive to gastric fluid
- Offers effective GI tract and colon targeting
- Provides increased drug effectiveness
- Offers either pH or time dependent release profiles through solubilisation profile
- Good storage stability

Eudragit S100 was selected for this work due to it being soluble above pH 7.0 and therefore an ideal polymer for targeted drug release to the distal small intestine and beyond, the benefits of which were addressed in the previous chapter. A number of studies have used Eudragit S100 for colonic drug delivery (Khan *et al*., 1999; 2000), each of which show a release mechanism suitable for protection of the active ingredient through the stomach and small intestine with subsequent release occurring at the ileo-caecal junction and beyond.

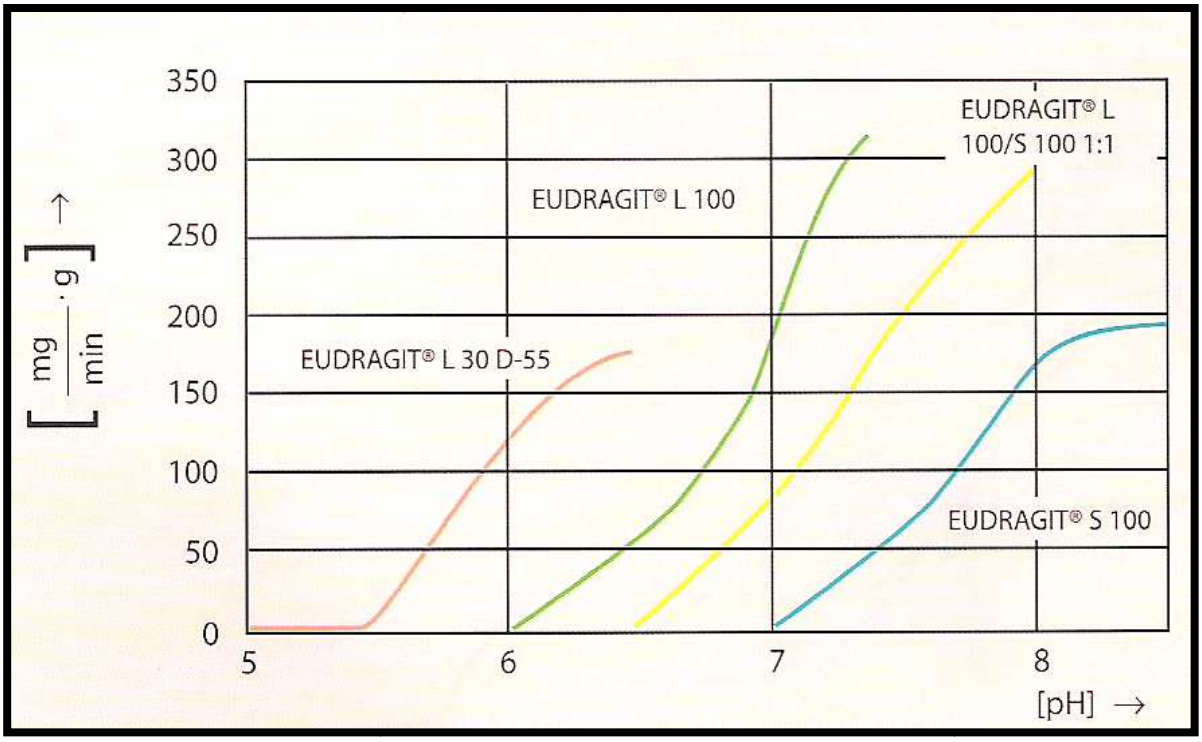


Figure 2.2. Dissolution profiles of varying grades of Eudragit polymers (evonik.com). It has been shown that different grades of Eudragits can be combined to produce polymers with different solubility profiles (Khan *et al*., 1999a; 1999b).

2.2.2.2. Chemical structure

Observing the specific chemical structure of Eudragit S100 is important for a number of reasons. The exact chemical structure of the Eudragit determines the solubility profile, more specifically that of the side chains and their ratio e.g. Eudragit L100 (>pH 6) has the ratio of 1:1 of free carboxyl groups to ester groups whilst Eudragit S100 (>pH 7) has the ratio 1:2. Furthermore, it may provide an insight into the possible interactions that could occur throughout the GI tract in relation to bile salts, enzymes etc. The specific chemical structure of Eudragit S100 is shown in Figure 2.3.

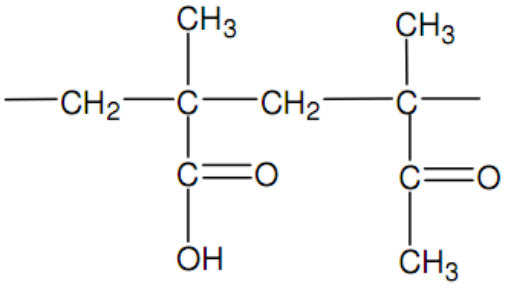


Figure 2.3. Molecular structure of Eudragit S100.

2.2.2.3. Mechanism for coating with Eudragit S100; organic vs aqueous

It has been shown that the specific coating technique used will alter the properties of Eudragit S100 when investigated *in vitro* (Ibekwe *et al*., 2006a; Ibekwe *et al*., 2006b; Bando *et al*., 2006). Traditionally film coatings are achieved by applying the polymer from a solution usually using Eudragit S100 dissolved in organic solvents, but there are associated environmental and health concerns. Film formation from organic polymer solutions results from the evaporation of the solvent which causes an increase in polymer concentration and subsequent inter-diffusion of the polymeric chains. Further solvent evaporation leads to a solvent free polymeric film being formed. Despite this, there will always be certain quantities of residual solvent within the system.

With this in mind a formulation suitable for processing as an aqueous dispersion was developed which was prepared by the partial neutralisation of the methacrylic acid group of

the polymer. Aqueous dispersions are created through the dropwise addition of a dilute ammonia solution to Eudragit S100 dispersed in water whilst undergoing high speed stirring. Film formation from aqueous polymer solutions occur with the polymer particles coalescing into a film that happens in conjunction with the evaporation of water. The addition of ammonia can be calculated to give a specific level of neutralisation and therefore predict the resultant solubility profile. A number of studies use the addition of ammonia leading to approximately 15% neutralisation of the methacrylic acid group and therefore altering the solubility profile of the resultant film (Ibekwe *et al*., 2006a and 2006b; Bando *et al*., 2006). The neutralisation of the acid group reduces the ratio of side groups (from 2:1 towards 1:1), therefore producing a polymer solution between that of L100 and S100, and subsequently producing a film that is soluble between pH 6 and 7.

The experimental results comparing the use of organic solutions and aqueous dispersions are considerably different. As expected both formulations produce a coating that provides an effective gastro-resistant barrier, where the pH is very low and therefore neither the organic or aqueous formulations show any sign of drug release. Ibekwe *et al*., (2006a) showed that in five out of eight patients the organic coated tablets reached the ascending colon, with the remaining three subjects showing signs of the tablet staying intact which was attributed to low patient GI tract pH. In comparison for aqueous coated tablets seven out of eight participants showed complete tablet disintegration in the proximal to mid small intestine and therefore indicated it would not be suitable for ileo-caecal drug delivery. Similarly, Bando *et al*., (2006) showed that two cast films, organic and aqueous, had negligible weight loss when exposed to 0.1 M HCl (simulated gastric conditions), but were considerably different during the simulated small intestine conditions. It was shown that during a 4 hour period in pH 6 PBS the aqueous film displayed approximately 40% weight loss in comparison to the negligible loss for the organic film. It was then shown that in pH 7 PBS that 100% weight loss occurred after 2 hours for the aqueous film whilst the organic took over 4 hours to completely dissolve. This indicates that the use of aqueous dispersions for coating, although favourable in terms of environmental and health concerns would considerably alter the solubility profile of the polymer and subsequently make it unsuitable for ileo-caecal drug delivery.

2.2.2.4. Solubilisation mechanism of Eudragit S100

In order to understand the specific properties of a drug delivery formulation it is essential to appreciate the solubility mechanism involved in the site specific release. In the case of Eudragit, a pH responsive polymer, any alteration in the chemical structure will lead to an alteration in solubility profile. The solubility mechanism for pH responsive polymers revolves around the ionisation of the functional group when a shift in pH occurs. The ionisation leads to a large number of groups with the same charge, therefore causing repulsion and subsequent expansion and solubilisation. The difference in the pH at which solubilisation occurs is due to the ester group which is evident with the difference between Eudragit L100 and S100. Eudragit L100 has a 1:1 carboxyl to ester group ratio, while S100 has a 1:2 ratio in favour of the ester group and therefore solubilisation occurs slower and at a higher pH.

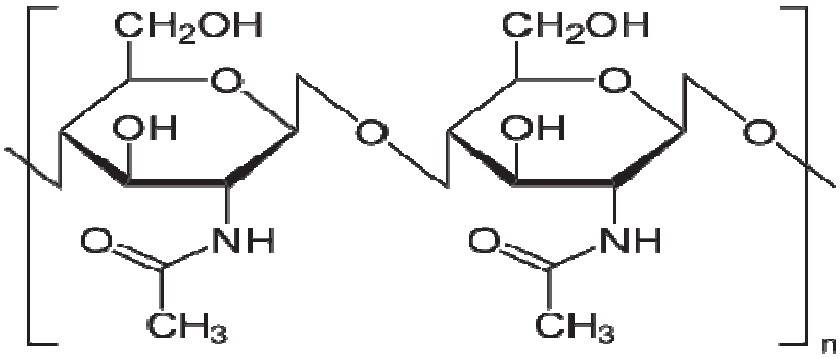
2.2.3. Chitosan

2.2.3.1. Introduction

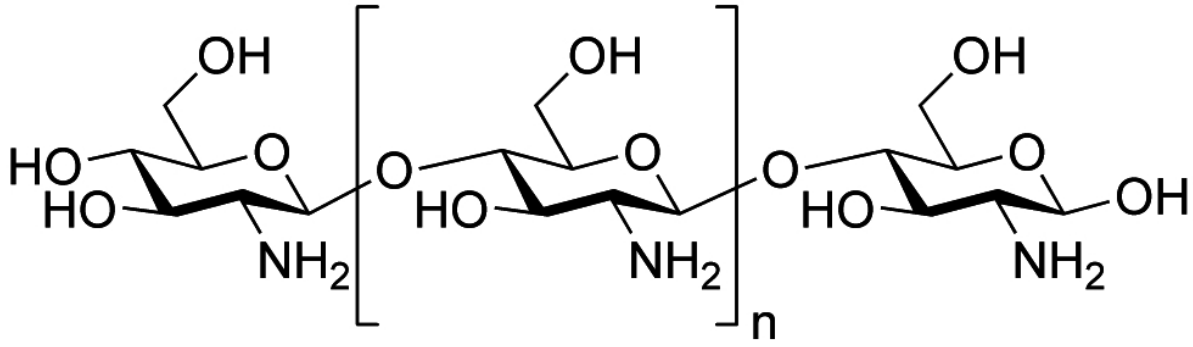
Chitosan was selected as the enzyme triggered polymer to be used as it is a naturally occurring polysaccharide, cost effective, and available in abundance. Chitosan is formed through the alkaline deacetylation of the mucopolysaccharaide chitin. The use of chitosan is an attractive proposition as it is solubilised by the enzymes present in the colonic microflora and therefore presents a characteristic for colonic drug delivery. Chitosan refers to a large number of polymers which differ in their degree of deaceylation (40-98%) and molecular weight (50,000-2,000,000 Da) which has been shown that the molecular weight of the chitosan influences the solubility, and therefore the release profile considerably (Hejazi and Amiji, 2003; Zhang and Neau, 2002). A chitosan grade termed by the manufacturers as low molecular weight (Sigma Aldrich; product code: 448869), was used throughout, however the specific molecular weight would be established through GPC. The manufacturer's data sheet indicated that the supplied chitosan was 75-85% deacylated which further influences the solubility of the chitosan. It has been shown that higher levels of deaceylation lead to a coating with in increased solubility in comparison to chitosan with a lower deaceylation percentage (Filipović-Grčić *et al*., 2001).

2.2.3.2. Chemical structure of chitosan and its derivatives

Chitosan is a hydrophilic, biocompatible and biodegradable polymer that is derived from chitin, which is a derivative of glucose predominantly obtained from the shells of crab and shrimp. To fully understand the structure of chitosan it is necessary to view the chemical structure of chitin and therefore the changes that occur during the alkaline deaceylation to produce chitosan (Figure 2.4). Chitin ($C_8H_{13}O_5N$) is a long chain polymer of N-acetylglucosamine with the repeat units being joined through a β (1-4) glycosidic bond. In comparison chitosan is composed of both N-acetylglucosamine (acylated) and D-glucosamine (deacylated) units joined by β (1-4) glycosidic bonds. The process of alkaline deaceylation, which is normally completed in 40% sodium hydroxide at 120°C for 1-3 hours, removes the acetyl groups (CH3-CO) leaving the amine groups (NH).



Chitin



Chitosan

Figure 2.4. Chemical structure of chitin and chitosan following the alkaline deaceylation of chitin.

2.2.3.3. Solubilisation of chitosan by the colonic microflora

As discussed in the previous chapter the human microflora is a complex system containing an enormous variety of both aerobic and anaerobic bacteria creating a hugely complex ecosystem. Polysaccharidases like glucosidases and glycosidases are released by the colonic microflora, which are responsible for the degradation of polysaccharides (Jain *et al*, 2007). These polysaccharidases are responsible for the random scission of the 1,4 glycosidic bonds observed between the single monomer units in chitosan. As the human microflora activity has been shown to not be affected by diet, age and geographic location it can be assumed that patient variability would not influence the ability of the colonic region to digest chitosan. Furthermore it has been shown that the anaerobic bacteria can react to changing mixtures of carbohydrates entering the colon, by recognising a variety of substrates the appropriate digestive enzyme can then be produced to ferment the specific bonds involved.

2.3. Materials

2.3.1. Polymers

Eudragit S100 was a gift from Evonik (Essen, Germany). Chitosan (low molecular weight – 34234) was purchased from Sigma Aldrich (Dorset, England). Polymers were used in their 'as received' state.

2.4. Apparatus and methodology

2.4.1. Gel Permeation Chromatography (GPC)

2.4.1.1. Introduction to GPC

GPC is a technique used to determine the molecular weight distribution of a polymer. GPC is a form of size exclusion chromatography which is predominately used for sizing polymers. The technique uses the size exclusion principle whereby a particle is defined by its hydrodynamic radius and therefore may or may not be able to enter small pores in a bed of cross-linked polymer particles (Sperling, 1999). The technique uses a column packed with gel (stationary phase) which has a specific pore size. A solvent is then passed through the column (mobile phase) at a controlled speed. The sample is then injected into the mobile phase where

it begins to interact with the stationary phase (Figure 2.5). Larger particles are unable to interact with the pores of the gel and therefore pass through the column relatively uninhibited, while the smaller particles diffuse in and out of the pores via Brownian motion and are therefore delayed. The molecular weight can then be related to the specific retention time of the polymer within the column.

All GPC was completed by Rapra Smithers Technology Ltd. (Shawbury, UK). Due to the difference in samples, two different methods were required for the GPC of Eudragit S100 and chitosan which are outlined in sections 2.3.1.1 and 2.3.1.2 respectively.

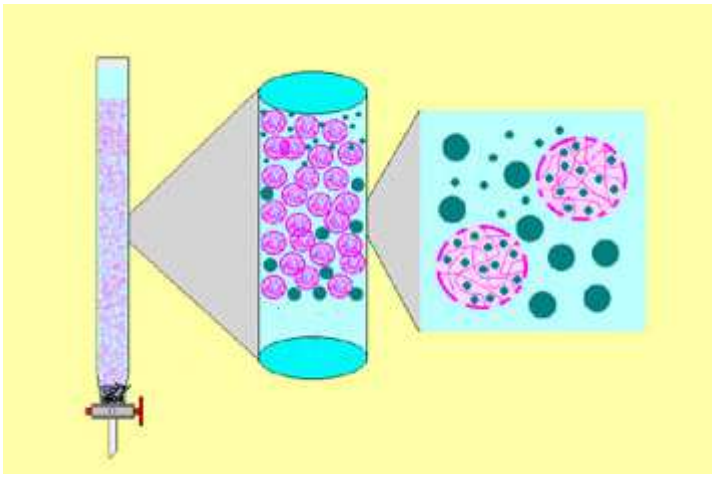


Figure 2.5. Diagrammatic representation of GPC (http://whale.wheelock.edu/bwcontaminants/analysis.html).

2.4.1.2. GPC conditions for Eudragit S100

Eudragit S100 had been proven insoluble in chloroform or tetrahydrofuran and therefore N,N'dimethylsulphoxide was adopted as the solvent. A solution was prepared by adding 15 ml of the solvent to 30 mg of chitosan, heated to 95°C, with shaking for 4 hours. The solution was left overnight to stabilize and then re-heated to 95°C for 30 minutes before filtering through a 1.0 µm glass-fibre pad. The instrument used for experimentation was the Polymer Laboratories PL-GPC 120 with PL-AS-MT autosampler. The columns used were PL PolarGel guard plus 2 x PolarGel-M, 30 cm, 8 µm. A flow rate of 1.0 ml/min and temperature of 95°C were adopted throughout the experimentation.

2.4.1.3. GPC conditions for chitosan

As chitosan is insoluble in solvents an aqueous based approach was adopted for GPC analysis. A chitosan solution was created by adding 10 ml of 10% acetic acid to 20 mg of chitosan and left overnight to stabilise. The solution was then warmed to 40°C and held for 30 minutes before being vortex mixed and filtered through a 0.45 µm PVdF membrane. GPC was conducted using the Viscotek triple detector array TDA301 with associated pump and autosampler. The column used throughout was PLaquagel OH guard plus PLaquagel OH-mixed, 30 cm, 8 µm. The eluent used was 0.5 M $NaNO_3$; 0.01 M NaH_2PO_4 adjusted to pH 2.0. A flow rate of 1.0 ml/min and temperature of 30°C were selected. The detector used observed refractive index with differential pressure and light scattering.

2.4.2. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is a branch of molecular vibrational spectroscopy whereby infra-red light is used to excite the covalent bonds into higher vibrations and subsequently the bonds absorb the light to a different extent. The specific wavelength at which the light is absorbed relates to specific bonds, and therefore is unique to that material and can be used to determine its structure, presence and quantification due to the specific absorbance level.

Infra red spectra of as received polymer samples 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) at a resolution of 2 cm^{-1}. Initially a background scan was completed to improve the signal-noise ratio. Each trace consisted of 200 scans. A small amount of dry polymer was then placed onto the stage. Each experiment was conducted in triplicate, with the mean of three independent trials being displayed.

2.4.3. Scanning Electron Microscopy (SEM)

SEM was conducted on the as received polymer samples to determine the surface morphology, homogeneity of the sample and indicate the general particle size. The samples were mounted on a SEM stub and subsequently coated with platinum using an Emscope SC500 sputter coater. The samples were coated for 2 minutes, depositing a layer of platinum

equivalent to 150 Ångström. Images were taken using a Phillips XL-30 FEG ESEM under vacuum conditions.

2.5. Results and Discussion

2.5.1. Eudragit S100 characterisation

2.5.1.1. GPC analysis of Eudragit S100

GPC analysis of Eudragit S100 indicates a molecular weight (M_w) in the region of 122,000 – 123,000 (Table 2.1), which is very similar to that of 125,000 published by the manufacturer. The M_w is obtained by dividing the chains into a series of size ranges and subsequently determining the weight fraction in each range (Callister, 2003). The corresponding number average molecular weight which is based on the number fraction within each size range (M_n) was shown to be between 72,400-75,000. The wider range observed for M_n is representative of the theory that polymers with relatively low molecular mass are more sensitive to M_n while Mw is more sensitive when observing high molecular mass polymers. The polydispersity (PDI) of the sample is calculated by dividing the M_w by the M_n to give a value representative of individual molecular masses in a wide ranging sample. The PDI for Eudragit S100 was recorded as between 1.6-1.7, with a value being closer to 1 indicating a uniform polymer chain length throughout the sample. The consistency of the results is further emphasised by the graph showing a normal distribution for both trials (Figure 2.6) which overlap each other very closely.

Table 2.1. Table showing the GPC results for two separate trials of Eudragit S100 indicating the range in M_w, M_n and polydispersity.

Sample	M_w	M_n	Polydispersity
Eudragit S100 (A)	122,000	72,400	1.7
Eudragit S100 (B)	123,000	75,000	1.6

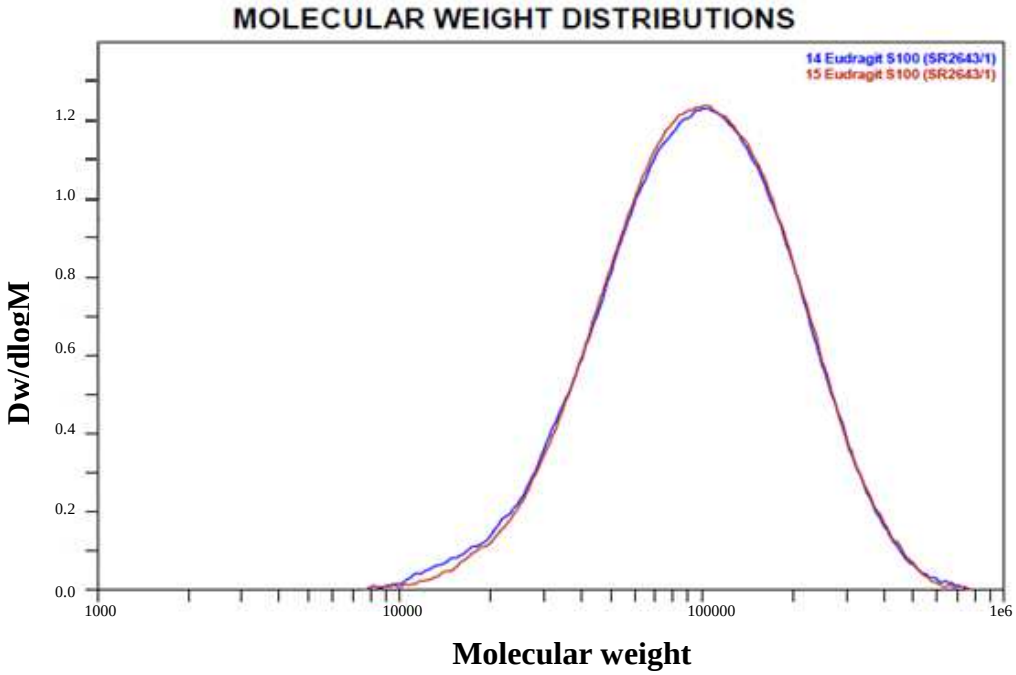


Figure 2.6. Molecular weight distribution for as received Eudragit S100 showing two separate trials overlapping each other determined through GPC.

2.5.1.3. FTIR analysis of Eudragit S100

The FTIR trace provided by the manufacturer is shown in Figure 2.7. The main peaks identified in the analysis shows the characteristic bands of the C=O vibrations of the carboxylic acid groups at 1705 cm^{-1} and of the esterified carboxyl groups at 1730cm^{-1}. Further ester vibrations were identified at 1150-1160 cm^{-1}, 1190-1195 cm^{-1} and 1250-1275 cm^{-1}.

The FTIR analysis of as received Eudragit S100 is shown in Figure 2.8. The main peaks have been identified with their corresponding bonds being outlined in the table accompanying the spectra. The main bonds associated with Eudragit S100 and visible on the FTIR trace are the C=O vibrations of the carboxylic acid groups at 1705 cm^{-1} and the esterified carboxyl groups at 1726 cm^{-1}. Further ester vibrations can be observed at 1151, 1189 and 1249 cm^{-1}, with CH_x vibrations visible at 1480, 1451 and 1389 cm^{-1}. When compared with the trace published by

the manufacturer the main peaks are the same, with only the level of transmittance being different, which is due to the quantities of Eudragit S100 used in the testing method and the specific testing parameters used in each trial. The FTIR trace will prove useful in future experiments to indicate any change in chemical structure. The technique will prove beneficial to ensure that the polymer does not change during both formulation and experimental conditions and therefore its desired properties are maintained.

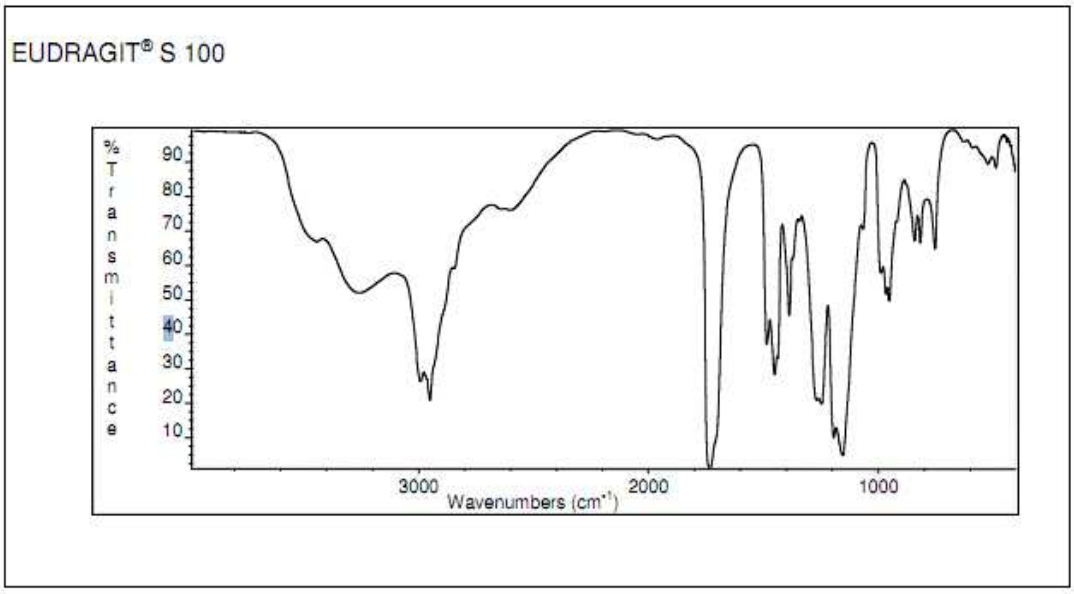
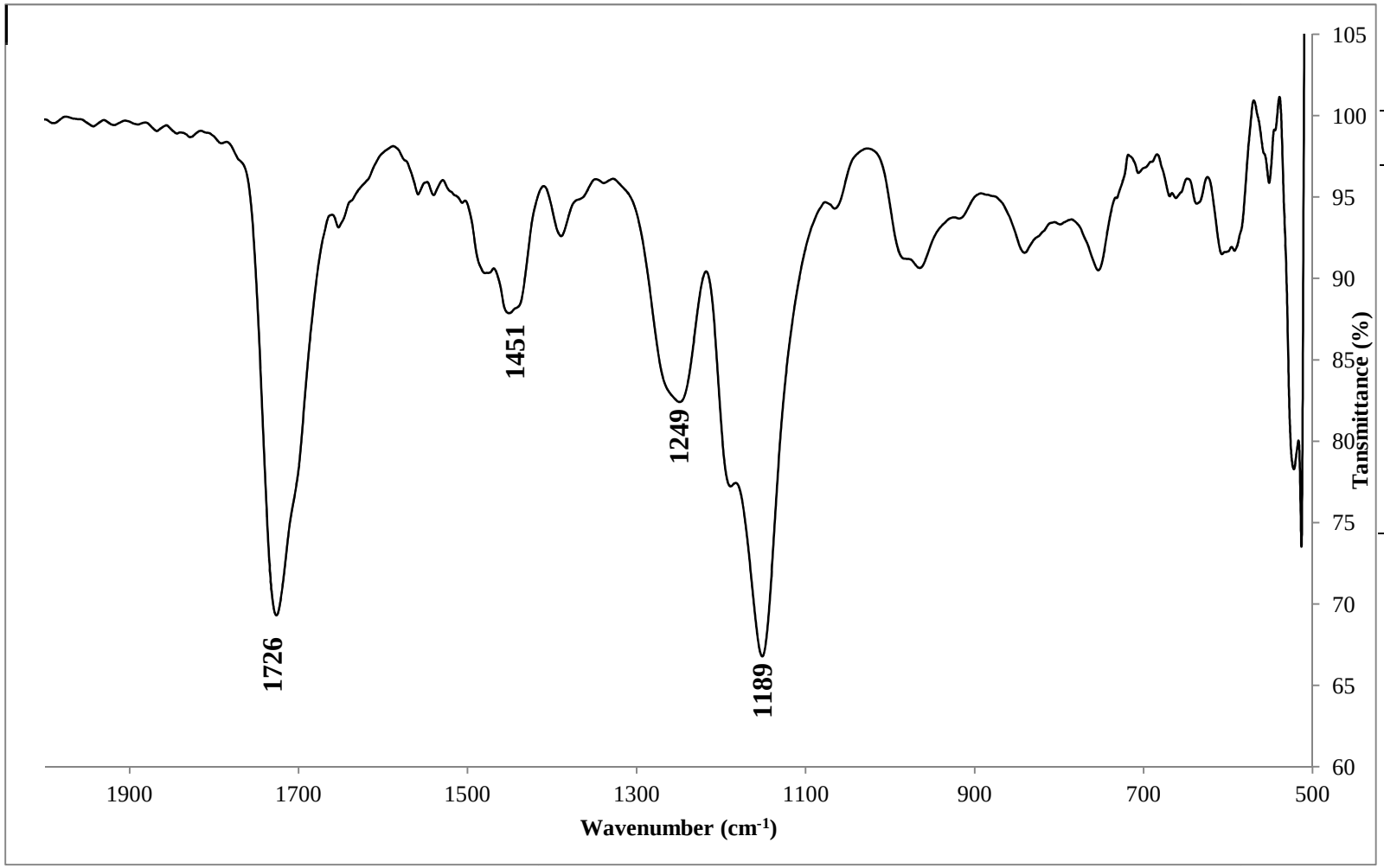


Figure 2.7. Manufacturers FTIR trace of Eudragit S100. Dry films of 15 µm thickness were produced by placing a few drops of the test solution on a crystal disc (KBr, NaCl) and then dried under vacuum conditions at approximately 70°C.



Wavenumber (cm^{-1})	Bond
1726	C=O
1480	CH_x
1451	CH_x
1389	CH_x
1249	C-O
1189	C-O
1151	C-O

Figure 2.8. FTIR trace for as received Eudragit S100 showing the main bonds associated with the polymer structure. The attached table shows the exact wavelength of the peak and the corresponding bond it represents.

2.5.1.4. SEM analysis of Eudragit S100

The SEM images for as received Eudragit S100 are shown in Figure 2.9. From the images it can be seen that the polymer is spherical in shape within in the size range 10-20 µm. The surface of the polymer appears to be relatively smooth with very few inclusions or abnormalities. The SEM images are important to future investigations as they will indicate whether the Eudragit S100 is present in its original form that has precipitated out of solution or whether a film has been formed through the interaction of the Eudragit solution and the liposomes. It has been shown in a number of studies that the use of chitosan produces a thin coating layer on liposomes (Mady *et al*., 2009; Laye *et al*., 2008; Wei and Lu, 2003), but as yet the direct coating of liposomes has not been completed using a Eudragit S100 solution.

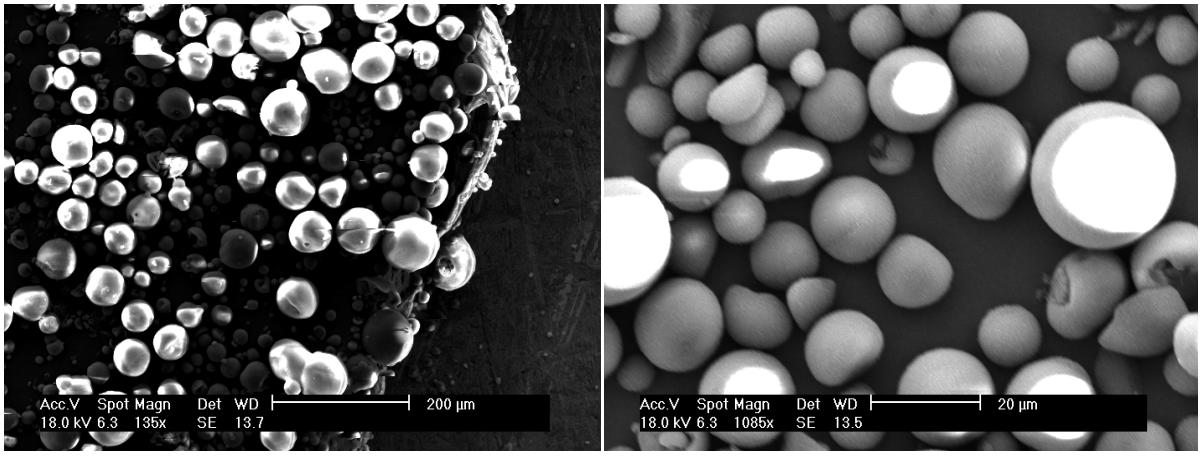


Figure 2.9. SEM images showing the uniform spherical nature of as received Eudragit S100.

2.5.2. Chitosan characterisation

2.5.2.1. GPC analysis of chitosan

A number of studies have shown that the specific molecular weight of chitosan has a profound effect upon the dissolution profile (Lorenzo-Lamosa *et al*., 1998). Theoretically it is known that an increase in M_w would lead to an increase in polymer viscosity/density and therefore a significant slowing in drug release times would be anticipated. It is therefore essential that the molecular weight of the chitosan being used is known to subsequently predict the *in vivo* response. From the resultant GPC data shown in Table 2.2 and corresponding distribution (Figure 2.10) it can be seen that the M_w of the chitosan was 237,000-238,000 with very little range in M_n and polydispersity being observed, which is

indicated by the closely overlapping distributions. Previous studies have shown the use of chitosan with a M_w in the region of 250,000 were suitably solubilised when exposed to colonic microflora. In contrast to this chitosan with an increased M_w in the region of 400,000 showed very little solubilisation in the same conditions. Not only is the molecular weight important to know for *in vivo* predictions but it has also been shown that during *in vitro* analysis the molecular weight is an important factor when producing simulated colonic fluid to ensure chitosan solubilisation will occur.

Further advantages of knowing the specific M_w of the chitosan used relates to the surface interactions between the polymer and liposomes. It has been observed that chitosan grades with lower M_w have shown a more uniform adsorption when coating liposomes (Henriksen *et al*., 1997), which in turn reduces the probability of interaction between different particles and subsequently reduces agglomeration. Knowledge of the exact M_w would therefore allow predictions to be made of the processes involved during the coating of liposomes with chitosan with a lower M_w chitosan having shorter chains and therefore produce a charge reversal of particles, whilst a larger M_w would lead to bridging effects dominating the process (Henriksen *et al*., 1994).

Table 2.2. Table showing the GPC results for two separate trials of chitosan indicating the range of M_w, M_n and polydispersity

Sample	M_w	M_n	Polydispersity
Chitosan (A)	238,000	50,300	4.7
Chitosan (B)	237,000	50,200	4.6

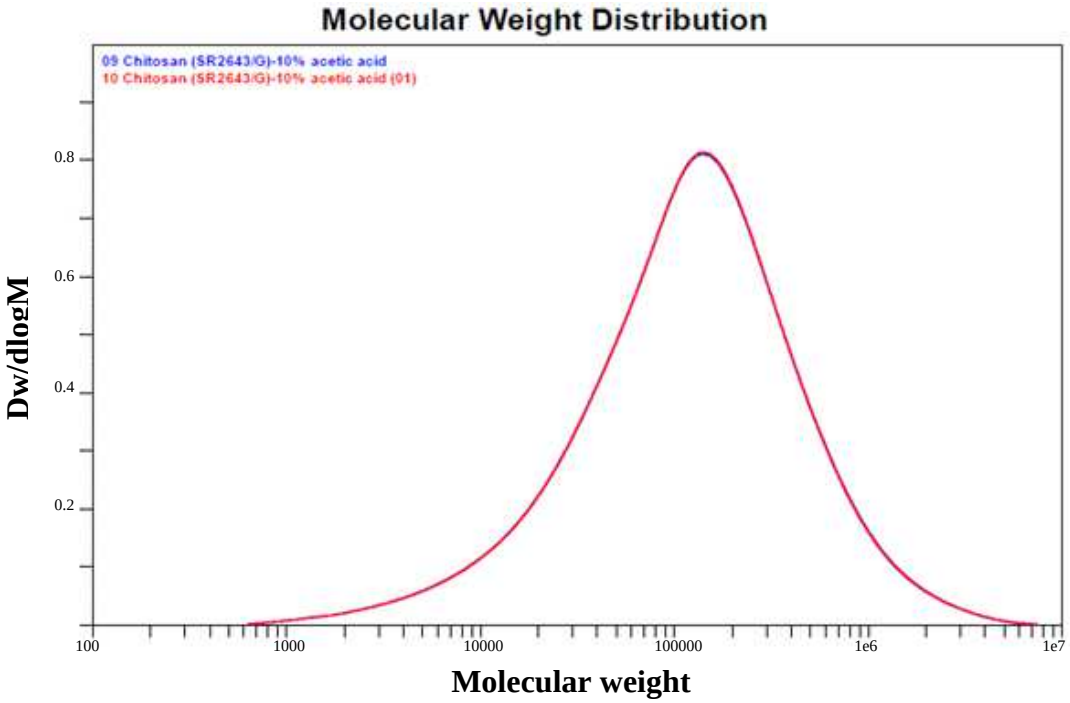
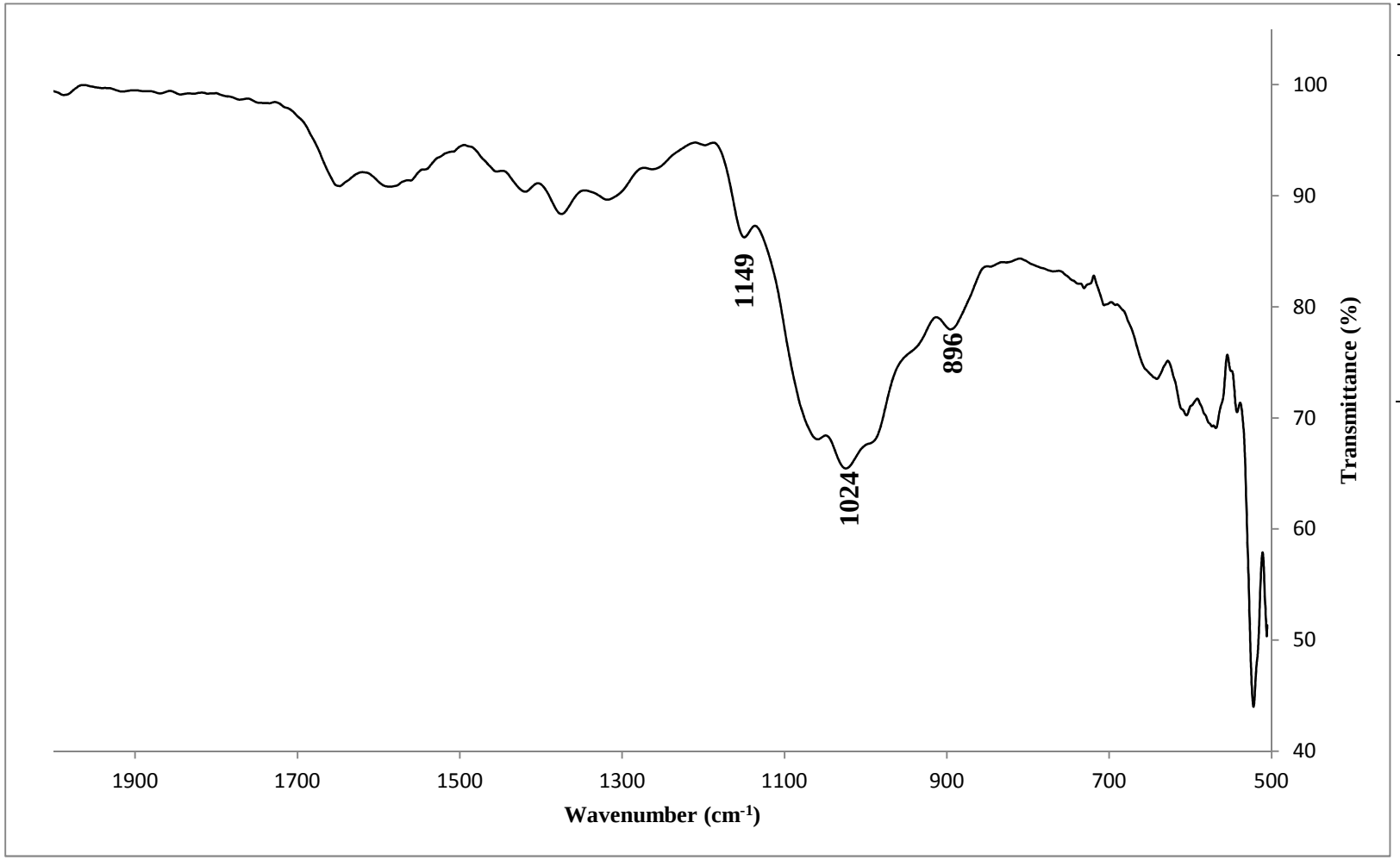


Figure 2.10 – Molecular weight distribution for as received chitosan showing two separate trials overlapping each other determined through GPC.

2.5.2.3. FTIR analysis of chitosan

The FTIR analysis of as received chitosan is shown in Figure 2.11. The main peaks have been identified with their corresponding bonds being outlined in the table accompanying the spectra. The main bonds associated with chitosan visible on the FTIR spectra are the NH_2 bending at 1648 cm^{-1}, 1585 cm^{-1}, 896 cm^{-1} and 641 cm^{-1}. CH_2 bending is visible at 1375 cm^{-1} and O-C stretching vibrations of the carboxylic acid group can be seen at 1149 cm^{-1}, 1059 cm^{-1} and 1024 cm^{-1}.



Wavenumber (cm^{-1})	Bond
1648	NH_2
1585	NH_x
1375	CH_x
1149	O-C
1024	O-C
896	NH_x
641	NH_x

Figure 2.11. FTIR trace for as received chitosan. The attached table shows the exact wavelength of the peak and the corresponding bond it represent

2.5.2.4. SEM analysis of chitosan

The SEM imaging of as received chitosan particles are shown in Figure 2.12. It can be seen that the chitosan particles vary in both size and shape, showing a number of plate-like particles.

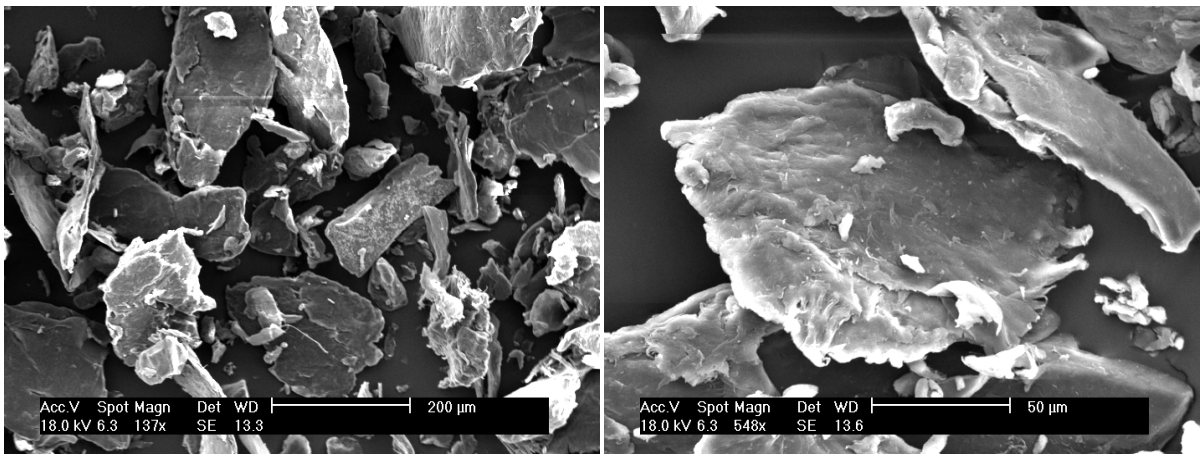
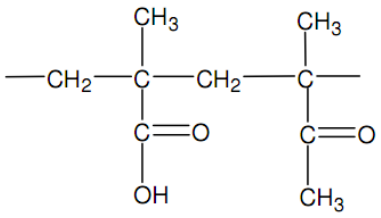
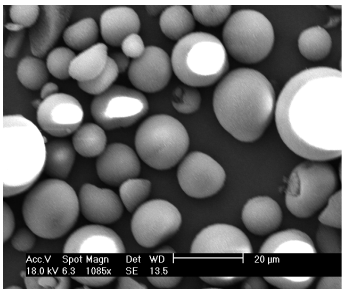
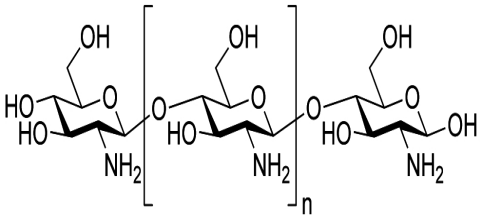
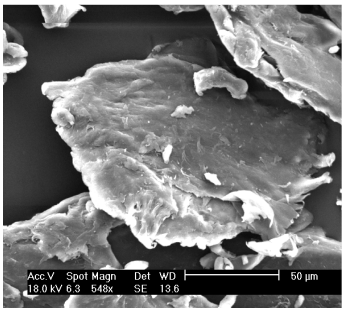


Figure 2.12. SEM images showing as received chitosan with irregular particle size and shape throughout the sample.

2.6. Conclusions

The current chapter provides an introduction to both the polymers being used throughout this thesis (Table 2.3). The chemical structures, physical properties and solubility mechanisms have been discussed. Through GPC the specific molecular weights of Eudragit S100 (122,500) and chitosan (237,500) were experimentally derived. The use of FTIR built up a picture of each of the polymers specific characteristics including identifying the specific bonds within the chemical structure which lead to their solubilisation through different triggers. SEM showed a smooth spherical structure to Eudragit S100 whilst in comparison chitosan showed an irregular shape with both abnormalities and inclusions throughout the sample.

Table 2.3. Summary table displaying properties of Eudragit S100 and chitosan.

Polymer	Molecular weight	Chemical Structure	SEM image
Eudragit S100	122,500		
Chitosan	237,500		

Beyond the characterisation of the polymers, the specific solubilisation mechanisms have been outlined with the specific triggers and subsequent bond cleavage being identified. This chapter acts as an introduction to the polymers involved in this thesis and subsequently provides a framework to analyse any future results for formulations produced using either Eudragit S100 or chitosan.

3.0 Exploring direct coating of liposomes with Eudragit S100

ABSTRACT

Liposomes have been coated with the pH responsive polymer Eudragit S100, and the formulation's potential for lower GI tract targeting following oral administration has been 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. The results show an association between the liposome and polymer through a significant change in zeta potential, increased size and visual indication of a coating layer. 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. Further drug release trials were conducted whereby the simulated small intestine condition included the model bile salt sodium taurocholate. Whilst 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. It is thought the interaction between liposomes and Eudragit S100 may follow a non-linear adsorption regime and therefore a solid coat is improbable.

3.1. Introduction

A number of studies have shown that the direct coating of liposomes with chitosan is possible through electrostatic interactions (Henriksen *et al*., 1994 and 1997; Perugini *et al*., 2000; Guo *et al*., 2003; Mady *et al*., 2009). The strategy involves the simple mixing of the liposomal formulation with a chitosan solution usually dissolved in acetic acid. It has been decided that a direct coating method through stirring of a liposome solution with a polymer solution would be simplest and therefore the first investigation that will be carried out. As chitosan is soluble in the acidic environment in the stomach it was decided that attempting to directly coat liposomes with the pH responsive polymer Eudragit S100 would provide the simplest and most effective formulation at targeting the ileo-caecal junction for drug delivery.

The main aim of the studies described in this chapter was to investigate whether liposomes could be directly coated with Eudragit to produce a formulation that specifically targets the colon. It was hypothesised that cationic liposomes could be coated through mixing with the anionic polymer Eudragit through electrostatic interactions which has been observed in a variety of other polymers. It was hypothesised the resultant formulation would be pH responsive and subsequently begin release in the ileo-caecal junction for drug release to occur in the colon. Characterisation of the coating mechanism involved was undertaken through a variety of methods including imaging, zeta potential and size distribution. The suitability of the formulation was assessed by simulating the conditions representative of the GI tract. Once the liposomal coating has been confirmed the formulation was then assessed *in vitro* in buffer systems representative of those experienced *in vivo.*

3.2. Materials

3.2.1. Phospholipids and cholesterol

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. EPC and CH were stored at -20°C and SA at room temperature in accordance with the manufacturer's guidelines.

3.2.2. Model drug

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 sink conditions could be readily maintained during release studies). Vitamin B_{12} was stored at 4°C in accordance with the manufacturer's guidelines.

3.2.3. Buffers for simulating GI tract conditions

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*., 2006a), small intestine (Ibekwe *et al*., 2006a) and ileocaecal junction (Khan *et al.*, 1999), respectively. All components for the release media were purchased from Sigma Aldrich (Dorset, UK).

3.2.4. Other chemicals and reagents

All other chemicals and solvents used were of an analytical grade and used as received.

3.3. Apparatus and methodology

3.3.1 Production of cationic liposomes

Liposomes were prepared using EPC and CH in the molar ratio 1:1. Initially, a number of molar ratios of SA were investigated (0, 5, 10, 15, and 20%) to determine the minimum amount required to produce a consistently cationic liposomal formulation. The results of subsequent zeta potential analysis (section 3.4.1) indicated that the use of 5 mol% would be most suitable for the application. The conventional thin film hydration method (Bangham *et al*., 1965) was used to produce multilamellar vesicles (MLVs) for the study. The lipids were dissolved in 5 ml chloroform in a 50 ml round bottom flask. The chloroform was then removed using a rotary evaporator (Rotavapor-Buchi), leaving a thin lipid film on the side of the flask which was then dried under nitrogen for 2 hours to remove any 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 by vortex mixing. Excess drug was

removed by centrifugation using a Sigma 3K30 centrifuge maintained at 4°C to prevent the sample heating up. Centrifugation was carried out at 26,000 rpm (63,000g) using a 12111 angled rotor with the capacity of 10 x 10 ml tubes. The liposomes were centrifuged at 26,000 rpm (relative centrifugal force (rcf) 63,000) and washed three times by removing the supernatant and replacing with fresh PBS (Figure 3.1). The final pellet was then re-suspended in 10 ml of PBS.

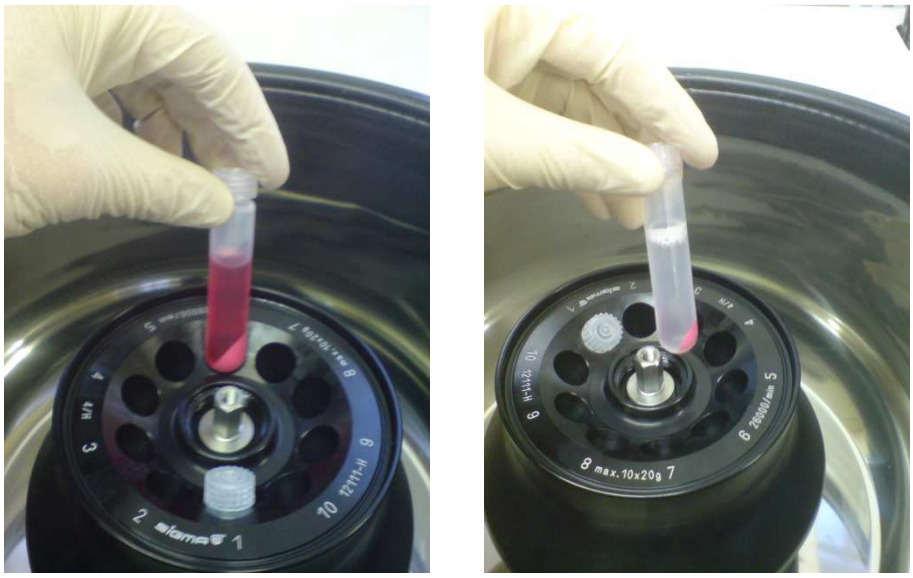


Figure 3.1. Vitamin B_{12} loaded liposomes before and after centrifugation and washing.

3.3.2. Production of Eudragit S100 coated liposomes

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 pH 7.4 PBS) were combined and hand-shaken for 2 minutes.

3.3.3. Liposomal characterisation techniques

3.3.3.1. Zeta potential

Zeta potential measurements can be used to determine the stability of a colloidal system. The measurements indicate the overall surface charge of a particle and therefore provide

information as to whether the system may remain stable or consequently undergo aggregation or flocculation. Particles with a zeta potential more positive than + 30 mV, or more negative than -30 mV are considered stable as they will repel each other. The zeta potential of a colloidal system can be affected by a number of factors including pH and conductivity.

Electrophoresis refers to the movement of a charged particle relative to the liquid it is suspended in under the influence of an applied electric field, and can be used to calculate the zeta potential of a system. It is possible to measure the electrophoretic mobility by using a zeta potential instrument. The relationship between zeta potential and electrophoretic mobility is shown through the Henry equation:

$$U_E = \frac{2\varepsilon.z.f(Ka)}{3\eta} \quad (1)$$

Where U_E = electrophoretic mobility, ε = dielectric constant, z = zeta potential, f(Ka) = Henry's function and η = viscosity.

The instrument used to calculate zeta potential measures the electrophoretic mobility by applying an electric field across a capillary cell. Particles then move towards the electrode with their velocity being measured and expressed in unit field strength as their mobility. A laser source is used to illuminate the particles in the sample, with scattered light being detected after it has passed through the sample. The movement of particles in the cell, when the potential is applied, will cause a fluctuation in light intensity which can then be interpreted by a digital signal processor and subsequently fed to a computer.

Initially zeta potential was used to characterise the amount of SA required to produce a liposomal formulation which has a plateau in zeta potential and therefore suitably cationic for coating with the anionic polymer Eudragit S100. This also allowed for the minimum amount of SA to be ascertained and therefore reduce the amount of excess used within the liposomal system. This would be essential for the formulation due to the knowledge that SA has certain levels of toxicity when used in high quantities *in vivo* (Campbell 1983; Senior *et al*., 1991).

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 3.3.2.) 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. The calibration of the instrument was periodically confirmed using a zeta master transfer standard (Malvern Instruments - DTS1230) that had a charge of –68 mV ± 6.8 mV at 25ºC.

3.3.3.2. 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.

3.3.3.3. Cryo-scanning electron microscopy (cryo-SEM)

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 sputter coated with gold for 2 minutes 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.

3.3.3.4. Laser diffraction particle sizing

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). The instrument has a quoted measurement range of 0.02 – 2000 µm and therefore enabled the full range of particles to be analysed produced in this study. Measurements are taken when a helium-neon laser passes through a flow cell based within a column. Particles within the flow cell scatter the light at an angle which is inversely proportional to their size. Detectors are used to

measure light and subsequently calculate the particle using the Mie scattering model. The Mie model takes into account both diffraction and diffusion of the light around the particle in its medium. Measurements were carried out in pH 6.3 Hanks' and pH 7.4 PBS where the polymer is insoluble and soluble respectively. Three independent formulations were produced and subsequently measured five times with a mean value for each trial given.

3.3.4 Drug release studies

3.3.4.1. UV spectral analysis and calibration curves of vitamin B_{12}

Initially, a UV spectra was completed to determine a suitable wavelength which Vitamin B_{12} has a suitable absorbance to analyse drug release media for B_{12} content (Figure 3.2). All UV spectra were completed on a Jasco V-530 UV/Vis spectrophotometer. A small amount of vitamin B_{12} was dissolved in water with the spectra being taken in the range 250-500 nm, at 5 nm/second.

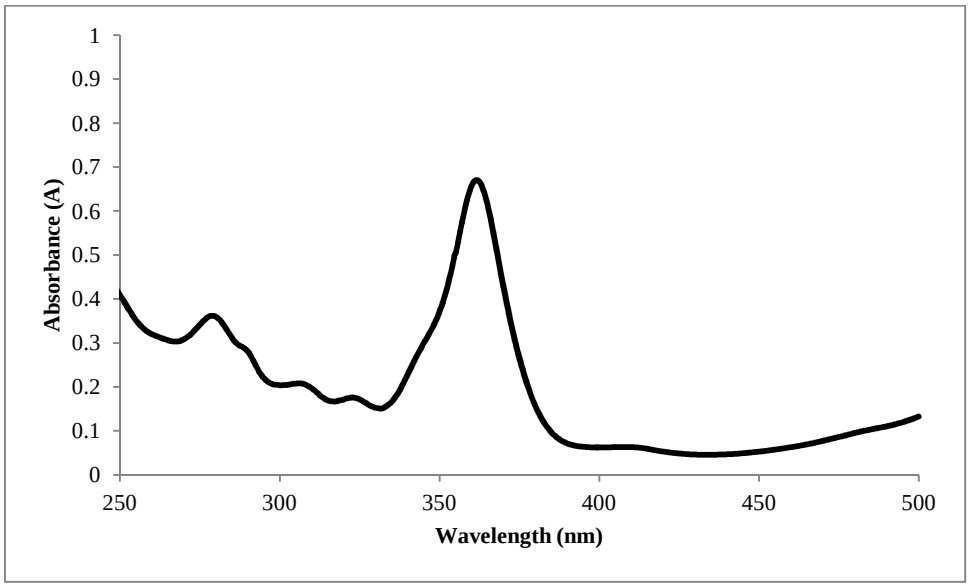
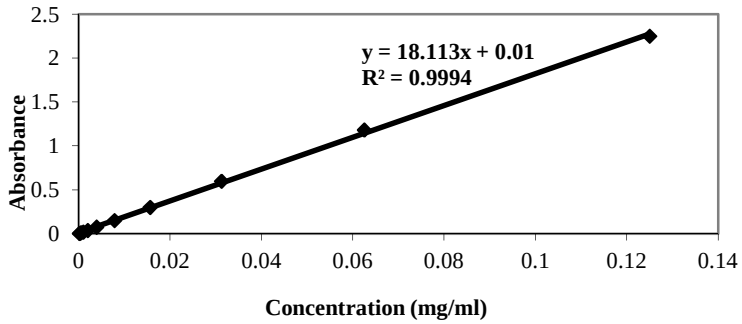
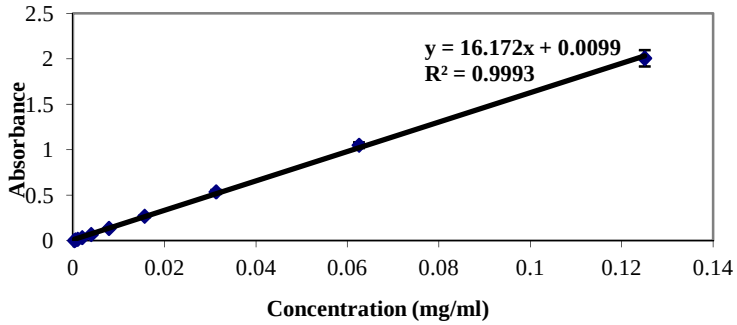


Figure 3.2. UV spectra of Vitamin B_{12} dissolved in water.

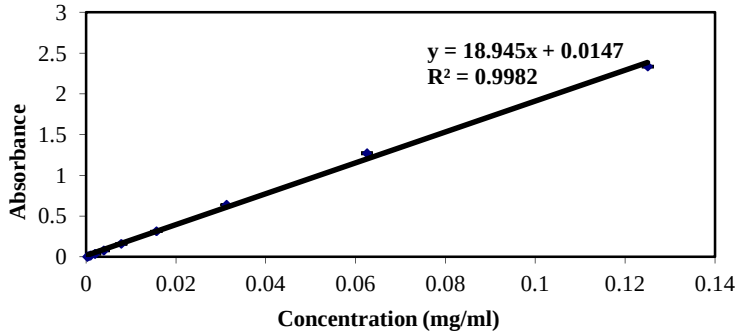
Calibration curves were then constructed for vitamin B_{12} dissolved in each of the buffers to be used during the release trials (thus allowing for concentrations to be calculated from the specific absorbance measured during the release trial). For each calibration curve a solution of 1 mg/ml of vitamin B_{12} was made up in the required buffer, and then a serial dilution was completed to a point where the absorbance level became negligible. All measurements were completed in triplicate on independent samples at the λmax = 361 nm.



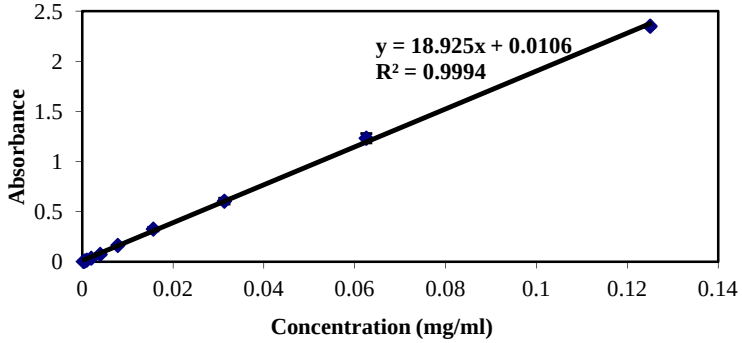
(A)



(B)



(C)



(D)

Figure 3.2. Calibration curves for vitamin B_{12} dissolved in (A) 0.1M HCl, (B) Hanks' solution, (C) PBS and (D) ethanol at 361 nm.

3.3.4.2. Quantification of encapsulated vitamin B_{12}

The amount of drug present within the liposomal sample prior to coating was quantified by lysing the liposomes in ethanol and therefore releasing the entrapped drug. Briefly, 0.1ml of liposome sample was added to 0.9ml ethanol and vortex mixed to ensure all the liposomes had been destroyed. The solution was then analysed spectrophotometrically with the concentration being calculated by referring to the calibration curve in ethanol (Figure 3.2 D) and accounting for any dilutions.

3.3.4.3. Drug release studies in pH conditions representative of the GI tract

Drug release studies with uncoated and coated liposomes (liposomes + polymer will be referred to as coated liposomes throughout) were conducted in each of the different pH media described in section 3.2.3. For each release experiment, 1 ml of liposomal suspension was added to 40 ml of preheated (37˚C) release medium and well-agitated (100rpm) in an incubator maintained at 37°C. Sink conditions were maintained throughout each experiment, ensuring the maximum released concentration was less than 10% of the saturated solubility of vitamin B_{12} in accordance with the British Pharmacopoeia (BP). 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. 1 ml of pre-heated fresh buffer was replaced to maintain sink conditions throughout. 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.

For all drug release studies throughout this thesis statistical methods have been adopted to ascertain whether there is any difference between the samples. The Mann-Whitney U test was adopted as it is a non-parametric statistical hypothesis test for assessing whether one of two independent observations is significantly different to the other. The Mann-Whitney U test adopts a ranking system which attributes a score to each data point between two samples. The sum of these scores is then used to calculate a U value for both sets of data. The lower U

value is then used and compared to critical values from a set of values found in specific tables. If the U value is lower than that of the tabulated value for that level of significance it can be deemed that there is a statistical difference between the two samples.

3.3.4.4. Drug release studies with the addition of the model bile salt sodium taurocholate

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 (Iwanaga *et al*., 1997)). These trials aimed to test the liposomal formulations beyond their response to pH alone. Over a period of 4 hours (the upper estimation of small intestine transit time (Rouge *et al*., 1996; Wilding, 2001; Davis *et al*., 1986) samples were removed and analysed spectrophotometrically at λ=361nm against a reference sample of the release medium.

3.3.4.5. Investigation into bile salt interaction through size distribution and FTIR analysis

The inclusion of a model bile salt to simulate the conditions experienced in the small intestine offers a significant challenge to liposomal formulations. The presence of a bile salt may influence both the drug release but also may have some bearing on the chemistry of the coating polymer, Eudragit S100. 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) at a resolution of 2 cm^{-1}. 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 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.

3.4. Results and Discussion

3.4.1. Investigation into stearylamine concentrations required for cationic liposomal formulation

The 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 (Table 3.1). Higher levels of SA were not found to significantly increase zeta potential, and therefore it was decided to use a 5 mol% throughout. It was imperative to obtain a balance between producing a stable cationic liposomal formulation (minimum 30 mV) but also to maintain the SA levels at their lowest point required due to its reported toxicity. The toxicity of SA (LD_{50} 2395 mg/kg in rats) is so low that for the purposes of the current formulation the levels are negligible. If the levels of SA were to become an issue then it may be resolved through the use of a synthetic cationic lipid for example DOTAP, DOPE and therefore eradicating the need for SA. The use of these lipids would however alter the methodology required due to the higher transitional temperatures seen in synthetic lipids.

Table 3.1. Table showing the change in zeta potential when increasing the concentration of stearylamine in the liposomal formulation.

SA Concentration (mol %)	**Zeta Potential (mV)**
0	**-12.3 ± 10.7**
5	**63 ± 3.6**
10	**63 ± 6.3**
20	**64 ± 4.7**

3.4.2. Investigation into the evolution of the Eudragit S100 coating

3.4.2.1. Zeta potential analysis to determine the presence of a Eudragit S100 coat

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; Takeuchi *et al*., 2005). Guo *et al*. (2003) observed the evolution of a chitosan coating on liposomes of differing purity (Epikuron 170 and 200) through zeta potential analysis. Both samples showed a marked increase in zeta potential, with the lipid of lowest purity showing the greatest change in zeta potential due to the electrostatic interactions dominating as opposed to the high purity lipid where hydrophobic interactions dominate. Similarly, Takeuchi *et al*. (2005) observed an increase in zeta potential with the increasing concentration of chitosan in the presence of DSPC liposomes to a certain concentration where the zeta stabilised and a complete coating had evolved. Figure 3.3 shows the vesicle zeta potential as a function of polymer concentration, where the polymer concentration shown is that of the 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 then used in all further studies.

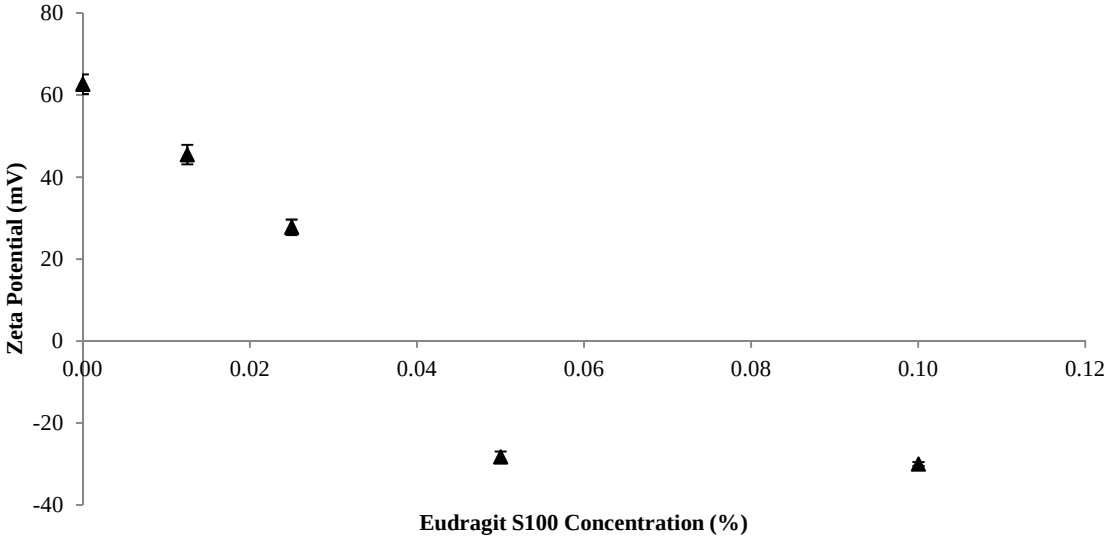
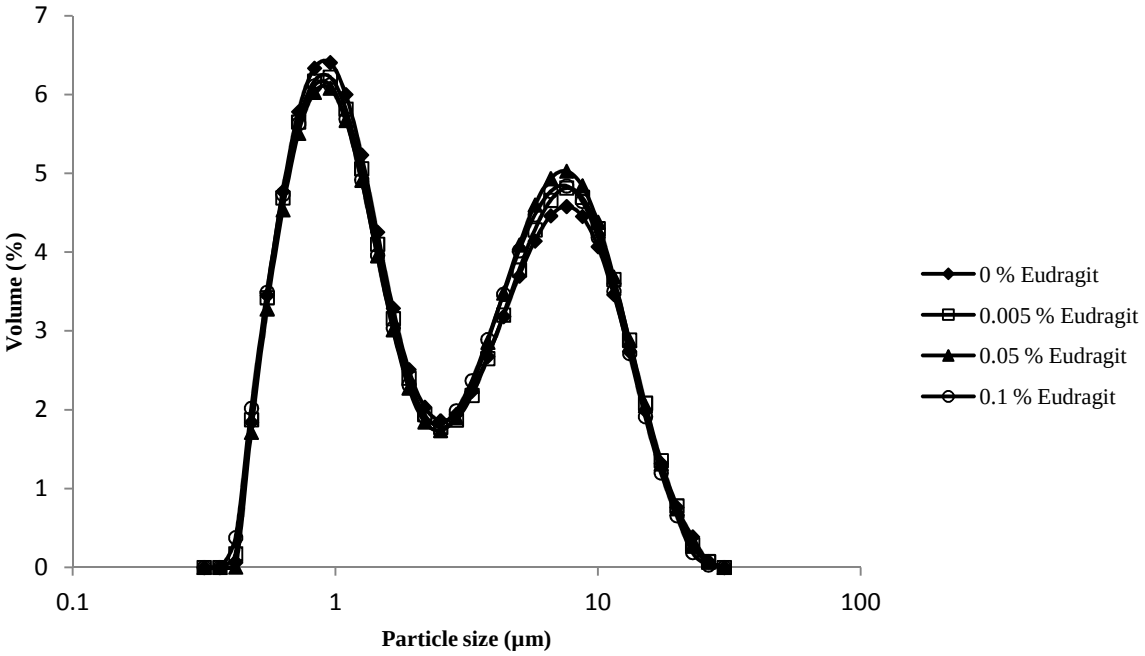


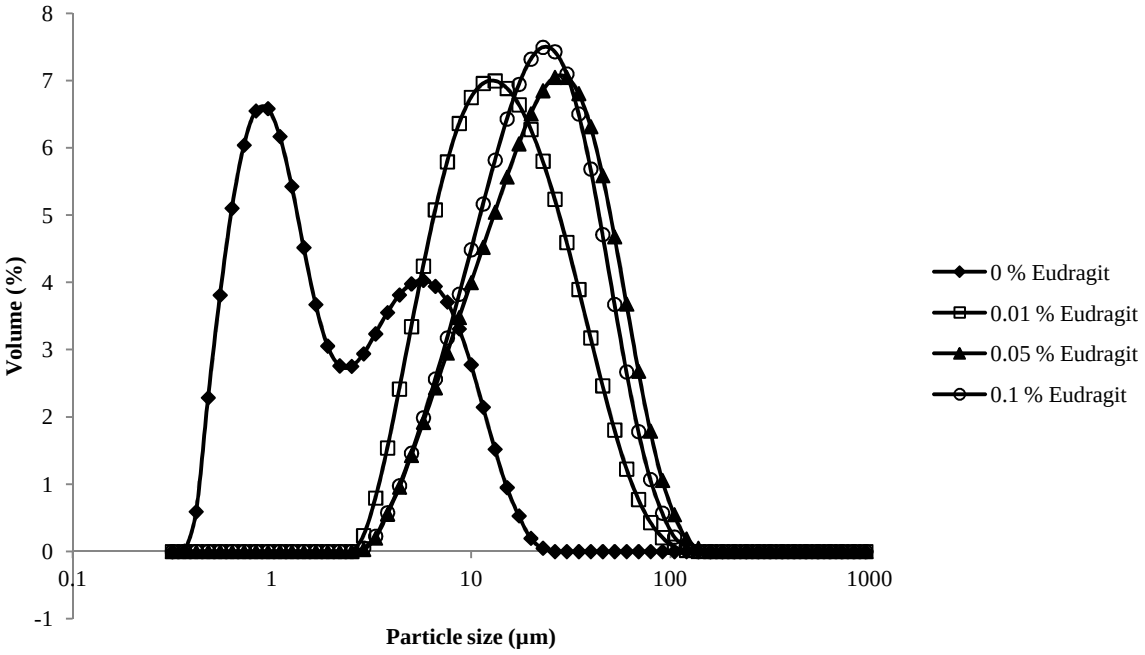
Figure 3.3. Change in zeta potential with the increased concentration of Eudragit S100 in water. Error bars represent the mean ± the standard deviation of three independent trials.

3.4.2.2. Laser diffraction particle sizing to investigate presence of Eudragit S100 coat

Size distribution analysis was completed at two pH values; one at which the Eudragit S100 was insoluble (pH 6.3) and one at which the polymer was soluble (pH 7.4). It was hypothesised that the coating layer should only be observed at a pH lower than 7 due to the solubility profile of Eudragit S100. Size distribution in pH 7.4 PBS, where the Eudragit S100 is soluble, showed no change with the inclusion of Eudragit S100 (Figure 3.4A). The size distribution profile observed for uncoated liposomes is bimodal and therefore expected due to the heterogeneous nature of a MLV formulation. For the size distribution completed in pH 6.3 Hanks' solution, where the polymer is insoluble, increased vesicle size (Figure 3.4B) correlated with a higher concentration of polymer. This increase continued until 0.05% at which point there was a plateau, similar to that seen for the zeta potential results.



(A)



(B)

Figure 3.4. Laser diffraction particle sizing distributions for Eudragit S100 coated liposomes in pH 7.4 PBS (A) and pH 6.3 Hanks' solution (B).

3.4.2.3. Imaging techniques to observe Eudragit S100 coat

Evidence of an association between the polymer and liposomes was also seen using light microscopy. Figure 3.5A 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 3.5B), 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 3.5D). A control experiment (Figure 3.6) in which liposomes were excluded showed that polymer 'particles' resulting from precipitation at pH 6.3 were considerably smaller (approximately 500 nm) than the liposomes used in this study. In this way, the agglomerates seen in Figure 3.5C were assumed to be liposomes + polymer and not precipitated polymer alone.

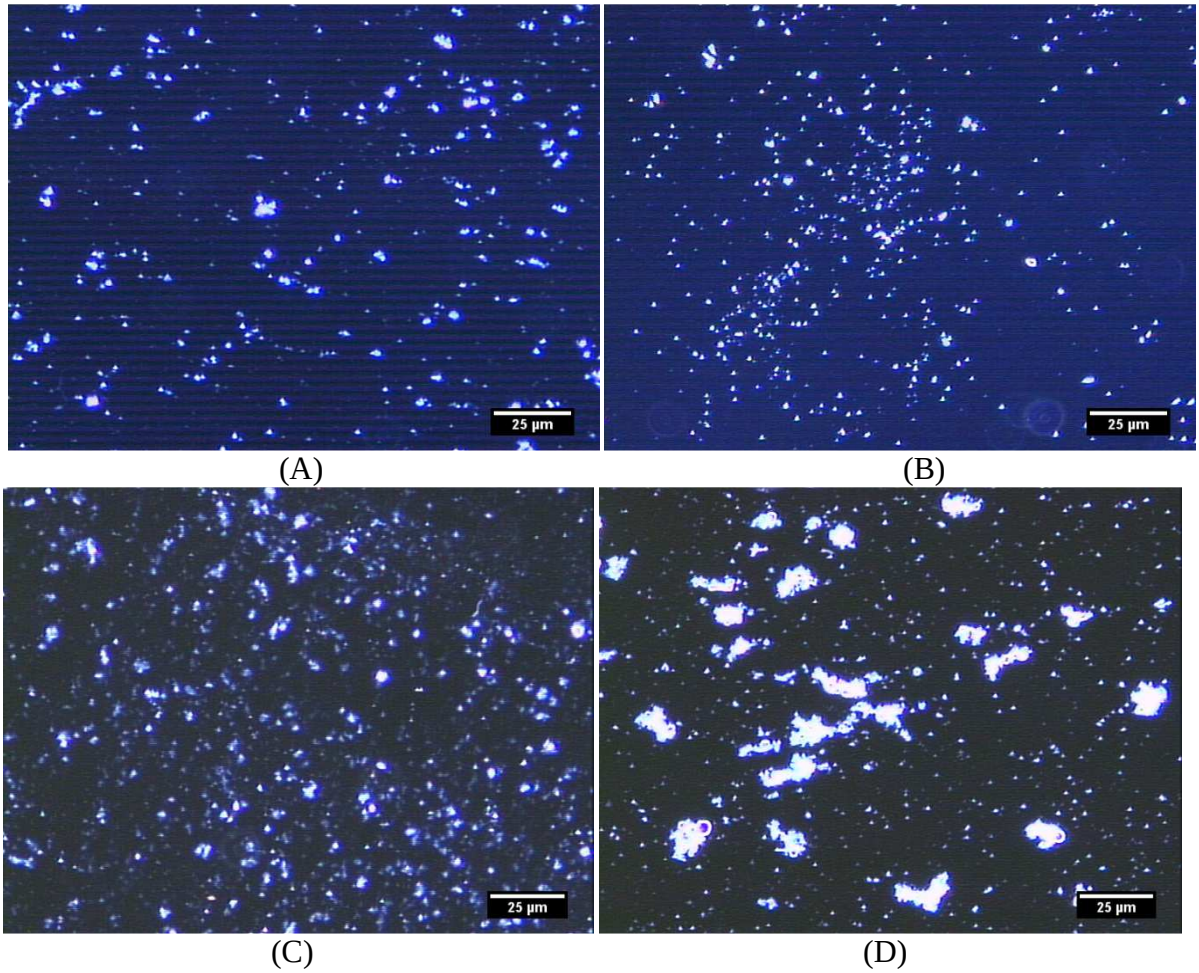


Figure 3.5. Light microscope images of (A) uncoated and (B) Eudragit S100 coated liposomes in pH 7.4 PBS. Further light microscope images of (C) uncoated and (D) Eudragit S100 coated liposomes in pH 6.3 Hanks' solution.

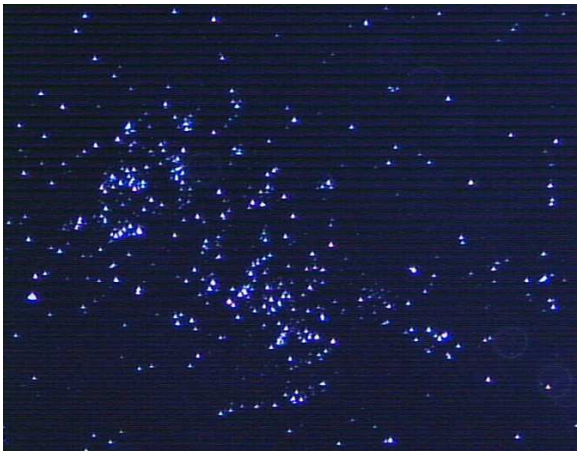


Figure 3.6. Light microscope image of control experiment showing precipitated Eudragit S100 at pH 6.3 not in the presence of liposomes.

Cryo-SEM imaging was used to image the liposomal formulations in their 'natural' state as opposed to lyophilisation and rehydration, which has been shown to alter liposome characteristics (particle size, drug retention) when produced without a cryoprotectant (Glavas-Dodov *et al*., 2005). Throughout all experiments, liposomal formulations were investigated whilst still in a wet dispersion, having undertaken no form of drying. This further indicates the importance of cryo-SEM as imaging can be completed using liposomes in the same state that all other experiments have been completed.

In Figure 3.7 typical images of uncoated liposomes from cryo-SEM are shown. The images clearly show the lamellae with each bilayer being visible. The central aqueous core can also be identified and has been labelled accordingly. The range in size of MLVs can also be observed, as a number of smaller vesicles, which can be seen around the larger fractured liposomes.

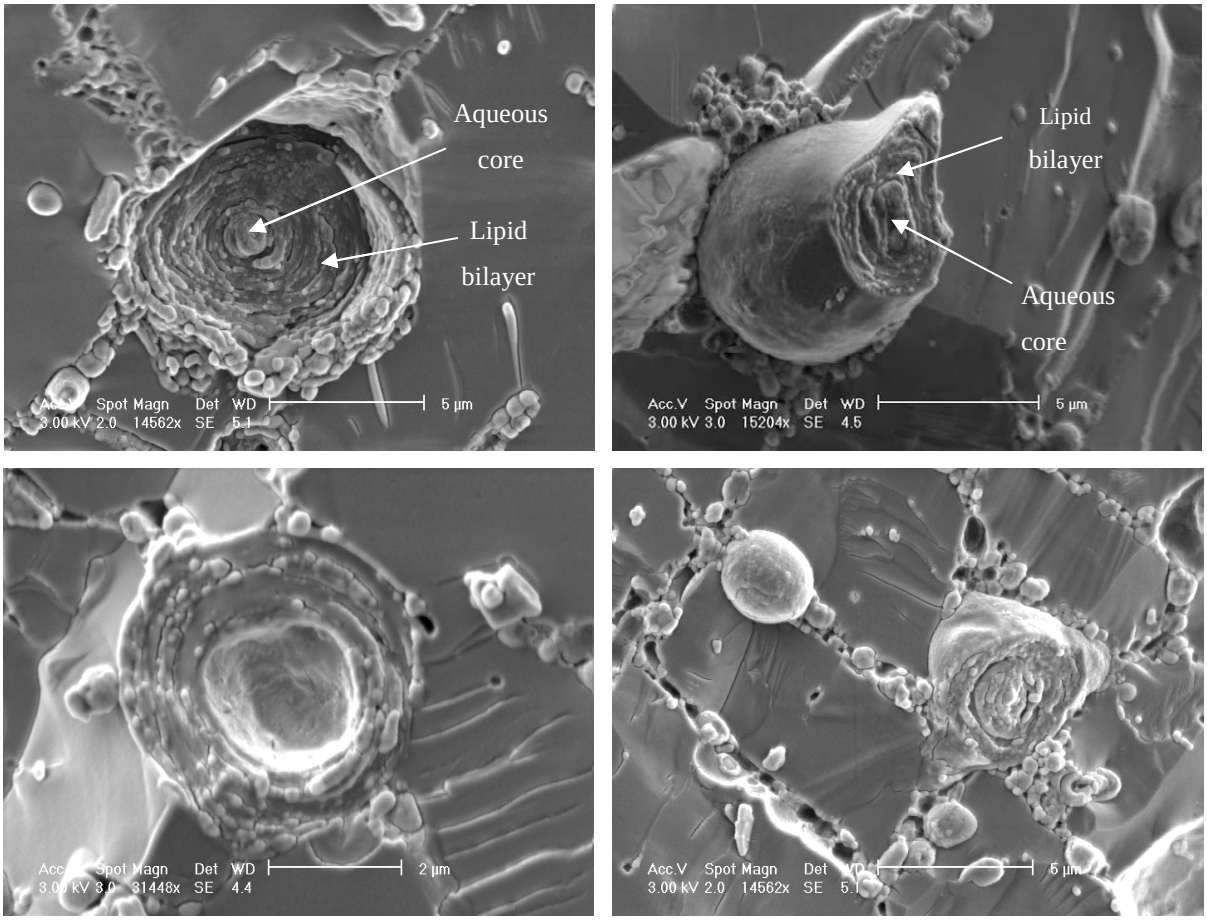


Figure 3.7. Cryo-SEM images of uncoated liposomes. The aqueous core and lipid bilayers are labelled showing the internal structure of uncoated MLVs.

Cryo-SEM images were taken of Eudragit S100 coated liposomes in conditions where the polymer was insoluble (Figure 3.8) and where the polymer was soluble (Figure 3.9). Figure 3.8 displays images taken with the Eudragit S100 coated liposomes suspended in pH 6.3 Hanks' buffer. It can be seen that a polymer crust forms around the liposomes, which was not observed in the uncoated samples (Figure 3.7). The coating on the surface of the liposomes is spherical in nature, which would indicate an agglomeration of precipitated Eudragit S100. The structure of the coat adopts the appearance of Eudragit S100 in its natural state (Figure 2.9), indicating the possibility that Eudragit S100 shows no change in form through the coating process. This indicates that when the polymer precipitates on the surface of the liposomes very little morphological change can be seen, which would indicate that a single coating layer may not have been achieved as with other coating techniques (Guo *et al.*, 2003; Davidsen *et al.*, 2001). The images indicate more of an agglomeration of Eudragit S100 on the surface of the liposomes as opposed to the previously hypothesised single layer

coating. This agglomeration occurs due to the electrostatic interactions between the polymer and liposomes, but the actual form of Eudragit S100 indicates no significant polymer bridging has occurred, which would be seen when coating liposomes with chitosan; for example Henriksen *et al.*, 1994, 1997. The images showing coated liposomes in pH 7.8 PBS (Figure 3.9) where the polymer is soluble display no visible agglomeration around the liposomes, but merely show similar characteristics to the uncoated formulations shown in Figure 3.7.

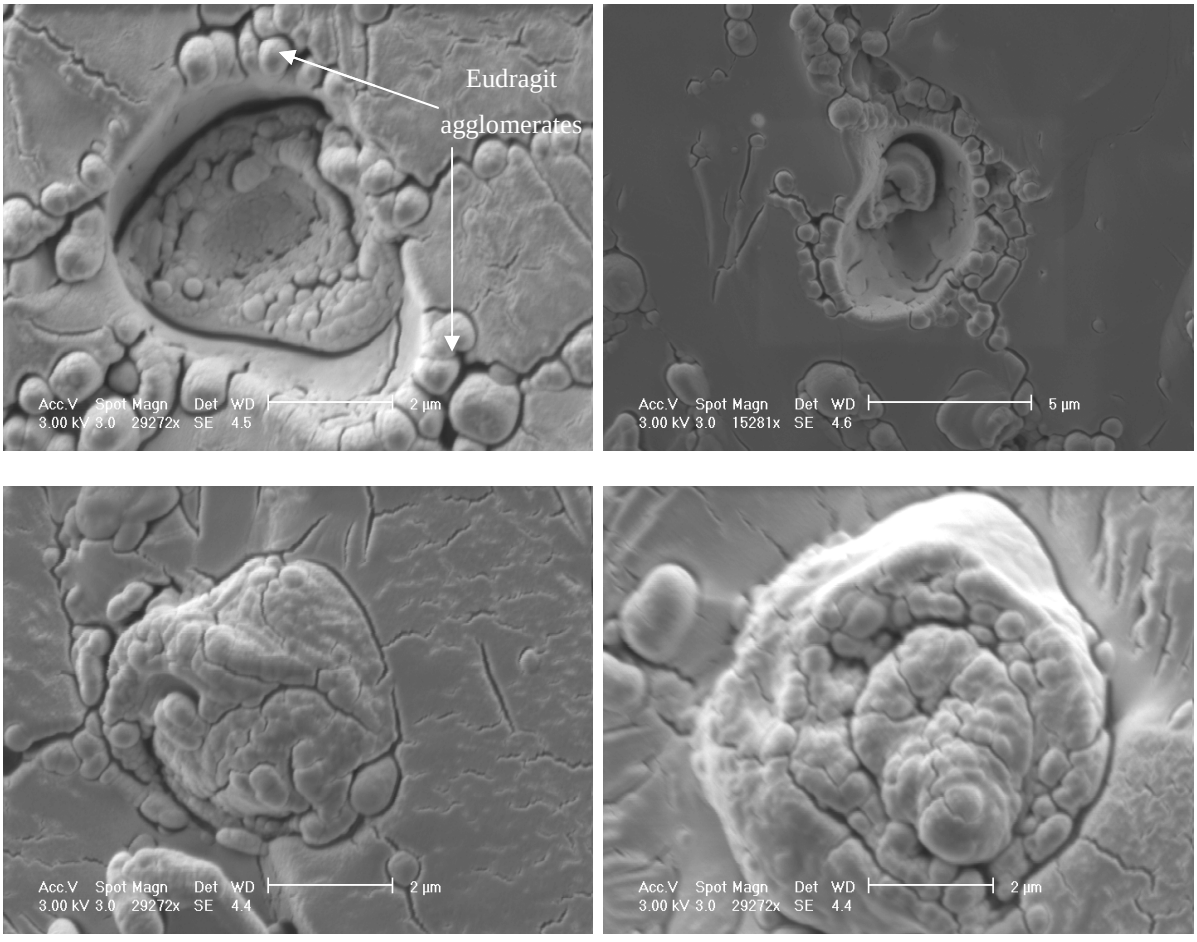


Figure 3.8. Cryo-SEM images of Eudragit S100 coated liposomes dispersed at pH 6.3 at which the polymer is insoluble. A Eudragit S100 'crust' is shown to be present around the liposome.

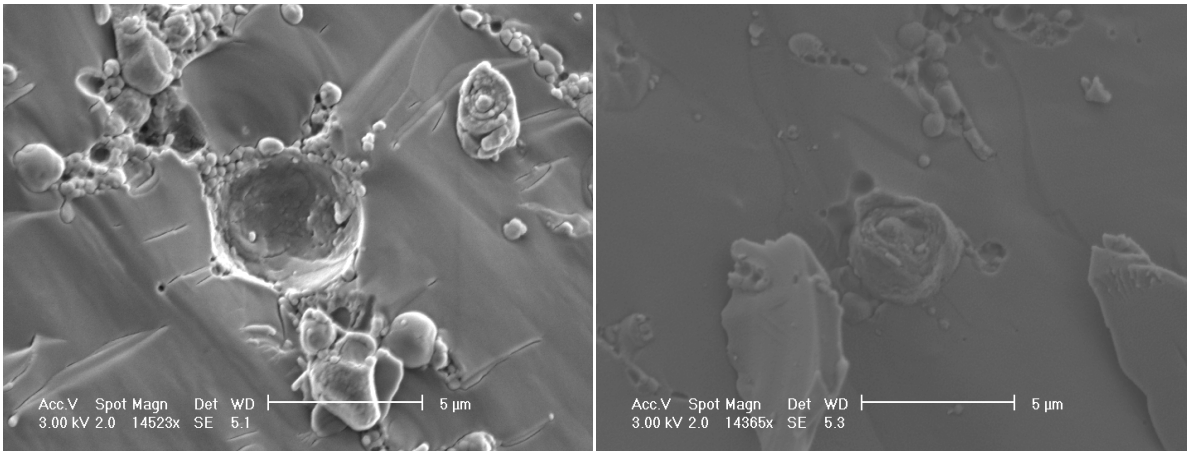


Figure 3.9. Cryo-SEM images of Eudragit S100 coated liposomes at pH 7.4 at which the polymer is soluble. The liposome structure can be seen without the presence of a polymer 'crust' similar to that seem for the uncoated liposomes.

3.4.2.4. Discussion of coating mechanism for Eudragit S100 coated liposomes

A number of studies have successfully coated both neutral and anionic liposomes with the cationic polymer chitosan (Henriksen *et al*., 1994 and 1997; Perugini *et al*., 2000; Guo *et al*., 2003; Mady *et al*., 2009). In each of these studies the coating mechanism was attributed to electrostatic interactions, which was proven through the distinct change in surface charge observed on the liposomes. The main issue is whether the same coating mechanism can be attributed to the use of Eudragit S100 as is observed for chitosan. Perugini *et al*. (2000) was the only study which expanded on the concept of electrostatic interactions, when looking at the coating of neutral liposomes with chitosan. It was hypothesised that the coating of neutral liposomes was through hydrogen bonding between the polysaccharide and the phospholipid head group. It can be assumed this would not be taking place in the current conditions due to the significant charge of the liposomes and the opposite charge of the Eudragit S100, therefore indicating that electrostatic interactions would dominate.

Few studies have attempted to directly coat liposomes with any of the Eudragit brand polymers, although Hasanovic *et al*., (2010) appeared to have some success in improving the stability of DPPC liposomes through coating with Eudragit EPO. Eudragit EPO is a Poly (butyl-methacylate-co-(2-dimethylaminoethyl) methacrylate-co-methyl methacrylate polymer which is soluble in gastric fluid up to pH 5 and swellable and permeable above pH 5. The technique used to coat the liposomes involved the mechanical stirring of the Eudragit solution (varying concentrations) with liposomes for 30 minutes (1:1 ratio). Similar to the current

study a significant decrease in zeta potential was observed along with an increase in particle size. It was hypothesised that the coating mechanism is similar to that observed for chitosan-coated liposomes with hydrogen bonding between the polymer and liposome surfaces. The main purpose of stabilising the liposomes was for the transdermal application of liposomes and therefore the specific integrity of the coat was not investigated.

Whilst it may be assumed that the coating mechanism would be similar when using an oppositely charged polymer to coat liposomes, there have been a number of papers discussing the complexities involved with particle adsorption and the role of electrostatic interactions (Adamczyk, 2003). Adamczyk outlined a distinct difference between linear and non-linear adsorption regimes whereby a linear adsorption regime would involve the ordered coverage of a particle through the sequential coating layer. To support this idea Perugini *et al*., (2000) observed a number of chitosan layers surrounding the liposomes indicating the presence of layer by layer deposition as described by Adamczyk (2003). Non linear adsorption regimes occur when adsorbed particles exert specific and hydrodynamic forces on adsorbing (moving) particles, excluding them from the part of the volume near the interface (Adamczyk, 2003). A non linear adsorption regime would provide a more complicated coating mechanism which is governed by a number of factors including particle size and shape, surface properties (charge), ionic strength, transport mechanism etc. The presence of non linear adsorption regimes can lead to a number of blocking effects preventing a solid adsorption layer on a particle surface. The cryo imaging of the coated liposomes would indicate that certain areas of the liposomes may not have been completely coated which may in theory be due to a non linear adsorption regime.

3.4.3. Drug release studies for Eudragit S100 coated liposomes

3.4.3.1. Drug loading and subsequent encapsulated aqueous volume calculations

Drug loading calculations were completed for each batch prior to coating and subsequent drug release trials. Each batch of MLVs produced was used as a source for both uncoated and coated drug release trials, therefore any differences between batches would be the same for both formulations. The range of drug loading spanned from 0.28 – 0.33 mg/ml which can be attributed to a number of differences occurring due to the self assembly nature of liposomes

which will lead to differences in size distribution and lamellarity. The encapsulated aqueous volume (ml) can then be calculated using the following formula:

$$Encapsulated\ aqueous\ volume\left(\frac{Drug\ loading\ concentration(mg/ml)}{Hydrating\ drug\ stock\ solution(mg/ml)}\right)x1ml \qquad (2)$$

This value can then be expressed in relation to the amount of lipid within the system and expressed in terms of litres aqueous space/mol lipid (l/mol). The mean captured volume in relation to lipid concentration was calculated as 1.28 l/mol which is very similar to the value quoted by Perkins *et al.,* 1988 of 1.2 µl/µmol for MLVs produced from dried film with a lipid concentration of 6 mg/ml.

3.4.3.2. Drug release profiles in simulated GI tract conditions representative of pH changes

In Figures 3.10, 3.11 and 3.12 drug release profiles for liposomes with and without polymer are shown in the different release media representing each stage of the GI tract. At pH 1.4 and 6.3 (Figures 3.10 and 3.11) 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 3.10) only 2.5% was released from the coated formulation compared to 10% for the uncoated. Similarly for a transit time representative of the small intestine (4 hours) the release for uncoated liposomes is approximately 12% while for the same time period only 3% release is observed for the coated formulation. It is perceived that the agglomeration of Eudragit S100 around the liposomes significantly reduces the diffusion of drug from the liposome bilayers in comparison of that observed for the uncoated formulations. However, it can clearly be seen that although drug release was significantly reduced it was not completely prevented. It is thought this may be due to the small channels still present between the Eudragit S100 particles, therefore allowing for a slower rate of drug diffusion to take place into the dissolution media. In comparison the coated formulations in both pH 1.2 and 6.3 show a gradual drug release which would possibly indicate a form of 'leaking' through the polymer channels formed as the Eudragit precipitates on the surface of the liposomes. As the

Eudragit S100 is insoluble throughout it is assumed that the polymer remains intact, and therefore a relatively constant release rate should be observed.

In contrast to this the release profile in simulated ileo-cecal junction dissolution media of pH 7.8 show similar release rates for both uncoated and coated formulations (Figure 3.12). This would be expected as previous studies looking into the solubility of Eudragit S100 in pH 7.0 PBS have shown thin films to form pores and partially break down within 2 minutes (Sabot and Krause, 2002). This would account for the very similar release profiles observed, and therefore little or no delay being seen in the presence of Eudragit S100. The uncoated formulations in the three media show a similar total release of approximately 80% after 120 hours, which is also observed for the coated formulation in pH 7.8 PBS, indicating the solubility of Eudragit S100 in pH 7 and above.

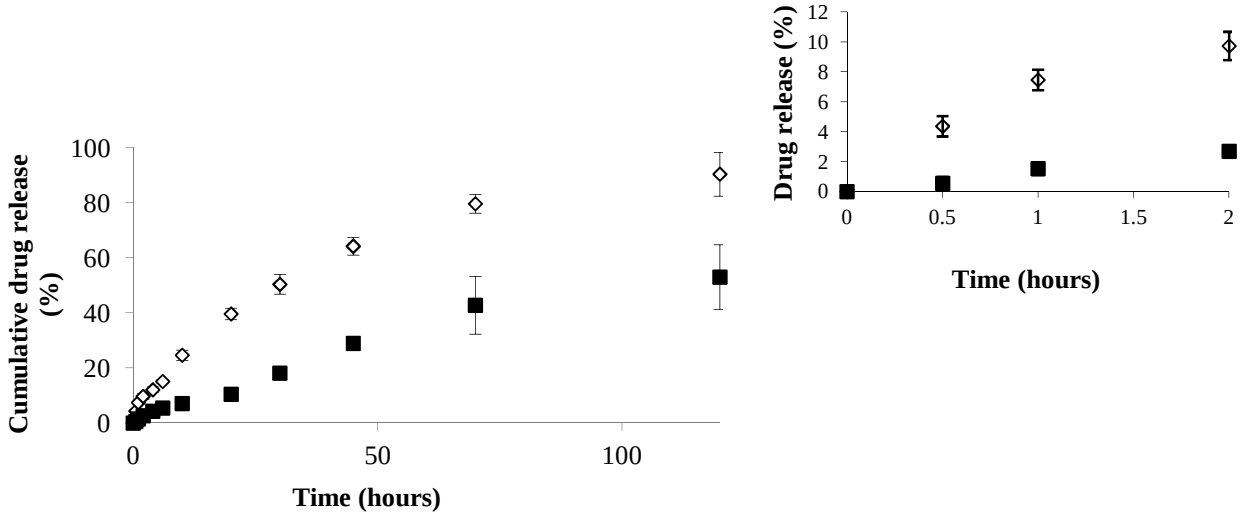


Figure 3.10. Drug release profile for uncoated (◊) and Eudragit S100 coated (■) liposomes in 0.1M pH 1.4 HCl. Each value represents the overall mean of three independent experiments ± the standard error of the mean.

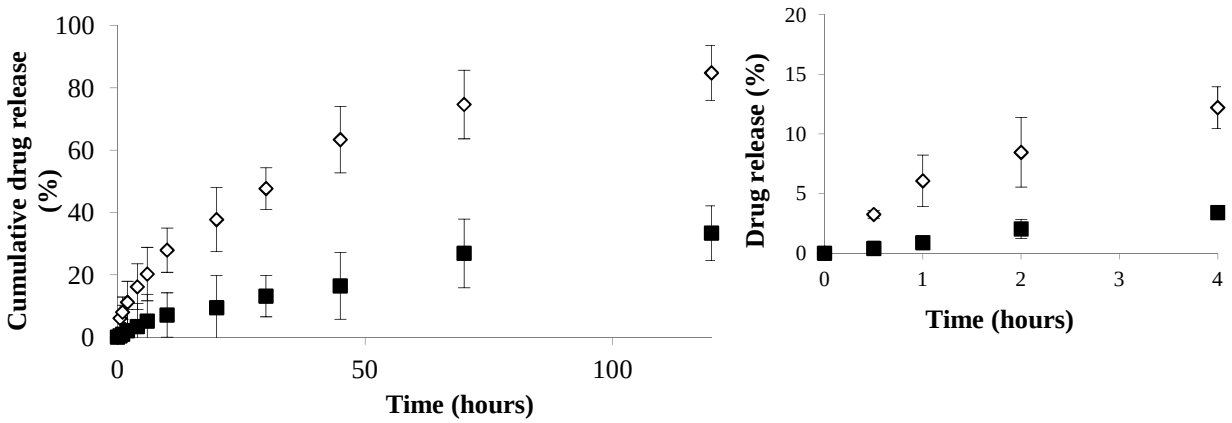


Figure 3.11. Drug release profile for uncoated (◊) and Eudragit S100 coated (■) liposomes in pH 6.3 Hanks' solution. Each value represents the overall mean of three independent experiments ± the standard error of the mean.

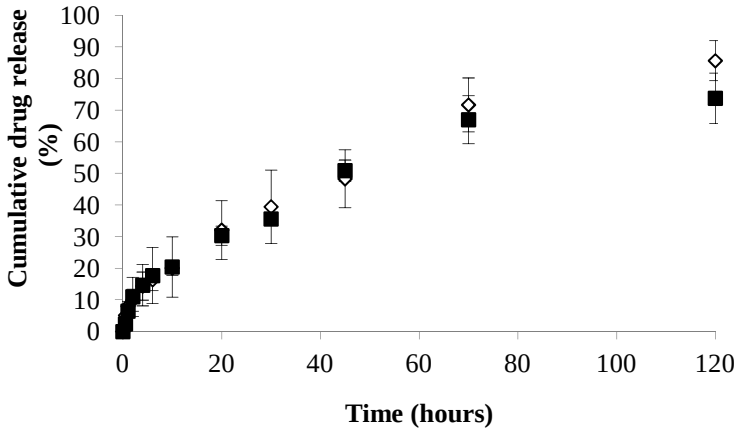


Figure 3.12. Drug release profile for uncoated (◊) and Eudragit S100 coated (■) liposomes in pH 7.8 PBS. Each value represents the overall mean of three independent experiments ± the standard error of the mean.

3.4.3.3. Drug release profile with the inclusion of the model bile salt sodium taurocholate

The addition of bile salts to the release media significantly increased the drug release rate for both uncoated and coated liposomes (Figure 3.13). There was also a significant increase in release rate in comparison to the uncoated formulation without the presence of bile salts showing that the inclusion of bile salts has a marked effect on the release rates of EPC liposomal formulations. 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. This observation can be seen at the first time point of 30 minutes, indicating that the presence of the polymer had very little effect on slowing the drug release from the liposomes. As the

results differ considerably to those observed without the model bile salt it is hypothesised that the polymer agglomeration had previously acted as a diffusional barrier, slowing drug release from the coated formulations in both the stomach and small intestine simulated conditions. However, it was unable to protect against bile salts which indicates that premature drug release and liposomal degradation could be expected *in vivo*.

Drug release results in Figure 3.13 indicate 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. The cryo-SEM images of coated formulations in pH 6.3 Hanks' (Figure 3.8) indicate that there are points within the polymer agglomeration that would be susceptible to bile salt penetration. This would result in the lysis of the liposome and therefore cause the Eudragit S100 to disperse, as there is no central core for it to adhere to. This would then allow for similar release profiles to be observed as the uncoated liposomes in the presence of bile salts.

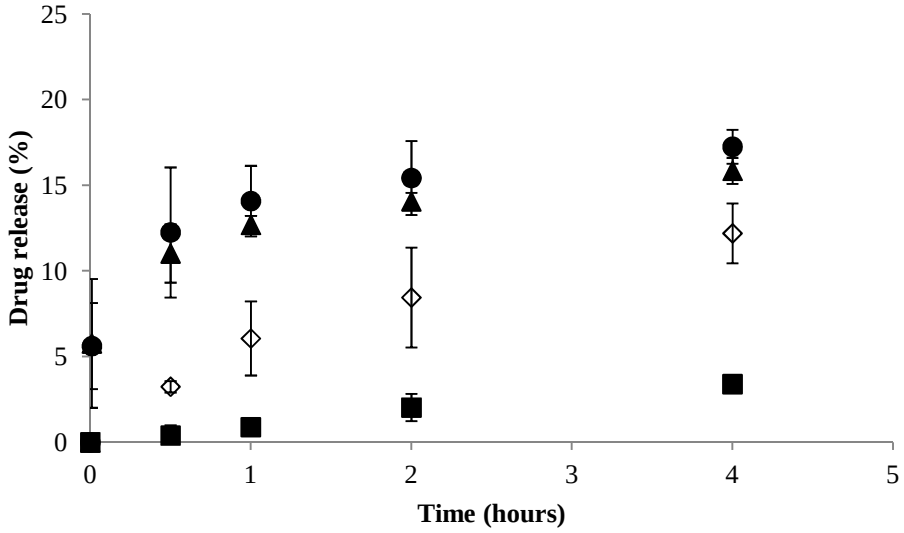


Figure 3.13. Drug release profiles for Eudragit S100 coated (●) and uncoated (▲) liposome formulations at pH 6.3 in the presence of 10mM sodium taurocholate. Release data from Figure 3.10 (no bile salts) are shown for comparison Eudragit S100 coated (■) and uncoated (◊). Each value represents the overall mean of three independent experiments ± the standard error of the mean.

The mechanism by which bile salts interact with EPC liposomal formulations has been well investigated (Nagata *et al*., 1990; Ramaldes *et al*., 1996; Andrieux *et al*., 2004), with each study showing that a simple EPC liposomal formulation would not withstand the rigors of bile salt exposure experienced *in vivo.* The disintegration of lipid bilayers by bile salts is modelled as a three stage process which can be dependent on a number of factors including vesicle size, surfactant concentration and rate of addition (Ramaldes *et al.*, 1996; Annesini *et al*., 2000).

Stage one begins with the penetration of the outer bilayer by the surfactant. This penetration has been described by Walde *et al.*, (1987) and Schubert *et al.*, (1986, 1988) as a two stage process that involves the aggregation of the sodium taurocholate molecules in the external leaflet of the bilayer, where no change in permeability is observed. The second stage occurs when the molecules flip to the internal leaflet as the concentration increases and the subsequent formation of transient membrane pores can take place. The sodium taurocholate molecules then redistribute within the membrane and increase in concentration to lead to the destabilisation of the vesicles.

Stage two involves the further incorporation of sodium taurocholate into the bilayer subsequently producing mixed micelles.

Stage three refers to a reduction in size of mixed micelles due to the increasing ratio of surfactant/phospholipid (Nagata *et al*., 1990). The process of solubilisation for MLVs is considered to be stepwise, with each (outer) bilayer being penetrated by the surfactant sequentially. It can therefore be assumed that the process is governed, in this case, by the permeability of the liposomal bilayer and therefore the specific CH concentration used. The current study uses a high CH concentration leading to a slow drug release in the presence of bile salts, compared to previous studies where ratios of 7/3 and 10/2 EPC to CH were used (Ramaldes *et al*., 1996; Rowland and Woodley 1980).

The marked difference in how the Eudragit S100 coated liposomes react in the presence of the model bile salt sodium taurocholate is a very significant result, as it shows that the coating would be inadequate for use *in vivo* due to the concentrations and variability of bile salts observed throughout the small intestine. The finding also 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

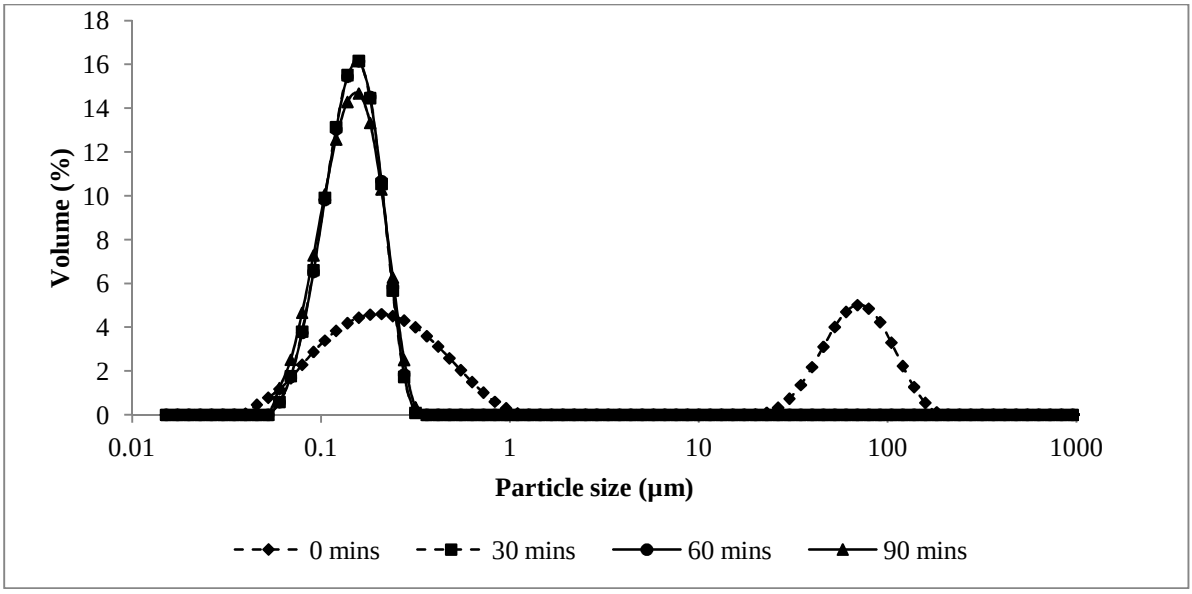
pursued by others (e.g. Guo *et al*., 2003; Filipović-Grčić *et al*., 2001). Whilst the coating mechanisms in these studies may be different to those observed for Eudragit S100 coated liposomes, assumptions cannot be made that the coat and liposomes will remain intact in the presence of bile salts without the necessary investigations being undertaken.

3.4.3.4. Influence of sodium taurocholate on the size distribution of Eudragit S100

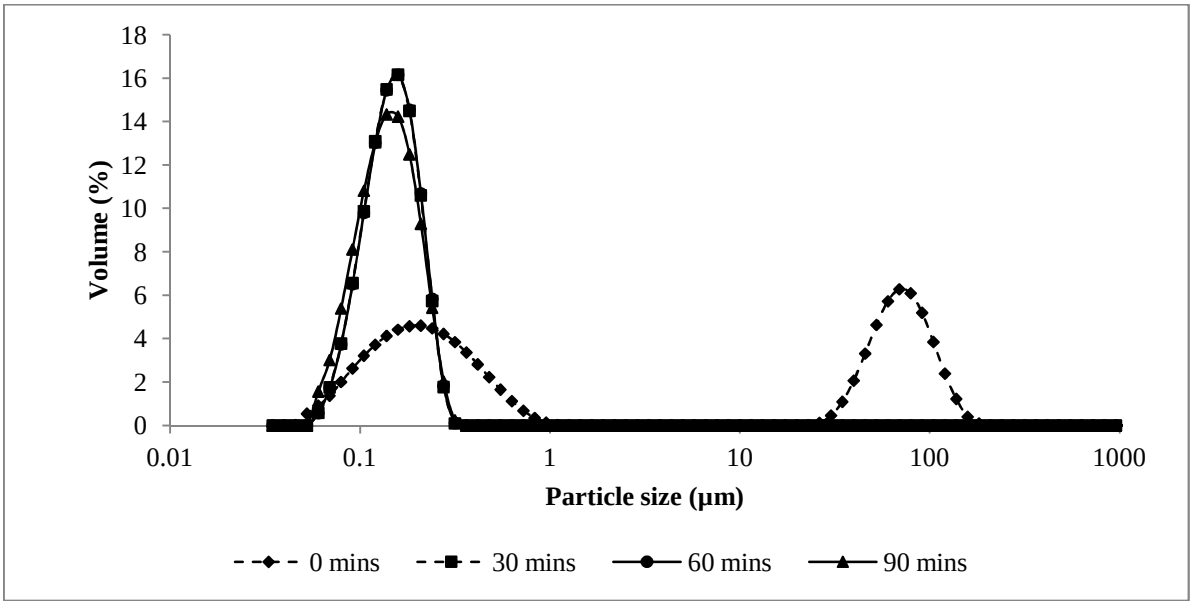
To explore the cause of the increased drug release in the presence of bile salts, an initial study was conducted, investigating if any change in particle size was observed in Hanks' solution containing bile salts. This would indicate whether any polymer solubilisation/degradation was taking place. The resulting polymer particle size distributions are shown in Table 3.2 and Figure 3.14, which show identical results in both dispersion media, indicating that the bile salts did not facilitate polymer solubilisation or dissolution. The initial high value was recorded as the sample was being stirred and can therefore be attributed to dispersing the polymer throughout the sample chamber. This would explain the large drop in size distribution observed in both the media, as the polymer was added to the instrument prior to the first reading taking place and therefore not allowing for complete polymer dispersion to occur. The possibility of initial agglomerates in the chamber would then lead to skewed results due to the light scattering technique of the instrument thereby indicating a larger size distribution than is plausible. Beyond this point no significant change in particle is observed in both conditions, it can therefore be assumed that the bile salts have no marked effect on the Eudragit S100. This would be expected due to the nature of Eudragit S100 regarding its use as an enteric coating material for colonic release and its solubility above pH 7. This reinforces the use of Eudragit S100 as a pH triggered release polymer as opposed to a polymer triggered by bile salts as there were no obvious interactions when included.

Table 3.2. Size distributions for Eudragit S100 powder dispersed in pH 6.3 Hanks' solution and in the presence of model bile salt sodium Taurocholate (10mM).

	Eudragit S100 in pH 6.3 Hanks'			**Eudragit S100 + bile salt in pH 6.3 Hanks'**		
Minutes	d(0.1)	d(0.5)	d(0.9)	d(0.1)	d(0.5)	d(0.9)
0	0.094	0.352	83.66	0.102	0.399	86.053
15	0.079	0.129	0.199	0.087	0.137	0.201
30	0.087	0.136	0.201	0.087	0.137	0.201
45	0.087	0.137	0.201	0.087	0.137	0.202
60	0.087	0.137	0.202	0.087	0.137	0.202
75	0.079	0.13	0.201	0.087	0.137	0.202
90	0.082	0.135	0.206	0.079	0.13	0.201



(A)



(B)

Figure 3.14. Size distribution traces for Eudragit S100 powder dispersed in (A) pH 6.3 Hanks' solution and (B) pH 6.3 Hanks' solution with 10mM sodium Taurocholate.

3.4.3.5. FTIR investigation into possible interactions between Eudragit S100 and sodium taurocholate

The results for the FT-IR analysis of Eudragit S100 dispersed in Hanks' and Hanks' plus bile salt is shown in Figure 3.15. Examination of the spectra revealed no variation in peak position; in fact, the spectra could be superimposed. Due to the standard resolution of 2 cm^{-1} any minor shifts observed (< 2 cm^{-1}) could be attributed to measurement error as opposed to significant changes in bonds. The main areas where changes in the bonds may be expected have been highlighted on the trace (C=O stretch, -C=C- stretch, C-C stretch and the C-O stretch), all of which show no significant shift in peak position. These peaks are specific to the Eudragit S100 trace and any direct influence on the chemical structure of the polymer would be indicated through a peak shift. Differences were observed for the relative absorbance of the peaks which can be attributed to the varying components of the mixtures and the actual concentrations in contact with the optical unit. Furthermore, a change in peak height would indicate a change in morphology, which would indicate there is no form of interaction between the Eudragit S100 and sodium taurocholate. The results for the size distributions and FT-IR therefore indicate it seems likely that disruption to the coat was due to the loss of liposome structure, through bilayer disintegration.

Whilst liposomes can be designed to increase their resistance to bile salts through the inclusion of CH or the use of synthetic lipids (Rowland and Woodley, 1980; Kokkona *et al*., 2000; Andrieux *et al*., 2009), it would also be necessary to improve the integrity of the coat to prevent bile salt ingress. Rowland and Woodley (1980) showed that when a number of liposomal formulations were exposed to 10mM bile salts it caused the release of over 80% of the entrapped marker. Only one formulation proved promising which was produced using the synthetic lipid DSPC, which only released 16% of the entrapped marker in the one hour period. It was hypothesised that the increased level of retention was due to the 'solid' nature of the liposomal bilayer due to the high transition temperature of DSPC being 58°C therefore resisting bile salt disruption. In comparison the formulations produced using lipids with a lower transition temperature (EPC, DMPC) will have a fluid like bilayer at test conditions of 37°C and therefore allow for bile salt disruption and subsequent drug release. Similar results were observed by Kakkona *et al*. (2000) where DSPC liposomal formulations combined with CH showed improved drug retention properties when exposed to sodium taurocholate. The inclusion of CH (1:1 molar ratio) in EPC liposomes was shown to delay drug release in the

presence of sodium taurocholate in comparison to EPC alone, but significant release was still observed within the first hour (~80%). The inclusion of CH was shown to improve drug retention in the case of lipids with higher transition temperatures to that of EPC (DPPC, DSPC) throughout the 5 hours of the study, which exceeds the average small intestine transit time. It was therefore concluded that the lipid used, and its specific transition temperature, has more of an influence on the stability of liposomes in the presence of bile salts compared to the CH concentration.

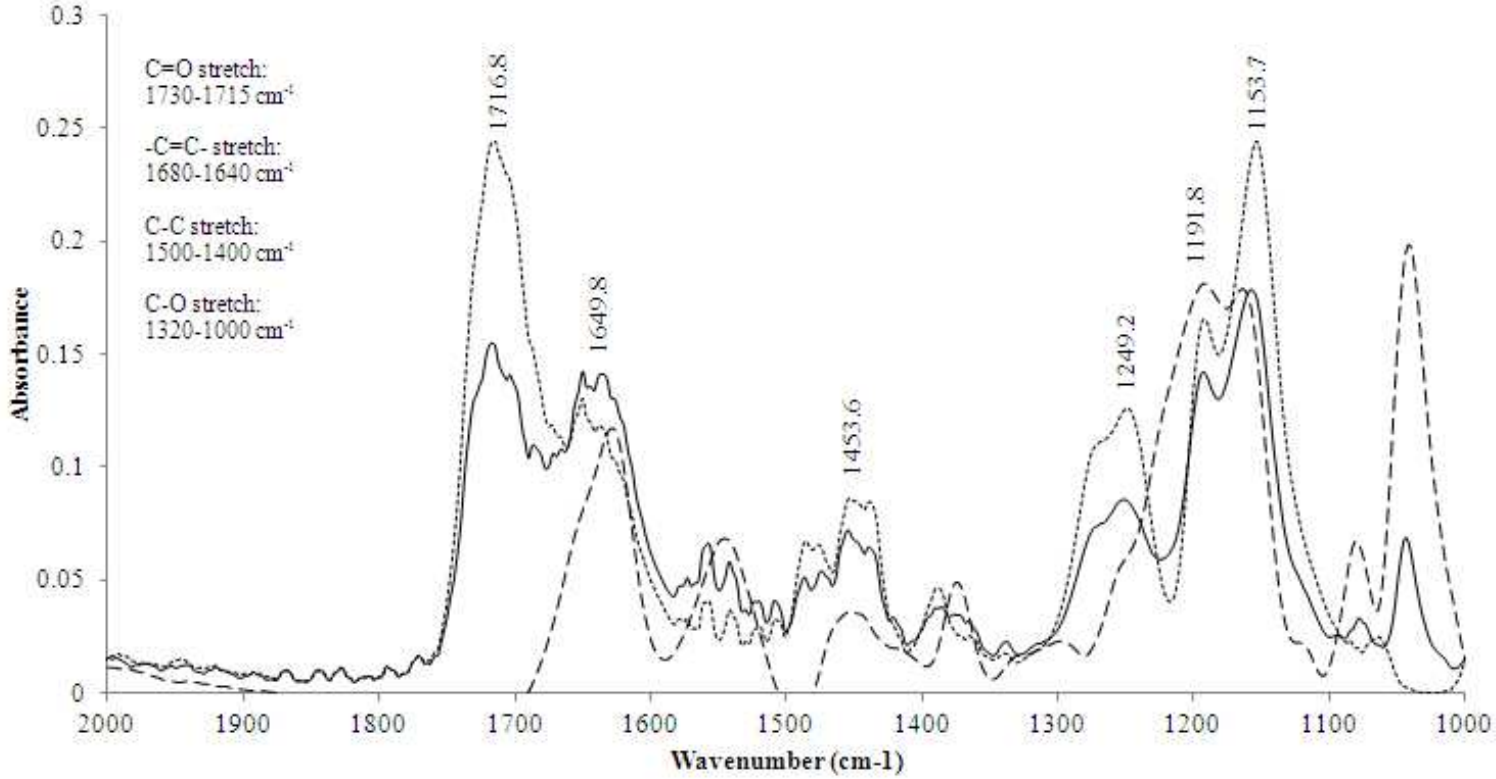


Figure 3.15. FT-IR spectra of pastes comprising: Eudragit S100 + Hanks buffer (·····), Eudragit S100 + Hanks buffer + bile salts (——), and Hanks buffer + bile salts (– – –).

3.5. Conclusions

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. Initial results were promising, with characterisation techniques showing a shift in zeta potential, an increase in size and light microscope images showing the association between the polymer and liposomes. Despite the cryo-SEM images not showing a single distinctive coat around the liposomes there was enough of a barrier to significantly slow drug release through the stomach and small intestine conditions. As expected, the coated formulation drug release profile in pH 7.8 conditions was very similar to that of the uncoated, due to the solubility of the polymer in these conditions. Further rigorous testing, whereby the coated formulation was exposed to bile salts, led to a significant drug release in line with that of the uncoated formulation at the same conditions, indicating that the coat was not as stable as the initial release trials suggested. It is hypothesised that the bile salts could penetrate the liposomes through the channels between the polymer aggregates and therefore facilitate drug release through the normal digestion of lipids, as would be seen for the uncoated formulation. The importance of evaluating coated liposomes for oral drug delivery beyond pH shift studies has been demonstrated with the addition of bile salts.

4.0 Investigation into the production of Eudragit S100 microspheres

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ABSTRACT

Eudragit S100 microspheres have been produced using a double emulsion - solvent evaporation method, with a view to encapsulating liposomes in the internal structure. A number of parameters were investigated including polymer concentration, homogenisation speed, homogenisation time and surfactant concentration. The microspheres were characterised using a number of methods including laser diffraction particle sizing and SEM. It was found that the specific properties including size, homogeneity and yield of the microspheres could be controlled by altering one or more process variables.

An increase in polymer concentration initially led to an increase in microsphere size, but above a certain concentration (6% w/w) a non-homogenous sample was observed with a number of polymer agglomerates. Increasing both the homogenisation speed and time led to a smaller microsphere population, with a narrower size distribution being observed. The use of Span 85 and Tween 85 was deemed the most successful surfactant combination yielding a large batch of discrete microspheres.

4.1.1. Introduction

It was shown in the previous chapter that Eudragit S100 coated liposomes could not withstand the attack of bile salts that would be found *in vivo* in the small intestine. It was concluded that the coating around the liposomes allowed for the ingress of bile salts and therefore led to the solubilisation of the liposomes. It is hypothesised that producing a solid microparticulate system will significantly improve the stability of a liposomal system developed for colonic drug delivery. With this in mind the key aims of the current chapter were to;

- Produce a solid Eudragit S100 microparticulate system that would protect the liposomes through the stomach and small intestine to begin release at the ileo-caecal junction.
- Identify the most suitable processing parameters to produce a formulation with discrete microspheres of a high yield.
- Evaluate the formulation for its suitability to survive varying pH conditions and subsequently degrade above pH 7.

4.1.2. Introduction to the production of microspheres suitable for the encapsulation of liposomes

Over the past thirty years a number of researchers have investigated degradable microspheres as a technique for controlled drug release (Freiberg and Zhu, 2004). The advantages of these systems include;

- Their small size enables administration through either ingestion or injection.
- They can be targeted for specific release profiles and even target specific organs.
- Microspheres can be produced to enable prolonged release which not only reduces the number of administrations but also increases patient compliance.
- Microsphere properties can be manipulated through adjustment of a number of production variables.
- Microspheres can also be used as a solid protective barrier against specific enzymes, bile salts during drug delivery.

To be able to encapsulate a liposomal dispersion within microspheres it has been shown that an adapted double emulsion solvent evaporation (W/O/W) (Figure 4.1) technique can be utilised (Feng *et al*., 2004). This technique is usually used for the production of polymeric microspheres to encapsulate water soluble agents. For this production method the primary emulsion is made up by homogenising the polymer dissolved in an organic solvent with the encapsulant (liposomes) and an emulsifier. At this point it is important that a stable emulsion is formed therefore ensuring no phase separation occurs prior to the formation of the secondary emulsion. The use of sonication is normally undertaken to produce the primary emulsion but due to the presence of liposomes and the effect it would have of their structure it is proposed that homogenisation is used throughout.

The secondary emulsion (W/O/W) is then produced by adding the primary emulsion to a large quantity of aqueous volume containing an emulsifier (e.g. PVA) which then leads to the formation of microspheres. Through continued stirring the microspheres harden and solvent evaporation occurs. The stirring is then continued for a prolonged period to ensure the maximum amount of residual solvent has been removed and subsequent microsphere harvesting can take place.

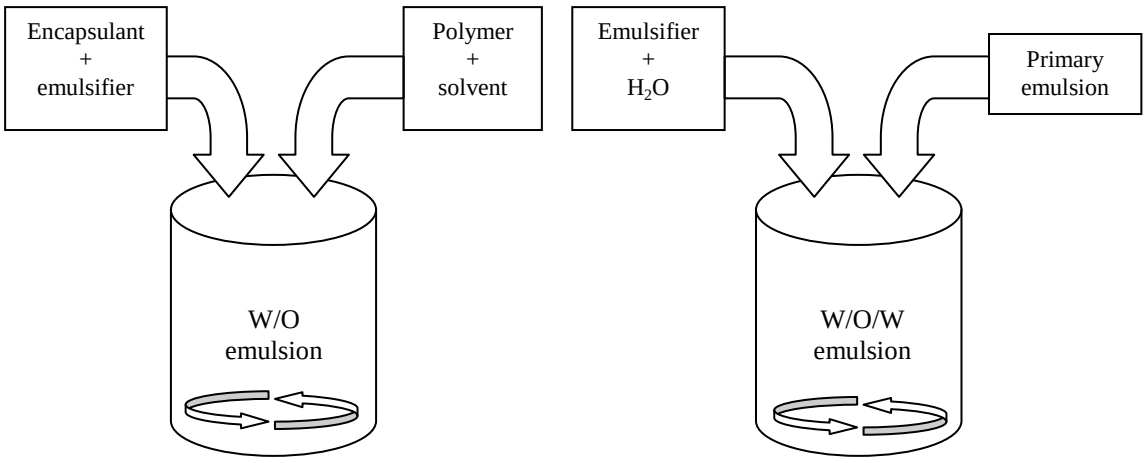


Figure 4.1. Schematic diagram representing double emulsion method (W/O/W) for microsphere production. The primary emulsion (W/O) is formed by homogenising a polymer solution and a aqueous solution containing the drug. The W/O/W is then formed by adding the primary emulsion to a large quantity of aqueous volume containing the emulsifier. Microspheres are then stirred for a period of time to allow for hardening and subsequent solvent evaporation.

4.1.3. Eudragit S100 microspheres

Whilst Eudragit S100 has been used extensively as an enteric coating for a number of other microsphere formulations (Maestrelli *et al*., 2008; Paharia *et al*., 2006; Oosegi *et al*., 2008), the direct production of Eudragit S100 microspheres has only come to the fore more recently. A number of studies have successfully produced Eudragit S100 microspheres whilst observing the influence of varying polymer concentrations, surfactant concentration, stirring speeds, and internal/external aqueous phase ratios (Jain *et al*., 2005; Rawat *et al*., 2007; Sipos *et al*., 2005). The Eudragit S100 microspheres produced in the current chapter have been formulated by adapting a double emulsion - solvent evaporation method previously used by Lee *et al*. (2001) and Jain *et al*. (2005). The initial microsphere production method is described in section 4.3.1., but further studies were undertaken to establish the method to produce the most suitable microspheres in terms of particle size, morphology and homogeneity for oral colonic drug delivery. The variables that were investigated were that of polymer concentration, homogenisation speed and homogenisation time as these are all known to significantly influence particle size (O'Donnell and McGinity, 1997).

4.2. Materials

4.2.1. Polymers

Eudragit S100 (Evonik - Essen, Germany) was used to produce the microspheres due to its specific pH solubility profile making it suitable for ileo-caecal drug delivery. PVA (molecular weight: 125,000 Sigma Aldrich.) was also used in a variety of concentrations from 1 – 3% (w/w) for the secondary emulsion and subsequent microsphere hardening.

4.2.2. Surfactants

Polysorbate 20 (Sigma Aldrich) was used in a variety of concentrations from 1-3% (w/w).

4.2.3. Other chemicals and reagents

The solvents including DCM, ethanol and HCl used were all of an analytical grade and used as received.

4.3. Apparatus and methodology

4.3.1. Production of Eudragit S100 microspheres

Eudragit S100 microspheres were produced using a double emulsion - solvent evaporation method adapted from a number of studies that have previously produced Eudragit S100 microspheres (Jain *et al*., 2005; Rawat *et al*., 2007; Sipos *et al*., 2005). The different experimental conditions used for the investigation of production variables are outlined in Table 4.1. The three variables of polymer concentration, homogenisation speed and homogenisation time were investigated therefore variables such surfactant concentrations and aqueous volumes were maintained. A standard method was used (outline in the following section), which then allowed for the single variable to be investigated whilst keeping all other variables the same.

4.3.2. Standardised Eudragit S100 microsphere production method

The internal aqueous phase consisted of 0.8 ml water vortex mixed (3,400 rpm, 20 seconds) with 0.2 ml 3% polysorbate solution. The primary emulsion (W_1/O) was then formed by homogenising (3,400 rpm, IKAT25 homogeniser, Fisher Scientific) the internal aqueous phase with 5 ml 6% w/w Eudragit S100 dissolved in a solvent mixture of DCM:ethanol:propanol (5:6:4) for 2 minutes. The primary emulsion (W_1/O) was then poured into 100 ml 1% PVA solution to create the double emulsion ($W_1/O/W_2$), whilst under magnetic stirring at 125 rpm (Fisher Scientific). The emulsion was then left stirring for 3 hours to allow for the microspheres to harden through solvent evaporation. The microspheres were then washed using vacuum filtration with a filter membrane of 1.6 µm. The microspheres were then collected and allowed to dry at room temperature for 48 hours.

Table 4.1. Experimental conditions employed in the production of Eudragit S100 microspheres to investigate the influence of polymer concentration, homogenisation speed and homogenisation time.

Polymer concentration (% w/w)	Homogenisation speed (rpm)	Homogenisation time (minutes)
2	3400	1
6		
10		
15		
6	400	1
	3400	
	7400	
6	3400	1
		2
		4

4.3.3. Scanning Electron Microscopy (SEM)

SEM was conducted on the dried microsphere samples to determine the surface morphology, homogeneity of the sample and indicate the general particle size. These specific characteristics will be important to the final product as they will influence the specific solubility profile of the microspheres and therefore possibly impact the drug release profile. The dried samples were mounted on a SEM stub and subsequently coated with platinum using an Emscope SC500 sputter coater. The samples were coated for 2 minutes with a deposition pressure of 20 barr depositing a layer of platinum equivalent to 150 Ångström. Images were taken using a Phillips XL-30 FEG ESEM under vacuum conditions.

4.3.4. Particle size distribution

Particle size distribution was measured using the method outlined in section 3.3.3.4. The microsphere sample, in its dried form, was added to the dispersion unit until the obscuration level was in the range of 14-18% (Mastersizer 2000 handbook, Malvern Instruments). The sample was dispersed in pH 7.0 distilled water.

4.3.5. Cryo SEM imaging of microspheres

Microsphere samples were dispersed in 5 M sucrose solution to ensure they remained well dispersed throughout, therefore preventing aggregation at the base of the sample well. The method used for sample sectioning and imaging was the same as for the liposomal samples as described in section 3.3.3.3.

4.3.6. Microsphere solubilisation in buffers representative of the GI tract

A degradation study of blank Eudragit S100 microspheres was conducted to investigate what happens to the microspheres at a pH above 7 and therefore gauge their suitability for ileo-caecal release. 50 mg of blank microspheres were placed in 40 ml of three different buffers; pH 1.4 0.1M HCl, pH 6.3 Hanks' buffer and pH 7.4 PBS, maintained at 37°C and shaken at 100 rpm in an incubator. As in chapter 3 the pH 6.3 Hanks' buffer contained the model bile salt sodium taurocholate to assess the resistance to bile salt attack as would be present in the small intestine. 1 ml samples were taken at pre-determined intervals simulating the transit times of the stomach, small intestine and large intestine respectively. 1 ml of pre-heated fresh buffer was replaced after each sample extraction. The sample was then spun down at 26,000 rpm (63,000 g) for 10 minutes, with the supernatant being removed. The remaining microspheres could then be completely dried at room temperature for 48 hours, ready for subsequent SEM analysis as described in section 4.3.2.

4.4. Results and Discussions

4.4.1. Effect of polymer concentration on Eudragit S100 microspheres

The influence of polymer concentration on the morphology of the final microsphere sample can be observed in the SEM images seen in Figure 4.2. The images show the successful production of microspheres for 2 and 6% polymer concentrations, but for 10 and 15% the results are less consistent. From the images it can be clearly seen that the microspheres have a broad size range indicating the possible need for alteration of processing conditions. A sieving technique could be used to ensure a homogenous sample is obtained but this will lead to loss of sample and therefore a reduced drug encapsulation efficiency. The 6% polymer solution appears to produce the most consistent microspheres in both size and morphology, with 2% showing a number of microspheres with deformed morphology. In addition to this,

increasing the polymer concentration to 10 and 15% produced a large amount of polymer aggregates both throughout the sample but also deposited on the magnetic stirrer, thus reducing the production efficiency of this method. These observations can be attributed to the increased viscosity of the organic phase and therefore an increase in interfacial tension between the organic and aqueous phases. This theory is further reinforced by the second image in Figure 4.2 (D) where a microsphere has formed but is still held within a polymer agglomerate. As the microsphere still has a number of fibrous connections it is thought the shear forces involved during the homogenisation stage are not large enough to create a disperse, stable emulsion and subsequent polymer agglomerations are seen throughout the sample.

Further evidence for the use of 6% polymer concentration is supported by the findings of Jain *et al*. (2005) where it was shown that 6% Eudragit S100 PVA-stabilised microspheres could be produced with a good spherical form and smooth surface. A similar solvent mix was used for the organic phase (DCM-ethanol-isopropyl alcohol) and in the majority of formulations 100 ml of 2% PVA was used as the external aqueous phase, therefore making it comparable to the current study. In comparison, Jain *et al.* (2005) adopted the same method to produce polyvinyl pyrrolidone (PVP) stabilised microspheres which displayed fibrous-threadlike structures on the surface and therefore PVP was deemed an inferior stabiliser in comparison to PVA for the production of Eudragit S100 microspheres.

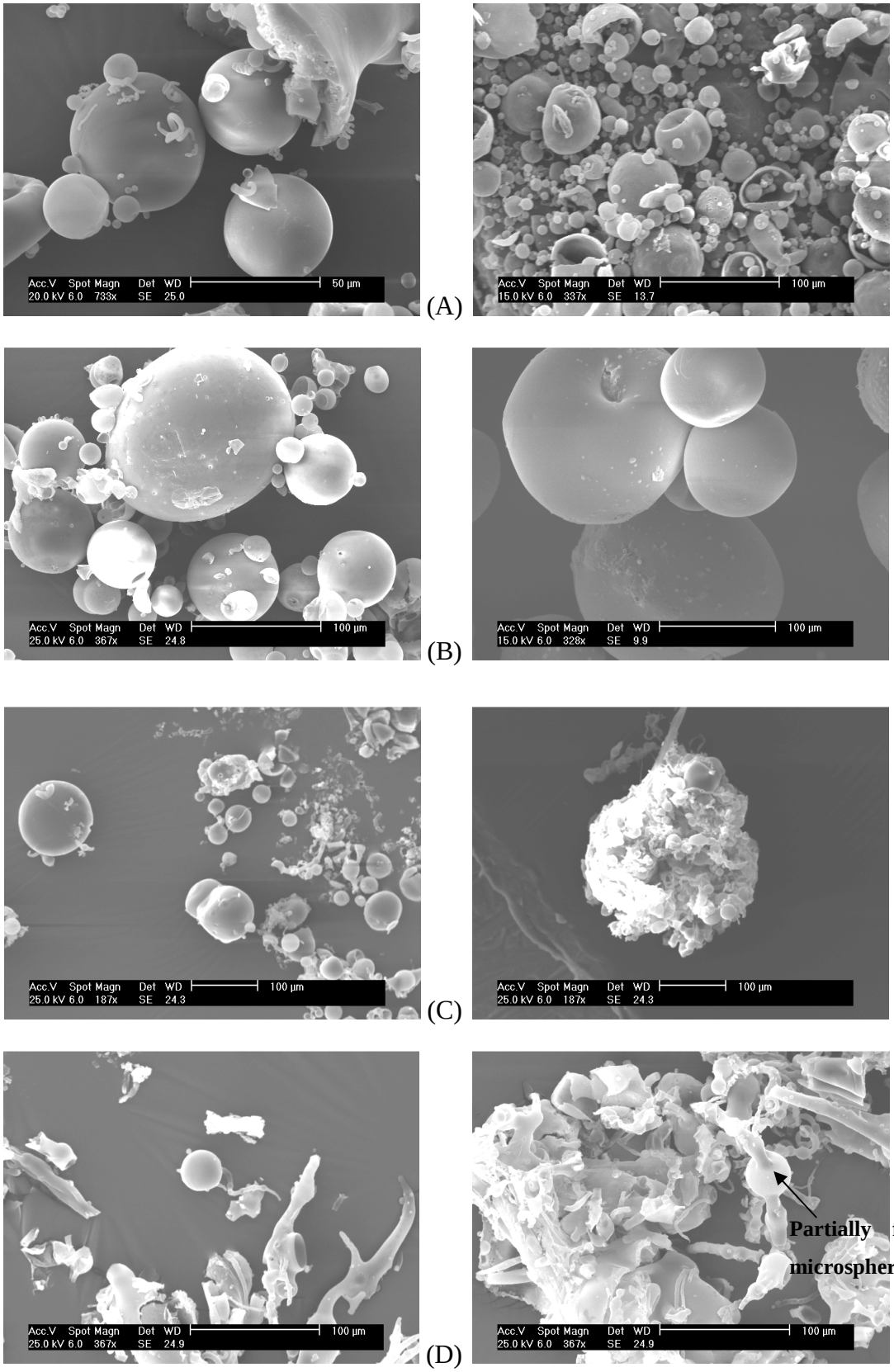


Figure 4.2. SEM images showing Eudragit S100 microspheres produced using (A) 2%, (B) 6%, (C) 10% and (D) 15% Eudragit S100 (% w/w).

Despite the 6% polymer concentration showing the promising results for the formulation of Eudragit S100 microspheres, it has been shown through a number of studies that altering a number of variables can produce a successful formulation. Rawat *et al*. (2007) successfully produced Eudragit S100 microspheres using a range of Eudragit S100 concentrations (1.9, 2.2 and 2.7%) and subsequently compared a variety of surfactants (Tween 20, 40, and 80) and relative concentrations. It was shown that Tween 80 was the most successful surfactant in terms of enzyme entrapment efficiency for this system, with an increase in concentration showing an increase in drug loading but a subsequent decrease in microsphere size. In comparison increasing the DCM concentration was shown to have a negative effect on both drug loading and size which was attributed to the drug leakage in the continuous aqueous phase as the droplets would stay in the liquid form for a longer period of time in comparison to lower DCM concentrations. Eudragit S100 microspheres have been successfully produced using a 10% polymer concentration by Lee *et al*. (2001) with the notable difference being that the external aqueous phase consisted of 1000 ml 0.4% PVA which would subsequently reduce the viscosity of the double emulsion and increase the likelihood of a homogenous dispersion.

The effect of polymer concentration on particle size distribution is shown in Figure 4.3. The results fall very much in line with those presented for the SEM imaging, but a note of caution is required due to the nature of the sizing technique. The principle of measurement of the mastersizer assumes the particles to be spherical, polymer agglomerates that can be seen for both 10 and 15% Eudragit S100 may provide false readings of large microspheres being formed. With this in mind it can be observed that the curve with the narrowest size distribution is seen for the 6%, which is also indicated by the SEM imaging. In contrast to this the higher polymer concentrations provide a broader size distribution with a high percentage of large particle sizes which could be attributed to the agglomerations of polymer observed during processing.

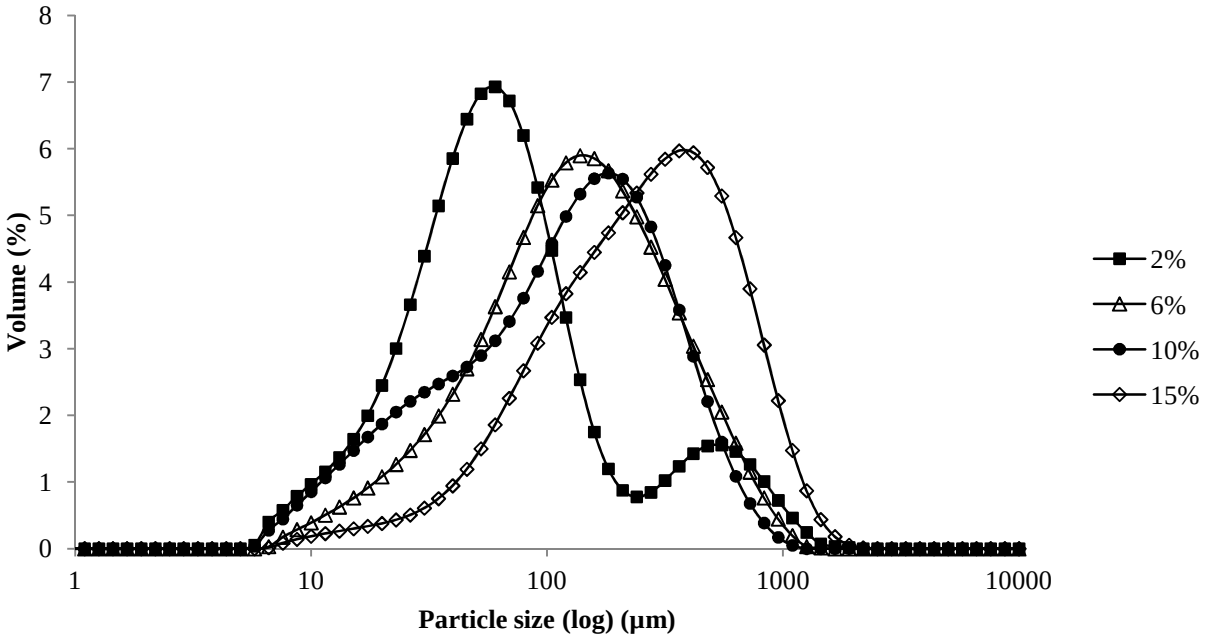


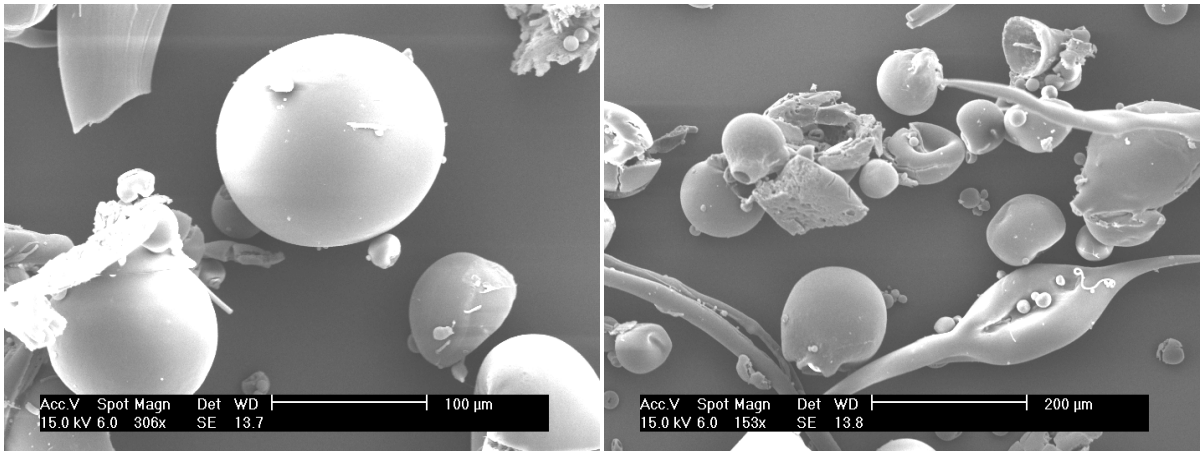
Figure 4.3. Size distribution showing influence of Eudragit S100 concentration (% w/w) on size distribution of blank Eudragit S100 microspheres.

4.4.2. Effect of homogenisation speed on Eudragit S100 microspheres

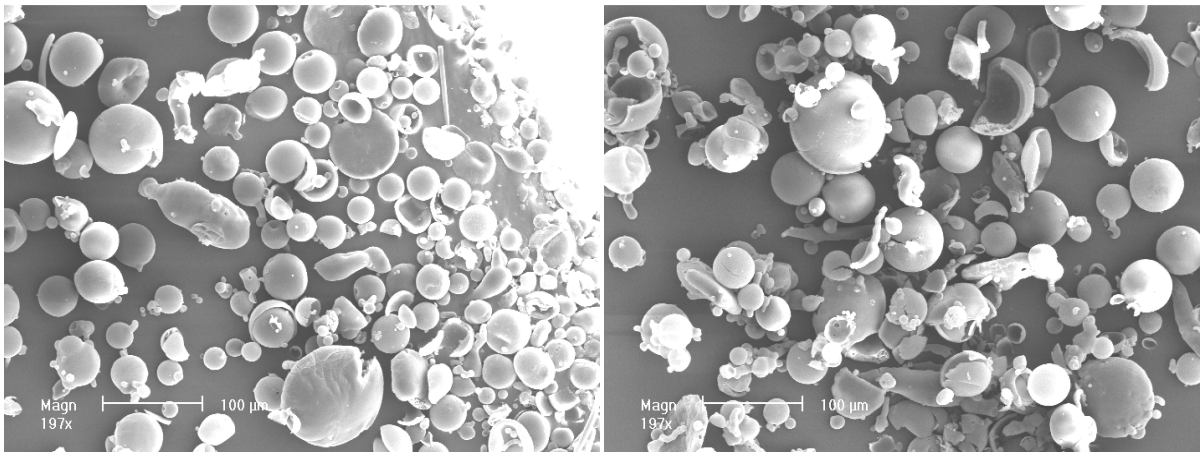
As mentioned previously, a primary emulsion needs high enough shear forces to create a stable emulsion that can then be dispersed into the aqueous phase. It has previously been shown that the homogenisation speed can significantly alter the final microsphere product (Dunne *et al*., 2000). It can be seen from the current study that the case of Eudragit S100 microspheres are no different (Figure 4.4.). The SEM images show a range of microsphere formulations produced using homogeniser speeds of 400, 3400, and 7400 rpm. The microsphere formulation produced homogenising at 400 rpm shows a number of fully formed microspheres with a diameter in the region of 100 µm. These microspheres are largely spherical in shape but do have a number of imperfections which can be attributed to the low shear forces produced at 400 rpm. Furthermore, a number of polymer agglomerates can be seen where a stable primary emulsion would not have been produced causing the droplets to agglomerate and subsequently harden when in the aqueous phase. At 3400 rpm the microsphere population appears to be more homogenous and in the majority, smaller than those observed at 400 rpm. A larger yield of microspheres can also be observed which can be attributed to the increased number of droplets formed in the primary emulsion, leading to

fewer polymer agglomerates being formed. Finally, the microspheres produced by homogenising at 7400 rpm display the most homogenous distribution with the majority of microspheres being approximately 60 µm or less. Furthermore, the image shows very few, if any unformed microspheres indicating the higher homogenisation speed offers the most polymer efficient production route for Eudragit S100 microspheres.

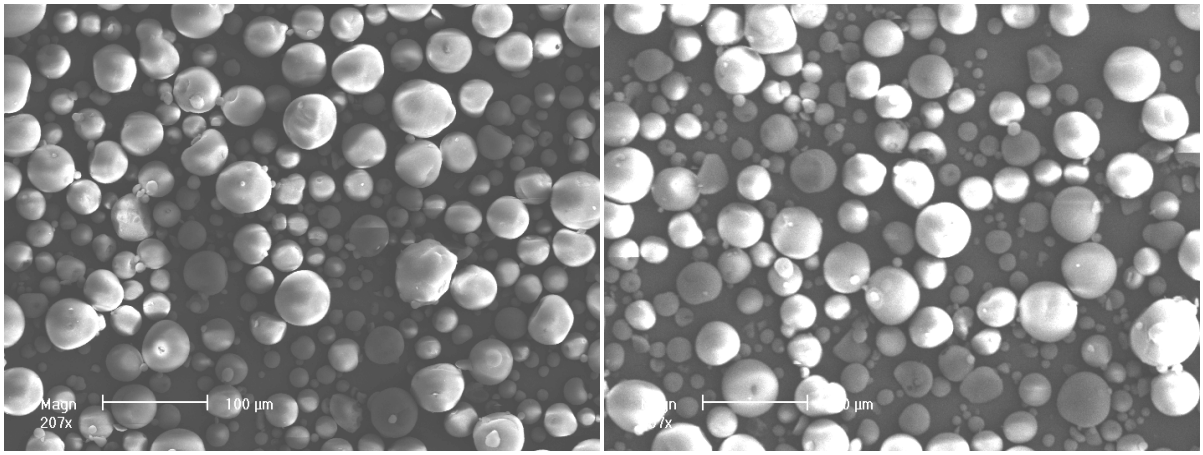
The use of increasing homogenisation speed specifically in the production of Eudragit microspheres has been identified in a number of studies. Initially Mateovic *et al*. (2002) investigated the influence of stirring rate on the properties of Eudragit RS microspheres and found that increasing the stirring rate subsequently decreased the microsphere size due to a finer dispersion of droplets in the primary emulsion. Similarly, Sipos *et al*. (2005) reported a decrease in particle size with an increase in stirring rate with homogenisation speeds of 14,400 and 20,800 rpm providing microsphere diameters of 250 and 116 µm respectively. Sipos *et al*. (2005) then went on to observe the drug release kinetics of the microspheres, showing the formulation produced using the highest homogenisation speed gave the fastest release rate due to the increased specific surface area.



(A)



(B)



(C)

Figure 4.4. SEM images showing blank Eudragit S100 microspheres produced by homogenising for 2 minutes at (A) 400 rpm, (B) 3,400 rpm, and (C) 7,400 rpm.

The results for the size distribution of Eudragit S100 microspheres produced using various homogenisation speeds are shown in Figure 4.5. The three peaks for the 400 rpm formulation indicate the heterogeneous nature of the sample which, coupled with the SEM images, indicate that the shear forces created by the low speed are too small to produce a disperse emulsion. The distribution for 3400 rpm displays a single peak, with a relatively broad span and a significant 'shoulder' at the lower end of the curve. This can be attributed to the majority of the formulation producing well formed spherical microspheres as seen by the SEM, but there is evidence of a number of smaller deformed microspheres which add a bias towards the smaller particle size distribution. In accordance with the SEM images the 7400 rpm microsphere sample show a narrow particle size distribution in the region of 60 – 70 µm.

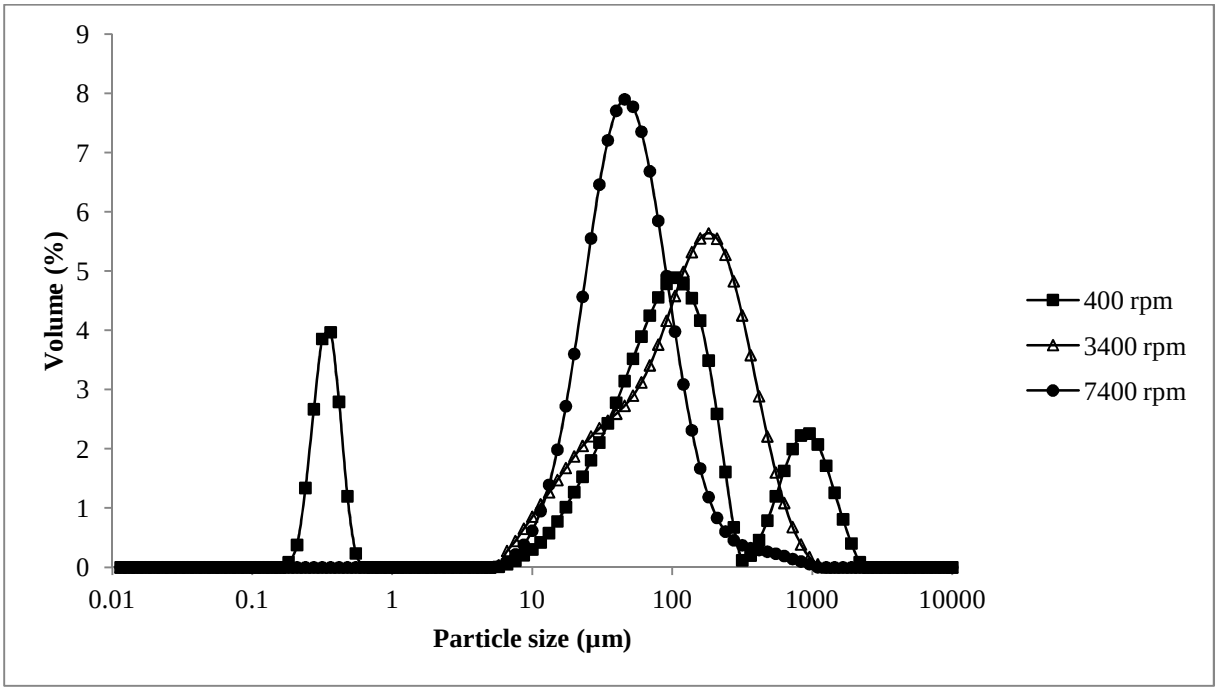
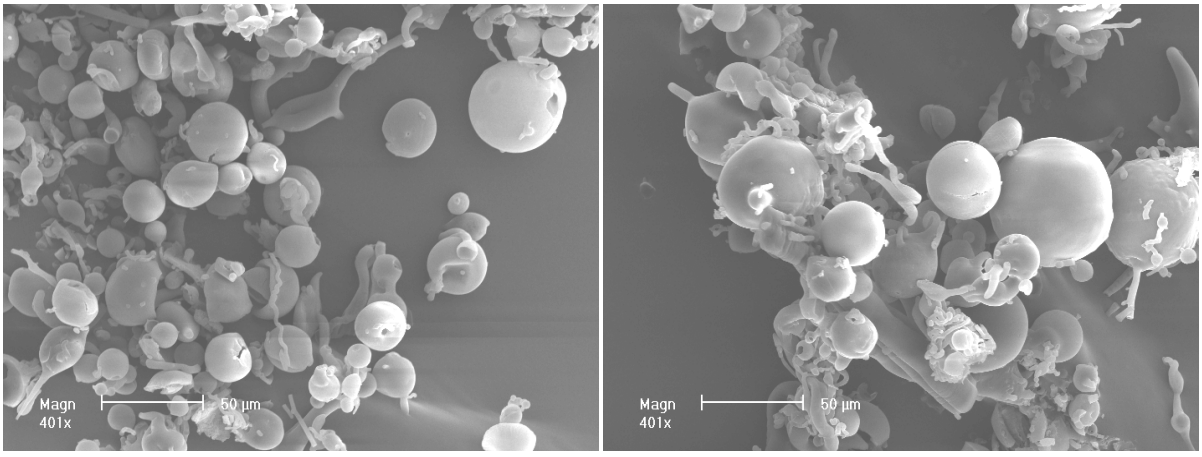


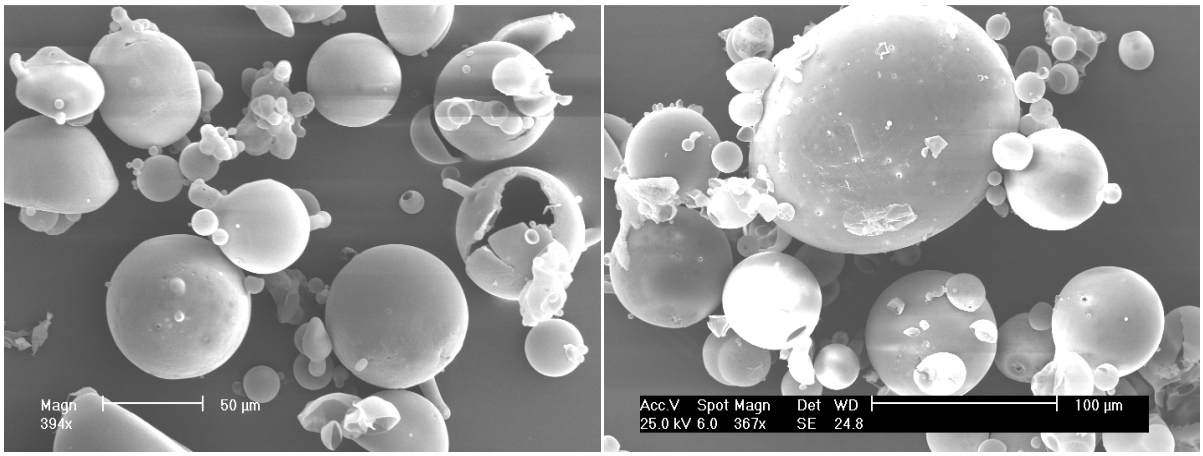
Figure 4.5. Size distribution showing the influence of primary emulsion homogenisation speed on size distribution of blank Eudragit S100 microspheres.

4.4.3. Effect of homogenisation time on Eudragit S100 microspheres

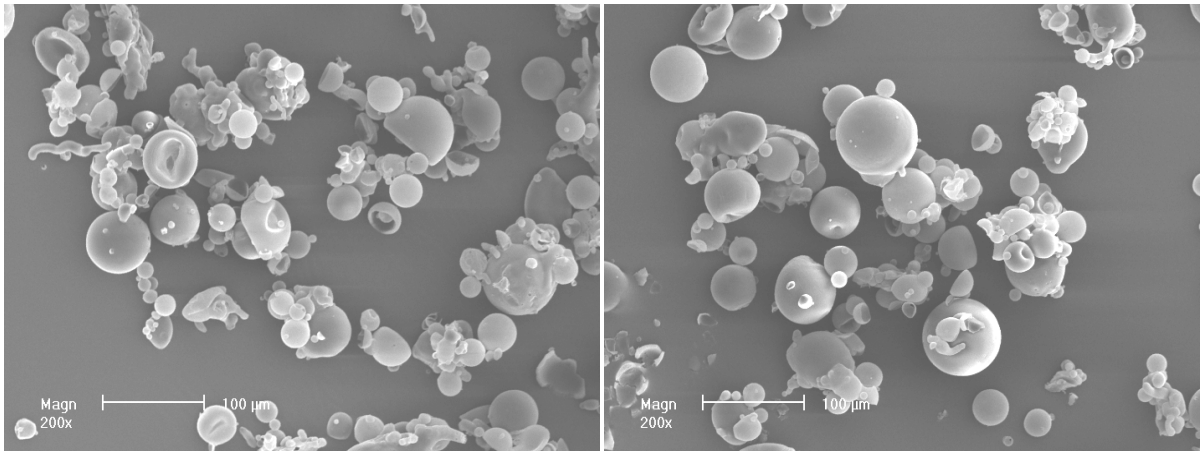
The final parameter investigated is that of homogenisation time for the primary emulsion. A similar theory is applied to this parameter as that of homogenisation speed, where the ability to create a disperse primary emulsion through increased shear forces should lead to a more homogenous sample size. In the previous section the homogenisation speed of 3,400 rpm created a varied microsphere sample population and should therefore give a good indication into the effect of homogenisation time. SEM images for the three homogenisation time scales of 1, 2 and 4 minutes are shown in Figure 4.6. In each of the images the formation of microspheres is clearly evident. For 1 minute, there appears to be a range of microsphere size, with a number of polymer complexes which would indicate that a completely disperse primary emulsion has not been created during this time frame. As indicated previously this will subsequently increase the particle size distribution which may not give an accurate indication of the actual microsphere size formed. No real change in microsphere size was observed for the microsphere formulation produced using 2 minutes, the main difference being that of a reduced number of complexes being observed throughout the sample. The microsphere morphology and sphericity are maintained throughout, with each increase in homogenisation time showing little difference in microsphere characteristics. The final sample produced using 4 minutes homogenisation indicates a marginal decrease in microsphere size, with a number of small (<40 µm) microspheres being visible. Once again no polymer agglomerates can be observed in comparison to that of Figure 4.6 A, and it can therefore be assumed that a homogenous primary emulsion is being formed. Although the changes in microsphere sample are not as significant as those observed in Figure 4.4 the increase in homogenisation time appeared to improve the microsphere sample by producing smaller organic droplets and allowing for a more homogenous emulsion to be formed therefore producing smaller particles and fewer polymer agglomerates through the coalescence of droplets.



(A)



(B)



(C)

Figure 4.6. SEM images showing blank Eudragit S100 microspheres produced by homogenising the primary emulsion for (A) 1 minute, (B) 2 minutes and (C) 4 minutes at 3,400 rpm.

The size distribution results (Figure 4.7) for homogenisation time reflect that of the SEM images, with a more narrow distribution being observed for microspheres being produced using longer homogenisation times. The 1 minute homogenisation time does not show a symmetrical size distribution which may be due to the effect of the polymer agglomerates seen in the SEM images and therefore skewing the sample. In comparison, the 2 and 4 minute samples show a symmetrical distribution which becomes narrower, showing a decrease in particle size as the homogenisation time increases. This once again is in accordance with the concept of increasing the shear forces present during homogenisation and therefore creating a more disperse primary emulsion. In comparison to other studies, Sipos *et al.* (2005) produced Eudragit RS microspheres by high shear mixing for 4 minutes and Jain *et al.* (2005) produced Eudragit S100 microspheres by using an ultrasonic disruptor for 1 minute. This indicates that although homogenisation time has an effect on the final microsphere product, the influence of homogenisation speed has a much greater significance.

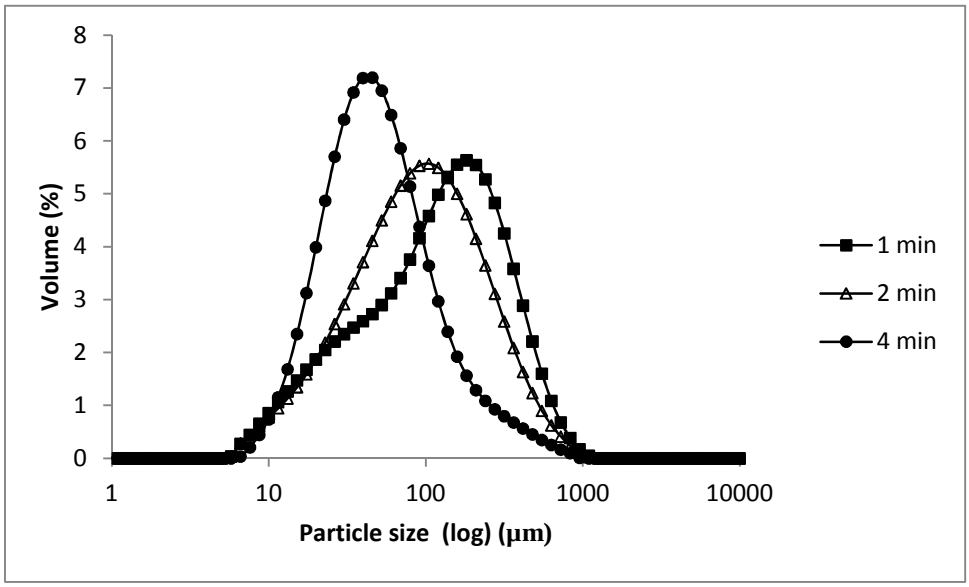


Figure 4.7. Size distribution showing the influence of primary emulsion homogenisation time on size distribution of blank Eudragit S100 microspheres.

4.4.4. Microstructure of fractured Eudragit S100 microspheres using SEM

Eudragit S100 microspheres produced using the standard protocol as outlined in section 4.3.2 were fractured under cryogenic conditions. The resultant SEM images are shown in

Figure 4.8. From the images a wide microsphere outer (approximately 15 µm) can be identified in relation to the complete microsphere diameter. The wall is relatively dense with a few minor pores being visible towards the inner part of the shell. This is similar to that observed by Lee *et al*. (2001), where a smooth outer was observed, but the internal part of the microsphere wall had larger pores visible. This was attributed to the fact that firstly, the outer surface makes contact with the aqueous phase and therefore solidifies more rapidly in comparison to the internal part where solidification is a longer process. The formation of the skin layer then governs the subsequent solvent removal rate and therefore the shell structure (Crotts and Park, 1995). It is hypothesised that a porous skin formation would form with a high inner aqueous volume, therefore allowing for rapid solvent evaporation to occur. In comparison formulations with a low internal aqueous phase would create a nonporous skin layer and due to the low level of aqueous inclusion in the formulation, would lead to a slow rate of polymer solidification, providing a dense microsphere shell. The skin layer can therefore be governed by both the amount of internal aqueous phase and its relative homogeneity within the emulsion. The current methodology produces hollow microspheres that provide adequate aqueous space to encapsulate any form of liposomal formulation (MLVs, LUVs etc.). From these images it would be assumed that the microspheres would be able to protect its contents from bile salt attack that would be observed in the small intestine, in comparison to that observed for Eudragit S100 coated liposomes (Figure 3.7). For the Eudragit S100 microspheres a solid single structure of can be observed whilst for the coated liposomes an intermittent ‘crust’ could be observed.

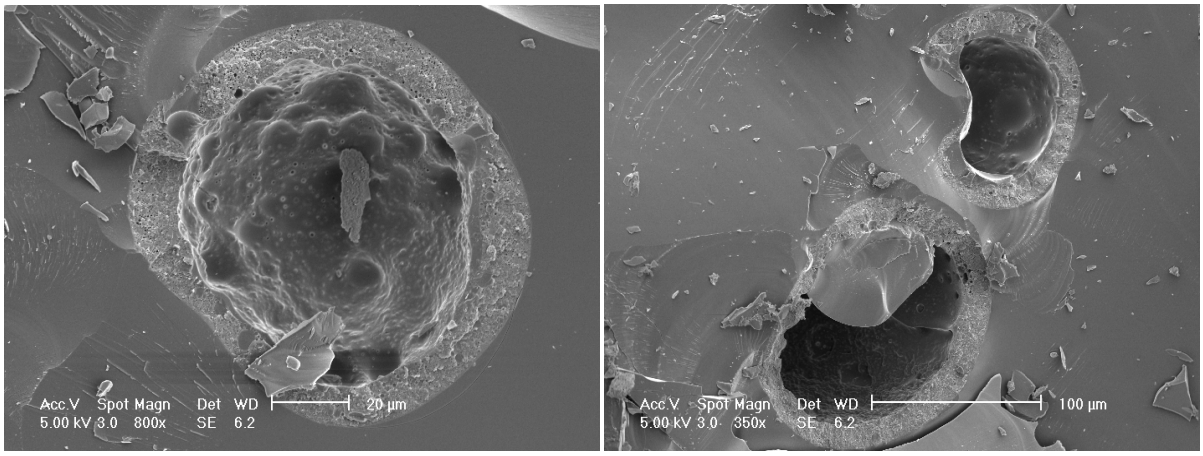
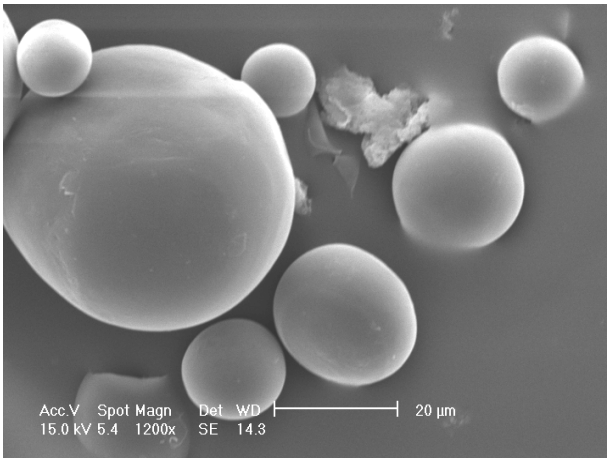


Figure 4.8. Cryo-SEM images showing the internal morphology of Eudragit S100 microspheres. Eudragit microspheres were produced using the standardised protocol as outlined in section 4.3.2.

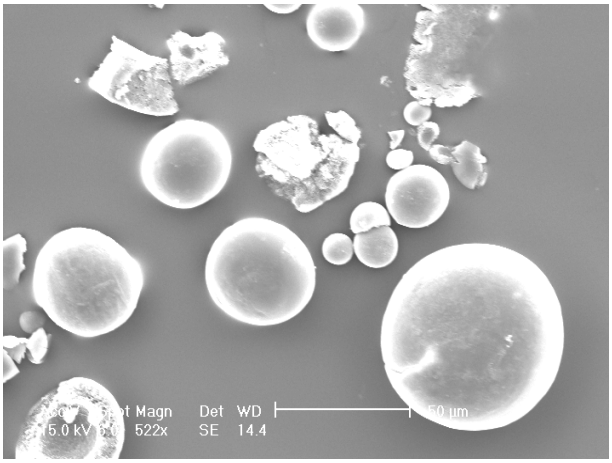
4.4.5. Degradation of blank microspheres in simulated GI tract conditions

It can be seen that Eudragit S100 microspheres maintain their structure and morphology whilst in pH 1.4 0.1M HCl for 2 hours, which is representative of the transit time found in the stomach (Figure 4.9.). No differences can be observed over the 2 hour period with some 'broken' microspheres visible which are attributed to the processing conditions (3400 rpm) used, as discussed previously in this chapter. The important observation is that there is no visible surface erosion and therefore no obvious signs of microsphere degradation when in contact with the simulated stomach conditions.

Similarly, the microspheres observed in Figure 4.10 can be seen to withstand the increased pH of 6.3 and the possibility of bile salt attack by maintaining a smooth surface morphology and spherical form. As no indication of polymer degradation can be observed this would indicate that the microspheres would be able to withstand any possible bile salt ingression, and therefore protect a liposomal formulation within the core.

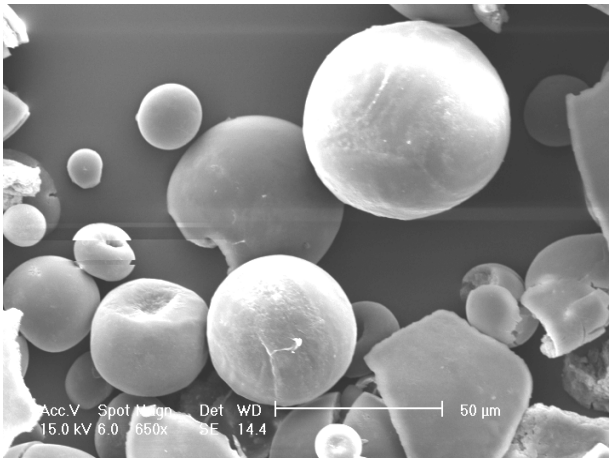


(A)

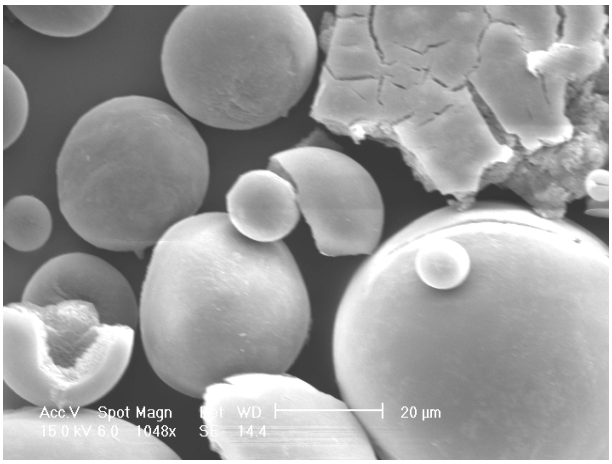


(B)

Figure 4.9. SEM images showing Eudragit S100 microspheres in pH 1.4 HCl at (A) 1 hour and (B) 2 hours.



(A)



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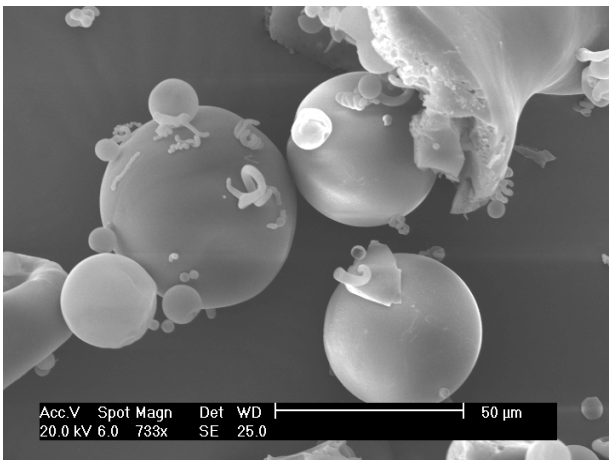


(C)

Figure 4.10. SEM images showing Eudragit S100 microspheres in pH 6.3 Hanks' buffer containing the model bile salt sodium taurocholate at (A) 1 hour, (B) 2 hours and (C) 3 hours.

The SEM analysis of Eudragit S100 microspheres in pH 7.4 PBS are shown in Figure 4.11. The onset of dissolution could be observed within 30 minutes of exposure to pH 7.4, with certain areas of pitting being observed on the microsphere surface. The rapid onset of dissolution conforms to studies that have observed the dissolution rates of Eudragit S100 films, which have shown that within a matter of minutes dissolution has been observed (Lee *et al*., 2001; Rawat *et al*., 2007). The dissolution of the microspheres continues at these inclusions exposing the microsphere wall and internal surfaces, subsequently increasing the surface area and therefore encouraging an increased rate of dissolution (Figure 4.11. B and C). The final images at 4 hours show mainly polymer remnants as opposed to any form of microsphere which would collect during the centrifugation stage. Samples were taken at 6 and 8 hours, but nothing was observed under SEM and therefore it is assumed that complete dissolution of the microspheres had taken place within 6 hours of exposure to pH 7.4 PBS.

As previously described in chapter 2, Eudragit S100 dissolves at a pH above 7 due to the ionization of the carboxylic acid groups within its structure. The specific type of degradation experienced has been evaluated in a number of studies (Sabot and Krause, 2002; Krause *et al*., 1997; McNeil *et al*., 1995). These studies use either impedance spectroscopy or quartz crystal microbalance impedance measurements to determine the specific dissolution associated with Eudragit S100. In each of the studies Eudragit S100 dissolution was attributed to a complex pore formation mechanism on the surface of the polymer. Further dissolution was then shown to be caused by the rigidity of the film and therefore lack of tendency to swell. Further dissolution was said to have taken place under the surface once the polymer had been penetrated through pore formation (Sabot and Krause, 2002). The images in Figure 4.11 give an indication that pore formation is occurring with Eudragit S100 microspheres, with large pores being visible after only 30 minutes.

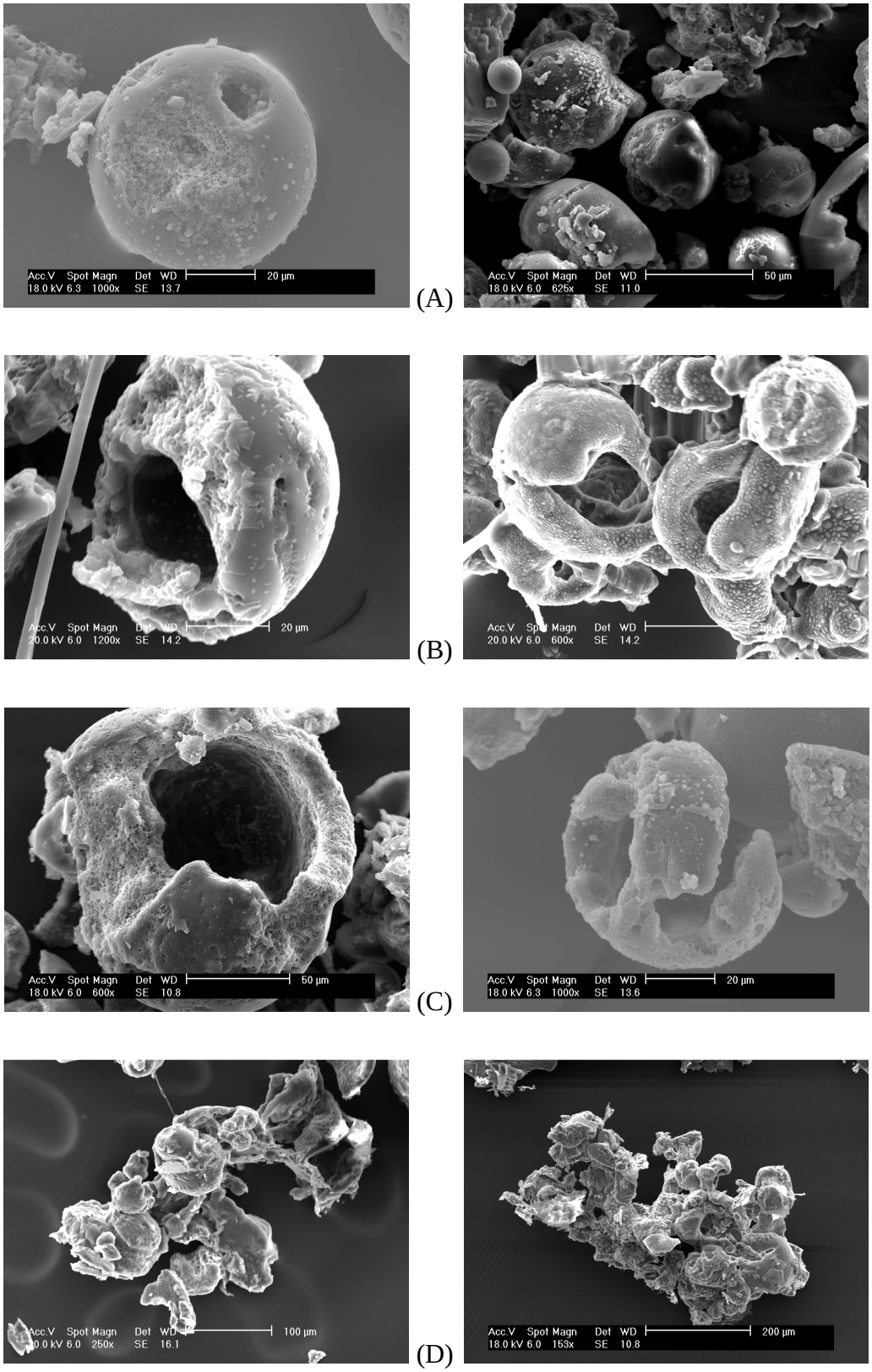


Figure 4.11. SEM images showing Eudragit S100 microspheres in pH 7.4 PBS at (A) 0.5 hours, (B) 1 hour, (C) 2 hours and (D) 4 hours. Microsphere degradation can be observed after just 30 minutes with only small polymer complexes remaining after 4 hours.

Looking at previous studies completed on the dissolution of Eudragit S100 microspheres, Lee *et al*. (2001) observed 90% drug release after 1 hour of exposure to pH 7.4 for ketoprofen loaded Eudragit S100 microspheres. Corresponding SEM micrographs of the formulations showed a microsphere wall thickness in the region of 30 µm, therefore supporting the current formulation and its possible release profile. Similarly, Jain *et al*. (2005) achieved up to 74% protein release (depending upon formulation parameters) in 6 hours for Eudragit S100 microspheres in pH 7.4 PBS. It was also shown that altering the processing variables (internal and external aqueous volumes) could determine not only the cumulative release but also change the initial burst release observed. This is attributed to the altering the internal aqueous volume where a large quantity is considered to produce large droplets which have high amounts of surface associated protein in comparison to those produced using smaller volumes. Rawat *et al*. (2007) showed very little release of serratiopeptidase (STP) in HCl (6.56% in 2 hours) which was attributed to the adsorption of STP on the surface of Eudragit S100 microspheres. It was then shown that approximately 80% STP release was achieved in pH 7.4 PBS, which although still within the required times for colonic drug delivery is slower than that observed by Lee *et al*. (2001) despite having an average microsphere diameter of 56 µm.

4.5. Conclusions

Hollow Eudragit S100 microspheres have been produced using a double emulsion – solvent evaporation technique. Investigating a number of processing conditions showed that increasing the homogenisation speed and time led to a more homogenous population of microspheres.

Subsequent analysis in simulated GI tract conditions have shown that the microspheres can withstand *in vitro* environments representative of the stomach and small intestine. The smooth surface and spherical morphology was maintained throughout the 2 hour exposure to 1 M HCl and 3 hour exposure to Hanks' buffer. The onset of microsphere dissolution was observed in pH 7.4 PBS after as little as 30 minutes, with continued polymer degradation taking place up to 4 hours. The microspheres were completely dissolved within 6 hours of exposure to pH 7.4 PBS which is well within the estimated transit time required for colonic drug delivery (Rawat *et al*., 2007).

The current chapter has addressed the problems that arose during chapter 3 where a single Eudragit S100 liposomes coating could not withstand the attack of bile salts. The solid Eudragit S100 microsphere has shown stability in the presence of bile salts and therefore the formulation can evolve by encapsulating drug loaded liposomes within the microspheres to produce an effective oral colonic drug delivery vehicle.

5.0 Chitosan-coated liposomes

ABSTRACT

A method for producing chitosan-coated liposomes to protect them during encapsulation within Eudragit microspheres was investigated. The technique was required to protect the liposomes from the organic solvents used when forming the primary emulsion of a proposed double emulsion solvent evaporation technique. Both neutral and anionic LUVs were produced and subsequently coated with chitosan. The chitosan coating layer was confirmed through a number of techniques including zeta potential analysis, cryo-SEM and fluorescence microscopy.

The coated liposomes were then investigated for their suitability for colon targeted drug delivery by completing drug release profiles simulating the stomach, small intestine and colon. The small intestine conditions included the model bile salt sodium taurocholate which had previously been shown to cause drug release in insufficiently coated liposomes (chapter 3). The colonic conditions contained β-glucosidase to simulate the action of the colonic microflora and subsequently show its effect on the chitosan coating. It was shown that the chitosan coating slowed drug release through the stomach and small intestine compared to uncoated formulations with significant release being shown in the colonic conditions. A chitosan-coated liposomal formulation has been created suitable of withstanding the processing conditions of microsphere production and capable of significant release in the colon.

5.1. Introduction

It has been previously shown that the direct coating of liposomes with Eudragit S100 did not prove successful at protecting the liposome bilayer from bile salt attack. It is therefore suggested that a liposome-in-microsphere (LIM) formulation could be produced encapsulating liposomes within solid Eudragit S100 microspheres. It was shown in the previous chapter that Eudragit S100 microspheres have the potential to remain intact through the stomach and small intestine, to subsequently begin degradation in the ileo-caecal junction. It is hypothesised the microspheres will begin to release the liposomes at the ileo-caecal junction, therefore allowing for sustained drug release at the site of the colon. The proposed method to produce LIMs is an adapted double emulsion solvent evaporation technique, therefore exposing the liposomes to a solvent mixture (DCM, ethanol, propanol) which would rapidly destroy the bilayer of the vesicles. With this in mind it is essential that the liposomes are protected through the microsphere production stages. Furthermore not only do the liposomes need to be protected during the production of LIMs but they must also show a suitable release profile that would enable colonic drug delivery. It is hypothesised that using a polysaccharide to coat the liposomes that will initially protect them from the organic solvent and then be solubilised by the colonic microflora when released by the Eudragit S100 microspheres. The overall aim of this chapter was to devise a method of coating liposomal formulations with chitosan that would protect the vesicles during their exposure to the solvent mix when encapsulating them into the microspheres and provide a suitable release profile for colonic drug delivery.

A number of researchers have shown that liposomes can be coated with the polysaccharide chitosan, which would not only protect the liposomes during transition through to the colon, but would also provide an enzyme controlled component to the system, which would therefore make the formulation with a two-trigger system. It has been demonstrated that a system with a two trigger formulation has a number of advantages over single trigger systems (Kaur and Kim, 2009), including site specificity and formulation stability. Kaur and Kim (2009) showed that producing a double coated formulation with a chitosan inner coat and Eudragit outer coat enables the chitosan to control drug release to the colon; the Eudragit coating would provide an enteric protective layer for the formulation, thus preventing solubilisation in the stomach. The use of an enzyme controlled release system is considered

to provide improved site specificity, as variability associated with transit time and pH through the GI tract does not influence the colonic microflora and the enzymes specifically found in the large intestine (section 1.4.3.4). This use of a double trigger system would allow for more flexibility in the Eudragit coating, as less emphasis is required on the site specificity due to the controlled release of the chitosan coating i.e. if the microspheres were to degrade in the proximal small intestine the chitosan coating would ensure that drug release is limited until arrival into the colon. This would enable a variety of Eudragits to be used, or even a combination of two different Eudragits (L100 and S100), in order to produce a solubility profile suitable for possible patient variability i.e. patients with a GI tract pH profile lower than 'normal' (Khan *et al*., 1999; 2000).

5.1.1. Chitosan

It has been proposed that liposomes can be coated with the polysaccharide chitosan (Laye *et al*., 2008; Guo *et al*., 2003; Mady *et al*., 2009; Henriksen *et al*., 1994; 1997), which is dissolved by bacteria present within the microflora of the colon (section 1.4.3.4). Investigations into chitosan and its suitability for resistance to solvents, and its ability to coat liposomal formulations has been well documented (section 2.3.2). As chitosan has a number of amine groups it also dissolves in weak acidic conditions and would therefore need an enteric coating to remain intact through the acidic conditions found within the stomach (Shimono *et al*., 2002).

A number of studies have successfully looked at coating liposomes through electrostatic interactions, by producing anionic liposomes and subsequently coating with the cationic polymer (Laye *et al*., 2008; Wei and Lu, 2003; Henriksen *et al*., 1994; 1997). Laye *et al*., (2008) investigated the ideal chitosan concentrations for production of chitosan-coated liposomes through the electrostatic deposition of chitosan onto the surface of an anionic liposome formulation. It was concluded that below a minimum critical chitosan concentration (C_{min}) large aggregates formed that phase separated in minutes which was attributed to the formation of coacervates. In comparison a maximum critical chitosan concentration (C_{max}) was observed whereby concentrations above this value produced large flocs that sedimented within hours, which was attributed to depletion flocculation. It was shown that the C_{min} and C_{max} were dependent upon the liposome concentration and size. Wei and Lu (2003) showed that negatively charged, positively charged and neutral liposomes could all be coated with

chitosan. The resultant formulations, with an outer enteric coating, showed very little (< 7%) release in simulated gastric and enteric fluid (4 and 8 hours respectively) whilst simulated colonic fluid containing β-glucosidase showed considerable release with a $t_{1/2}$ of 3.63 hours. Henriksen *et al*., (1994, 1997) closely investigated the interactions between chitosan and liposomes also taking into account the numerous factors involved including addition speed, stirring speed and the specific molecular weight of chitosan used.

In contrast a number of studies have shown that neutral liposomes can be coated with the cationic polymer through hydrogen bonding between the polysaccharide and the phospholipid head groups (Mady *et al*., 2009; Guo *et al*., 2003; Perugini *et al*., 2000; Henriksen *et al.*, 1994 and 1997). It was suggested that coating neutral liposomes provided a more stable system that would be influenced less by any changes in pH and buffer upon dilution (Guo *et al*., 2003). It has been shown that the charge of the liposomes can significantly alter the chitosan coating process (Henriksen *et al.*, 1994; 1997) and therefore this chapter will look at the coating of both neutral and negatively charged liposomes to compare the resultant formulations. As discussed in the introduction (section 1.7.5.1), there are advantages to colonic delivery of both neutral and negatively charged liposomes, and therefore if both can be coated with chitosan it would provide the opportunity to specifically attach to inflamed or healthy mucosa, depending upon the condition.

5.1.2. The advantages of using LUVs over MLVs

Previous experiments (chapter 3) attempted to utilise MLVs for drug delivery to the colon due to their multilayer structure and therefore slower release rates in comparison to LUVs and SUVs. With a view to the liposomes being coated and then subsequently encapsulated in Eudragit S100 microspheres a prolonged release rate is not required in comparison to MLVs directly coated with Eudragit S100 where the coating would be solubilised at the distal small intestine exposing the liposomes to drug release. It is therefore proposed that LUVs are encapsulated within the microspheres due to the size of MLVs being problematic for the microspheres produced in the previous chapter. The use of LUVs will also lead to an increased encapsulation efficiency in comparison to MLVs which should balance out with the losses associated with polymeric membrane extrusion.

The overall aims of the work described in the current chapter were to:

- Investigate the extrusion of EPC MLVs with a view to producing LUVs with a narrow size distribution.
- Develop a coating strategy to coat either neutral or negatively charged liposomes with chitosan.
- Determine the size distribution and uniformity of coating through a variety of techniques.
- Investigate the mechanism of the coating and assess its effects on the drug release profile of liposomes in buffers representative of the pH range observed within the GI tract. The buffers will include a model bile salt and enzyme for the small and large intestine respectively. This will provide a more detailed indication of the formulation's properties *in vivo* and will ensure that the chitosan coat will withstand the distal region of the small intestine and subsequently release the active ingredient in the colon.

5.2. Materials

5.2.1. Lipids

EPC was a gift from Lipoid GmbH, which had a purity of 99.2%. Cholesterol (≥99%) was purchased from Sigma Aldrich (Dorset, UK). Dicetylphosphate (DCP) (≥99% purity), a negatively charged lipid, was used to produce anionic liposomes and was also purchased from Sigma Aldrich. All lipids were stored at -20°C ± 5 °C in sealed containers.

5.2.2. 5-Aminosalicylic acid (5-ASA)

The model drug used throughout the investigations was 5-ASA which is an anti-inflammatory drug used in the treatment of ulcerative colitis and Crohn's disease. The active dose of 5-ASA is 400mg, the current formulation does not set out to deliver this but merely uses 5-ASA as a model drug that is delivered to the colon as a reference for the possibility of delivering specific peptides and proteins to the colon.

5.2.3. Chitosan

Chitosan (Sigma Aldrich-medium molecular weight) had a molecular weight of approximately 237,000 as measured by GPC (section 2.4.2). A medium molecular weight chitosan was chosen as previous studies have shown that low and high molecular weight chitosan can promote extensive particle aggregation (Mun *et al*., 2006). Chitosan solutions (0.01, 0.25, 0.5, 1, 2 and 3%) for coating were created by dissolving the appropriate amount of chitosan in 1% acetic acid through vortex mixing and sonication.

5.3. Apparatus and methodology

5.3.1. Production of neutral and anionic LUVs

MLVs were produced in the same way as outlined previously in section 3.3.1, using the original method published by Bangham *et al*. (1965). Neutral liposomes were produced using a EPC:CH ratio of 7:2 with it being hypothesised the lower CH concentration would lead to a faster drug release rate due to the increased bilayer mobility (Lemmich *et al*., 1996). Anionic liposomes were produced by including DCP in a ratio of 7:2:1 (EPC:CH:DCP). Blank MLVs were hydrated with PBS buffer whilst drug loaded MLVs were hydrated with a 1mg/ml 5-ASA in PBS solution.

LUVs were produced by polymeric membrane extrusion of MLVs through membranes with a reducing pore size (1 µm, 0.4 µm and 0.2 µm) usingthe Mini-Extruder (Avanti Polar Lipids, Inc., Alabaster). The mini extruder allows for small samples sizes (≤ 10ml) to be extruded using a variety of polycarbonate membranes (0.03 µm to 5 µm). The materials used in the production of the mini extruder are heat resistant, therefore allowing for a variety of lipids (with a phase transition temperature above room temperature) to be extruded up to a maximum of 80°C. Due to the low transition temperature of EPC (-5 to -15˚C), there was no requirement to heat the extrusion apparatus, therefore all extrusions were performed at room temperature. MLV samples were extruded through the membrane a number of different times (5, 11, 15 and 19) to investigate how many extrusions were required to achieve a homogenous size distribution of LUVs. The manufacturers recommend that a minimum of 11 passes are required, but sample requirements are governed by the lipids being used. The extrusion process relies on pressure being applied to the syringe to force the sample through

the membrane and therefore may be open to user variability (unless a peristaltic pump is used). With this in mind a full study on the number of passes required to obtain a homogenous sample size was necessary, with a minimum of three individual samples to be assessed.

5.3.2. Characterisation of LUVs

The size distribution using each membrane after the specific number of passes was measured by laser diffraction particle sizing as previously outlined in section 3.3.3.4. Three independent samples were produced with each sample being measured a total of five times.

Liposomes were labelled with the lipophilic dye Nile red. The use of a lipophilic stain means the lipid will become fluorescent under a fluorescent light microscope therefore allowing the liposomes to be viewed non – invasively in their natural state with no need for freeze drying to view under SEM. The same thin film hydration method was adopted as before (section 5.3.1) with 2mg of the Nile red being dissolved with the other lipids in the solvent. Samples were then imaged using a Leica microscope with attached fluorescence unit.

5.3.3. Production of chitosan-coated liposomes

The coating of liposomes was completed by adopting a technique used by Henriksen *et al.* (1994; 1996). Liposomes were added drop-wise to chitosan solution (0.01, 0.25, 0.5, 1, 2, 3% w/w) in equal volumes whilst under magnetic stirring (400 rpm). The sample was then left stirring for a further 2 minutes and allowed to stabilise at 4˚C for 24 hours. The chitosan-coated liposomes were then washed three times by centrifugation (26,000 rpm, 63,000 g) and replacing the supernatant with fresh 1% acetic acid.

5.3.4. Characterisation of chitosan-coated liposomes

5.3.4.1. Zeta potential analysis for chitosan-coated liposomes

A number of studies have used the change in zeta potential to indicate the evolution of a chitosan coating on both neutral and anionic liposomes (Mady *et al.,* 2009; Laye *et al*., 2008; Guo *et al*., 2003; Takeuchi *et al*., 2005). 1 ml of washed chitosan-coated liposomes was added to 20 ml of distilled water. The sample was then measured using the method described

in section 3.3.3. The average of 10 measurements was calculated with three independent samples being measured overall.

5.3.4.2. Influence of chitosan concentration on size distribution of liposomal formulations

Chitosan-coated liposomes were measured using the laser diffraction method outlined in section 3.2.3. Both anionic and neutral liposomes were coated with 1% chitosan solution with the resultant formulations being dispersed in distilled water.

5.3.4.3. Cryo-SEM analysis of chitosan-coated liposomes

Chitosan-coated liposomes were imaged using the cryo-SEM method described in section 3.2.4. Initial images of chitosan-coated LUVs proved inconclusive due to the small particle size (approximately 200nm) and the low accelerating voltage required to maintain cryo chamber temperature throughout. With this in mind images of chitosan-coated MLVs were also taken as it has been observed the initial liposome diameter has little effect on the coating properties (Laye *et al*., 2008).

5.3.4.4. Fluorescence labelling of chitosan-coated liposomes

To visualise the chitosan coating layer on the liposomes a method used by Amin *et al*. (2009) was adopted. FITC-labelled chitosan was synthesized by adding 100ml dehydrated methanol followed by 50 ml of FTIC in methanol (2.0 mg/ml) to 100 ml of chitosan (1% in 0.1 M acetic acid) in the dark and at ambient temperature. After 3 hours, the labelled polymer was precipitated in 0.2 M sodium hydroxide. The precipitate was pelleted at 40,000 x g (10 min) and washed with methanol-water (70:30, v/v) until no fluorescence was observed in the supernatant. The labelled chitosan was then re-dissolved in 20 ml of 0.1 M acetic acid and suitable for coating as in section 5.3.2. Further trials were completed using Nile red dyed liposomes (section 5.3.2.2.) in combination with labelled chitosan to so that the lipid and aqueous regions of the liposomes could be observed. All images were taken using the fluorescence method described in section 5.3.2.2.

5.3.5. Drug release properties of chitosan-coated liposomal formulations

5.3.5.1. UV spectral analysis and calibration curves for 5-ASA quantification

Initially, a UV spectrum was completed to determine the wavelength at which 5-ASA has a suitable absorbance to analyse drug release media for release (Figure 5.1). All UV spectra were completed on a Jasco V-530 UV/Vis spectrophotometer. A small amount of 5-ASA was dissolved in water with the spectra being taken in the range 250-500 nm, at 5 nm/second.

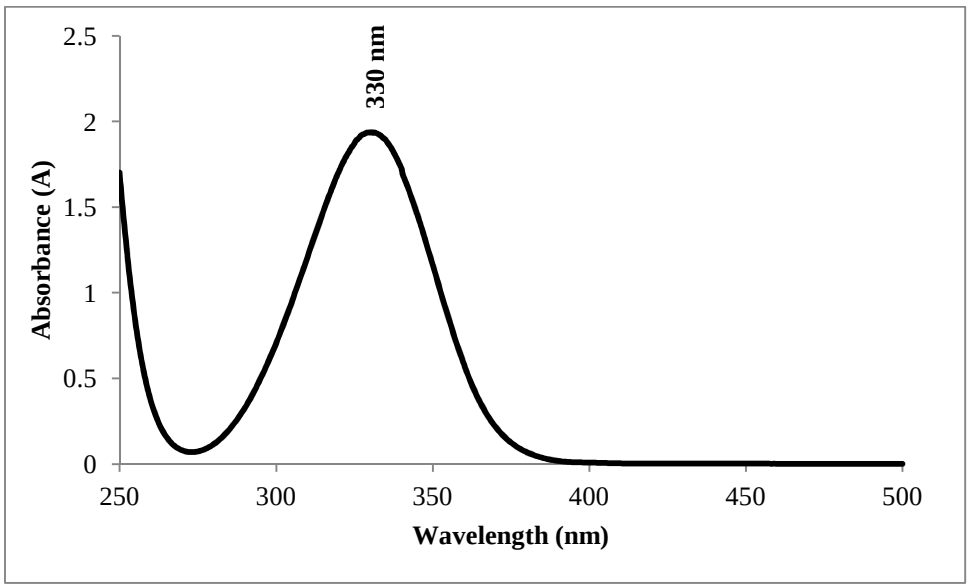


Figure 5.1. UV spectra of 5-ASA dissolved in distilled water.

Calibration curves were then constructed for 5-ASA dissolved in each of the buffers to be used during the release trials (outlined in section 5.3.4.3). For each calibration curve a solution of 1 mg/ml of 5-ASA was made up in the required buffer, and then a serial dilution was completed to a point where the absorbance level became negligible. All measurements were completed in triplicate on independent samples at the λ_{max} = 330 nm. The resultant calibration curves are shown in Figure 5.2.

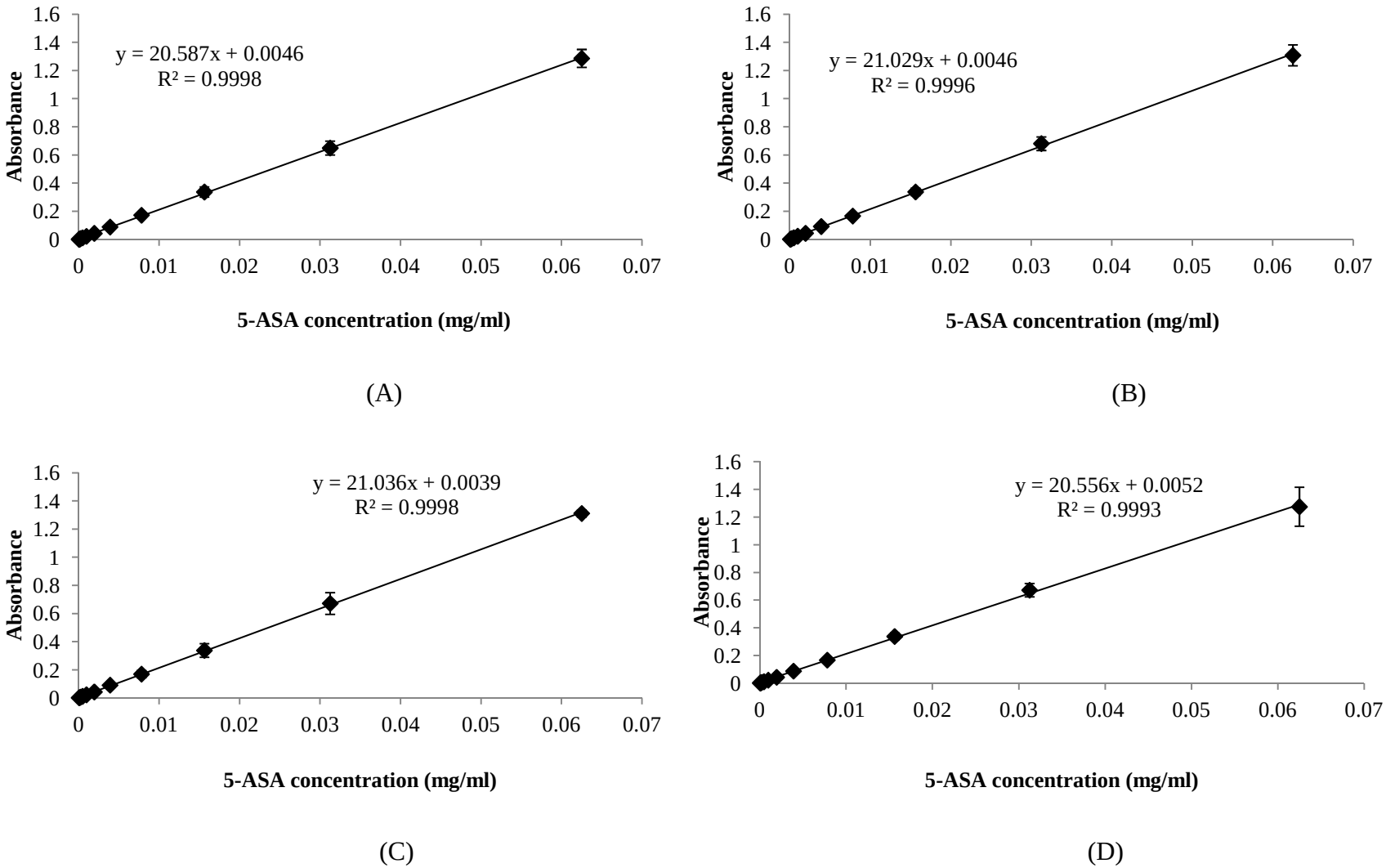


Figure 5.2. Calibration curves for 5-ASA dissolved in (A) 0.1M HCl, (B) Hanks' solution, (C) PBS and (D) ethanol at 330nm.

5.3.5.2. Quantification of encapsulated 5-ASA

The amount of drug present within the LUVs was quantified by lysing the liposomes after extrusion to give a total encapsulation for uncoated formulations. 0.1 ml of liposome sample was added to 0.9 ml ethanol and vortex mixed to ensure all the liposomes had been destroyed. The solution was then analysed spectrophotometrically with the concentration being calculated by referring to the calibration curve in ethanol (Figure 5.2 D). For all calibration curves and release experiments, initial trials were performed to ensure none of the individual components of the formulation would influence the UV absorbance when measuring for specific drug concentrations.

5.3.5.3. Drug release studies in conditions representative of the GI tract

As outlined previously the GI tract has a range of pHs and transit times. The release media used for the stomach and small intestine were the same as outlined in section 3.2.3. The use of 10 mM sodium taurocholate in the Hanks' solution was continued as it not only provides similar conditions to those observed in the GI tract but was also shown to be a rigorous test when used for trials of Eudragit S100 coated liposomes (chapter 3).

The method for the large intestine release media was adapted from previous experiments. The inclusion of a specific enzyme that is present in the colon is necessary to solubilise the chitosan as would be expected *in vivo* (described in section 2.3.3). A number of studies have evaluated the solubilisation of the chitosan coating layer of liposomes by assessing drug release within buffers containing either rat or human faecal slurries (McConnell *et al*., 2008; Zhang and Neau, 2002). Due to the number of safety considerations and complications associated with using faecal samples, a number of studies have investigated *in vitro* methods that utilise commercially available enzymes that will solubilise chitosan in a similar way to *in vivo*, whilst maintaining human relevance. Fang *et al*. (2009) showed that β-glucosidase (4% w/w) gave comparable results to rat cecal contents (4% w/w) for theophylline release of chitosan-coated tablets. Furthermore, Orienti *et al*. (2002) showed that in the presence of β-glucosidase (1 mg/ml) an increased drug release was shown from a variety of chitosan salts in comparison to a standard pH 7.0 PBS to simulated colonic fluid. Wei and Lu (2003) observed that chitosan-coated liposomes released very little in simulated gastric and enteric fluids, but showed that there was a significant increase in percentage released and release rate when the liposomes were subjected to simulated colonic media containing β-glucosidase (concentration

unknown). It was decided to use a 4% (w/w) concentration of β-glucosidase in PBS with a pH of 7.4 which represents the average pH found at the ileo-caecal junction (outlined in section 1.4.3.4).

5.3.5.4. Drug release profiles for uncoated and chitosan-coated LUVs

Drug release trials with uncoated and 1% chitosan-coated neutral LUVs were conducted in the release media described in section 5.3.4.3. For each experiment 1 ml of liposome sample was injected into a 20,000 molecular weight cut off (MWCO) Slide-A-Lyzer dialysis cassette (Piercenet, Rockford). The cassette had been previously assessed that it maintained its integrity within the simulated media to be used. This assessment was completed by injecting a sample of 5-ASA in PBS and assessing the release media over a number of time points. It was shown that the dialysis cassette maintained its integrity over the required time periods with the indication of very little or no release being observed (<2%). The cassette had been previously hydrated in 0.1 M HCl (maintained at 37˚C) for 2 minutes. The dialysis cassettes were then attached to the rotating shaft of a USP Type II dissolution apparatus (Varian DS 705, Agilent Technologies, England) dissolution apparatus and lowered into a 400 ml beaker containing 250 ml HCl. The beaker was held in a water bath within the 1 litre vessels of the apparatus which was maintained at 37˚C throughout. The stirring speed of the apparatus was maintained at 100 rpm in accordance with the British Pharmacopoeia's guidance on dissolution testing (Appendix XII B). Aliquots of 1 ml were removed from the buffer at pre-determined time intervals and replaced with 1 ml of pre-heated fresh buffer. The concentration of released 5-ASA was determined using UV spectrophotometry against a standard curve obtained at λ=330 nm. All measurements were taken against reference samples of the appropriate dissolution medium. After two hours the cassettes were moved from the HCl to beakers containing Hanks' buffer with 10mM sodium taurocholate and the trial was continued with samples being taken at pre-determined time intervals for the next three hours. Finally the samples were moved from beakers containing Hanks' buffer into beakers containing PBS and β-glucosidase. As the dialysis cassettes are cellulose based it was hypothesised that the inclusion of β-glucosidase in the release media would lead to the degradation of the membrane over time. A trial experiment was conducted whereby a blank cassette was placed into the release media. Samples were taken at pre-determined time points to observe if any alteration in the UV absorption occurred. It was shown that the absorption increased at approximately 7-8 hours and therefore it was decided every 5 hours the sample

was removed from the cassette and placed in a new cassette into the same buffer. The 5 hour time frame ensured membrane degradation would not influence the absorption readings obtained for the specific drug release of the liposomes.

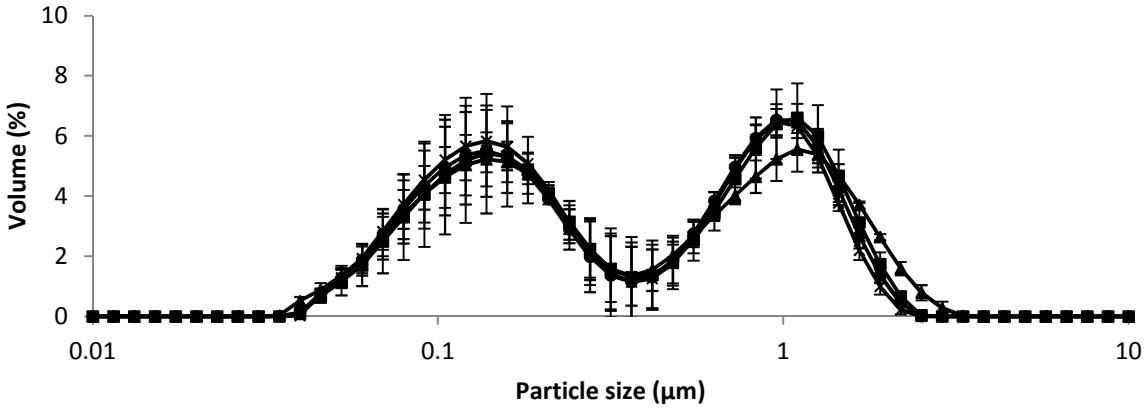
5.4. Results and Discussion

5.4.1. Investigation into size distribution of extruded MLVs

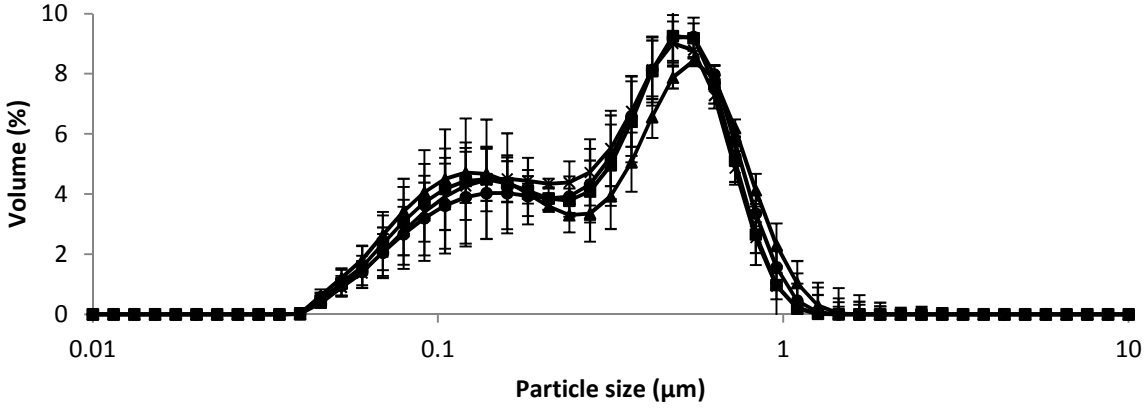
5.4.1.1. Investigation into the number of extrusions required for a homogenous sample

Size distribution results for liposomes exposed to different numbers of passes through the extruder are shown in Figure 5.3. The results shown are for the three membrane sizes; 1, 0.4 and 0.2 µm, for 5, 11, 15, and 19 passes. The size distributions are similar to those observed in previous studies (Macdonald *et al*., 1991; Subbarao *et al*., 1991) for this apparatus whereby a bimodal size distribution is observed initially, then as the number of passes increases and the membrane size decreases a unimodal, normal distribution is observed. The unimodal distribution is not observed until the 0.2 µm membrane is used which is also in accordance with the manufacturer's literature whereby it is suggested a membrane pore size ≤ 0.2 µm is required for the production of LUVs. The membrane pore sizes above 0.2 µm are recommended to produce a polydisperse suspension of MLVs.

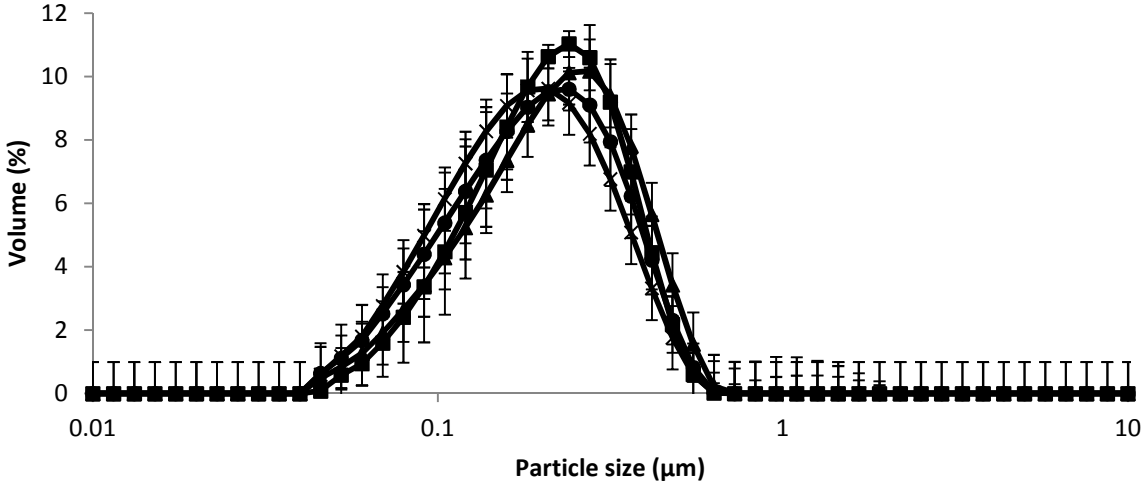
The size distribution statistics (d_{10}, d_{50}, d_{90}) also offer an insight into the change in size distribution with increasing passes through the membrane (Table 5.1.). The majority of difference can be seen between the samples produced using 5 passes and the samples produced using the recommended minimum number of passes of 11.



(A)



(B)



(C)

Figure 5.3. Size distributions for liposomes extruded using (A) 1 µm, (B) 0.4 µm and (C) 0.2 µm polycarbonate membrane. Samples were extruded through the membrane 5 (▲), 11 (■), 15 (●) and 19 (x) times.

Table 5.1. Table showing size distribution statistics (d_{10}, d_{50}, d_{90}) of extruded liposomes using various membrane pore size filters and varying number of extrusions.

	d_{10}			d_{50}			d_{90}		
	Membrane size			Membrane size			Membrane size		
Number of Passes	1 µm	0.4 µm	0.2 µm	1 µm	0.4 µm	0.2 µm	1 µm	0.4 µm	0.2 µm
5	0.0903	0.0927	0.109	0.338	0.361	0.230	1.611	0.783	0.407
11	0.0990	0.097	0.0937	0.376	0.360	0.197	1.450	0.706	0.367
15	0.0927	0.101	0.0890	0.339	0.382	0.197	1.396	0.742	0.378
19	0.0907	0.103	0.0873	0.286	0.347	0.187	1.338	0.699	0.365

5.4.1.2. Results showing effect of reducing membrane size at 15 extrusions

From the initial size distributions it was decided 15 passes through the polycarbonate membrane produced a suitably homogenous sample, and therefore it was decided to use this number of passes throughout the future studies. A size distribution comparing the change in liposomal size from MLVs to LUVs at 15 passes is shown in Figure 5.4. The transition from an MLV formulation into a low polydisperse LUV sample using the 0.2 µm can be observed. Further extrusions at lower membrane pore sizes could have been completed to produce a sample with a more narrow size distribution, but due to the drug losses associated with the extrusion process it was decided LUVs in the region of 0.2 µm would be suitable for coating and therefore maintain a high enough concentration of the original drug content (Berger *et al*., 2001; Bhardwaj and Burgess, 2010).

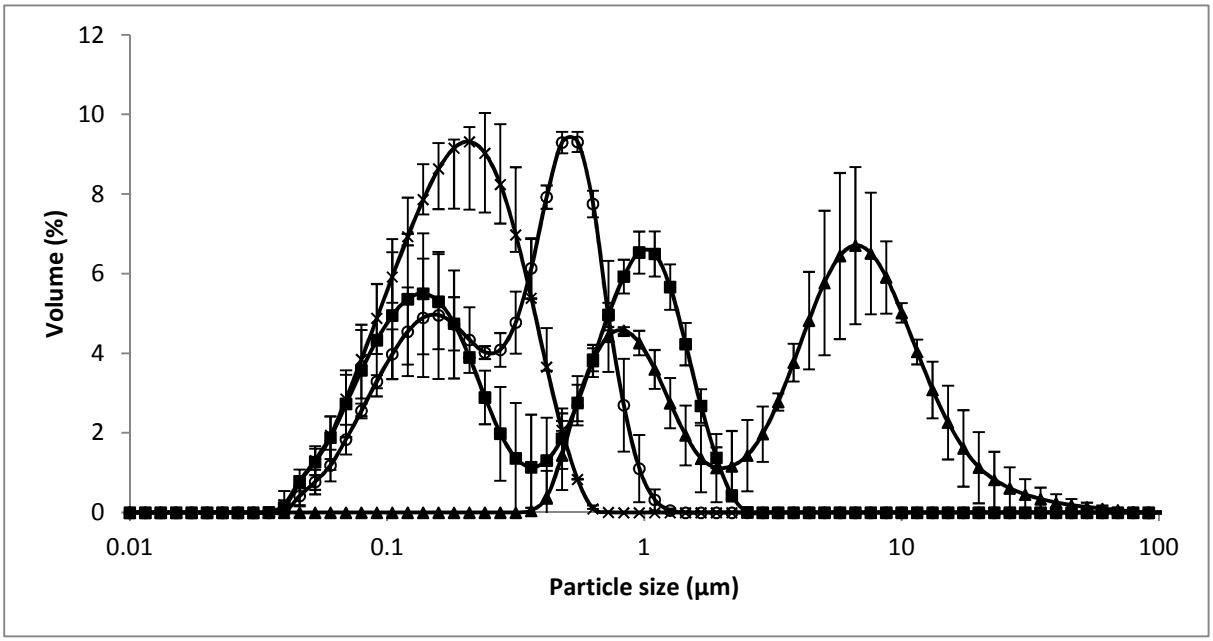


Figure 5.4. Size distribution for MLVs (▲) and liposomes extruded 15 times using 1 µm (■), 0.4 µm (○), and 0.2 µm (×) polycarbonate membranes.

Fluorescence imaging was undertaken on Nile red stained liposomes and the subsequent stages of extrusion through the various membranes (Figure 5.5). This method highlighted the phospholipid bilayers due to the staining and therefore offers a clear indication of liposome size and homogeneity. The stained bilayers are very clear for the MLVs, with the use of ever decreasing pore sized membranes being used showing an alteration in the vesicle size, thus reinforcing Figure 5.4. The vesicle size decreases significantly with the 1 µm membrane appearing to show a homogenous sample, but still with individual vesicles being visible. The 0.4 µm membrane shows less definition of vesicles which is a limitation of the current method using a light microscope. Finally, the 0.2 µm membrane shows no evidence of single vesicles, which is due to the resolution of the microscope and the subsequent small size of the LUVs. The lack of definition of vesicle observed for the 0.2 and 0.4 µm membrane is due to both the limitation of the microscope but also the nature of the fluorescent dye for small vesicles can cause excessive excitation which doesn't allow for definition between the unstained internal core to be observed in comparison to the stained bilayer.

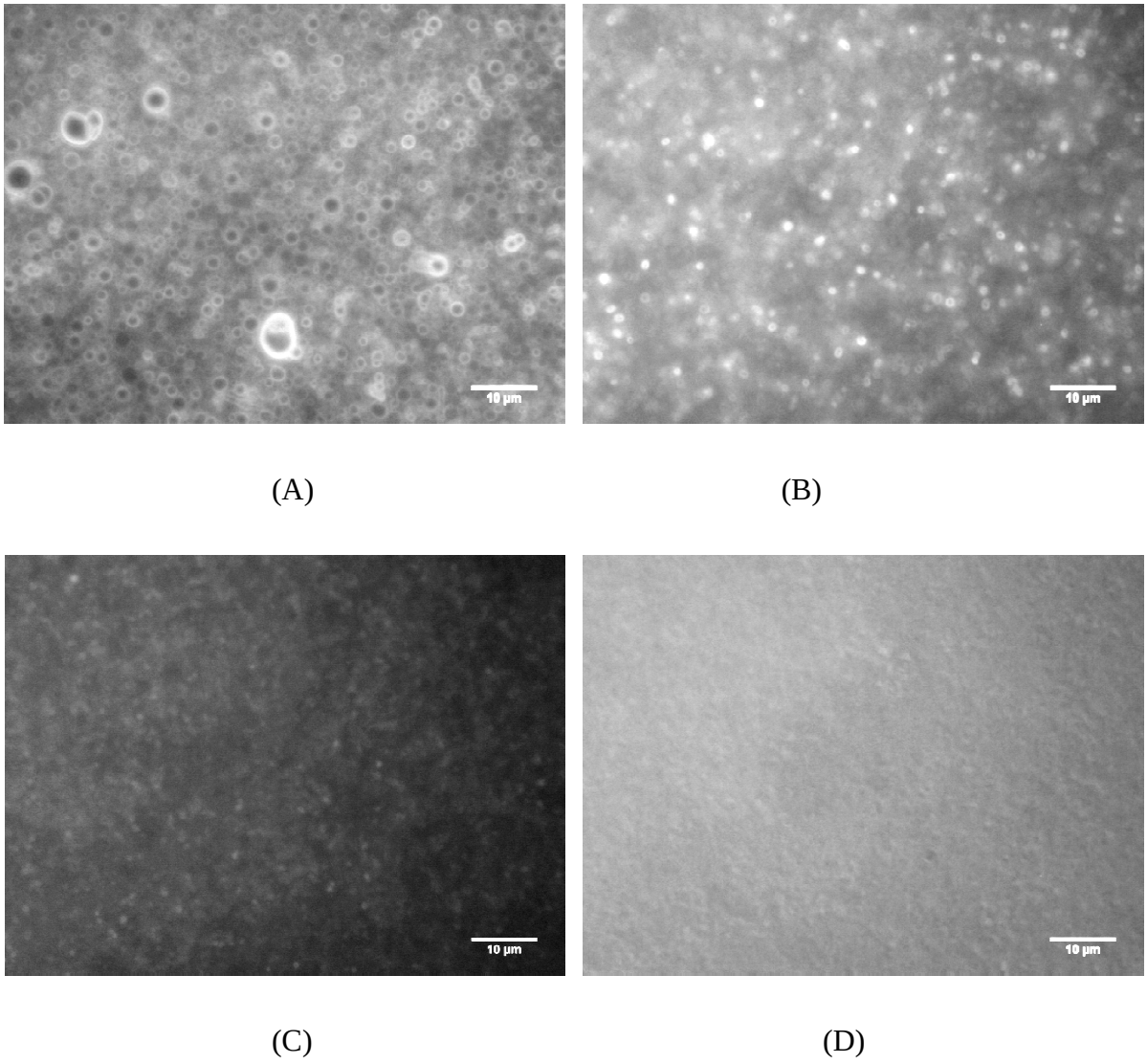


Figure 5.5. Fluorescence microscope images of Nile red stained (A) MLVs, and extruded liposomes using the (B) 1 µm, (C) 0.4 µm and (D) 0.2 µm polycarbonate membrane.

5.4.2. Investigation into chitosan-coated liposomes

5.4.2.1. Change in zeta potential for chitosan-coated liposomes

It has been widely documented that the evolution of a chitosan coating on liposomal surfaces can be monitored through analysis of zeta potential (Mady *et al.*, 2009; Laye *et al*., 2008; Guo *et al*., 2003; Takeuchi *et al*., 2005). Zeta potential values can be compared across most studies as the final coated liposomes should have a similar zeta potential regardless of the lipid used in the production of the liposomes. It would be difficult to compare specific concentrations used for chitosan coating as each study uses different ratios of chitosan to

liposomes, coupled with the variation in specific lipid and its concentration. The change in zeta potential observed for neutral and negatively charged LUVs is shown in Figure 5.6. For anionic liposomes an initial zeta potential in the region of -40 mV can be seen with neutral liposomes having a marginally negative charge of -1.2 mV, which is in accordance with previous studies (Mady *et al*., 2009; Makino *et al*., 1991). An increase in zeta potential can be observed for both liposomal formulations as the chitosan concentration increases. This would be expected as the cationic chitosan is absorbed onto the liposomal surface causing a change in overall surface charge. At 1% chitosan coating the zeta potential appears to plateau with no further increase observed for 2 and 3% coating in both liposome formulations. This is similar to that observed by Henriksen *et al*. (1997) where it was shown for washed liposomes the plateau occurred at similar zeta potentials for both neutral and negatively charged liposomes. A much higher zeta potential (~ +85 mV) was observed for unwashed due to excess chitosan polymer chains being loosely associated with the coated liposomes and therefore increasing the electrophoretic mobility. Henriksen *et al*. (1997) observed changes in zeta potential of -25 to +32 mV and -1.8 to +29 mV for negatively charged and neutral liposomes respectively when coating with chitosan, which are similar to those seen in Figure 5.6. Similarly, Guo *et al*. (2003) and Mady *et al*. (2009) observed a plateau in zeta potential at approximately 40 mV for liposomes produced using EPC and DPPC respectively when coated with chitosan.

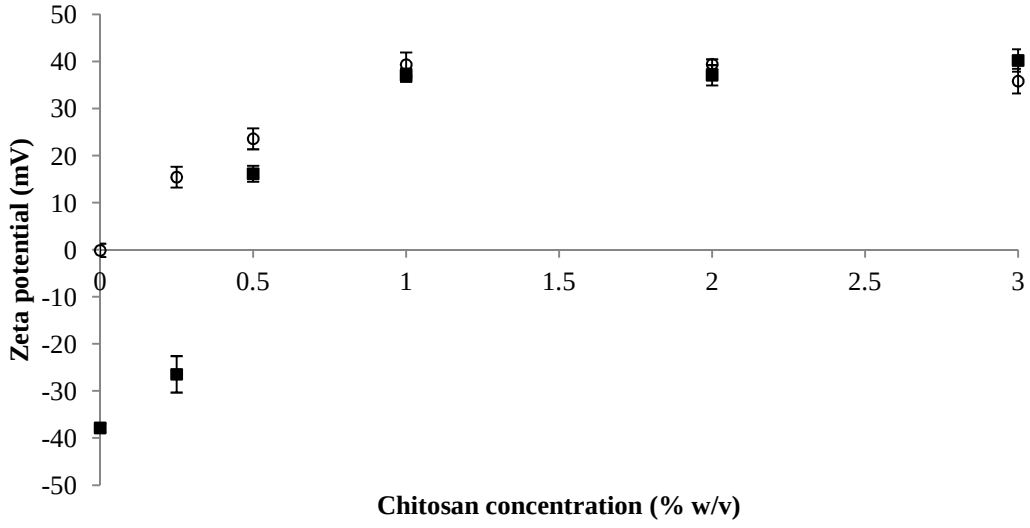


Figure 5.6. Influence of chitosan concentration on zeta potential of neutral (○) and negatively charged (■) chitosan-coated LUVs. Each point represents the mean of three independent trials, with the mean of 10 measurements completed for each trial.

Due to the plateau observed during the zeta potential trials it was decided that a sufficient chitosan coating would be achieved through the addition of 1% chitosan. This was not only to reduce the excess quantities of chitosan being removed during the washing process but also to attempt to reduce the possibility of aggregation and subsequent phase separation observed by Laye *et al*. (2008) when going below or above the saturation concentration. The saturation concentration was shown to have a linear relationship with liposomal concentration as an increase in liposomes leads to an increase in exposed surface area and therefore an increased amount of chitosan is required for coating.

5.4.2.2. Influence of chitosan concentration on size distribution of LUVs

Size distribution analysis was completed on uncoated and 1% chitosan-coated samples to observe the influence the chitosan coating had upon the particle diameter (Figure 5.7). It can be seen that for both coated formulations the size increases marginally compared to that of the uncoated formulations. This size increase, above the saturation concentration as defined by Laye *et al*. (2008), is expected for chitosan-coated formulations. Interestingly, there is a small volume of the anionic coated formulation with a much larger particle diameter above 10 µm which is indicative of possible aggregation through interactions between uncoated and coated sections of liposomes.

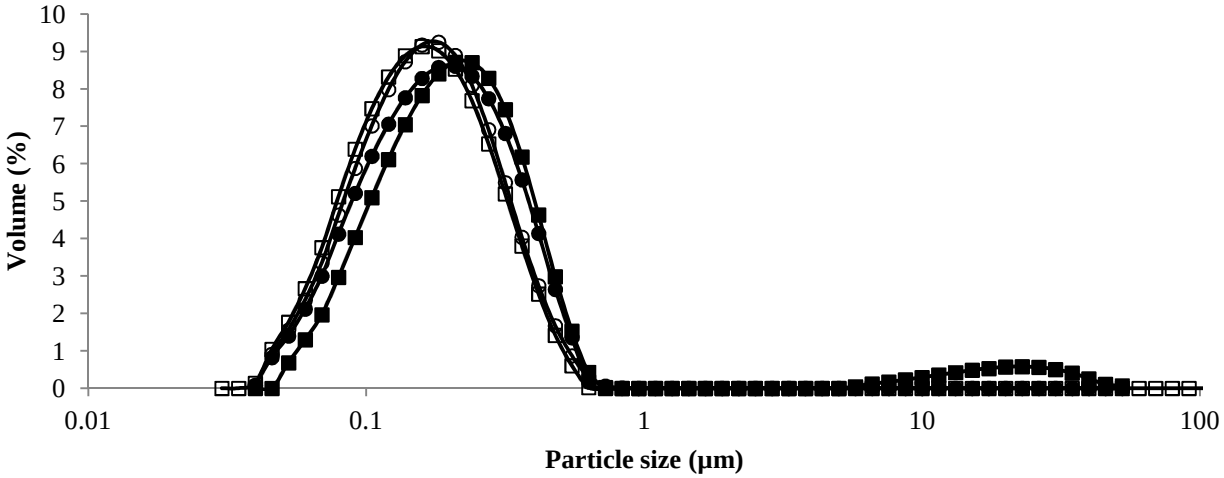


Figure 5.7. Size distribution of uncoated anionic (□) and neutral (○) LUVs, with 1% chitosan-coated anionic (■) and neutral (●) LUVs.

The coating mechanism for anionic liposomes combined with chitosan is predominantly attributed to electrostatic interactions which has been extensively investigated (Henriksen *et al*., 1994; 1997; Laye *et al*., 2008; Guo *et al*., 2003). The coating of liposomes through electrostatic interactions is often interpreted through the charge mosaic theory or adsorption coagulation (Gregory, 1973; Mabire *et al*., 1983). A diagram published by Mabire *et al*., (1983) suggesting the possible interactions during the coating of anionic particles with cationic macromolecules is shown in Figure 5.8. The diagram indicates that the uncoated formulation (interactions b) would not flocculate due to the repulsive forces between the two anionic particles. Similarly for two coated particles (interactions a) it can be seen that the two positive charges would also repel each other, therefore maintaining a homogenous dispersion of coated particles. The situation whereby flocculation could be observed is when a partially coated formulation is attracted to an uncoated particle (interactions c), therefore resulting in the aggregation of particles, which further supports the size distributions observed in Figure 5.7. This further supports Henriksen *et al.*'s (1994) strategy of adding liposomes to excess chitosan as this allows for particles to become completely coated and thus reducing the possibility of flocculation compared to if the chitosan solution was added to the liposomes.

Henriksen *et al*. (1997) indicated that a number of mechanisms of flocculation would be present (polymer bridging, double layer coagulation etc.), but identified that there were two main reactions taking place between chitosan and liposomes during the coating process. The two reactions, where C indicates chitosan and L liposomes, are;

$$C + L \rightarrow CL \quad (3)$$

$$CL + L \leftrightarrow LCL \quad (4)$$

Where (3) represents the irreversible coating of the liposome with chitosan and (4) represents a partly coated liposome interacting with a part of an uncoated liposome causing an increase in the measured particle size. If chitosan is present in excess then (3) should be able to proceed until a stable positive charge is formed therefore preventing (4) from occurring. This supports the need to maintain consistency in the production of chitosan-coated liposomes through both the stirring rate used and the rate of liposome addition as indicated by Henriksen *et al*. (1997).

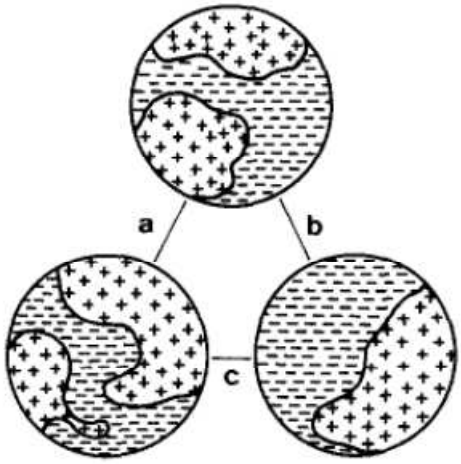


Figure 5.8. Diagram representing the possible interactions through the addition of chitosan to anionic liposomes. Interactions a and b are repulsive whilst c is attractive. (Mabire *et al.*, 1983).

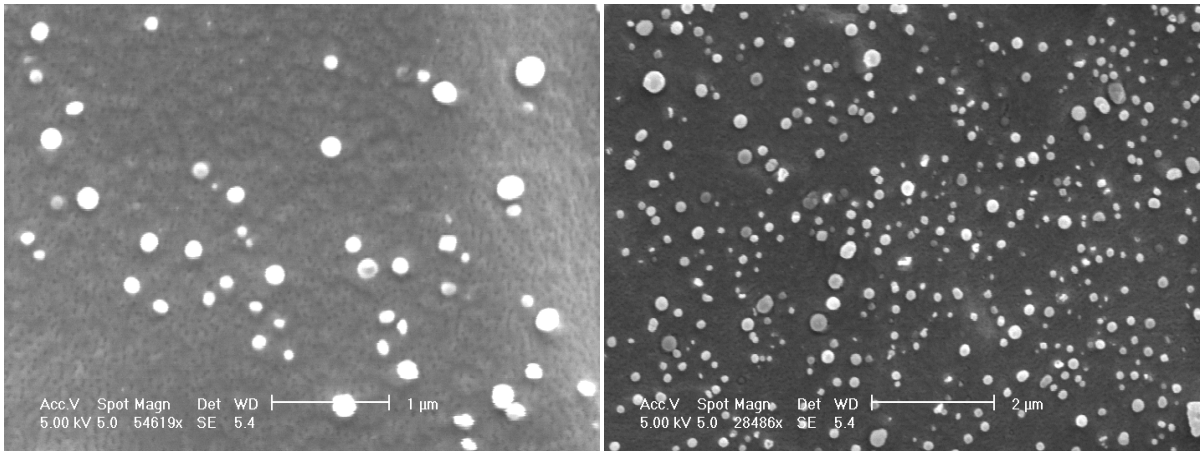
In comparison to the electrostatic interactions observed for the coating of anionic liposomes, the mechanism of coating neutral liposomes has not been as well documented. It has been hypothesised that hydrophobic interactions dominate the coating process (Perugini *et al.*, 2000; Guo *et al*., 2003), through hydrogen bonding taking place between the chitosan and the phospholipid head groups. Guo *et al*. (2003) compared the same lipid of two different purities to compare the possible interactions involved; Epikuron 170 and 200. A lipid of low purity was used, which produced anionic liposomes, to investigate the electrostatic interactions whilst a high purity lipid was used, which produced marginally positive liposomes, to investigate the possibility of hydrophobic interactions. An increase in zeta potential, size and stability upon dilution was observed for both batches of liposomes, but a number of differences were observed. The lipid of low purity (electrostatic interactions dominate) produced a coated liposomal formulation four times the size compared to that of the high purity lipid. Furthermore, Guo *et al.* (2003) observed a significant difference for the zeta potential measurements where the low purity lipid reached a plateau at approximately 40 mV whilst the high purity lipid at approximately 20 mV. This does however contradict the work of Henriksen *et al*. (1994) where similar zeta potentials were observed for both neutral and negatively charged liposomes. Guo *et al.* (2003) hypothesised that the addition of electrostatic interactions provided the increased affinity for the liposomes to the chitosan and therefore produced a thicker coating. It was concluded that chitosan coating through

hydrophobic interactions would be more favourable as it is considered more stable and was affected negligibly by the changes in pH and buffer upon dilution. It was also hypothesised that the thickness of coating could be problematic as it may restrict the release of the active ingredient at the target site.

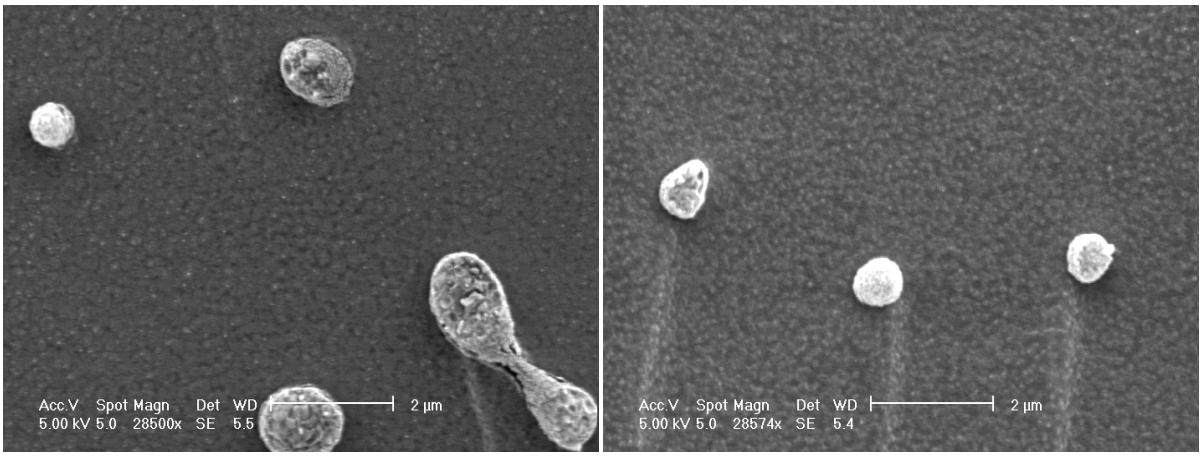
5.4.2.3. Imaging techniques used to investigate the chitosan coating of neutral and negatively charged liposomes dispersed in water.

Cryo-SEM

Initially uncoated and chitosan-coated anionic LUVs were investigated using cryo-SEM (Figure 5.9). The images of uncoated LUVs show a large population of small vesicles with a wide size range, which could not be seen during light microscopy (Figure 5.5). This variation is more representative of the size distribution observed during the extrusion process, which is indicative of using the largest membrane pore size suitable for the production of LUVs (0.2 µm). The subsequently coated LUVs showed a significant increase in size, with a much lower population of liposomes being observed (Figure 5.9 B). It can be assumed that due to the size change and significant reduction in liposome quantity that a number of chitosan-liposome complexes were produced, therefore not producing a single liposome with a defined coating layer. If this is the case it can be assumed that some sort of flocculation may be occurring during the production of chitosan-coated liposomes, as outlined by the mosaic charge theory.



(A)

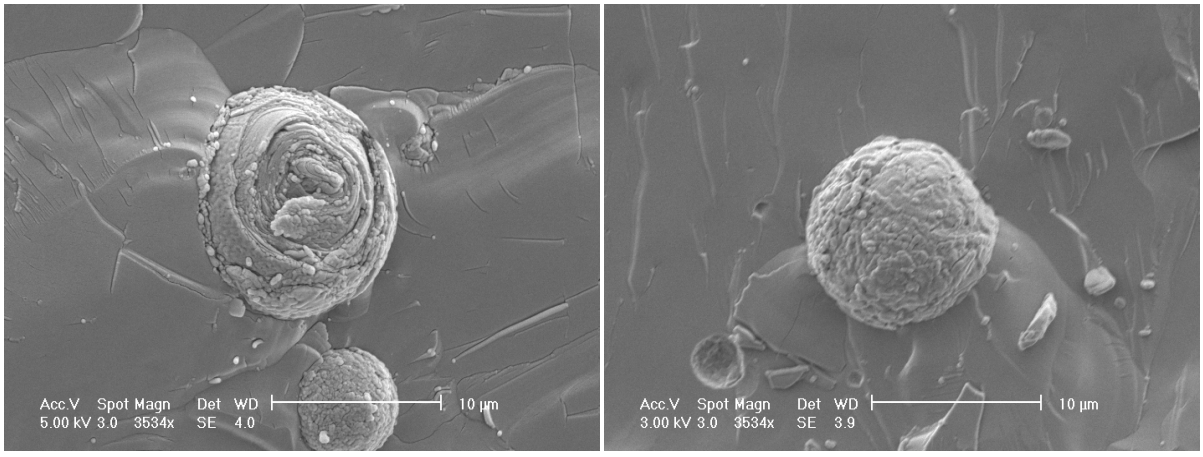


(B)

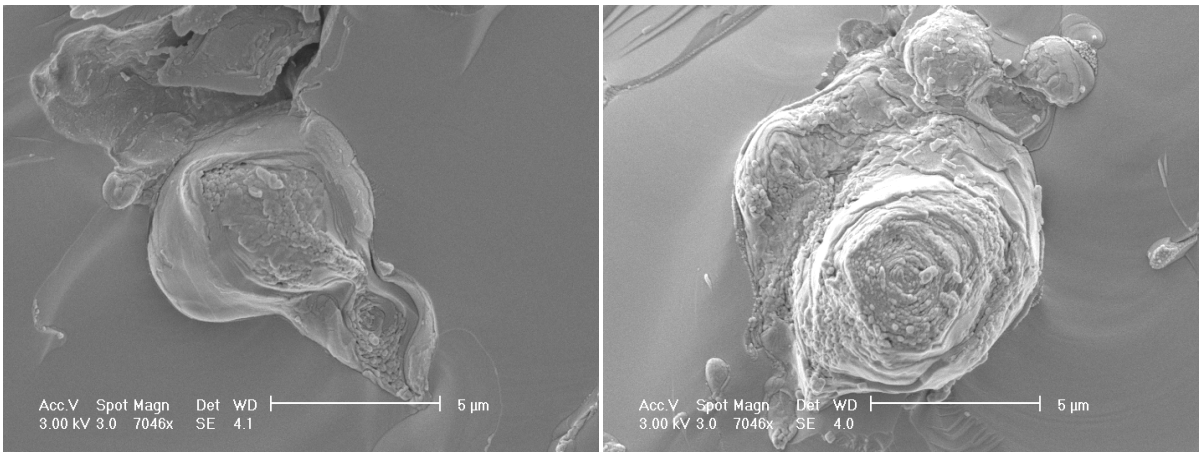
Figure 5.9. Cryo SEM images showing (A) uncoated and (B) chitosan-coated anionic LUVs.

Due to having to work at a lower accelerated voltage during cryo-SEM to maintain the temperature, the resolution possible did not give a clear indication of the coating layer and therefore chitosan-coated MLVs were observed. As it has been shown that there is little difference between the chitosan coating of varying size vesicles it was assumed that the same coating would take place for MLVs as LUVs (Laye *et al.*, 2008). As mentioned previously, Laye *et al.* (2008) observed a linear relationship between the lipid concentration and required amount of chitosan to reach saturation, but as the same lipid concentrations were maintained throughout it was decided no change in chitosan concentration would be required to coat MLVs. Figure 5.10 shows the resultant cryo-SEM images of uncoated and chitosan-coated

anionic MLVs. The uncoated MLVs display an uneven surface representing the phospholipid outer layer of the vesicle. The individual bilayers can also be observed in one of the images where the actual liposome has been fractured. In comparison the coated formulation show a smooth outer shell which would indicate the presence of an outer chitosan shell having been formed. The first image shows the presence of two liposomes in what appears to be a single chitosan complex, which further indicates that flocculation occurs during the processing. The second image of chitosan-coated MLVs shows what appear to be individually coated liposomes that have agglomerated into a single complex. This may be partially due to some electrostatic interactions still being prominent between uncoated sections of a liposome with coated sections of liposomes, similar to that observed by Laye *et al*. (2008). Laye *et al*. (2008) observed small aggregates forming when coating liposomes, which were visible as flocculations to the naked eye, but did not sediment to form a separate layer. It can be assumed that when measuring the size distribution of chitosan-coated anionic liposomes that the stirring speed (1750 rpm) was sufficient to disrupt these agglomerations and produce a homogenous formulation throughout.



(A)

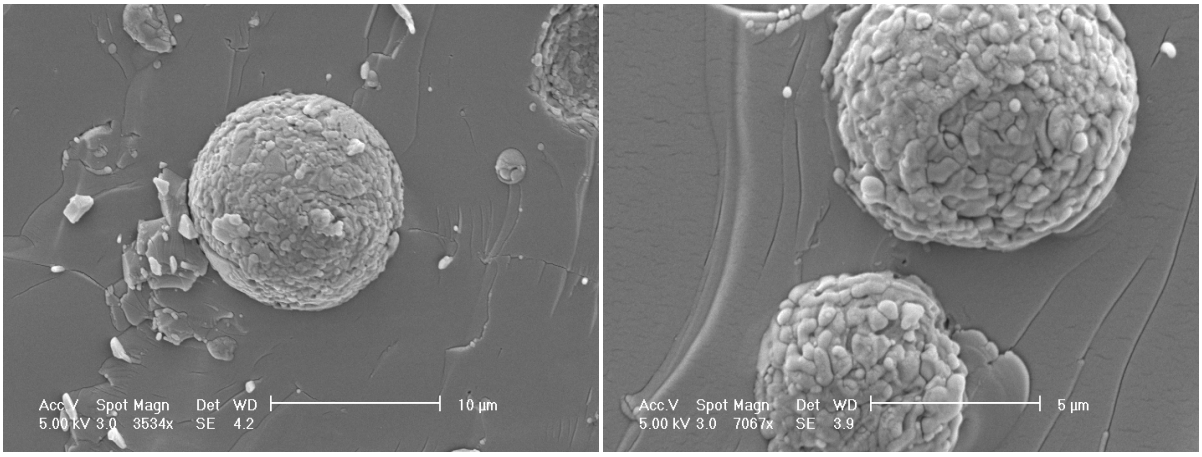


(B)

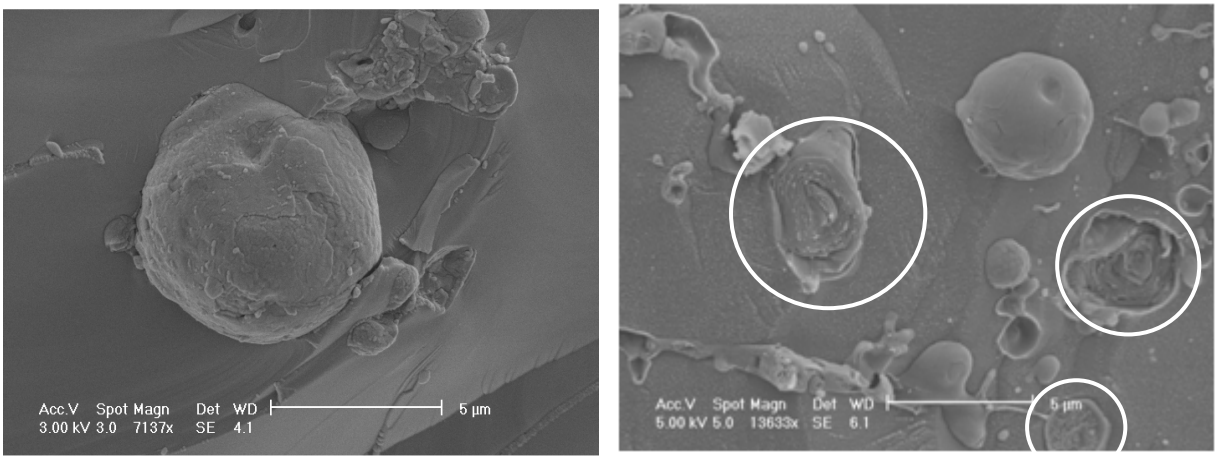
Figure 5.10. Cryo-SEM images showing (A) uncoated and (B) 1% chitosan-coated anionic MLVs. Samples are dispersed in distilled water.

Cryo-SEM images for uncoated and chitosan-coated neutral MLVs are shown in Figure 5.11. Uncoated neutral MLVs show a similar uneven, 'globular' surface morphology as seen for uncoated anionic MLVs. Chitosan-coated neutral MLVs show the same smooth outer shell as seen for anionic coated MLVs with the main difference being the neutral MLVs appear to remain as individual vesicles. The first image shows a complete liposome with a smooth outer shell whilst the second image shows a number of fractured vesicles. The fractured liposomes show a solid outer shell (circled on diagram) which indicates the presence of a solid chitosan coating. The appearance of this coating differs significantly to that observed

for Eudragit S100 coated liposomes where the polymer appeared to agglomerate on the liposome surface. This solid layer observed is indicative of a permanent coating that has a number of interactions including electrostatic and hydrogen bonding.



(A)

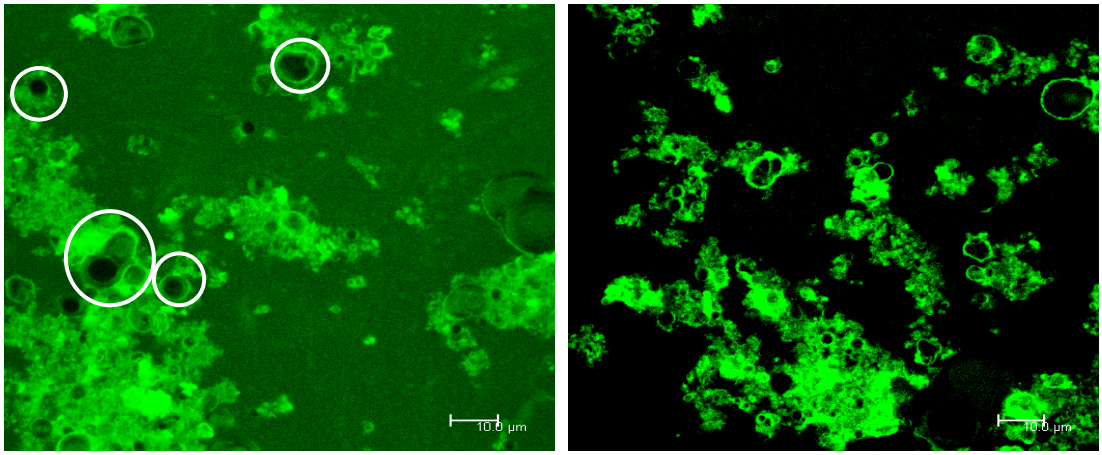


(B)

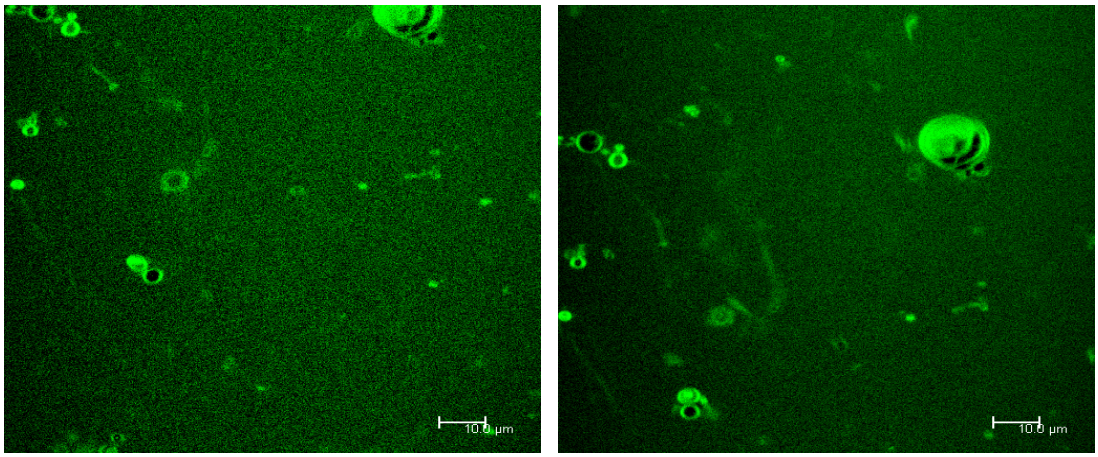
Figure 5.11. Cryo-SEM images showing (A) uncoated and (B) 1% chitosan-coated neutral MLVs. Samples are dispersed in distilled water.

Fluorescence Imaging

Cryo-SEM indicated the presence of a chitosan coat as the surface morphology changes from an uneven to smooth surface, with an outer shell being visible. As the Eudragit S100 coated liposomes also showed a form of coating but could not withstand bile salt attack, it was necessary to further investigate the nature of the coat and therefore its suitability to prevent liposome lysis when exposed to the solvent mixture during microsphere production. By labelling the chitosan with FITC it was assumed that the nature of the coat could be investigated by observing whether a single layer had been produced or if an agglomeration on the surface of the liposomes had occurred as observed for the Eudragit S100 coating. Figure 5.12 shows the effect of FITC labelling of chitosan prior to the coating of both anionic and neutral liposomes. The anionic liposomes (Figure 5.12 A) can be clearly seen with a black inner core indicating the internal structure of the liposomes and therefore where the FITC does not penetrate. The coating layer itself appears to be a uniform single layer around each liposome. There is also a clear indication of agglomeration in the sample with an excess of chitosan surrounding the liposomes. The images in Figure 5.12 provide a more conclusive indication of the chitosan coating thickness in comparison to the previous FITC labelled chitosan coating study by Amin *et al*. (2009). The images obtained are very similar to the cryo-electron micrographs presented by Henriksen *et al*. (1994) which show what he describes as a liposome double membrane having formed with the coating of LUVs. The neutral liposomes (Figure 5.12 B) show examples of single chitosan-coated liposomes with no chitosan agglomerations around the liposomal structure, which is in accordance with the cryo-SEM images. This further reinforces the use of the charge mosaic theory whereby chitosan agglomerations can be observed for anionic liposomes but not for neutral, which also supports the hypothesis that different coating mechanisms are involved.



(A)



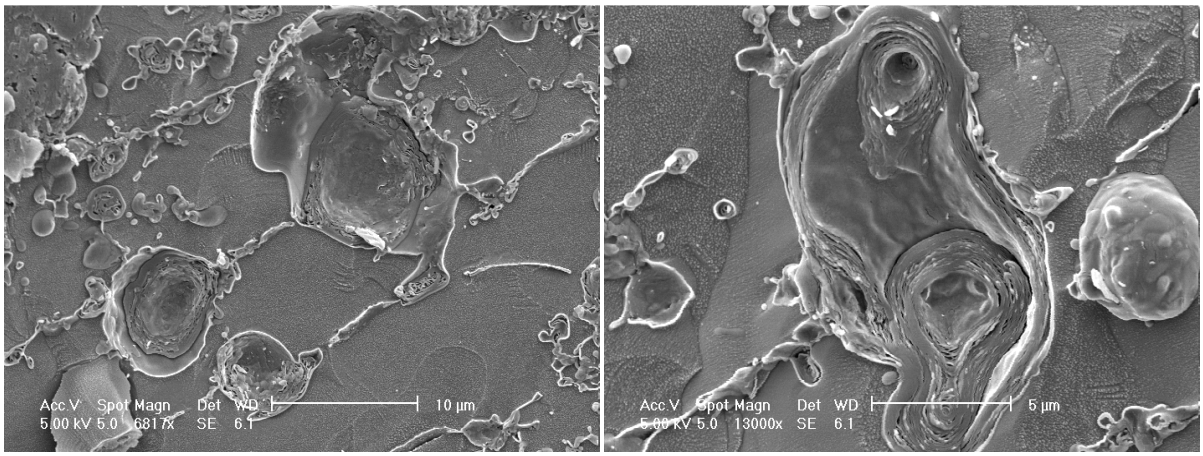
(B)

Figure 5.12. Fluorescence microscope images showing FITC labelled 1% chitosan-coated (A) anionic MLVs and (B) neutral MLVs dispersed in water. Each scale bar represents 10.0 µm.

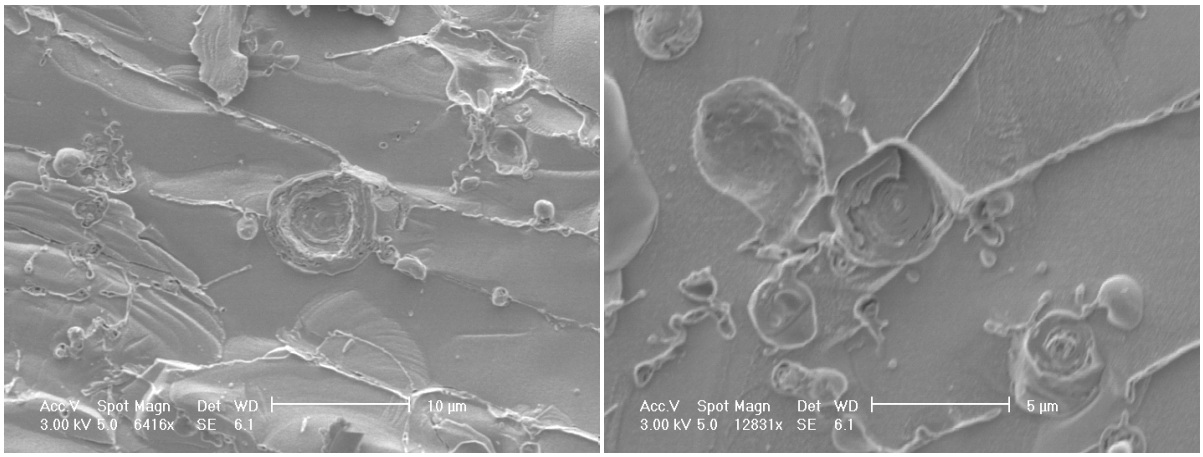
5.4.2.4. Cryo-SEM investigation into chitosan-coated liposomes stability when exposed to microsphere processing conditions.

Previous images of chitosan-coated liposomes (section 5.4.2.2.) have shown what appears to be a solid layer around the liposomes. The presence of the coating layer has been confirmed through a number of techniques (zeta, size and imaging), but the initial reason for coating the liposomes was to protect them during microsphere production and therefore it still remains to be seen whether the liposomes remain intact when exposed to the solvent mixture. Figure 5.13 shows cryo-SEM images of both anionic and neutral chitosan-coated MLVs

homogenised for 4 minutes at 7,400 rpm in the solvent mixture used for microsphere production. From both sets of images it can be seen that the chitosan coating and the internal liposomal bilayers have remained intact throughout the processing. No major differences in chitosan-coated structure or morphology can be observed between those dispersed in water and those dispersed in the solvent mix. This indicates that the liposomes would therefore be able to be successfully encapsulated within the Eudragit S100 microsphere.



(A)



(B)

Figure 5.13. Cryo-SEM images showing chitosan-coated (A) anionic MLVs and (B) neutral MLVs dispersed in solvent mixture of DCM:ethanol:propanol (5:6:4).

To provide further evidence of the protection provided by the chitosan coat in the solvent mix, Nile red stained liposomes were coated with FITC labelled chitosan. The resultant images of the sample homogenised in the solvent mix are shown in Figure 5.14. A number of liposomes can be observed with a definite chitosan coating surrounding them. If the coating had not protected the liposomes then the Nile red dye would not be visible as it would have leaked into the background. These images further support the conclusion that chitosan coating offers significant protection for both anionic and neutral liposomes to enable their encapsulation into Eudragit S100 microspheres using a double emulsion – solvent evaporation method.

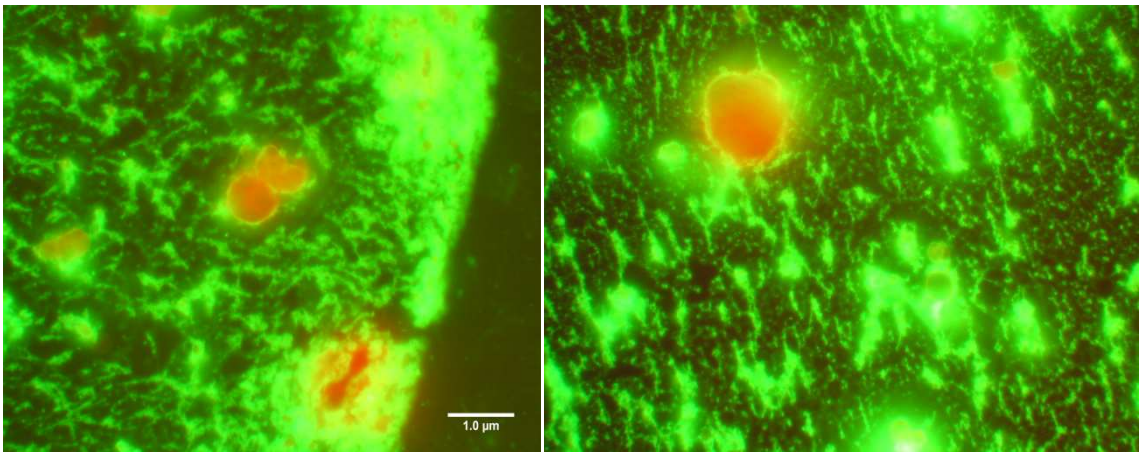


Figure 5.14. Fluorescence microscope images of neutral Nile red stained MLVs, coated with 1 % FITC labelled chitosan and subsequently homogenised in solvent mix (DCM:ethanol:propanol).

5.4.3. Drug release profiles for uncoated and chitosan-coated LUVs

A number of studies have investigated the drug release profiles of chitosan-coated liposomes and shown it is necessary for an enteric coating to be used as the coat is soluble within the acidic conditions of the stomach. Therefore, the current drug release profiles are to assess the release rates of the liposomes and their suitability for the application of colonic drug delivery whilst encapsulated within Eudragit S100 microspheres. The drug release profiles produced are shown in Figure 5.15. It was shown that the drug release curves were not statistically different (Mann Whitney U test), and therefore indicates that the presence of a chitosan coating layer would not sufficiently protect liposomes through the upper GI tract. The release data has been split into the three sections which correspond to the average transit time in the

GI tract and therefore the point where the dialysis cassettes were moved to the next release media. The uncoated liposomes show a continual steady release which leads to over 50% of drug release occurring in the first 10 hours. The release appears to slow considerably in the PBS with a plateau occurring at approximately 40 hours. Although continued release is observed for the uncoated formulation the actual timings of release may be subject to a short lag time whereby the drug has to diffuse through the dialysis membrane of the cassette. With this in mind though, both formulations will be subject to the same lag time and therefore allows for a comparison to be made.

The chitosan-coated liposomes follow a similar shaped curve to that of the uncoated formulation, the main difference being, the initial drug release in HCl and Hanks' buffer appearing to be considerably slower. After exposure to both HCl and Hanks' buffer only 15% of 5-ASA had been released, whereas 5 hours in the large intestine condition increased the cumulative release to 50%. The delayed drug release could be due to the slow solubilisation of the chitosan coating in the HCl and therefore preventing the diffusion of 5-ASA across the liposomal membrane. The results for chitosan-coated liposomes are comparable to that of Wei and Bin (2003) who observed that chitosan-coated liposomes released 13.1% of the drug when exposed to 4 hours in pH 1.2 and 8 hours in pH 6.8, with the majority of release (approximately 80%) taking place when exposed to pH 7.8 containing β-glucosidase for 12 hours. This further indicates the suitability of using β-glucosidase *in vitro* due to its specific ability to solubilise the 1-4 glycosidic bonds within chitosan (as described in section 2.3.2). The release of drug from chitosan-coated liposomes in HCl has been observed by Guo *et al*. (2003), where it was shown that over a two hour period chitosan-coated liposomes showed significant leakage of leuprolide when diluted in both HCl (pH 1.2) and PBS (pH 6.9).

The release profiles comparing uncoated and chitosan-coated liposomes indicate that the presence of the chitosan does slow the drug release considerably through the simulated GI tract conditions. Despite these findings the leakage that has been observed during these *in vitro* conditions further indicate the need for an enteric coating to protect the chitosan layer and therefore prevent premature drug release in the upper GI tract (Kaur and Kim, 2009).

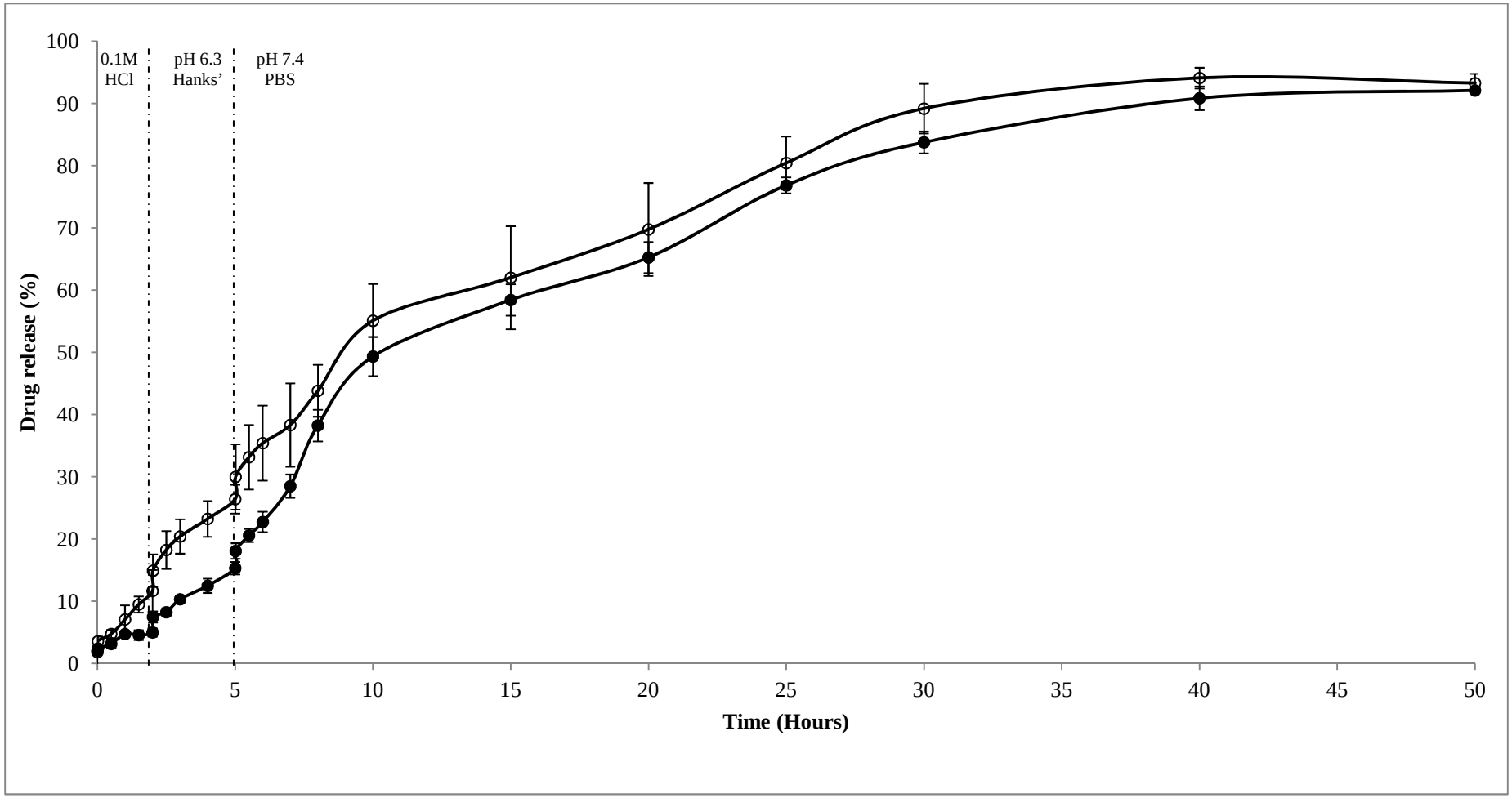


Figure 5.15. Drug release profiles for uncoated (○) and 1% chitosan-coated (●) neutral liposomes in 0.1 M HCl, Hanks' buffer containing sodium taurocholate and pH 7.4 PBS containing β-glucosidase.

5.5. Conclusions

Both anionic and neutral liposomal LUVs were produced through membrane extrusion. It was shown that 15 extrusions produced a homogenous sample size, which would then be suitable for coating. The effect of chitosan concentration on zeta potential was investigated when coating LUVs, with a 1% concentration proving adequate to coat the LUVs and reach a plateau therefore being at the saturation point of the liposomes. The presence of a chitosan coating layer in both liposomal formulations was confirmed through size distribution, cryo-SEM and fluorescence microscopy. The coating appeared as solid single coating which contrasts that of Eudragit S100 coated liposomes observed in chapter 3. The stability of the coat was subsequently investigated by exposing the liposomes to the solvent mixture and processing conditions involved in the production of Eudragit S100 microspheres. Both cryo-SEM and fluorescence microscopy confirmed that the liposomes still remained in the dispersion, therefore indicating that the chitosan coating had protected them during solvent exposure. 5-ASA release from uncoated and chitosan-coated neutral LUVs was investigated in simulated GI tract conditions. The coated formulation showed an improved drug retention in comparison to that of the uncoated formulation for the stomach and small intestine conditions, with both showing significant release in PBS containing the model enzyme β-glucosidase. Despite the improved drug retention it is evident that an enteric coating is required to protect the chitosan coating in the strong acidic conditions found within the stomach and therefore their incorporation into Eudragit S100 microspheres is essential for colonic drug delivery.

6.0 Development of a liposome in microsphere (LIM) formulation

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ABSTRACT

A liposome in microsphere (LIM) formulation has been created. Eudragit S100 microspheres were successfully used to encapsulate liposomes containing the model drug 5-ASA. The liposomes were coated with the natural polysaccharide chitosan, to protect the vesicles during the production stages of the LIM formulation. Characterisation of the LIMs was completed by laser diffraction particle sizing, cryo-SEM and drug release studies. Drug release studies were carried out in three media simulating the stomach, small intestine and large intestine. The simulated small intestinal fluid contained a model bile salt and the simulated large intestinal fluid contained a model enzyme produced within the human colon. The drug release trials showed that LIMs prevented drug release within the simulated stomach and small intestine conditions with subsequent drug release occurring in the large intestine conditions. The microspheres were solubilised by the pH 7.4 PBS which then exposed the chitosan-coated liposomes. The chitosan coating was then solubilised by the presence of the colonic enzyme β-glucosidase therefore releasing the LUVs and allowing drug release. It was concluded that Eudragit S100 microspheres encapsulating chitosan-coated LUVs was a successful formulation and has the potential for targeted colonic drug delivery.

6.1. Introduction

It has been previously shown that Eudragit S100 coated liposomes did not provide suitable protection to prevent bile salt ingress and subsequent exposure to *in vitro* small intestine conditions (chapter 3). It was then shown that solid Eudragit S100 microspheres produced using a double emulsion-solvent evaporation technique were stable through both the stomach and small intestine simulated conditions, with solubilisation taking place in conditions representative of the ileo-caecal region (chapter 4). It was then shown that liposomes could be coated with the natural polysaccharide chitosan, to protect the lipid vesicles during microsphere production (chapter 5). The next stage was to encapsulate chitosan-coated LUVs into the Eudragit S100 microspheres and therefore produce a formulation controlled by two separate factors within the GI tract. The dosage form would consist of the pH controlled Eudragit S100 microspheres followed by the enzyme dependant chitosan coating. The use of a microflora-activated system is seen as the most promising current trigger as the increase in bacteria population and enzyme activity represent a non-continuous event which is independent of GI transit time and therefore eradicates any problems associated with patient transit time variability (Yang *et al*., 2002).

A number of LIM systems have been reported (Stenekes *et al*., 2001; Feng *et al*., 2004; Park *et al*., 2006), all of which comprise encapsulated liposomes within microspheres produced from biodegradable polymers. With this in mind each of these formulations are susceptible to variations in patient transit times and therefore specific targeted release of the active ingredient could not be guaranteed. The use of a pH responsive polymer (i.e. Eudragit S100) as the microsphere material not only provides a suitable enteric coating that will protect the liposomal formulation through the acidic conditions in the stomach but will also provide a pH trigger that will begin to solubilise within the ileo-caecal junction. This will then enable the chitosan-coated liposomes to continue to the colon where enzyme triggered drug release will occur.

Feng *et al*. (2004) encapsulated both chitosan-coated LUVs and MLVs into PLA/PLA-PEG-PLA microspheres. The initial release of liposomes from PLA microspheres was shown to be significantly slower than GI transit times (50% release in 30 days) but the inclusion of PEG into the polymer matrix increased the liposome release rate showing approximately a 20% increase in the same time-frame. This release rate would not be suitable for the targeting

within the GI tract due to the mis-match with transit times but changes in polymer molecular weight and PEG concentration could further increase the rate at which the liposomes are released. The size of encapsulated liposome was also shown to significantly influence the release rate from the microspheres with the use of LUVs (<200 nm) showing the highest percentage release in comparison to larger liposomes. It is hypothesised the use of chitosan-coated LUVs in a pH responsive microsphere should produce a more specifically targeted system for the use in colonic drug delivery.

The main aims of the work described in this chapter were to:

- Successfully encapsulate chitosan-coated LUVs into Eudragit S100 microspheres and confirm their presence through a number of characterisation techniques.
- Perform a degradation study observing the LIMs and their response in different simulated GI tract conditions.
- Perform a drug release investigation into the ability of the LIM system to remain intact through simulated stomach and small intestine buffer solutions, and subsequently release the active ingredient (5-ASA) in simulated colonic conditions.

6.2. Materials

6.2.1. Lipids

Neutral liposomes were produced using a 7:2 molar ratio of EPC:CH. EPC was a gift from Lipoid (Ludwigshafen, Germany) and CH was purchased from Sigma Aldrich (Dorset, UK).

6.2.2. Polymers

Chitosan-coated liposomes were produced using medium molecular weight chitosan (Sigma Aldrich) as investigated in chapter 2 (approximate molecular weight 236,000). Microspheres used to encapsulate liposomes were created using Eudragit S100 (Evonik Industries, Essen) as investigated in chapter 2 and used for the production of blank microspheres in chapter 4.

6.3. Methods

6.3.1. Production of chitosan-coated LUVs

MLVs were initially produced using the Bangham *et al*. (1965) thin film hydration method. A detailed method for liposomal production can be found in chapter 3. Briefly, the lipid components were dissolved in 5ml chloroform and then evaporated to leave a thin film on the bottom of a round bottomed flask. The film was then hydrated with a 1mg/ml 5-ASA in PBS solution and vortex mixed to create MLVs. The subsequent MLVs were then membrane extruded through to a pore size of 0.2 µm as investigated in section 5.4.1. The subsequent LUVs were then washed three times through centrifugation at 26,000 rpm (63,000 g) for 10 minutes with the supernatant being replaced with fresh buffer. The LUVs were then coated with chitosan by adding LUVs dropwise to a 1% chitosan solution whilst under magnetic stirring as outlined in section 5.3.3.

6.3.2. Production of LIMs

LIMs were produced by using 5-ASA loaded chitosan-coated LUVs in the internal aqueous phase (IAP) during microsphere production. 0.8 ml of chitosan-coated LUVs were vortex mixed with 0.2 ml 3% (w/w) polysorbate 20 solution to form the internal aqueous phase (IAP). The primary emulsion (W_1/O) was then formed by homogenising (7,400 rpm, 2 minutes) the IAP with 5 ml 6% w/w Eudragit S100 dissolved in a solvent mixture of DCM:ethanol:propanol (5:6:4). The primary emulsion was then poured into 100 ml 1% PVA whilst under magnetic stirring at 125 rpm. Stirring was continued for three hours to ensure complete microsphere hardening and solvent evaporation. The microspheres were then washed using vacuum filtration with a filter membrane with a pore size of 1.6 µm. The microspheres were then subsequently harvested and primarily used in their ‘wet’ state for release trials.

6.3.3. Characterisation of LIMs

6.3.3.1. Size distribution analysis

Particle size distribution was measured using the method outlined in section 3.3.3.4. Samples were measured in their ‘wet’ state immediately after filtration as this was the state in which the LIMs would be used for drug release trials. The samples were added to the dispersion unit

until the obscuration level was in the range of 14-18% (Mastersizer 2000 handbook, Malvern Instruments). The sample was dispersed in pH 7.0 distilled water.

6.3.3.2. SEM imaging of microsphere morphology

The surface morphology of dried LIMs could be analysed using SEM. It not only gave an indication of the size of the LIMs but also supplies information regarding the homogeneity of the sample and if there is any excess polymer or unformed microspheres. LIMs were allowed to dry out in an oven at 37°C for 48 hours and then subsequently mounted, coated and imaged according to the method used for blank microspheres outlined in section 4.3.2.

6.3.3.3. Cryo-SEM

Cryo-SEM imaging was undertaken to establish the internal structure of the microspheres and confirm the presence of encapsulated chitosan-coated liposomes. The microspheres were suspended in a highly viscous 5M sucrose solution to reduce mobility within the sample well and therefore ensure the sample remained suspended for the duration of the sectioning. Sectioning and imaging was then completed as previously described in section 4.3.5.

6.3.3.4. Degradation study of LIMs in GI tract simulated conditions

An SEM study was designed to observe the effect of varying conditions on the morphology and structure of LIMs. 10 mg of LIMs were placed in 20 ml (concentrations representative of drug release trials) of each of the release media representing the three sections of the GI tract (outlined in section 6.3.4.2) including both the model bile salt state and colonic enzyme state. The vessels were then maintained at 37˚C and agitated at 100 rpm in a New Brunswick G25 incubator-shaker. 1 ml samples were removed at pre determined time intervals: stomach (0.5, 1, and 2 hours), small intestine (1, 2, and 3 hours) and large intestine (1, 2, 4, 6, and 40 hours). In each case, the maximum time in each buffer system was representative of average normal transit time. 1 ml of fresh pre-heated buffer was added after each sample was taken to maintain sink conditions throughout. The samples were centrifuged at 26,000 (63,000 g) for 10 minutes, with the supernatant being removed. The remaining microspheres could then be completely dried in an oven at 37°C for 48 hours, ready for subsequent analysis as described in section 4.3.2.

6.3.4. Investigation into drug loading and release profiles for LIMs

6.3.4.1. Establishing drug loading of LIMs

The specific drug loading of the LIMs was investigated by 'stripping' off each layer (as would be experienced during drug release trials) protecting the liposomes, followed by lysing the liposomes in solvent. Initially 10 mg of LIMs were placed in 2 ml of the solvent mixture (DCM: ethanol: propanol) used in microsphere production. The solution was vortexed for 5 minutes to ensure that the Eudragit S100 microspheres had been completely dissolved. The solution was then centrifuged at 26,000 rpm (63,000g) for 10 minutes to form a pellet of chitosan-coated LUVs at the bottom of the Eppendorf tube. The supernatant was then removed, with 2 ml of 1% acetic acid being added to the liposomes to solubilise the chitosan coating. 5 minutes of further vortex mixing ensured the chitosan coating was solubilised and therefore exposing the LUVs. The LUVs were then pelleted through centrifugation (26,000 rpm, 10 minutes). The liposomes were then lysed through the addition of 2 ml of ethanol, releasing the encapsulated 5-ASA. The resultant solution was then measured spectrophotometrically at a wavelength of 330 nm and related to the calibration curve in Figure 5.2D to calculate the total 5-ASA encapsulated in the LIMs. This technique would also indicate whether the liposomes were intact in the microspheres as each supernatant was analysed when 'stripping off' the layers. The levels of drug within the supernatant would indicate whether the liposomes were intact within the microspheres or whether considerable leaking had taken place during production.

6.3.4.2. *In vitro* conditions to simulate GI tract pH, bile salt and enzyme activity

The *in vitro* conditions were the same as those described in section 5.3.4.3. 0.1 M HCl was used to simulate the stomach condition. pH 6.3 Hanks' buffer containing 10 mM sodium taurocholate was used to simulate the small intestine and finally pH 7.4 PBS containing 4% w/w β-glucosidase was used for large intestine simulation.

6.3.4.2. Drug release profiles for LIMs in simulated GI tract conditions

In order to accommodate the large particle size of the LIMs, it was not possible to use the 20,000 MWCO dialysis cassettes used in chapter 5 as they were too large to be injected into the syringe ports at each corner of the cassette. A similar drug release method was adopted by

using 14,000 MWCO dialysis membrane (BioDesign, New York) that would allow suitable LIM insertion due to the wider end opening. 50 mg of LIMs were placed inside the hydrated dialysis membrane and sealed. The membranes were then placed in 100 ml of release media in a 250 ml conical flask. The flasks were then placed in a New Brunswick G25 incubator maintained at 37˚C. The flasks were agitated at 100 rpm with 1 ml samples being removed at pre-determined time intervals. 1 ml of fresh pre-heated buffer was then added to the flask to maintain sink conditions throughout. The removed sample was analysed spectrophotometrically at a wavelength of 330 nm and related back to the calibration curves in Figure 5.2 to determine the amount of 5-ASA that has been released. The release curve was calculated by taking into account the drug that had been released at each time point and the effect of the dilution through the addition of the fresh buffer.

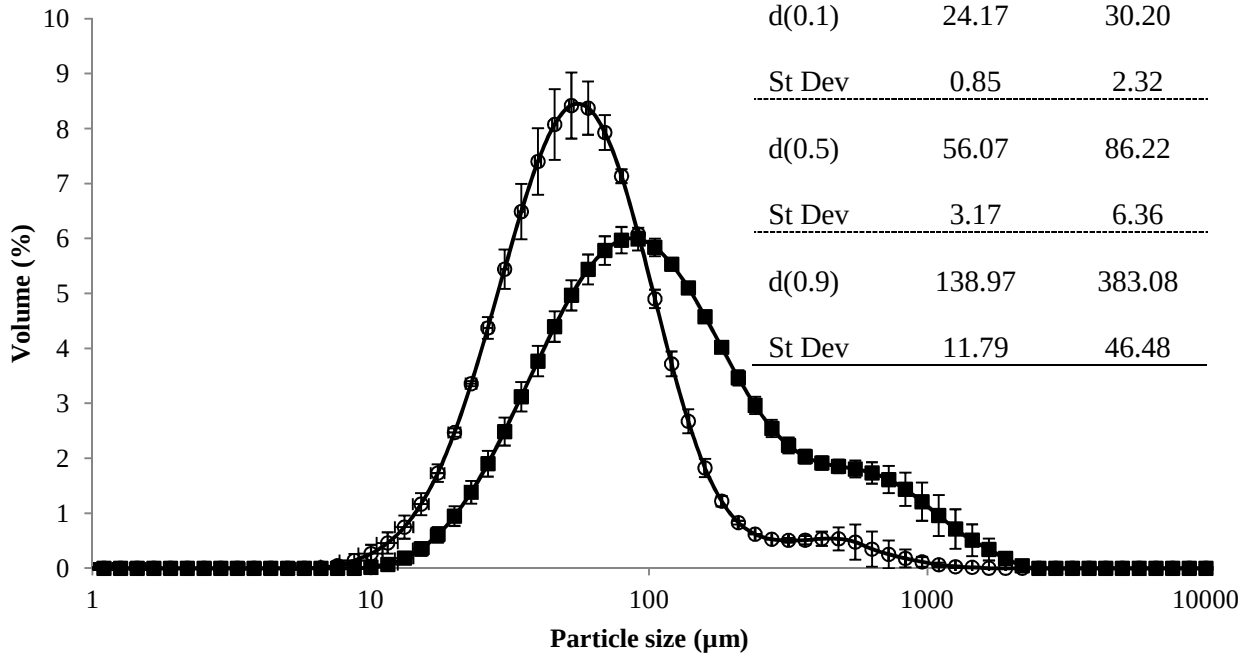
6.4. Results and Discussion

6.4.1. LIM characterisation

6.4.1.1. Size distribution analysis

The average volumetric mean size of the LIMs was found to be 86 µm (± 6.36 µm) which is approximately 30 µm larger than the blank Eudragit S100 microspheres (Figure 6.1). Despite the apparent large size difference there are a number of similarities between the two size distributions. Both distributions have a similar shape, with the LIMs showing a broader distribution in comparison to the narrow peak observed for the blank microspheres. A small 'shoulder' can be seen for both formulations towards the larger particle size, which may be an indication of agglomeration or coalescence of the IAP. The 'shoulder' is more pronounced for the LIMs which may be an indication of the increased electrostatic and hydrophobic forces present when the liposome/chitosan complexes come into contact with the solvent mixture. In comparison Feng *et al*. (2004) showed very little change between blank PLA microspheres and LIMs with the general particle size being 60-70 µm for both formulations. The difference observed between the current findings and those of Feng *et al*. (2004) can be attributed to a number of factors most importantly the materials used. Feng *et al*. (2004) used ethyl acetate for the organic solvent and therefore could produce a more stable W_1/O and subsequently maintain that stability when the chitosan-coated liposomes were introduced. The use of a three solvent system may also have a considerable impact when chitosan-coated

liposomes are introduced due to the specific miscibility of the solvent mix in comparison to the single solvent used by Feng *et al*. (2004).



	Blank microspheres	LIMs
d(0.1)	24.17	30.20
St Dev	0.85	2.32
d(0.5)	56.07	86.22
St Dev	3.17	6.36
d(0.9)	138.97	383.08
St Dev	11.79	46.48

Figure 6.1. Size distribution comparison between blank Eudragit S100 microspheres (○) and drug loaded LIMs (■). Each point is the mean of three independent measurements ± the standard deviation. Particle size statistics (d_{10}, d_{50} and d_{90}) are displayed with their standard deviations.

6.4.1.2. SEM imaging of LIMs

SEM imaging

SEM images of air dried LIMs are shown in Figure 6.2. In comparison to the blank Eudragit S100 microspheres (Figure 4.2 B) the LIMs are similar in sphericity and surface morphology having a smooth appearance with very few of the polymer strands visible that were seen for less successful formulations (higher polymer concentrations and lower homogenisation speeds). This indicates that although the size may have altered with the incorporation of chitosan-coated liposomes that the general emulsion formation and subsequent polymer hardening takes a similar form to that of the blank microspheres.

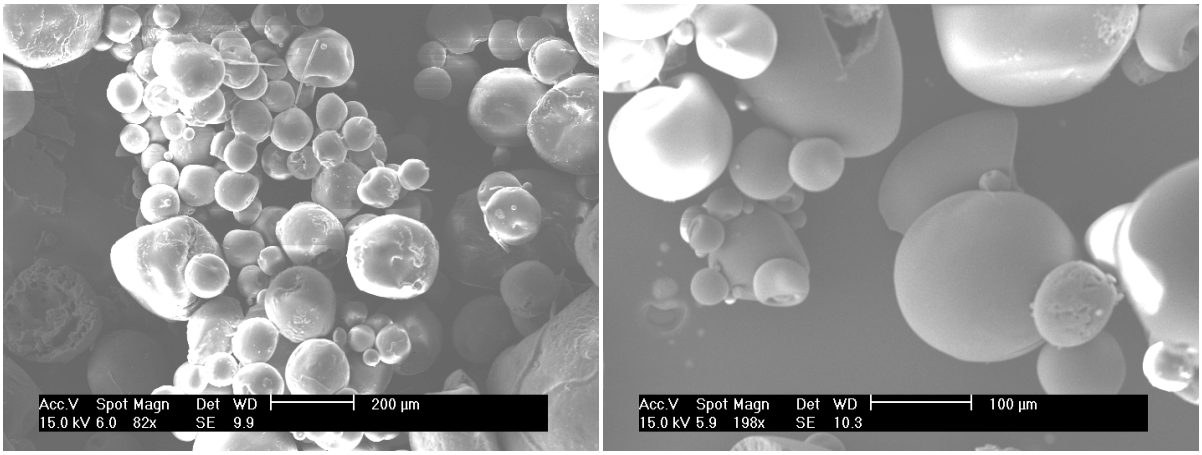
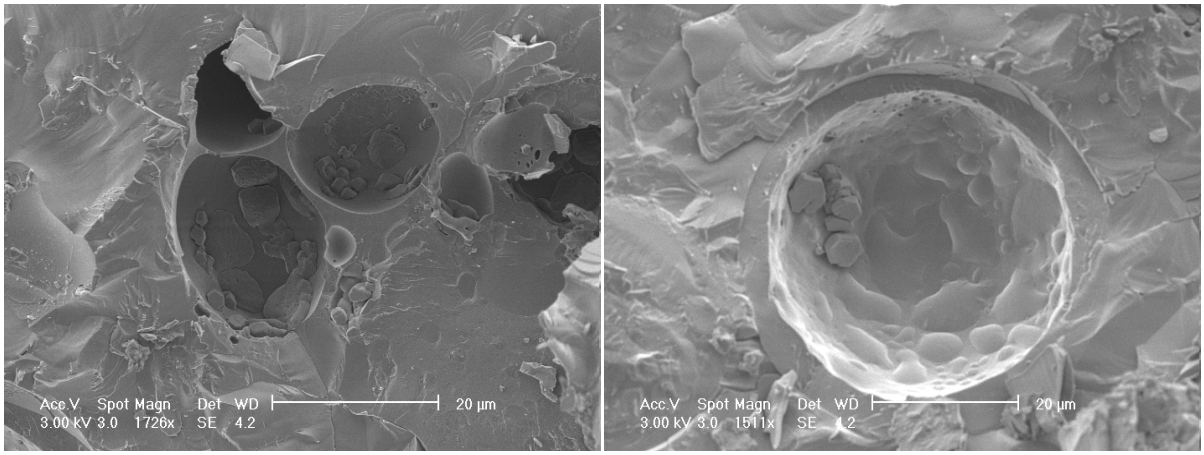


Figure 6.2. SEM images of air dried LIMs. 5-ASA loaded chitosan-coated LUVs were encapsulated within Eudragit S100 microspheres using a double emulsion-solvent evaporation technique.

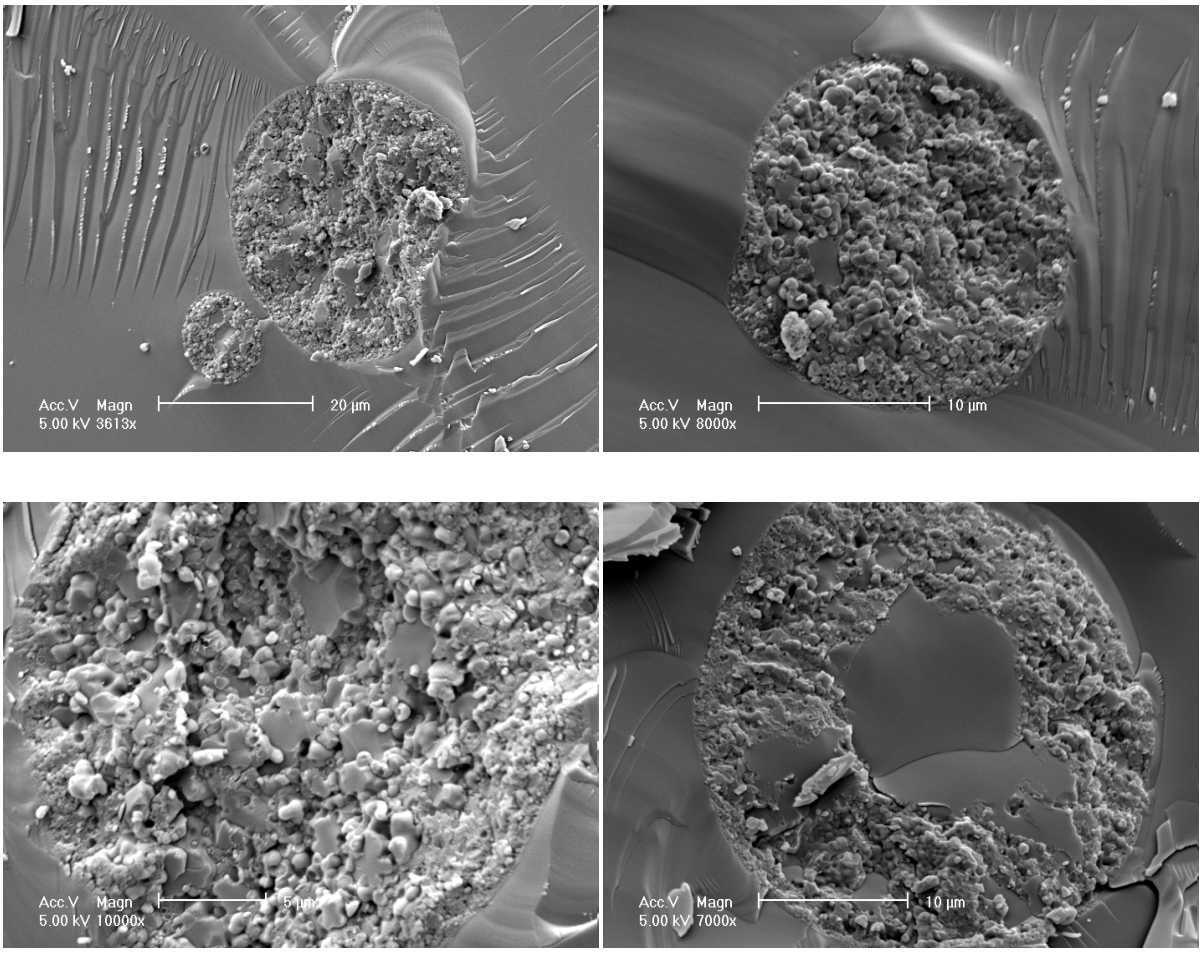
Cryo-SEM

A comparison of the microsphere internal structures for blank microspheres and LIMs show a stark difference (Figure 6.3). From the blank microspheres an empty shell can be seen, which would be expected due to the use of distilled water alone for the IAP. The blank Eudragit S100 microspheres shown in Figure 6.3A differ internally to the PLA microspheres produced by Feng *et al*. (2004) in that the internal structure of the PLA microspheres comprised a porous polymer matrix throughout, whereas the Eudragit S100 microspheres show a definite shell with an empty core. The observed shell thickness appears to be variable between microspheres, with only a thin shell seen in the first image whilst the second shows a shell wall with a thickness of over 2 microns in places.

In contrast, the LIMs show a solid internal structure with the presence of chitosan-coated liposomes throughout. Some larger particles are also visible within the microspheres which are thought to be liposome/chitosan complexes which form when the chitosan-coated liposomes are exposed to the solvent mixture, therefore causing agglomeration. The images of LIMs are very similar to those observed by Feng *et al*. (2004) where individual liposomes could also be seen within the porous polymer matrix of PLA microspheres.



(A)

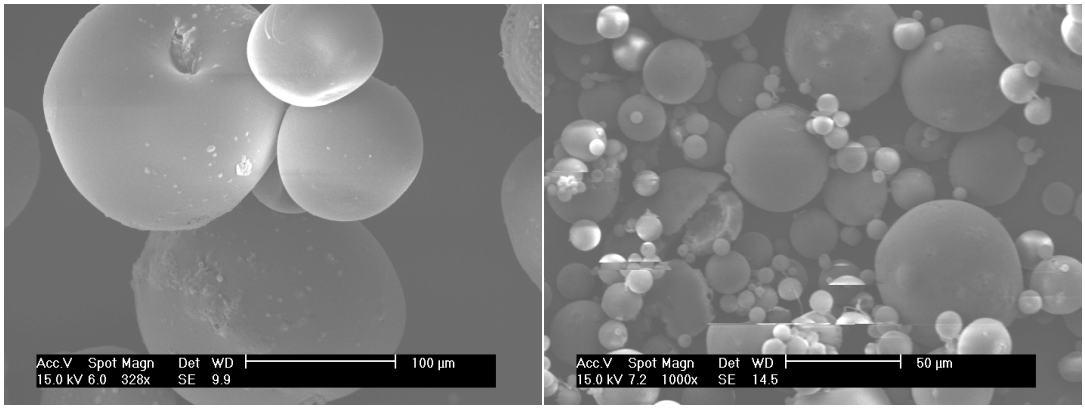


(B)

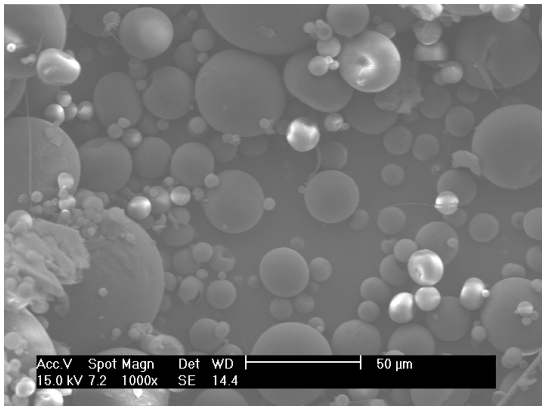
Figure 6.3. Cryo-SEM images comparing (A) blank Eudragit S100 microspheres and (B) chitosan-coated LIMs. The liposomes are visible in the internal structure of the LIMs compared to the blank interior of the microspheres.

6.4.1.3. Degradation of LIMs in simulated GI tract conditions

It has been previously shown that blank Eudragit S100 microspheres can maintain their stability when subjected to the *in vitro* conditions of the stomach and small intestine (section 4.4.5.). It is therefore essential that the incorporation of chitosan-coated liposomes does not influence the stability of the microspheres and subsequently protect the liposomal contents through to the proposed site of microsphere solubilisation at the ileo-caecal junction. SEM images of LIMs subjected to *in vitro* conditions representative of the stomach are shown in Figure 6.4. The images show exposure up to 2 hours which is the widely regarded average transit time within the stomach (Wilding, 2001). From the three time points it can be seen that the LIMs maintain their stability and have a consistent surface morphology throughout. The stability of the LIMs during exposure to the acidic environment of the stomach is essential as the chitosan coating is soluble in acidic conditions and therefore any exposure to the HCl would lead to drug release as seen in section 5.4.3.



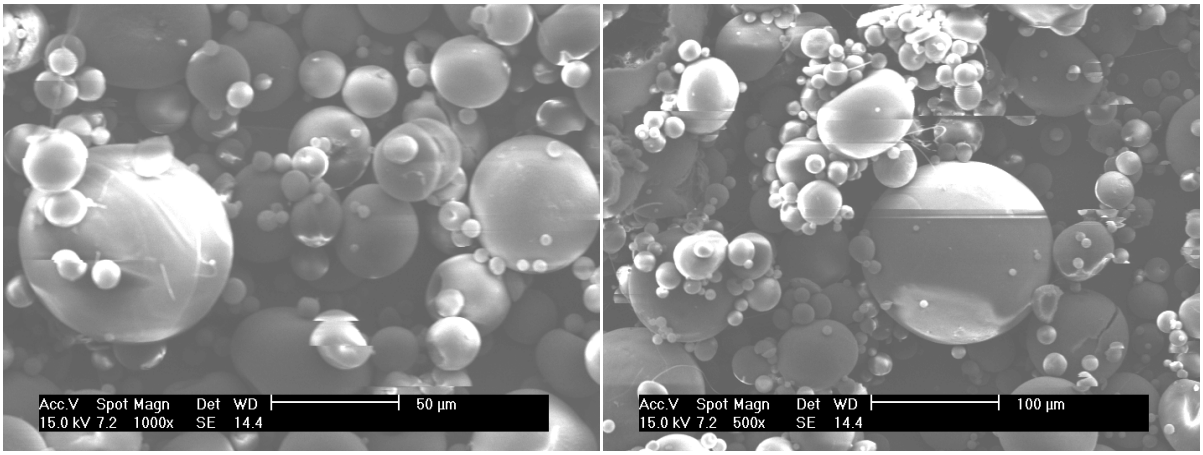
(A) (B)



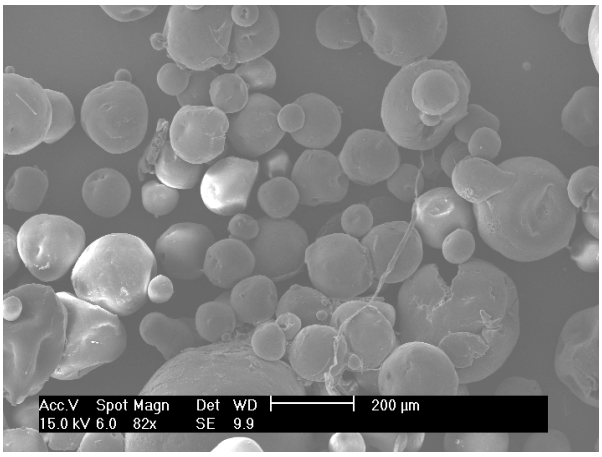
(C)

Figure 6.4. SEM images showing LIMs suspended in 0.1 M HCl for (A) 0.5, (B) 1 and (C) 2 hours. Samples show very little change in surface morphology through the time periods.

SEM images for LIMs exposed to pH 6.3 Hanks' buffer containing sodium taurocholate are shown in Figure 6.5. The exposure is up to 3 hours which is widely accepted as the average transit time observed in the small intestine (Wilding, 2001). Once again the LIMs appear to maintain their stability as observed for blank Eudragit S100 microspheres which would indicate the protection of the chitosan-coated liposomes and therefore drug retention through both the stomach and small intestine.



(A) (B)



(C)

Figure 6.5. SEM images showing LIMs suspended in pH 6.3 Hanks' buffer containing sodium taurocholate (10 mM) for (A) 1, (B) 2 and (C) 3 hours.

Figure 6.6 shows SEM images from the LIM formulation suspended in large intestine conditions of pH 7.4 PBS containing β-glucosidase. Due to the solubility profile of Eudragit S100 it would be expected that the microspheres would solubilise within this media similar to that of the blank microspheres. The images show times up to 6 hours, a sample was taken after 8 hours but after centrifugation only a small amount of 'sticky' residue could be seen at the bottom of the eppendorf which was thought to be lipid from the released liposomes. The solubilisation of the microspheres can be seen within one hour of exposure to the PBS, with pore formation being observed due to the mechanism described in chapter 4. The continued solubilisation of the microspheres can be seen after 2 hours with the microsphere shape still being identifiable but the internal area is becoming greater and therefore providing a larger contact area for further solubilisation. The images after 1 and 2 hours show a number of small particles exposed at the surface where solubilisation is occurring. These vesicles are assumed to be chitosan-coated liposomes that are being released from the internal aqueous core of the microsphere upon solubilisation of the solid Eudragit S100 shell. Each stage of solubilisation can be seen through the different time points with very little spherical form being observed after 4 hours. At 6 hours only remnants of microspheres can be seen with no distinctive spherical shape remaining or smooth surface morphology that was observed initially in Figure 6.2.

In comparison to the blank Eudragit S100 microspheres the LIMs remain intact for a longer period of time, with blank microspheres completely degrading after 6 hours while LIMs degraded after 8. It is thought that the stability of the internal aqueous phase of chitosan-coated liposomes offers increased stability to the microspheres upon solubilisation in comparison to the aqueous core of distilled water in the blank microspheres.

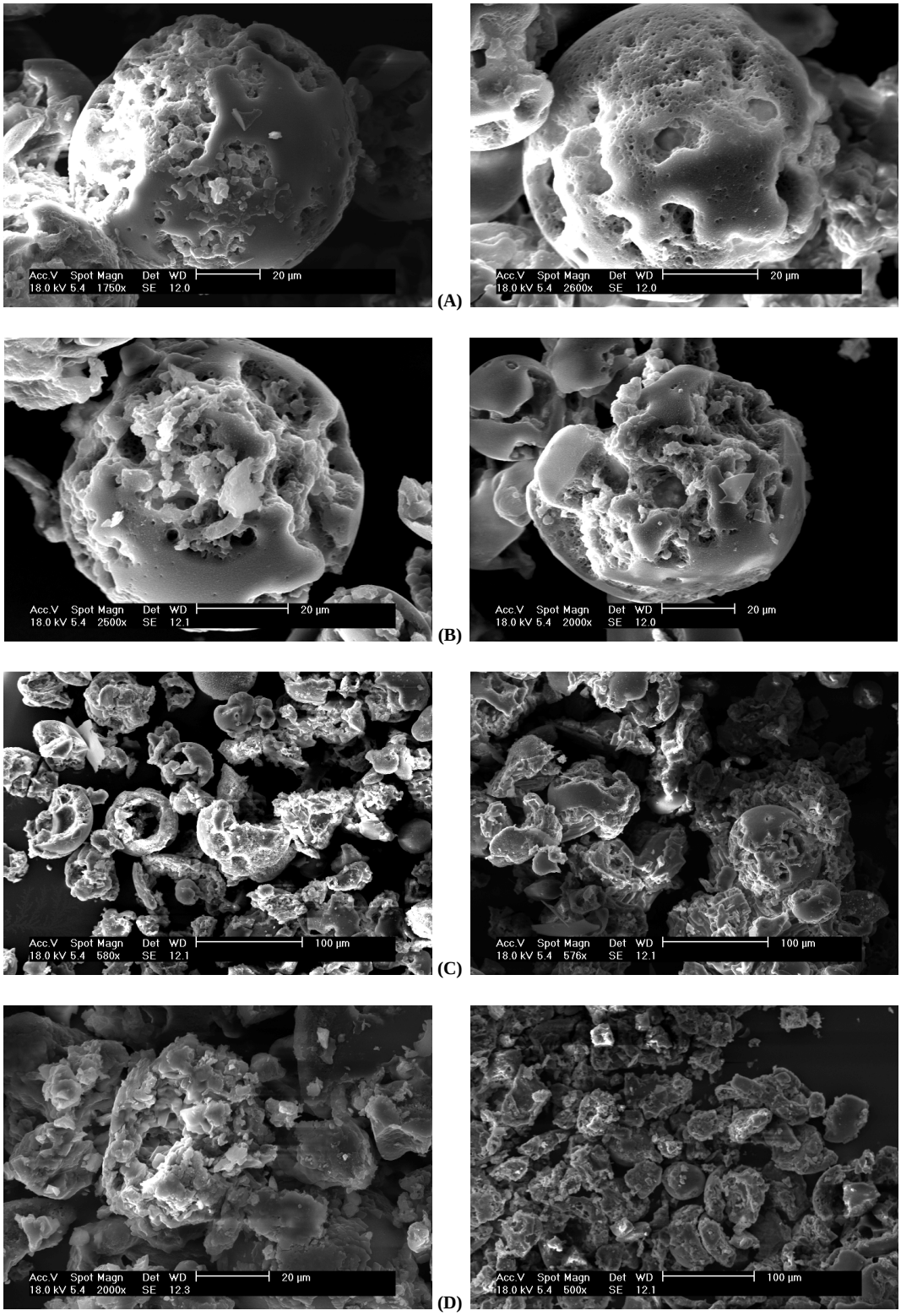


Figure 6.6. SEM images showing LIMs suspended in pH 7.4 PBS containing β-glucosidase (4% w/w) for (A) 1, (B) 2, (C) 4, and (D) 6 hours. Significant microsphere solubilisation can be observed from the first time point with further solubilisation taking place throughout the experiment.

6.4.2. Drug release profiles for LIMs in simulated GI tract conditions

When establishing the drug loading of the LIMs it is to be noted that when each individual layer was sequentially 'stripped off' by exposing each to an appropriate solvent, the supernatant was analysed for drug. Very little drug was found in the microsphere layer (<2%) and the vast majority was observed in the internalised liposomes. The drug release profiles for both chitosan-coated LUVs and LIMs are shown in Figure 6.7. The drug release profile for chitosan-coated LUVs differs considerably compared to that investigated using the dialysis cassettes in chapter 5. For overall drug release across all three media it was shown that there is a statistical difference between chitosan coated LUVs and LIMs (Mann-Whitney U test (chosen level of significance α=0.02). Drug release within the dialysis membrane is thought to be much quicker due to a number of factors including the increased surface area in comparison to the dialysis cassettes and the presence of the release media within the membrane therefore facilitating drug release without the need to pass through the membrane pores. Drug release from chitosan-coated LUVs can be observed in all three release media similar to that in chapter 5 with the 2 hours in HCl attributing to the solubilisation of the chitosan coating and therefore allowing the sustained drug release associated with liposomal formulations to continue throughout. The mechanisms involved in chitosan solubilisation have been discussed in chapter 2, therefore indicating the necessity of the Eudragit S100 micropshere to protect the chitosan coating and therefore prevent any drug release until the target site of the colon.

The drug release profile for the LIMs shows high levels of drug retention in both the stomach and small intestine (0-5 hours). This is due to the Eudragit S100 microsphere staying intact which has been previously shown in Figures 6.4 and 6.5. The stability of Eudragit S100 in release media less than pH 7 reflects that the microspheres maintain their stability until they reach the distal small intestine where the pH rises above pH 7. The nature of the release observed for LIMs in the PBS reflects that observed through SEM where after only 2 hours large channels had formed which would lead to extensive liposome release and therefore subsequent drug release. Furthermore, after 6 hours it was shown that very little of the microsphere structure remained, therefore indicating that the liposomes would be completely released and susceptible to solubilisation through the colonic enzyme. The release mechanism for LIMs is defined through both the diffusion of liposomes and the dissolution of the polymer matrix (Feng *et al*., 2004), therefore in the case of Eudragit S100 microspheres the

speed at which the polymer dissolves would therefore allow for the rapid diffusion of the liposomes into the bulk media. When producing LIMs using biodegradable polymers the formation of water channels to allow the liposomes to diffuse out has been seen as the rate limiting step, but once again due to the nature of Eudragit S100 the polymer dissolves indicates that liposomes can diffuse out much more rapidly.

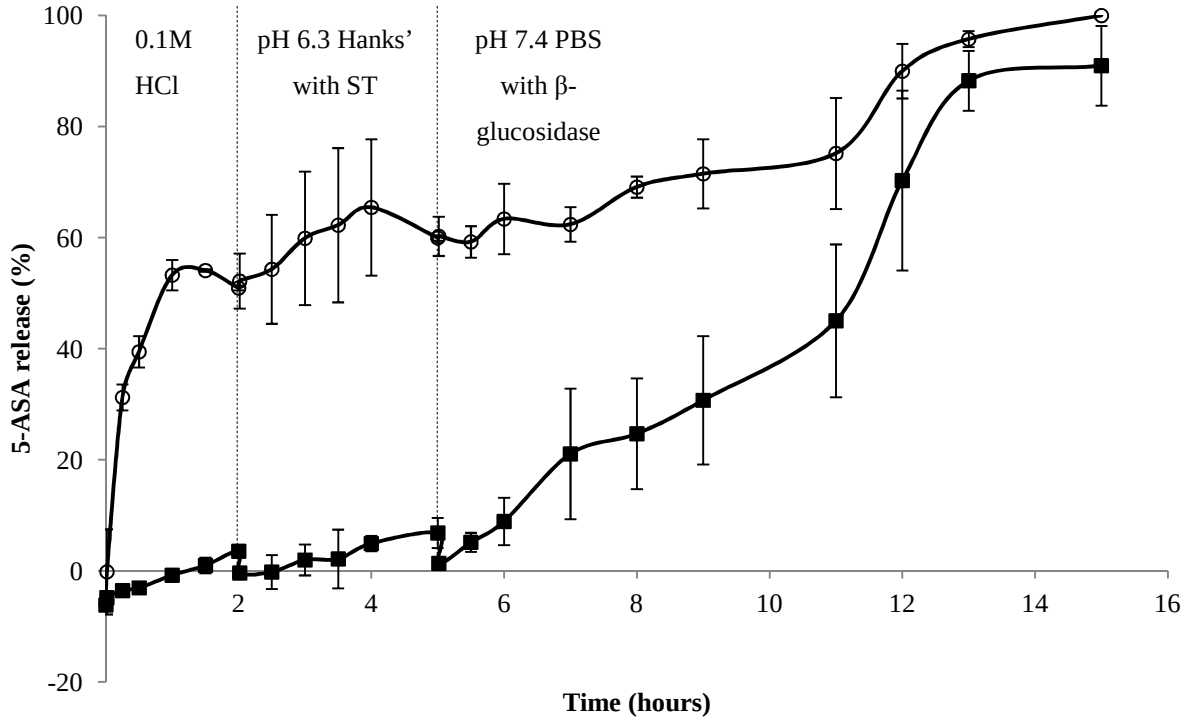


Figure 6.7. Drug release profiles comparing chitosan-coated LUVs (○) and LIMs (■). Each point represents the mean of three independent measurements ± the standard deviation. Release profiles were conducted in HCl for 2 hours, Hanks' buffer with sodium taurocholate for 3 hours and finally PBS with β-glucosidase for 10 hours.

Due to the novel nature of LIM formulations there are very few similar studies to compare the drug release profiles to, especially studies that use pH responsive polymers for the microsphere production. Feng *et al*. (2004) used PLA and PLA-PEG-PLA block copolymer and therefore the biodegradation rate is much slower than that observed for the pH responsive Eudragit S100. To further support the 'water channel' theory of liposomal diffusion Feng *et al*. (2004) showed that LUVs of a smaller size showed increased release rates compared to larger LUVs due to the ability of the smaller liposomes to diffuse through the

channels of the microspheres. Furthermore, it took 25 days to see any notable liposome release when MLVs were encapsulated within the microspheres, which is due to the time taken for suitably sized water channels to form. Increasing the percentage of PEG also led to an increased rate of liposomal release which provides a similar effect to the pores developing as the Eudragit was solubilised in the PBS as shown in Figure 6.6. The specific drug release of PLA-PEG-PLA is not described in Feng *et al*.'s (2004) study but it can be presumed that as the liposomes are said to be released intact that they would undergo the normal sustained drug release profile associated with chitosan-coated liposomes.

A similar system to the PLA-PEG-PLA microspheres produced by Feng *et al*. (2004) is that of the liposome loaded dextran microspheres produced by Stenekes *et al*. (2000). These microspheres had a similar liposome release profile with it taking approximately 20 days to see any notable liposome release. In contrast to the two delayed liposomal release formulations Park *et al*. (2006) produced LIMs using alginate microspheres to protect the liposomes through the stomach conditions and therefore begin drug release at the small intestine. The drug release showed an initial burst release within the first 15 hours then a sustained release for the following 35 hours to a relative plateau.

6.5. Conclusions

Chitosan-coated LUVs were successfully incorporated into Eudragit S100 microspheres. Characterisation techniques including laser drug loading experiments, SEM and cryo-SEM all indicated the presence of liposomes within the microspheres. Through simulated solubilisation experiments it was shown that the LIMs remained intact through both simulated stomach and small intestine conditions with subsequent microsphere solubilisation taking place in ileo-caecal junction conditions, with chitosan solubilisation taking place in the simulated colonic conditions containing β-glucosidase. Drug release studies were undertaken to investigate the LIMs for the application of colonic drug delivery. Drug retention levels in the simulated stomach and small intestine were very high (approximately 95%), with liposome and subsequent drug release occurring in pH 7.4 PBS containing the colonic enzyme β-glucosidase. Drug release was completed within 10 hours of exposure to the PBS and enzyme which would therefore indicate that the current formulation of LIMs has the potential as a colonic targeted drug delivery system.

7.0 Concluding remarks and suggestions for further work

Liposomal formulations for targeted drug delivery to the colon have been investigated. The techniques used in targeted delivery were researched with a view to producing a novel liposomal formulation capable of delivery to the colon. Various polymer systems were analysed in relation to the complexities of the GI tract. It was decided that the current studies would utilise the pH responsive polymer Eudragit S100 and the enzyme triggered chitosan throughout. Both polymers were introduced through their specific chemical structure, molecular weight, FTIR trace and SEM images. The solubilisation mechanism for each polymer was described with Eudragit S100 solubilising due to the ionisation of carboxylic acid side groups as the pH is increased and chitosan by the breaking of the 1-4 glycosidic bonds by the enzymes present in colonic microflora.

Initial trials producing Eudragit S100-coated liposomes proved promising with improved drug retention being observed in simulated stomach and small intestine conditions. The use of more complex simulated conditions proved unsuccessful at preventing bile salt ingress and subsequent bilayer solubilisation despite the obvious precipitation of the anionic polymer on the surface of the cationic liposomes. Investigations into the production of solid Eudragit S100 microspheres produced formulations capable of withstanding bile salts and showed suitable rates of solubilisation in the simulated ileo-caecal region of the GI tract to allow for colonic drug delivery.

Chitosan-coated liposomes were investigated to enable their encapsulation into the Eudragit S100 microspheres and protect the liposomes during exposure to the solvent mixture required during microsphere production. It was shown that both neutral and negatively charged liposomes could be coated with chitosan which would offer an enzyme controlled component to the system only found in the microflora of the colon.

The use of chitosan-coated liposomes within a Eudragit S100 microsphere offers the stability to navigate the stomach and small intestine for drug release to occur within the large intestine. Characterisation techniques have confirmed the presence of chitosan-coated liposomes within the microsphere core. Drug release trials have shown the relative stability of

the LIMs through the simulated stomach and small intestine with release being observed in the large intestine where the greater enzyme population is present.

Based on the results from the current investigation, the following areas of research are considered to have the most promise;

(1) To ensure a clinically relevant drug loading is possible and to ensure the optimum release is achieved. This may involve optimising both the liposomes and microspheres to ensure maximum drug encapsulation into the liposomes and subsequent maximum liposome encapsulation within the microspheres.
(2) Investigate a range of drugs that may be encapsulated into the liposomes whether it be in the aqueous core or the lipid bilayers.
(3) A long term stability study would be required to take the formulation further to ensure leakage does not occur over time. Further scope in this area would involve understanding the possibility of drying the formulation and subsequent rehydration and the effects this may have. This is an important factor as the effect of having the 'wet' liposome dispersion interacting with the hardened microsphere is not fully understood.
(4) As *in vitro* testing on the LIM formulation has been completed, continued development of the product would be completed through testing in cell cultures and then *in vivo* studies within animals.

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9.0 Appendix

Evaluation of liposomes coated with a pH responsive polymer

M. J. Barea [a*], M. J. Jenkins [b], M. H. Gaber [c], R.H. Bridson [a]

[a] Centre for Formulation Engineering, School of Chemical Engineering, University of Birmingham, Edgbaston, UK, B15 2TT, [b] School of Metallurgy and Materials, University of Birmingham, Edgbaston, UK, B15 2TT, [c] British University in Egypt, El-Sherouk City, Misr-Ismailia Road, Cairo, Egypt, 11837.

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

*Corresponding author. Tel.: + 44 (0) 121 4145082; fax: + 44 (0) 121 414 5324

Email address: mjb246@bham.ac.uk

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

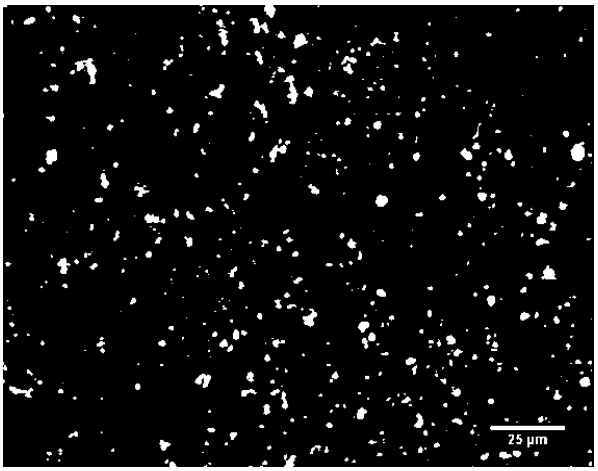
Figure 1. Light microscopy images showing liposomes: (A) without polymer, and in the presence of Eudragit S100 at (B) pH 7.8 and (C) pH 6.3.

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

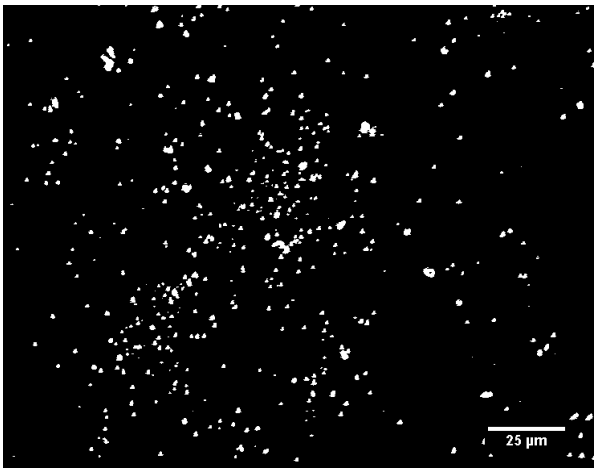
Figure 3. Drug release profiles for liposome formulations with (■) and without (◊) Eudragit S100 at (A) pH 1.4, (B) pH 6.3 and (C) pH 7.8. In Figure 4 (A) drug release over 2 hours is additionally highlighted, corresponding to the typical residence time in the stomach. Each data point represents the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



(C)

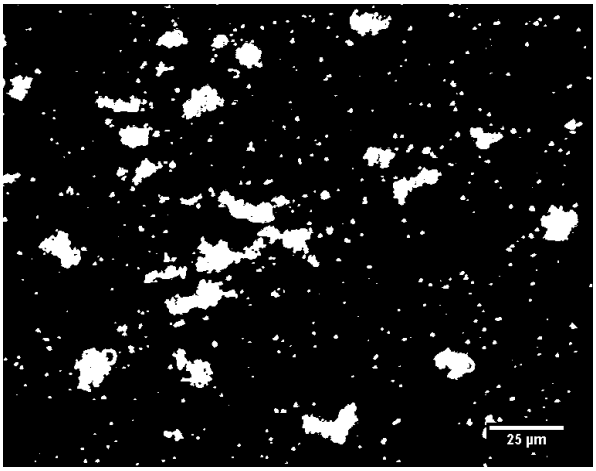
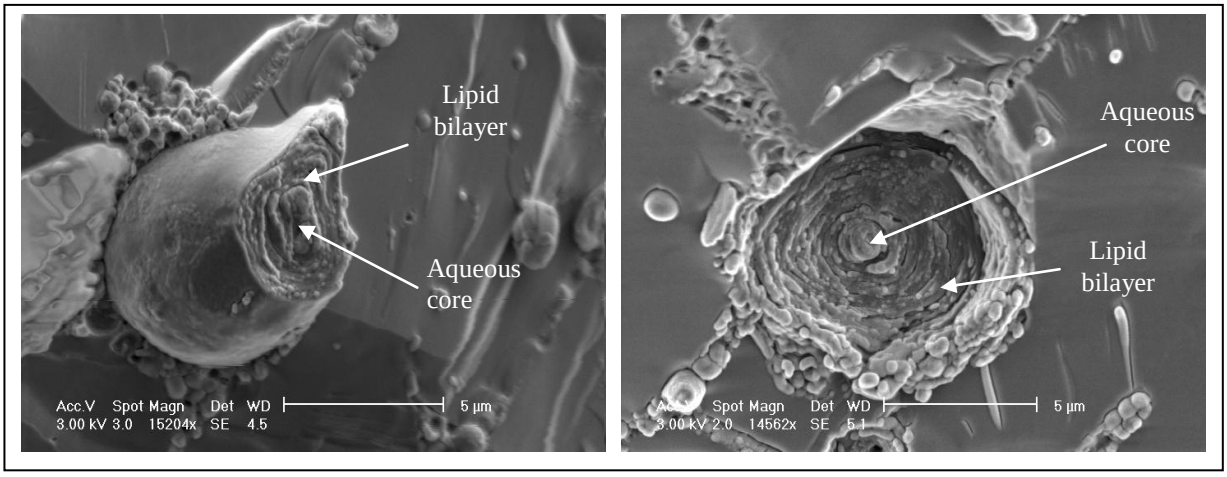


Figure 1.

(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)

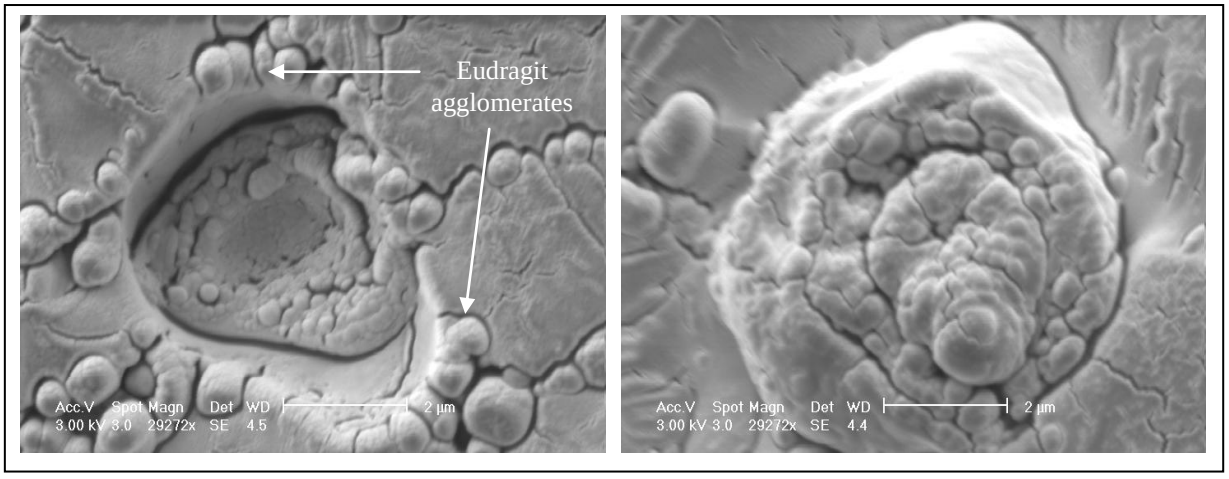

Eudragit agglomerates
Acc.V Spot Magn Det WD
3.00 kV 3.0 29272x SE 4.5
2 µm
Acc.V Spot Magn Det WD
3.00 kV 3.0 29272x SE 4.4
2 µm

Figure 2.

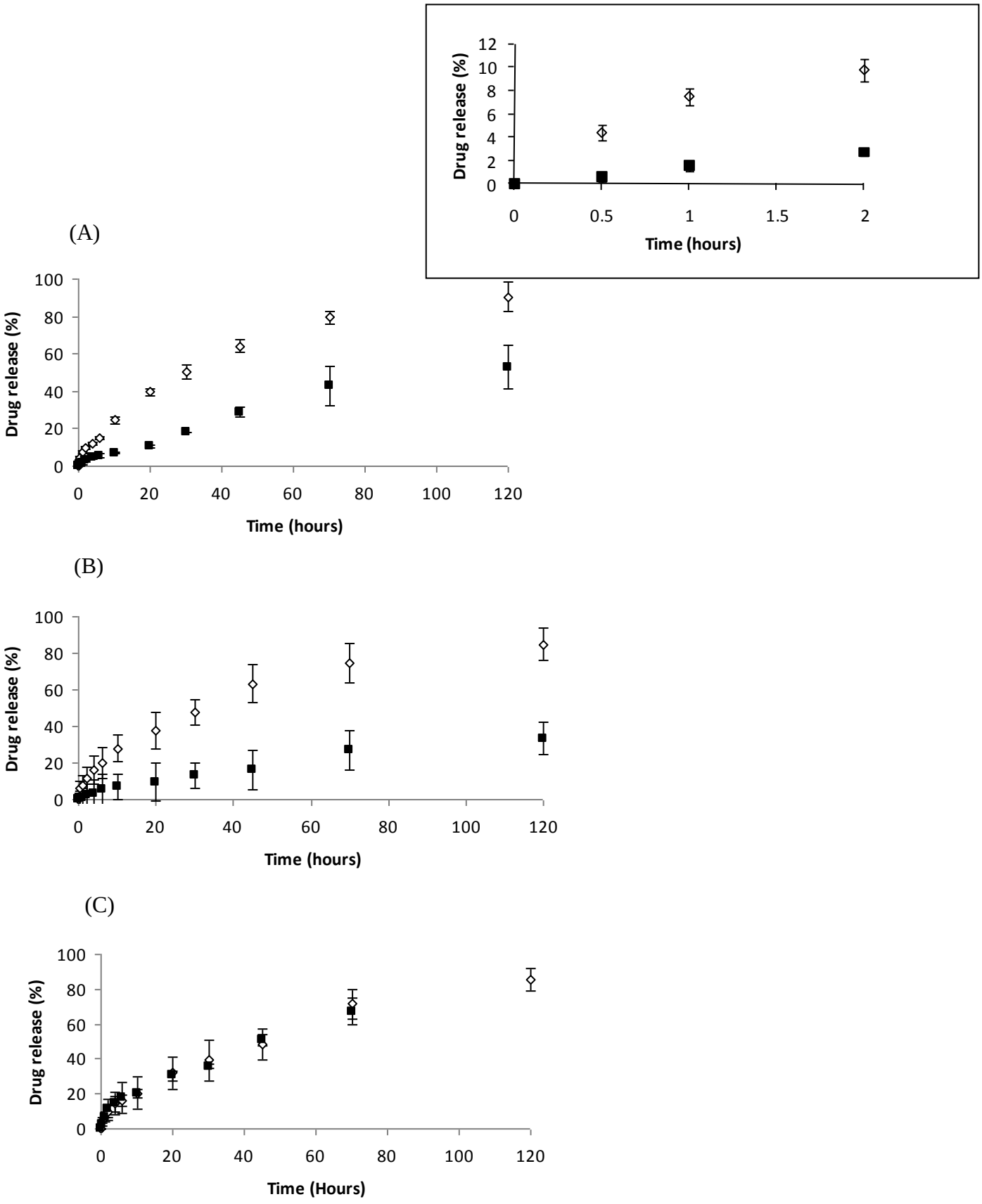

(A)
Drug release (%)
Time (hours)
(B)
Drug release (%)
Time (hours)
(C)
Drug release (%)
Time (Hours)

Figure 3.

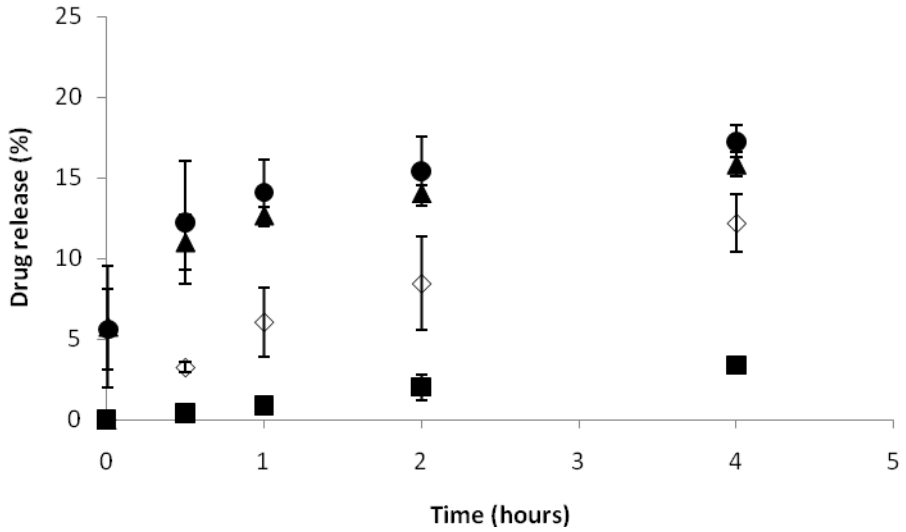


Figure 4.