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Developing oral formulations for protein and peptide drugs

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Plagiarism Statement

This thesis describes research conducted in the School of Pharmacy, University College London between 2008 and 2012 under the supervision of Dr. Simon Gaisford, Dr Satyanarayana Somavarapu and Professor Abdul Basit. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

_____ _____

Signature Date

Abstract

The vast majority of protein/peptide drugs are not available orally. Their oral delivery is hindered by intestinal instability and limited permeability. The aim of this project was to gain an understanding of the stability of a large protein, lactase, a small protein, insulin, and a newly discovered peptide throughout the intestinal tract. This was used for the rational design of their oral formulations.

Lactase was completely denatured at gastric pH within 10 minutes but was stable with intestinal enzymes. Encapsulation in enteric Eudragit L100 microparticles using a method previously used to encapsulate low molecular weight drugs produced small particles with a high yield and encapsulation efficiency, >90%. They restricted lactase release in acid but did not protect it from denaturation. Porosity and particle morphology investigations using an SEM with a new type of detector revealed surface structures which disappeared upon dispersal in acid and an inner porous structure which may allow acid entry and lactase denaturation. Co-encapsulation of an antacid preserved almost 10% of lactase activity in acid, superior to existing oral lactase supplements.

Insulin was not hydrolysed at gastric pH but was immediately and completely digested by gastrointestinal enzymes. To protect it from pepsin insulin was encapsulated in Eudragit L100 microparticles. The particles produced were <100µm with a yield and encapsulation efficiency of >70%. After incubation with pepsin they protected 80% of encapsulated insulin.

A small peptide, peptide 1 was gradually degraded in the intestinal fluids. To provide protection and increase its permeability peptide 1 was encapsulated in PLGA nanoparticles but 15% of the encapsulated peptide was immediately released *in vitro*. Encapsulation of the nanoparticles in Eudragit L100 microparticles successfully prevented any burst release in acid. This should minimise gastric digestion of peptide 1 and concentrate nanoparticle release in the small intestine providing a higher probability of permeation.

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List of abbreviations

AR	Androgen receptor
BNF	British National Formulary
BSA	Bovine serum albumin
CFLM	Confocal laser microscopy
DCM	Dichloromethane
DI	Deionised
EDTA	Ethylenediaminetetraacetic acid
ER	Estradiol receptor
GCSF	Granulocyte colony stimulating factor
GI	Gastrointestinal
GLP-1	Glucagon like peptide 1
GnRH	Gonadotrophin releasing hormone
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HPMCP	hydroxypropylmethylcellulose phthalate
K	Degradation rate constant
KDa	Kilodalton
LHRH	Luteinising hormone releasing hormone
LogP	Partition coefficient
NaOH	Sodium hydroxide
NDA	New drug application
ONP	o-nitrophenol
ONPG	o-nitrophenyl-β-D-galactopyranoside

o/o	Oil in oil emulsion
PA	Pharmacological availability
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PLA	Poly (lactic acid)
PLGA	Poly (lactic-co-glycolic) acid
PVA	Polyvinyl alcohol
SC	Subcutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEDDS	Self emulsifying drug delivery systems
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
Src	Proto-oncogene tyrosine-protein kinase
T max	Time of maximum plasma concentration
TRH	Thyrotropin releasing hormone
w/o/w	Water in oil in water double emulsion
ZOT	Zonula occludens toxin

Chapter 1

Introduction

1.1 Protein and peptide drugs

Protein and peptides are biological molecules consisting of amino acids. Typically peptides consist of 50 amino acids or less and proteins have more than 50 amino acids. The primary structure of proteins is the linear sequence of amino acids, held together by peptide bonds, and any disulphide bonds between them. Secondary structures are regular local substructures of either alpha helix or beta sheets held together by hydrogen bonds. Tertiary structure is the three dimensional structure of the folded protein and is held together by hydrogen bonds, hydrophobic interactions, ionic interactions, van der Waals forces and disulphide bonds. The three dimensional structure of a multisubunit protein, held together by non-covalent interactions and disulphide bonds, is defined as quaternary structure. Proteins and peptides are used as pharmaceuticals due to their specific and vital therapeutic activities.

A review of the British National Formulary (BNF) reveals numerous protein and peptide drugs are currently available for a wide variety of therapeutic applications. Most of these fall into the following categories; enzymes, hormones, cytokines or monoclonal antibodies, table 1.1. Productivity in research and development by large pharmaceutical companies has declined and with it focus has shifted from small molecule therapeutics to biologicals such as proteins and peptides (IMAP, 2011). Patent applications by leading pharmaceutical companies for biologicals now exceeds that of small molecules and this gap is widening. Reflecting this the top selling pharmaceutical drugs of 2012 are set to be protein drugs, more specifically antibodies; Humira (Adalimumab) (Abbot) and Remicade (Infliximab) (Janssen Biotech, Shering-plough, Mitsubishi Tanabe Pharma) (Genetic Engineering & Biotechnology News, 2013). This shift away from smaller molecules towards larger biologicals presents new challenges to formulators.

Table 1.1 *Examples of protein and peptide drugs in the BNF, their size and indications*

Protein/peptide drugs	Size (kDa)	Amino acids	Indications
Enzymes (eg lysosomal enzymes, pancreatic enzymes)	29-320	260-2928	Cardiac disorders, lysosomal storage disorders, leukaemia treatment, digestion aids
Hormones: (eg gonadotrophins, vasopressin analogs, oxytocin analogs, calcitonin, insulin)	0.4-37	3-244	Infertility treatment, hormonal cancer treatment, antidiuretic, osteoporosis, blood glucose maintenance, growth stimulation/inhibition, endometriosis
Cytokines: (interleukins, interferons)	15-19	132-165	Tumour shrinkage, rheumatoid arthritis, hepatitis B/C, multiple sclerosis, lymphomas
Growth factors (eg palifermin)	16-25	109-140	Ulcer treatment, oral mucositis
Antibiotics (eg vancomycin)	1-2	9-10	Cancer, bacterial infections, tuberculosis
Vaccines (antigens, toxins)	12-150		Immunisation
Monoclonal antibodies (eg infliximab, abciximab)	48-150		Coronary disease, Crohn's disease, psoriasis, rheumatoid arthritis, leukaemia, organ rejection, cancer treatment, irritable bowel disease, multiple sclerosis
Immunoglobulins (eg hepatitis B immunoglobulin)	150		Rheumatoid arthritis, psoriasis, prophylaxis against infection
Miscellaneous: cyclosporine A	1.2	11	Organ transplant, ulcerative colitis, rheumatoid arthritis, psoriasis

Peptide therapeutics is a dynamic and growing part of the pharmaceutical industry. Biotechnological advances have enabled their production on a commercial scale and new peptide chemical entities have increased from 9.7 per year in the 1990s to 16.8 per year from 2000-08 (Peptide Therapeutics Foundation, 2010). The therapeutic peptide market is predicted to grow from €5.3 billion in 2003 to €11.5 billion in 2013 (Pichereau and Allary, 2005). However this represents less than 2% of the global pharmaceutical market, estimated to reach €770 billion in 2012.

Despite the importance and growth of the protein and peptide therapeutic area, of more then 100 protein and peptide drugs listed in the BNF only four are available orally. The vast majority are only available by injection despite oral preparations being the most desirous dosage form from both the patient and pharmaceutical manufacturer's view. The first pure protein therapeutic molecule, insulin, was discovered 90 years ago and yet there is still no oral formulation available. Despite almost 100 years of research and development by academic groups and the pharmaceutical industry there are still very few protein and peptide drugs orally available. This introduction will explore the reasons for this, review the academic and industrial strategies that have been attempted for oral protein and peptide drug delivery and assess the probability of oral protein and peptide drugs becoming widely available.

1.2 Orally delivered protein and peptide drugs

The four orally available protein and peptide drugs listed in the BNF are listed in table 1.2. Oral delivery for these specific drugs, rather than for the majority, is due to unique characteristics of the drug or their delivery requirements.

Table 1.2 *Orally delivered protein and peptide drugs in the BNF*

Protein/peptide drug	**Size (kDa)**	**Amino acids**	**Action and indications**
Pancreatin: amylase, lipase, chymotrypsin	26-55	244-969	Compensates for reduced intestinal secretion, improves digestion
Desmopressin	1.1	10	Antidiuretic
Cyclosporine A	1.2	11	Immunosuppressant, organ transplants, ulcerative colitis, rheumatoid arthritis, psoriasis
Vancomycin	1.4	10	Gram positive infections, oral for colonic *Clostridium difficile* infection

Pancreatin and vancomycin are orally delivered as their site of therapeutic action is in the gastrointestinal (GI) tract. Pancreatin is delivered to the small intestine to replace a lack of pancreatic enzymes and vancomycin is delivered to the colon to treat *Clostridium difficile* infection. Pancreatin is generally enterically coated to protect the enzymes from denaturation while travelling through the stomach. Vancomycin is formulated in Macrogol 6000 (polyethylene glycol) filled capsules which may provide some stabilisation during GI tract transit. The glycosylated, tricyclic structure of vancomycin may also provide protection from enzymatic digestion, figure 1.1.

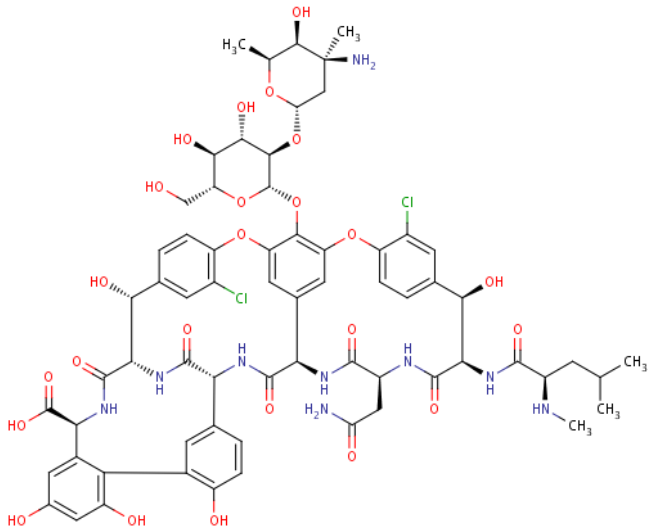


Figure 1.1 *Structure of vancomycin*

Desmopressin and cyclosporine A are orally delivered peptide drugs absorbed from the GI tract into the systemic circulation to elicit their therapeutic action. Cyclosporine A possess' some unique characteristics amongst peptides that make it suitable for oral delivery. It has an oral bioavailability of approximately 30%, most peptides are less than 5% orally available. Unlike most proteins and peptides it is highly lipophilic (logP 3) which may enable its partition across the lipid membranes of intestinal cells into the systemic circulation. Its bioavailability is actually limited by this high lipid solubility as its aqueous solubility is low. Formulations of cyclosporine A are pre-concentrates of oil and surfactant that upon contact with GI fluid form emulsions or microemulsions. Cyclosporine A also has a cyclic structure which may provide protection from digestive enzymes, figure 1.2.

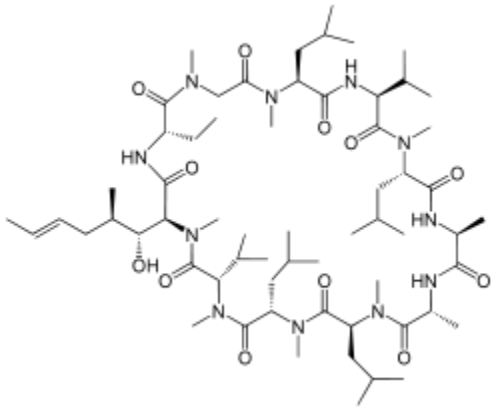


Figure 1.2 *Structure of cyclosporine A*

Desmopressin does not possess the lipophilicity of cyclosporine A, logP -1.95 (Ito et al., 2011), and is only part cyclised, figure 1.3, so may have less enzymatic protection, yet it is orally delivered. Its oral bioavailability is less than 1% but as only a very low dose is required to elicit its therapeutic effect only a very low oral bioavailability is required. Its wide therapeutic window also minimises any problems encountered by variations in oral absorption.

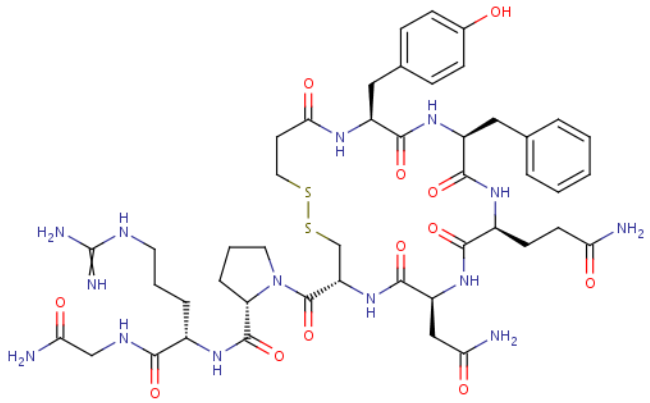


Figure 1.3 *Structure of desmopressin*

1.3 Advantages of oral delivery

Oral delivery of protein and peptide drugs is preferable to injections for patients as it eliminates any pain, discomfort or fear associated with needles. A survey of breast cancer patients found that 63% would prefer daily tablets to monthly intramuscular injections (Fallowfield et al., 2006). The reasons given for this were dislike of needles and greater convenience. There may also be greater compliance as taking an oral dosage form is generally considered less daunting than having an injection. Oral delivery is also preferable from a pharmaceutical manufacturer's point of view as costs of producing injectables under sterile conditions are higher than for oral dosages.

1.4 Oral and intestinal bioavailabilities of protein and peptide drugs

Currently the vast majority of protein and peptide drugs are not delivered orally due to their low oral bioavailability. Table 1.3 lists the bioavailabilities of protein and peptide drugs when administered orally or directly to various intestinal segments of humans and animals. With the exception of cyclosporine A, due to the reasons discussed above, all of the proteins and peptides listed have bioavailabilities of less than 5% in conscious animals, relative to intravenous or subcutaneous delivery, regardless of their size and location of administration. The reason why so few protein and peptide drugs are available as oral dosage forms is that sufficient drug would not be able to reach its therapeutic target and elicit an effect by this route.

The low bioavailabilities displayed in table 1.3 reveal that even if protein/peptide delivery is targeted to a specific part of the GI tract it will still experience huge obstacles to its stability and absorption into the bloodstream. Relatively higher oral bioavailabilities of thyrotropin releasing hormone (TRH), vancomycin and octreotide may be due to their small size, providing less opportunity for digestion, stabilising effects of glycosylation and a tricyclic structure of vancomycin, and the cyclic structure of octreotide.

Table 1.3 *Bioavailabilities of protein/peptide drugs when administered orally or to intestinal segments in humans/animals*

Protein/peptide	**Site of administration-bioavailability relative to intravenous/subcutaneous dose (%)**					
(amino acids, size)	**Oral**	**Stomach**	**Duodenum**	**Jejunum**	**Ileum**	**Colon**
TRH–rat (3, 0.4kDa)	1.6%[1]					
Vancomycin-rat (7, 1.4kDa)	1.7%[2]					
Octreotide-human (8,1kDa)		0.2% sc[3]	0.1% sc[3]	0.2% sc[3]	0.06% sc[3]	
Octreotide-rat (8, 1kDa)	4.3%[4]			0.3%-3.1%[5-6]		
Leuprolide-rat (9, 1.2kDa)	0.02-0.3% 1.2% sc[7-9]		0.08%[7]	1.3*% [10]	0.6-5.6*% [10]	0.4-9.6*%[10]
Buserelin-rat (9,1.3kDa)			0.1-0.8%[11-12]			
Vasopressin analogs-rat (9/10, 1.1kDa)	<0.1%[13]			0.9%[5]		
Desmopressin-human (10, 1.1kDa)		0.2%[14]	0.09-0.2%[14-15]	0.2%[14]	0.03%[14]	0.04%[14]
Cyclosporine A-human (11, 1.2kDa)	20-50%[16]					
Calcitonin-rat (32, 3.4kDa)	0-0.2%[17-20]		0.02-0.15%[17, 21, 22]	0.2-3.3%[22, 23]	0.06%[17]	0.02-0.9%[17, 20, 23, 24]

Calcitonin-human					0.22%[25]
Calcitonin-dog		0.04%[26]		0.06%[26]	0.02%[26]
Exenatide-rat (39, 4.2kDa)	0%[27]	0.005%[28]			
Insulin-rat (51, 5.8kDa)	0.7%, <1%[19, 29]				
Parathyroid hormone-rat/monkey (84, 9.4kDa)	0%[30, 31]			0%[30]	
Erythropoietin-rat (106, 18kDa)			0.6%[32]		
Interferon α-rat/rabbit (165, 19kDa)	0%[33]	<1% [33]			
GCSF-rat (175, 19kDa)		0%[34]			

* In anesthetized rats, thyrotropin releasing hormone (TRH), granulocyte colony stimulating factor (GCSF). Bioavailabilities are relative to an intravenous dose, unless specified as relative to a subcutaneous dose (sc).

[1] (Sasaki et al., 1997), [2] (Anderson et al., 2001), [3] (Kohler et al., 1987), [4] (Fricker et al., 1991), [5] (Michael et al., 2000), [6] (Thanou et al., 2000b), [7] (Adjei et al., 1993), [8] (Iqbal et al., 2011), [9] (Iqbal et al., 2012), [10] (Zheng et al., 1999b), [11] (Luessen et al., 1996), [12] (Thanou et al., 2000a), [13] (Lundin et al., 1994), [14] (d'Agay-Abensour et al., 1993), [15] (Fjellestad-Paulsen et al., 1996), [16] (Jaiswal et al., 2004), [17] (Hee Lee et al., 2000), [18] (Ogiso et al., 2001), [19] (Tozaki et al., 2001), [20] (Fetih et al., 2006), [21] (Sinko et al., 1995), [22] (Tozaki et al., 1998), [23] (du Plessis et al., 2010), [24] (Hastewell et al., 1992), [25] (Hastewell et al., 1995), [26] (Sinko et al., 1999), [27] (Jin et al., 2009), [28] (Gedulin et al., 2008), [29] (Takeuchi et al., 1996), [30] (Guo et al., 2011), [31] (Leone-Bay et al., 2001), [32] (Venkatesan et al., 2006a), [33] (Bayley et al., 1995), [34] (Jensen-Pippo et al., 1996)

Of the intestinal segments the jejunum appears to be the most favourable for successful absorption. Possibly the enzyme concentration is lower here than in other segments, there is greater surface area for absorption or it is more permeable to protein and peptide drugs. Despite the lack of secretion of intestinal enzymes in the colon bioavailabilities of drugs administered here were less than 1% in conscious animals. This suggests they are vulnerable to microbial mediated fermentation and enzymes. The smaller surface area for absorption and the tighter intercellular channels, compared to the small intestine, may also have restricted bioavailability.

All the proteins and peptides listed in table 1.3 are hormones, with the exception of interferon α, cyclosporine A and vancomycin, and therefore not completely representative of all therapeutic protein and peptide drugs. Research may have focused on these due to their relatively small size and they may be easier to obtain than some of the larger and more expensive proteins such as monoclonal antibodies. Most of the studies have not been conducted in humans so they may not represent what would happen upon oral administration to humans. However, the results show that generally, regardless of size, structure, site of administration and subject, protein and peptide drugs have very low oral bioavailabilities, <5%, and therefore cannot be administered in standard oral tablet or capsule formulations.

1.5 Barriers to oral protein/peptide drug delivery- instability

The major barriers to oral protein/peptide drug delivery are illustrated in figure 1.4. The GI tract is designed to digest proteins and peptides and thus it plays a major role in the bioavailability of orally delivered protein and peptide drugs. Protein and peptide digestion is initiated by acid and pepsin in the stomach and continued throughout the small intestine by a multitude of proteases and peptidases in its lumen and along the intestinal wall. Should any proteins or peptides enter the large intestine intact they may be digested or fermented by its large population of bacteria. These processes necessary for protein digestion work antagonistically to the oral delivery of intact protein/peptide drugs.

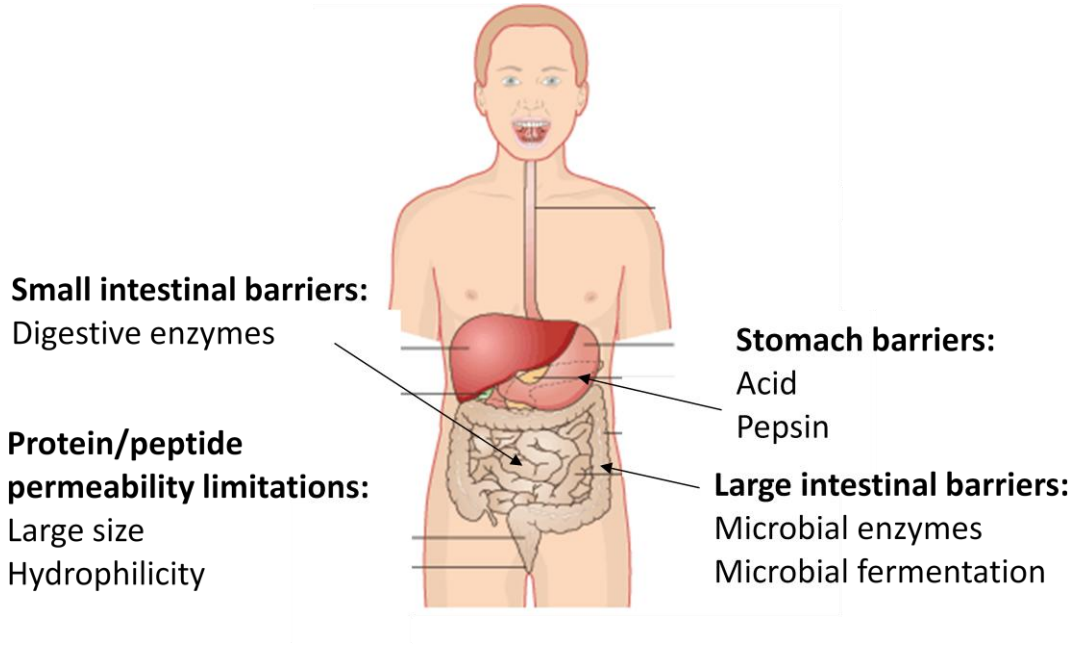


Figure 1.4 *The stability and permeability barriers to oral protein and peptide drug delivery, adapted from www.encognitive.com*

1.5.1 Gastric instability

The first major barrier faced by an orally delivered protein or peptide drug is the stomach. The pH of a human fasting stomach has been measured as pH 1-2.5 by a pH sensitive radiotelemetry capsule (Evans et al., 1988). Exposure to this low pH can alter the ionisation of amino acids which can affect the bonds that hold together the secondary and tertiary structure of larger peptides and proteins. Disruption of these bonds may cause a loss of specific structure and function.

Acid can also break peptide bonds between amino acids. Acid hydrolysis is used to determine the amino acid constituents of proteins and peptides (Berg, 2002). Peptides/proteins are placed in acid and heated to 100-110°C for 24 hours to break all peptide bonds. Due to the necessity of a high temperature and prolonged exposure peptide bonds are unlikely to be completely disrupted in the stomach. Therefore small peptides with no secondary or tertiary structure may not lose their structure at gastric pH.

As well as the acidic pH there is an enzymatic obstacle present in the stomach. Pepsin is secreted here and cleaves peptide bonds within a peptide chain (endopeptidase) between hydrophobic, preferably aromatic amino acids.

Table 1.4 indicates that the gastric stability of peptides and proteins is at least partly dependent on their size. The smaller peptides, vasopressin, oxytocin and TRH analogues appear to be completely stable in simulated, human and animal gastric fluids and mucosa. This may be due to their lack of higher structure which could be disrupted by the low pH. They also appear to be stable in the presence of pepsin which may mean they lack specific pepsin cleavage motifs or are partially protected by disulphide bridges as in oxytocin and vasopressin. The larger peptides and proteins such as the digestive enzymes, insulin and teriparatide appear to be susceptible to gastric denaturation of their secondary and tertiary structures.

These results suggest for successful oral delivery gastric protection is necessary for larger peptides and proteins and those containing pepsin cleavage sites. However, protection in the stomach is not needed for all proteins and peptides. Assessment of gastric stability is beneficial to not only discover where protection is needed for orally administered protein/peptide drugs but also to assess where protection is not required as this will reduce manufacturing costs and make oral delivery more achievable.

Table 1.4 *Protein/peptide recovery after incubation in simulated gastric fluid (SGF), human/animal gastric fluid and mucosa*

Protein/peptide (amino acids, size)	**Proportion intact/active after incubation in simulated/human/animal gastric media**			
	SGF no pepsin	**SGF pepsin**	**Gastric fluid**	**Gastric mucosa**
TRH (3, 0.4kDa)		100% 2hours[1-2]	100%-2 hours rat[1-2]	
Oxytocin analogs (9, 1kDa)			100% human[3]	100% human[3]
Vasopressin analogs (9/10, 1.1kDa)			100% human[3]	100% human[3]
LHRH (10, 1.2kDa)		60%, 6 hours[4]		
Teriparatide (34, 4.1kDa)		0% 5 mins[5]		
Insulin (51, 5.8kDa)		3%-10% 2 hours[6-7]	0% 3 mins- pig[8]	
Lysozyme (128, 14.3kDa)	0% 30 mins[9]			
β-lactoglobulin A/B (162, 18.4kDa)	17.2-34.3% 60 mins[9]	0% 2 mins[9]		
Ovomucoid (28kDa)		0% immediate[9]		
Digestive enzymes: amylase, lipase, trypsin (244-969, 26-55kDa)		0-8% 30 mins[10-11]		
Chicken egg yolk immunoglobulin (150kDa)		0% 1 hour[12]		

Luteinising hormone releasing hormone (LHRH)

[1] (Sasaki et al., 1997), [2] (Khomane et al., 2011), [3] (Fjellestad-Paulsen et al., 1995), [4] (Kafka et al., 2011), [5] (Werle et al., 2006), [6] (Han et al., 2012), [7](Jain et al., 2012), [8] (Werle et al., 2008), [9] (Zheng et al., 2010), [10] (Scocca et al., 2007), [11] (Massicotte et al., 2008), [12] (Li et al., 2009)

1.5.2 Small intestinal instability

The small intestine is the major site of protein and peptide digestion and represents a barrier to oral protein and peptide drug delivery. The pH of the gastrointestinal tract rises to pH 6.6 in the proximal small intestine and rises to pH 7.5 in the terminal ileum (Evans et al., 1988). The small intestine contains luminally secreted proteases and membrane bound peptidases which can digest protein and peptide drugs. They may also be subject to degradation by thiol-disulfide exchange reactions if they contain cysteine residues

The major enzymes secreted into the small intestine are the endopeptidases trypsin, chymotrypsin and elastase and the exopeptidases aminopeptidase and carboxypeptidase. These cleave within or from the ends of peptide chains according to their cleavage specificities. Identification of a cleavage motif within a peptide chain may not definitely indicate digestion by a specific enzyme. Cleavage is not just dependent on the presence of a vulnerable peptide bond but the size and position of other amino acids in the peptide sequence.

Investigations of protein and peptide stability in simulated intestinal fluid and enzyme solutions are summarised in table 1.5. With the exception of TRH there is a general trend that proteins are more stable than peptides in these conditions. This may be because proteins do not have as easily accessible cleavable peptide bonds as their peptide chains are involved in complex secondary and tertiary structures. However, if orally delivered these structures may be unfolded in the stomach and so would be more vulnerable to enzymatic digestion.

Trypsin and chymotrypsin appear to be the most degradative towards peptides and proteins and so for successful oral delivery these enzymes should be specifically inhibited. A reduction of pH to pH 6 reduced the degradation of desmopressin by chymotrypsin so this may be used to inhibit intestinal enzymes (Fredholt et al., 1999).

Table 1.5 *Protein/peptide recovery and half lives (HL) in simulated intestinal fluid (SIF) with pancreatin and enzyme solutions*

Protein/peptide drug	**Proportion remaining after incubation and half lives in SIF/enzyme solutions**				
(amino acids, size)	**SIF**	**Trypsin**	**Chymotrypsin**	**Elastase**	**Aminopeptidase**
TRH (3, 0.4kDa)	100%, 3hrs[1]				
Desmopressin (10, 1.1kDa)			pH 7.4-20.2min HL pH 6- 105.6min HL[2]		
LHRH (10, 1.2kDa)	0%, immediate[3]	850 min HL, 100% 2hr[4-5]	2-2.7 min HL[4-5]	70-114 min HL[4-5]	
Calcitonin (32, 3.4kDa)	0%, 1hr[6]	1.5-8.6 min HL 0% 15min[7-9]	12.8-22.4 min HL[7, 10] 0% 30 min[8-9]	21 min HL[7,10], 0% 40 min[8-9]	4.8 hr HL[10]
Teriparatide (34, 4.1kDa)		0% 5 min[11]	0% 5 min[11]	85% 3 hr[11]	20% 6hr[11]
GLP-1 analogs (39, 4.2kDa)		0.6-1.9 min HL[12]			
Insulin (51, 5.8kDa)	10% 2hr[13]	2% 30 min[14] 0% 1hr[15]	0% 15 min[15], 8% 40 min[16], 4% 6hr[14]	35% 3hr[15]	
Lysozyme (128, 14.3kDa)	22.8%, 1 hr[17]				
GCSF (175, 19kDa)		50%[18]	100%[18]		
Ovomucoid (28kDa)	23.4%, 1 hr[17]				
BSA (583, 66.5kDa)	17.7%, 1 hr[17]				

Glucagon like peptide 1 (GLP-1), bovine serum albumin (BSA)

[1] (Khomane et al., 2011), [2] (Fredholt et al., 1999), [3] (Kafka et al., 2011), [4] (Wen et al., 2002c), [5] (Walker et al., 2001), [6] (Lee et al., 2010), [7] (Lu et al., 1999), [8] (Ryan et al., 2011), [9] (Shah and Khan, 2004), [10] (Youn et al., 2006), [11] (Werle et al., 2006), [12] (Jin et al., 2009), [13] (Jain et al., 2012), [14] (Agarwal et al., 2001), [15] (Werle et al., 2008), [16] (Schilling and Mitra, 1991), [17] (Zheng et al., 2010), [18] (Jensen-Pippo et al., 1996)

Glutathione, which is part of the antioxidant defence system of the GI tract, may also contribute to intestinal instability. Reduced glutathione plays a role in thiol-disulphide reactions so may metabolise proteins/peptides with disulphide bonds. Table 1.6 shows the vulnerability of some peptides with disulphide bridges to degradation in the presence of glutathione. There was no degradation of octreotide, possibly due to the presence of aromatic amino acids in the vicinity of its disulphide bridge.

Table 1.6 *Stability of disulphide bridge containing peptides with glutathione*

Peptide	**Media**	**Proportion intact after incubation**
Desmopressin	Glutathione pH 3/pH 5.5	100%/50% (Schmitz et al., 2006)
Oxytocin	Glutathione pH 3	20% 3 hrs (Huck et al., 2006)
Oxytocin	Human ileal mucosa/+glutathione	~100%/60% 3 hrs (Fjellestad-Paulsen et al., 1995)
Octreotide	Glutathione pH 3	100% 3hrs (Huck et al., 2006)

Investigations into the stability of peptides in animal and human small intestinal fluids, table 1.7, revealed that the smaller peptides TRH and hexarelin were more stable than the larger peptides tested. As they have fewer amino acids they have a lower probability of containing one of the specific cleavage sites of the small intestinal enzymes so may be more stable. Tests with small intestinal enzyme solutions suggested proteins may be more stable in this environment but as no proteins >6kDa were tested it cannot be determined if this stability would persist in actual small intestinal fluids. Lowering the pH of human small intestinal fluids reduced vasopressin degradation possibly by inactivating the enzymes present (Fjellestad-Paulsen et al., 1995).

Table 1.7 *Protein/peptide recovery and half lives (HL) after incubation in human/animal small intestinal fluid and mucosa*

Protein/peptide (amino acids, size)	**Proportion remaining and half lives after incubation in human/animal small intestinal fluid/mucosa**	
	Small intestinal fluid	**Small intestinal mucosa**
TRH (3, 0.4kDa)	Rat-100%[1]	Rat-100%[1] Rabbit-94% 65 min[2]
Hexarelin (6, 0.9kDa)	Rat jejuna/ileal- 80%/60% 1hr[3]	
Octreotide (8, 1kDa)		Rat-100%[4]
Oxytocin analogs (9, 1kDa)	Human ileal-0% 60mins[5]	Human jejunal/ileal 100%[5]
Vasopressin analogs (9/10, 1.1kDa)	Human 0-50% 35 mins (at pH 4 100%)[5] Rat-0.2-58.3% 30 mins[6] Pig 0% 5 mins[7]	Human jejunal-30% 3hrs, ileal 100% 3 hrs[5]
Gonadotrophin releasing hormone analogs (9/10, 1.2-1.3kDa)	Brushtail possum- 22 min HL[8]	Rabbit-1.1% 1hr Rat-24.8% 5 hr[9], 90 min HL[10]
Calcitonin (32, 3.4kDa)	Rabbit- 20 min HL[11] Rat-jejunal 0% 5min, 0.4 min HL[12]	Rabbit- 239 min HL[11] Rat- 0% 25 min[13], 4.1-10.6 min HL[12, 14]
Teriparatide (34, 4.1kDa)		Rat- 50% 3 hr[15]
GLP-1 analogs (39, 4.2kDa)	Rat-0.51-1.76 min HL[16-17]	Rat- 0.79 min HL[17]
Insulin (51, 5.8kDa)	Pig-0% 3 min[18]	

[1] (Sasaki et al., 1997), [2] (Thwaites et al., 1993), [3] (Fagerholm et al., 1998), [4] (Fricker et al., 1991), [5] (Fjellestad-Paulsen et al., 1995), [6] (Lundin et al., 1994), [7] (Lundin et al., 1989), [8] (Wen et al., 2002c), [9] (Guo et al., 2004), [10] (Zheng et al., 1999a), [11] (Lu et al., 1999), [12] (Ogiso et al., 2001), [13] (Tozaki et al., 1998), [14] (Youn et al., 2006), [15] (Werle et al., 2006), [16] (Jin et al., 2009), [17] (Youn et al., 2008), [18] (Werle et al., 2008)

Degradation of peptides was generally reduced in the intestinal mucosal homogenates (Fjellestad-Paulsen et al., 1995) and proteolytic activity was found to be reduced here compared to the lumen (Wen et al., 2002c). There may be fewer enzymes here capable of digesting larger peptide chains as usually when they arrive at the mucosal membranes they have been digested to tri and dipeptides. Protein and peptide drugs could be targeted to the intestinal mucosa for release as here they will encounter less digestion.

Different regions of the intestinal tract have been found to have differing proteolytic activities. The luminal and mucosal extracts from the jejunum of the rat and brushtail possum were found to have greater proteolytic activity than those from the ileum (Wen et al., 2002c, Tozaki et al., 1998). LHRH was most degraded in brushtail possum luminal and mucosal extracts from the jejunum than the ileum or duodenum (Wen et al., 2002a, Wen et al., 2002b). Despite this, bioavailability of protein and peptide drugs tended to be greater when administered to the jejunum than from the duodenum or ileum, table 1.3. Possibly the opportunity for absorption into the systemic circulation is far greater from the jejunum than the other intestinal segments and so overcomes the greater proteolytic activity.

1.5.3 Large intestinal instability

Proteolytic activity in the large intestinal fluids and mucosa has been found to be lower than in the small intestine of brushtail possums (Wen et al., 2002c) and rats (Tozaki et al., 1998). LHRH degradation was least in the colonic contents of a brushtail possum intestine (Wen et al., 2002a, Wen et al., 2002b). Glatiramer acetate degradation was also lower in rat colonic mucosa compared to the other intestinal segments (Haupt et al., 2002). Desmopressin degradation was lowest in the colonic contents from a rat compared to extracts from small intestinal segments, however vasopressin was degraded mainly in the colonic contents (Ungell et al., 1992). This reduced proteolytic activity may make the large intestine a more attractive target for oral protein and peptide drug delivery than the small intestine.

Table 1.8 shows that despite reduced proteolytic activity in the large intestine protein/peptide degradation still occurs and would need to be minimised for oral delivery strategies targeted here.

The colon is home to a large population of microbes which may secrete their own proteases. Proteins and peptides may also be fermented by microbes in the large intestine metabolising them to volatile fatty acids. The contribution of microbial mediated degradation of orally delivered TRH was demonstrated by an increase in oral bioavailability from 1.6% to 3.1% when administered with antibiotics (Sasaki et al., 1997).

Table 1.8 *Protein/peptide recovery and half lives (HL) after incubation in human/animal large intestinal, faecal fluid and mucosa*

Protein/peptide (amino acids, size)	**Proportion remaining and half lives (HL) in large intestinal and faecal fluids/mucosa**		
	Caecal	**Colonic**	**Faecal**
TRH (3, 0.4kDa)	Rat-degraded[1]	Rat-degraded[1]	Rat/human/dog degraded[1]
Hexarelin (6, 0.9kDa)	Rat fluid- 100% 1hr[2]	Rat fluid- 100% 1hr[2]	
Vasopressin (9, 1.1kDa)		Human mucosal- 40% 30min[3]	
Calcitonin (32, 3.4kDa)	Rat-2.5 min HL[4-5]	Rat-19.23 min Rabbit-128 min HL[6-7]	Human-2-132 min HL[7-8]
Insulin (51, 5.8kDa)	Rat-34 min HL[4-5]		

[1] (Sasaki et al., 1997), [2] (Fagerholm et al., 1998), [3] (Fjellestad-Paulsen et al., 1995), [4] (Tozaki et al., 1997), [5] (Tozaki et al., 1995), [6] (Tozaki et al., 1998), [7] (Lu et al., 1999), [8] (Hastewell et al., 1995)

1.5.4 Systemic instability

Short circulation half life is an issue for peptide and protein drugs delivered by injection and many strategies such as pegylation and sustained release depot formulations have sought to overcome this. Opsonins can bind to proteins/peptides in the blood stream enabling them to be cleared by macrophages reducing their systemic half life. Should orally delivered protein/peptide drugs be absorbed intact into the systemic circulation they may also require strategies to prolong their systemic half life.

1.6 Barriers to oral protein/peptide drug delivery-permeability

The very low oral bioavailabilities of protein and peptide drugs are not just the result of their instability but also poor permeability. The routes for protein/peptide drug absorption from the intestinal lumen into the systemic circulation are between cells, paracellular, through cells, transcellular, by endocytosis/pinocytosis or by carrier mediated transport. The relatively small peptide TRH appeared to be absorbed by carrier mediated transport (Yokohama et al., 1984). However carrier mediated transport for larger peptides and proteins is likely to be limited as peptide carriers in the intestinal membrane are present for the uptake of di/tri peptides produced by digestion.

The relatively large size of protein and peptide drugs hampers their absorption as it has been shown to be size dependent and decreases rapidly when molecular weight is greater than 700Da (Humphrey and Ringrose, 1986, McMartin et al., 1987, Donovan et al., 1990). The paracellular route is particularly limited by the tight junctions between cells and restricted to molecules less than 200Da (Humphrey and Ringrose, 1986, McMartin et al., 1987, Donovan et al., 1990). As even the smaller peptides are larger than 700Da their intestinal absorption is likely to be very limited.

The majority of protein and peptide drugs are hydrophilic and therefore not compatible with passive transcellular absorption across the lipid bilayer membranes of intestinal cells. The lipophilic nature of cyclosporine A is thought to account for its higher oral bioavailability as it can be absorbed by the transcellular pathway. However a study found no correlation between the lipophilicity of peptides and their uptake across the Caco-2 *in vitro* intestinal cell model (Conradi et al., 1991). Instead they found a correlation between the number of hydrogen bonds a peptide could make with water and its permeability. The more H-bonds it could form the less permeable it was, possibly due to the greater amount of energy required to break these bonds before absorption.

The paracellular route may be more compatible for absorption of hydrophilic protein and peptide drugs. However paracellular spaces contribute less than 1% of the total mucosal surface area and their small diameter, less than 10Å, (Jung et al., 2000) limits the passage of these large molecules.

Human colon carcinoma (Caco-2) cells are used to mimic the intestinal absorptive epithelium for studying transepithelial drug transport. Caco-2 cell uptake studies revealed more than 90% of TRH remained on the donor side and salmon calcitonin uptake was negligible (Yoo and Park, 2004). The low uptake of even the very small peptide TRH shows the inherent lack of permeability of intact peptide and protein drugs.

Most absorption takes place in the small intestine due to its large absorptive surface area and leakier paracellular channels than those in the large intestine. Vasopressin absorption in a rat was found to be greater from the small intestine than the large intestine or stomach (Ritschel, 1991). Absorption of calcitonin in rats and dogs (Tozaki et al., 1998, Hee Lee et al., 2000, Sinko et al., 1999) and desmopressin in humans (d'Agay-Abensour et al., 1993) was greater from the small intestine than the colon.

Table 1.3 indicated bioavailabilities were greater after administration to the jejunum, however the results were gathered from many different studies which may have used different parameters possibly making comparisons inaccurate. Studies conducted to compare the absorption of various protein and peptide drugs from small intestinal segments in rats, beagle dogs and rabbits found that calcitonin (Tozaki et al., 1998, Hee Lee et al., 2000, Sinko et al., 1999), insulin (Han et al., 2012), oxytocin, carbetocin (Lundin et al., 1991), desmopressin, vasopressin (Pantzar et al., 1995, Lundin et al., 1991) and leuprolide (Zheng et al., 1999b) were all more absorbed from the distal than proximal small intestine. This may be due to lower proteolytic activity of the ileum than the jejunum. The M cells of gut associated lymphoid tissue are known to sample macromolecules from the ileum and represent a potential portal for oral protein/peptide absorption. Increased absorption from this segment may be due to these cells.

In human studies, however, desmopressin (d'Agay-Abensour et al., 1993) and octreotide (Kohler et al., 1987) absorption was greater from the proximal small intestine than the ileum. This disparity with animal absorption studies could be due to their higher percentage of M cells in Peyer's patches, 10-50%, in rodents, and 46% in rabbits (Gebert et al., 2004) compared to humans, 5%.

Absorption of leuprolide from small intestinal and colonic segments was greater in anesthetized rats than conscious rats (Zheng et al., 1999b), table 1.3.

This may have been due to reduced GI motility and enzyme secretion. The absorption of desmopressin was increased by pretreatment with loperamide, which decreased intestinal motility, in human volunteers (Callreus et al., 1999). This increased desmopressin residence time and therefore its absorption. It may also have inhibited pancreatic secretions increasing its bioavailability by reducing its enzymatic digestion. An increased intestinal residence time may be used to increase the oral bioavailability of protein and peptide drugs.

1.7 Oral protein/peptide drug delivery strategies

To overcome the stability and permeability barriers to oral protein and peptide delivery many different strategies have been attempted, illustrated in figure 1.5 and described below. The main challenge for the oral delivery system is to increase oral bioavailability from less than 5% to at least 30-50% (Shaji and Patole, 2008). In addition to this the formulation preparation process must not denature fragile protein/peptide drugs.

The vast majority of oral delivery research has been conducted with calcitonin and insulin, possibly due to the frequency of their administration and clinical importance. Some research has also focused on improving the oral delivery of cyclosporine A, desmopressin and pancreatin. There has also been research conducted with the peptide hormones leuprolide, oxytocin and octreotide possibly due to their smaller size making them more compatible for oral delivery. There has been very little investigation into the oral delivery of larger proteins. This could be because of the inherent difficulties of maintaining their complex structure during GI transit and the greater difficulty of absorption of such large molecules into the bloodstream. Recently the oral delivery of GLP-1 and its analogues exenatide and liraglutide has been explored possibly due to their importance in the treatment of type 2 diabetes (Rekha and Sharma, 2013).

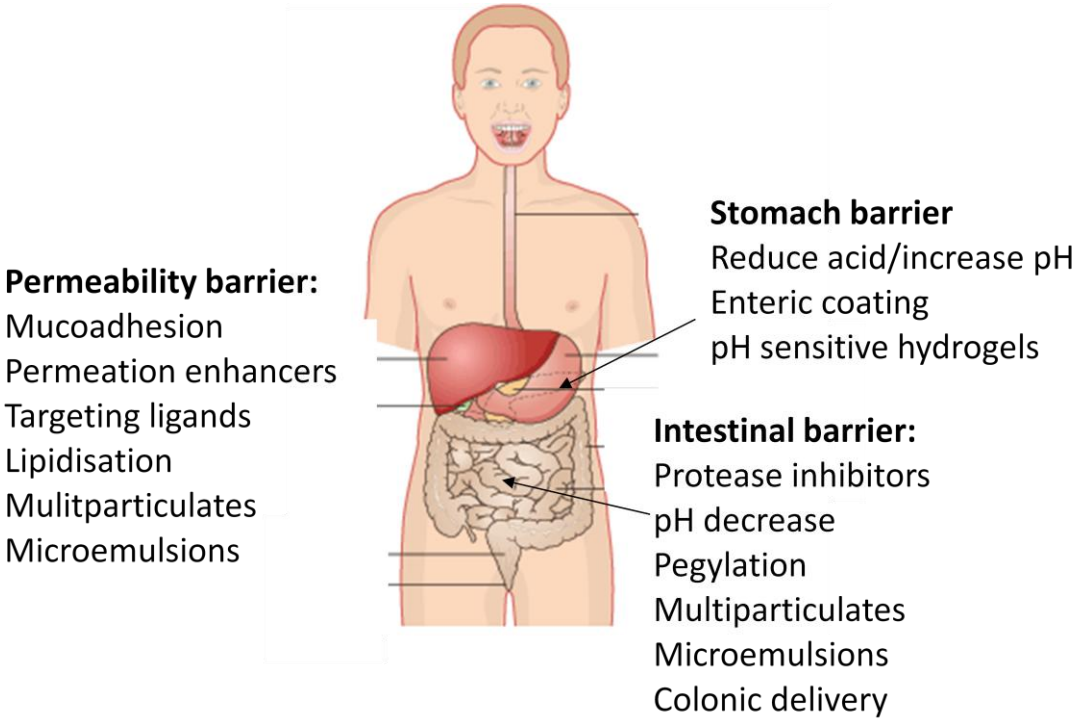


Figure 1.5 *Strategies to overcome the stability and permeability barriers to oral protein and peptide drug delivery, adapted from www.encognitive.com*

1.8 Overcoming the gastric barrier

Strategies to overcome gastric degradation of orally delivered proteins and peptides include excipients to raise gastric pH and encapsulation within enteric carriers, table 1.9. Reduction of stomach acid enabled the oral delivery of active pancreatic enzymes (Regan et al., 1977) and a murine monoclonal antibody (Ilan et al., 2010) in human volunteers. Local buffering provided by a carboxylated high amylase starch tablet in simulated gastric conditions enabled a 70% retention of pancreatic enzyme activity (Massicotte et al., 2008). However a prolonged and chronic raising of stomach pH may compromise this acidic barrier to ingested toxins and its role in digestion. Raising the pH may only partially or temporarily inhibit pepsin.

Table 1.9 *Oral protein/peptide drug delivery strategies to increase stability in gastric conditions*

Protein/ peptide drug	**pH modulation**	**Enteric micro/nanoparticles**	**pH sensitive hydrogels**
Oxytocin			Hydrogel of succinic derivatives of inulin[1]
Cyclosporine A		Eudragit L100, L100-55, S100 nanoparticles[2-3] *	
Bleomycin			P(MAA-g-EG) nanospheres[4-5]
Calcitonin		Eudragit L100 nanoparticles[6]* Eudragit P-1435F micropsheres[7]	P(MAA-g-EG)/ P(MAA-co-NVP) micro/nanospheres[8-10],
Insulin		Eudragit L100/S100/L100-55[11-17] *, Eudragit L30D coated PLGA[18] *, PLGA/HP55[19] *, HPMCP cross-linked chitosan[20] *, hyaluronic acid[21]* micro/nanoparticles	P(MAA-g-EG) microspheres [8, 22] * Poly N-vinyl caprolactam-co-methacrylic acid[23] *
Interferon β			P(MAA-g-EG) microspheres[8] *
Human growth hormone			P(MAA-co-NVP) microparticles[10]
Pancreatic enzymes	Cimetidine –H_2 receptor antagonist[24]	Eudragit L100[26] *,S100-Alginate[27], HPMCP[26] * micro/nanoparticles	

Pancreatic enzymes	Carboxylated high amylase starch tablet-local buffering [25]	
Murine monoclonal antibody, OKT3	Omeprazole –proton pump inhibitor[28]	
Egg yolk immunoglobulin		Chitosan alginate microcapsules[29] *

Poly(methacrylic acid-g-ethylene glycol) : P(MAA-g-EG), poly(methacrylic acid-co-N-vinyl pyrrolidone): P(MAA-co-NVP)

* Enteric strategy enabled retention of at least 75% encapsulated drug *in vitro* in acid

[1] (Mandracchia et al., 2010), [2] (Dai et al., 2004a), [3] (Dai et al., 2004b), [4] (Blanchette and Peppas, 2005a), [5] (Blanchette and Peppas, 2005b), [6] (Cetin et al., 2012), [7] (Lamprecht et al., 2004), [8] (Kamei et al., 2009), [9] (Torres-Lugo et al., 2002), [10] (Carr et al., 2010), [11] (Agarwal et al., 2001), [12] (Zhang et al., 2012b), [13] (Mundargi et al., 2011a), [14] (Jain et al., 2005), [15] (Jain et al., 2006), [16] (Jelvehgari et al., 2010), [17] (Morishita et al., 1993), [18] (Naha et al., 2008), [19] (Wu et al., 2012a), [20] (Makhlof et al., 2011b), [21] (Han et al., 2012), [22] (Torres-Lugo et al., 2002), [23] (Mundargi et al., 2011b), [24] (Regan et al., 1977), [25] (Massicotte et al., 2008), [26] (Naikwade et al., 2009), [27](Scocca et al., 2007), [28] (Ilan et al., 2010), [29] (Li et al., 2007)

Enteric formulations can prevent the release of orally delivered protein and peptide drugs in the stomach protecting them from acid and/or pepsin degradation, and can target their release to favourable regions of the intestinal tract for their absorption. Pancreatin is currently available as enteric coated tablets, granules and microspheres to prevent its gastric denaturation. Enteric micro and nanoparticles offer advantages over enteric tablets or capsules in terms of a faster drug release at small intestinal pH (Naikwade et al., 2009) and better *in vivo* control of symptoms (Littlewood et al., 1988).

Enteric micro/nanoparticles and most of the pH sensitive hydrogels, which swell in response to pH, were able to restrict the release of encapsulated protein/peptide drugs *in vitro* in acid, table 1.9, however some of the hydrogel particles prematurely released their loaded drug. Almost 40% of calcitonin and bleomycin were released from P(MAA-g-EG) hydrogels at pH 1.2 possibly due to their small size (Kamei et al., 2009, Blanchette and Peppas, 2005a, Blanchette and Peppas, 2005b).

While enteric particles prevented release of a high proportion of encapsulated protein/peptide drug in acid the proportion retaining activity after pepsin incubation was much lower, 26-60% (Scocca et al., 2007, Li et al., 2009, Makhlof et al., 2011b, Han et al., 2012). This disparity suggests they may not be able to prevent premature drug release, degradation of drugs exposed at the particle surface and/or influx of acid and pepsin which can degrade the encapsulated protein/peptide.

Encapsulation of pancreatin (Naikwade et al., 2009), calcitonin (Cetin et al., 2012) and insulin (Mundargi et al., 2011a, Jain et al., 2005, Naha et al., 2008, Han et al., 2012) in enteric particles increased their oral efficacy up to four times that of a drug solution in rabbits and rats. Pharmacological availabilities of insulin encapsulated in enteric micro/nanoparticulates, ranged from 0.8 to 11.4% relative to subcutaneous insulin (Zhang et al., 2012b, Morishita et al., 1993, Wu et al., 2012a, Makhlof et al., 2011b). An insulin solution produced negligible availability. Encapsulation of cyclosporine A in enteric nanoparticles increased its oral bioavailability in rats by 32.5% compared to the currently available microemulsion formulation Neoral (Novartis) (Dai et al., 2004a, Dai et al., 2004b).

Loading in pH sensitive hydrogels increased absorption of calcitonin and interferon β from rat ileum (Kamei et al., 2009). Insulin loaded hydrogels decreased blood glucose levels by 50% when orally administered to rats (Mundargi et al., 2011b).

Oral bioavailabilities of protein and peptide drugs, except cyclosporine A, solely encapsulated in enteric polymers were less than 5% (Han et al., 2012, Cetin et al., 2012, Lamprecht et al., 2004, Morishita et al., 1993). The vulnerability of the released protein/peptide to enzymatic degradation in the small intestine and poor permeability may restrict its bioavailability. Combining a pH sensitive polymer with a sustained release polymer, PLGA, increased insulin oral bioavailability to 11.4% (Wu et al., 2012a). Enteric polymers may need to be combined with other, sustained release polymers, enzyme inhibitors and permeation enhancers to form a successful oral delivery strategy.

1.9 Overcoming intestinal instability

1.9.1 Enzyme protection

To inhibit the enzymatic degradation of orally delivered protein and peptide drugs specific enzyme inhibitors and organic acids to lower the pH of the intestine, inactivating enzymes, have been used. These strategies inhibited degradation *in vitro* in enzyme solutions and in human, rat and brushtail possum intestinal fluids and mucosa, table 1.10. However, they may disrupt the normal digestion of proteins making them unsuitable for prolonged use. The attachment of polyethylene glycol (PEG) has also been used to stabilise proteins/peptides by shielding them from enzymes. Pegylation has been used to lengthen the circulatory half life and bioavailability of interferons (Pegasys, PegIntron) and GCSF (Neulasta) reducing the frequency of their injection.

Table 1.10 *Oral protein/peptide drug delivery strategies to overcome intestinal enzymatic degradation*

Protein/ peptide drug	**Protease inhibitors**	**pH reduction**	**Colonic formulation**
Leuprolide	Antipain, DCI [1] *		
Desmopressin	Aprotinin[2-4] *, chymostatin[2] *, bestatin[4] *,cyclodextrins[5] *		
LHRH	Soybean trypsin inhibitor, bestatin, bacitracin, sodium deoxycholate, carbopol, EDTA [6-7] *		
Calcitonin	Aprotinin[8-10] *, camostat [8, 10] *, soybean trypsin inhibitor[8, 10] *, chicken, duck, turkey ovomucoids[9] *, bacitracin[10], Bowman Birk inhibitor[11] *, elastatinal[11] *, sodium glycocholate[8] *, sodium taurocholate[12] *, carbopol[12] *	Citric acid [13-15]	Azopolymer coated pellets[16]
Insulin	Aprotinin[8] *, [17], camostat [8] *, soybean trypsin inhibitor [8] *, sodium glycocholate[8] *, chicken, duck ovomucoids[18] *, carbopol[19] *		Colonic release capsules[6] Azopolymer coated pellets[16]
GCSF		Citric acid [20]	

3,4 dichloroisocoumarin (DCI), ethylenediaminetetraacetic acid (EDTA). ***** Inhibited enzyme degradation *in vitro* in enzyme solutions and in human, rat and brushtail possum intestinal fluids and mucosa

[1] (Zheng et al., 1999a),[2] (Fjellestad-Paulsen et al., 1995), [3] (Fjellestad-Paulsen et al., 1996), [4] (Ungell et al., 1992), [5] (Fredholt et al., 1999), [6] (Wen et al., 2002a), [7] (Wen et al., 2002b), [8] (Tozaki et al., 1997), [9] (Shah and Khan, 2004), [10] (Tozaki et al., 1998), [11](Guggi and Bernkop-Schnurch, 2003), [12] (Ogiso et al., 2001), [13] (Lee et al., 2000), [14] (Lee et al., 1999), [15] (Wu et al., 2010), [16] (Tozaki et al., 2001), [17] (Kraeling and Ritschel, 1992), [18] (Agarwal et al., 2001), [19] (Bai et al., 1996), [20] (Ushirogawa et al., 1992)

In vivo the trypsin/chymotrypsin inhibitor aprotinin increased the bioavailability of desmopressin from 0.09 to 0.46%, when administered intraduodenally in human volunteers (Fjellestad-Paulsen et al., 1996), and of insulin from 5.02% to 6.15% when administered orally to dogs in a colonic formulation (Kraeling and Ritschel, 1992). Aprotinin attached to liposomes with calcitonin (Werle and Takeuchi, 2009) and insulin (Morishita et al., 1993) increased oral hypocalcemic activity 11 fold, compared to plain liposomes, and oral insulin bioavailability in rats from 1.3% to 3.6%.

Citric acid increased GCSF activity five fold when administered intraduodenally to rats, compared to a solution, (Ushirogawa et al., 1992). It also increased the oral bioavailability of salmon calcitonin in dogs, when loaded in enteric capsules, from 0.02% to 0.86% (Lee et al., 2000, Lee et al., 1999), and produced an oral calcitonin bioavailability of 1.8% in rats (Wu et al., 2010).

Pegylation increased GCSF bioavailability following intraduodenal administration to rats from undetectable to 1.8%, relative to intravenous GCSF (Jensen-Pippo et al., 1996). When pegylated calcitonin was administered intestinally in rats its activity was 5.8 fold greater than unmodified calcitonin (Youn et al., 2006) but when orally administered it was not as active as unmodified calcitonin (Cheng and Lim, 2009). Possibly PEG was detached from the drug before it reached the small intestine and may require additional GI protection.

Oral protein/peptide drug bioavailability of pegylated drugs or those formulated with protease inhibitors or citric acid alone was not more than 5%, relative to the intravenously administered drugs. These strategies alone may not be enough to enable oral protein and peptide drug delivery, however combined with other strategies they can increase oral bioavailabilities.

1.9.2 Colonic delivery

As the colon has been shown to be less proteolytically active than the small intestine (Tozaki et al., 1998, Wen et al., 2002c) some oral protein and peptide drug delivery strategies have been targeted there, table 1.10. However drugs released here will be vulnerable to microbial mediated degradation and any permeation enhancement could result in the uptake of potentially toxic material.

pH sensitive colonic release capsules loaded with an insulin microemulsion increased its oral pharmacological availability in dogs from 2.1%, for the microemulsion, to 5% from the capsules (Kraeling and Ritschel, 1992). Colon targeted azopolymer coated pellets loaded with eel calcitonin and insulin increased their oral pharmacological availabilities in rats from 0.16 to 1.04% and 0.67 to 3.38% respectively (Tozaki et al., 2001). The azo bonds of the polymer can be reduced by colonic microbiota triggering drug release. The increased bioavailability is still only 5% of that of an intravenous dose and therefore to reach desired oral bioavailability levels additional excipients may be required.

1.10 Overcoming the permeation barrier

1.10.1 Mucoadhesion

Mucoadhesive polymers such as chitosan, polyacrylic acids (carbopol, carbomer, carbophil) and thiomers have been used in oral protein and peptide drug formulations and increased *in vitro* mucoadhesion to cells and intestinal segments, table 1.11. Lectin attachment can increase binding to cells in the small intestine and therefore uptake of protein and peptide drugs. The lectin wheat germ agglutinin, WGA, has been most frequently used and can bind to intestinal cell surface receptors, table 1.11. Mucoadhesion can increase intestinal residence time and concentration gradient between the delivery system and intestinal membrane, increasing the absorption of the associated drug.

Chitosan is a natural, mucoadhesive polymer with a positive charge that allows it to interact with negatively charged intestinal mucosa. Chitosan coating of liposomes loaded with insulin increased its oral pharmacological availability five fold, compared to a solution, and produced a pharmacological availability of 5% relative to a subcutaneous dose (Takeuchi et al., 1996). Chitosan coated liposomes increased the oral efficacy of calcitonin in rats up to six times that of uncoated liposomes (Takeuchi, 1999). A dispersion of buserelin with chitosan increased its bioavailability from 0.1 to 5.1% when administered intraduodenally to rats (Luessen et al., 1996).

Table 1.11 *Oral protein/peptide drug delivery strategies to increase mucoadhesion and intestinal residence duration*

Protein/ peptide drug	**Chitosan**	**Poly (acrylic acids)**	**Lectins**	**Thiomers**
Leuprolide	Coated liposomes[1] *			Thiolated chitosan attached to gels[2]
Buserelin	Dispersion[3]			
Desmopressin	Coated minitablets/granules[4] *	Coated nanoemulsion[5]/particles[6]		
Cyclosporine A	Coated PLGA microspheres[7] *			
Calcitonin	Coated liposomes [8-9] *	Coated liposomes[9] *, emulsion[10]	WGA modified liposomes[11-12] *	Thiolated chitosan attached to liposomes[13] *
Insulin	Coated PLGA nanoparticles[14] *, liposomes[15] *	Coated liposomes[15] *	WGA attachment to hydrogel[16] *	Thiolated Eudragit L100 nanoparticles[17] *

* Increased *in vitro* mucoadhesion to cells and permeation

[1] (Guo et al., 2005), [2] (Iqbal et al., 2012), [3] (Luessen et al., 1996), [4] (van der Merwe et al., 2004), [5] (Ilan et al., 1996), [6] (Lehr et al., 1992), [7] (Malaekeh-Nikouei et al., 2008), [8] (Huang et al., 2011), [9] (Takeuchi et al., 1999), [10] (Ogiso et al., 2001), [11] (Makhlof et al., 2011a), [12] (Werle et al., 2010), [13] (Gradauer et al., 2012), [14] (Zhang et al., 2012a), [15] (Takeuchi et al., 1996), [16] (Wood et al., 2006), [17] (Zhang et al., 2012b)

Carbopol coated liposomes and emulsions increased the oral efficacy of calcitonin in rats 2.4 fold compared to uncoated liposomes (Takeuchi, 1999) and increased its oral bioavailability from 0.3 to 0.4% (Ogiso et al., 2001). Insulin loaded thiolated Eudragit L100 nanoparticles had an oral bioavailability of 7.3% in rats and a 2.8 fold greater efficacy compared to unmodified nanoparticles (Zhang et al., 2012b). Thiolated chitosan attachment to leuprolide loaded gels increased its oral bioavailability in rats from 1.2 to 4.5% (Iqbal et al., 2012). Attachment of WGA to carbopol coated liposomes increased oral calcitonin efficacy in rats six fold compared to those with no WGA (Werle et al., 2010).

Gastrointestinal mucoadhesive patches (GI MAPs) produced oral and intestinal availabilities of GCSF (Eiamtrakarn et al., 2002), interferon α (Ito et al., 2005), erythropoietin (Venkatesan et al., 2006a, Venkatesan et al., 2006b) and surfactant coated insulin (Toorisaka et al., 2012) of 2-23%, relative to an intravenous dose, in rats and dogs. In addition to a mucoadhesive layer, eg chitosan or polyacrylic acid, the patches had an enteric layer, citric acid to inhibit enzymes and permeation enhancers to aid absorption.

Mucoadhesion alone increased oral protein/peptide drug bioavailability to about 5% but with pH protection, protease inhibition and permeation enhancement, in the form of GI MAPs, oral bioavailability increased up to 23% (Eiamtrakarn et al., 2002). The use of mucoadhesive polymers to enhance oral delivery is limited however by the natural mucus turnover which is in the range of 12-24 hours in the human intestine (Shaji and Patole, 2008).

1.10.2 Permeation enhancers

To overcome the limited permeability of protein and peptide drugs excipients that disrupt the membranes of intestinal cells or widen the paracellular channels between them have been used, but they may cause the uptake of potentially toxic substances, limiting their prolonged use.

Chitosan (Luessen et al., 1997, Kotze et al., 1997a, Prego et al., 2005, Kotze et al., 1997b, Lin et al., 2007, Nguyen et al., 2011), polyacrylic acids (Kotze et al., 1997a), spermine (Makhlof et al., 2011c), sodium salts of medium chain fatty acids (Lindmark et al., 1995), acylcarnitines (LeCluyse et al., 1991) and bile salts (Michael et al., 2000) were able to reduce the transepithelial resistance (TEER) of Caco-2 cells and open paracellular channels. In formulations with protein and peptide drugs they increased their *in vitro* cell absorption, table 1.12.

Chitosan increased intraduodenal bioavailability of buserelin from 0.8% to 13%, (Thanou et al., 2000a), and octreotide intrajejunal bioavailability in rats from 3.1% to 15.9% (Thanou et al., 2000b). Exenatide encapsulation in γ-PGA nanoparticles shelled with chitosan increased its oral bioavailability in rats from undetectable to 14% (Nguyen et al., 2011). Spermine nanoparticles loaded with calcitonin were 15.2 fold more active than the free drug when orally administered to rats (Makhlof et al., 2011c) and acylcarnitine increased the oral bioavailability of salmon calcitonin in dogs from <0.5% to 1.3% (Sinko et al., 1999).

Bile salts increased desmopressin intrajejunal uptake in rats (Michael et al., 2000), and octreotide intrajejunal availability from 0.26 to 20.2% (Fricker et al., 1996). Bile salts formulated with insulin loaded liposomes (Niu et al., 2012) and PLGA nanoparticles (Sun et al., 2011a) produced an oral bioavailability of 11-12% in rats. Bile salts also increased the oral bioavailability of salmon calcitonin in dogs from less than 0.5% to 1.1% (Sinko et al., 1999) and produced an oral octreotide bioavailability in humans of 1.26% (Fricker et al., 1996).

Surfactants such as sodium dodecyl sulphate (SDS) can disrupt the cell membrane making it more permeable to protein and peptide drugs. SDS decreased the TEER of Caco-2 cells (Anderberg and Artursson, 1993). However exposure to SDS for two hours irreversibly altered the villi morphology, caused apical membrane wounds and structural separation of the tight junctions. Prolonged exposure of intestinal cells to surfactants like SDS could cause intestinal cell damage and unwanted absorption of harmful molecules.

Table 1.12 *Oral protein/peptide drug delivery strategies to increase intestinal permeation*

Protein/ peptide drug	**Permeation enhancer**	**Lipidisation**	**Targeting ligands**
Vancomycin			Folate coupled liposomes[1]
Octreotide	Chitosan[2] *, bile salts[3-4] *, cyclodextrins[5] * Intravail alkylsaccharides[6] *		
Buserelin	Chitosan[7-9] * and carbopol [7] *		
Desmopressin	Chitosan [7, 10] *, bile salts[3] *, SDS[11] *		
Cyclosporine A	Bile salts, cyclodextrins and medium chain fatty acids[12]		Vitamin B12 conjugation to micelles [13] *
LHRH			Vitamin B12[14] *
Calcitonin	Chitosan coated PLGA nanospheres[15,] chitosan nanocapsules[16], spermine nanoparticles[17] *, cyclodextrins[5] *, acylcarnitine[18] *, bile salts[18]	Palmitoylation [19]	Biotin[20]
Exenatide	Chitosan shelled γ-PGA nanoparticles[21] Cell penetrating peptides[22] *		Biotin[23]
GLP-1	Cell penetrating peptides[22] *		Biotin [24-25] *
Insulin	Chitosan[7] *, cell penetrating peptides[26] *, ZOT[27] * Bile salts with liposomes[28]/PLGA nanoparticles[29]		Vitamin B12 [30] *, attached to dextran nanoparticles[31], Folate coupled PLGA nanoparticles[32]

Erythropoietin		Vitamin B12[33]
GCSF		Vitamin B12[33] ,transferrin[34]
Interferon β	Cell penetrating peptides[22] *	
Human growth hormone		Fusion to transferrin [35] and Fc [36] *
Alpha-galactosidase		Attachment of antibody to ICAM-1 to polystyrene beads [37] *

* Increased *in vitro* absorption, Zonula occludens toxin (ZOT)

[1] (Anderson et al., 2001), [2] (Thanou et al., 2000b), [3] (Michael et al., 2000), [4] (Fricker et al., 1996), [5] (Haeberlin et al., 1996), [6] (Maggio and Grasso, 2011), [7] (Kotze et al., 1997a), [8] (Thanou et al., 2000a), [9] (Kotze et al., 1997b), [10] (Luessen et al., 1997), [11] (Anderberg and Artursson, 1993), [12] (Sharma et al., 2005), [13] (Francis et al., 2005a), [14] (Alsenz et al., 2000), [15] (Kawashima et al., 2000), [16] (Prego et al., 2005), [17] (Makhlof et al., 2011c), [18] (Sinko et al., 1999), [19] (Wang et al., 2003), [20] (Cetin et al., 2008), [21] (Nguyen et al., 2011), [22] (Khafagy el et al., 2009), [23] (Jin et al., 2009), [24] (Chae et al., 2008), [25] (Youn et al., 2008), [26] (Liang and Yang, 2005), [27] (Fasano and Uzzau, 1997), [28] (Niu et al., 2012), [29] (Sun et al., 2011a), [30] (Petrus et al., 2007), [31] (Chalasani et al., 2007), [32] (Jain et al., 2012), [33] (Russell-Jones et al., 1995), [34] (Bai et al., 2005), [35] (Amet et al., 2010), [36] (Lee et al., 2007), [37] (Ghaffarian et al., 2012)

Aegis Therapeutics have developed Intravail, an alkylsaccharide transmucosal absorption enhancing agent of hydrophilic saccharides linked to hydrophobic alkyl, non ionic surfactants. They transiently open tight junctions increasing paracellular permeability. They increased the oral bioavailability of octreotide four fold compared to subcutaneous delivery (Maggio and Grasso, 2011). This large increase in bioavailability may be due to limited perfusion of octreotide at the injection site or its retention in local tissues reducing its systemic uptake. Intravail has been tested preclinically with octreotide, parathyroid hormone, calcitonin, interferons and GLP-1 but not entered clinical trials yet.

Permeation enhancement increased oral protein/peptide drug bioavailabilities to more than 10%, and up to four fold greater than a subcutaneously delivered dose with Intravail (Maggio and Grasso, 2011), far greater than seen with previously assessed strategies. Despite the dangers of increasing intestinal permeability without it oral protein and peptide drug delivery may not be possible.

1.10.3 Lipidisation

As the hydrophilicity of proteins and peptides hampers their transcellular absorption attachment of fatty acids has been used to increase their lipophilicity and absorption, table 1.12. Palmitoylation of salmon calcitonin increased its lipophilicity and absorption 19 fold when administered orally to rats compared to unmodified salmon calcitonin (Wang et al., 2003). Modification of protein/peptide drugs to enhance their absorption may however alter their efficacy or toxicity.

1.10.4 Targeting ligands

Biotin, vitamin B12, folate and transferrin have been conjugated to protein/peptide drugs or their carriers to increase uptake by exploiting their intestinal receptor mediated endocytosis, table 1.12. Vitamin B12 conjugation to dextran nanoparticles and folate conjugation to PLGA nanoparticles increased encapsulated insulin oral pharmacological availability from 10.3 to 26.5% (Chalasani et al., 2007) and from 12.73% to 20.4% (Jain et al., 2012) respectively, relative to subcutaneous insulin, in rats.

Folate coupling of vancomycin loaded liposomes increased its oral bioavailability in rats from 6.7% for the uncoupled liposomes to 21.8% (Anderson et al., 2001). Biotin conjugation increased intrajejunal calcitonin absorption three fold compared to the unmodified drug in rats (Cetin et al., 2008) and exenatide oral bioavailability in rats from undetectable to 3.95% (Jin et al., 2009). Conjugation of transferrin however caused GCSF to lose 90% of its activity (Bai et al., 2005). Attachment of an antibody to a protein, ICAM-1, expressed on GI epithelium, to polystyrene beads with attached alpha-galactosidase enabled its uptake *in vitro* by Caco-2 cells (Ghaffarian et al., 2012). Fusion of human growth hormone to Fc increased *in vitro* uptake compared to a control by targeting the FcRn (receptor) found in the intestine of human adults (Lee et al., 2007). Neonatal FcRn mediates the transcytosis of intact immunoglobulins in milk in suckling rodents.

This strategy produced oral bioavailabilities of >20% when combined with encapsulation of the protein/peptide drug and doesn't involve membrane disruption. However conjugation to targeting ligands is a more complex procedure and could increase production costs. Conjugation may also cause a loss of therapeutic activity or increased toxicity (Bai et al., 2005). Absorption may also be limited *in vivo* by competitive binding of the natural ligand to the transporter.

1.11 Multiparticulates and emulsions

Encapsulation of protein/peptide drugs within microparticles, nanoparticles, liposomes, micelles and emulsions can provide protection from enzymes and acid, by a physical polymeric, lipid or oily barrier, and enhanced permeability, table 1.13. Their smaller size compared to tablets, capsules and granules can increase bioavailability by their rapid gastric emptying and dissolution due to their large surface area. Encapsulation of additional protease inhibitors and permeation enhancers and coating with enteric, mucoadhesive and/or permeation enhancing polymers can further increase oral bioavailability.

Table 1.13 *Oral protein/peptide multiparticulate delivery strategies*

Protein/ peptide drug	**Polymeric micro/nanoparticles**	**Lipid particles**	**Micelles**	**Liposomes**
Octreotide				Tetraether lipid[1]
Vasopressin			Polymeric micelles[2]	
Leuprolide	Poly (ethylcyanoacrylate) [3] * and polyacrylic acid nanoparticles [4]			Chitosan coated[5] * **
Cyclosporine A	PLGA micro/nanoparticles[6-8]	Glyceryl monooleate/poloxamer 407 nanoparticles[9], lipospheres[10] and solid lipid nanoparticles[11]	PEG-poly(lactide) [12-13] polysaccharide[14] **	Soybean phosphatidylcholine, sodium deoxycholate[15]
Calcitonin	PLGA[16] * **, polyacrylic acid[17] and Eudragit RSPO[18] nanoparticles	Trimyristin nanoparticles[19]		
GLP-1	PLGA microspheres[20]			
Insulin	PLGA nanoparticles [21-28] Polyisobutylcyanoacrylate nanopheres[29], poly (alkyl cyanoacrylate) nanospheres[30] *	Solid lipid nanoparticles[31-34] *	Dioctadecylamine-501 micelles[35], phosphatidylcholine micelles[36]	Hybrid silica[37]*, cationic liposomes[38], chitosan coated[39], double liposomes[40] *

* Increased *in vitro* stability, ** increased *in vitro* permeation, Poly (lactic-co-glycolic) acid (PLGA)

[1] (Parmentier et al., 2011), [2] (Ritschel, 1991), [3] (Kafka et al., 2011), [4] (Iqbal et al., 2011), [5] (Guo et al., 2005), [6] (Ankola et al., 2010), [7] (Italia et al., 2007), [8] (Fukata et al., 2010), [9] (Lai et al., 2010), [10] (Bekerman et al., 2004), [11] (Muller et al., 2006), [12] (Zhang et al., 2010a), [13] (Zhang et al., 2010b), [14] (Francis et al., 2005b), [15] (Guan et al., 2011), [16] (Yoo and Park, 2004), [17] (Makhlof et al., 2011c), [18] (Cetin et al., 2012), [19] (Martins et al., 2009), [20] (Joseph et al., 2000), [21] (Jain et al., 2012), [22] (Zhang et al., 2012a), [23] (Lin et al., 2007), [24] (Yang et al., 2012), [25] (Cui et al., 2006b), [26] (Sun et al., 2011b), [27] (Sharma et al., 2012), [28] (Kafka et al., 2011), [29] (Radwan, 2001), [30] (Damge et al., 1997), [31] (Fangueiro et al., 2011), [32] (Yang et al., 2011), [33] (Sarmento et al., 2007), [34] (Battaglia et al., 2007), [35] (Lin et al., 2011), [36] (Wang et al., 2010), [37] (Mohanraj et al., 2010), [38] (Park et al., 2011), [39] (Wu et al., 2004), [40] (Katayama et al., 2003),

1.11.1 Polymeric particles

Polymeric particles can protect encapsulated protein and peptide drugs and are more stable in the GI tract than liposomes or micelles. The encapsulated drug can be absorbed within the particles as the use of hydrophobic polymers can increase transcellular uptake. They have increased the *in vitro* stability and permeability of encapsulated protein/peptide drugs, table 1.13.

Salmon calcitonin loaded PLGA nanoparticles produced an oral bioavailability in rats of 0.4%, it was only negligible for a drug solution (Yoo and Park, 2004). Encapsulation of cyclosporine A in PLGA nanoparticles increased its oral efficacy in rodents and produced a 20% greater oral bioavailability than the commercial microemulsion formulation Neoral (Ankola et al., 2010). Encapsulation of insulin in PLGA nanoparticles produced oral bioavailabilities of between 7.6 and 12.7% in rats, relative to subcutaneous insulin (Jain et al., 2012, Zhang et al., 2012a, Sun et al., 2011b, Cui et al., 2006b). With co-encapsulated antacids to neutralise the acidic by-products of PLGA degradation oral insulin availability increased to 17% (Sharma et al., 2012).

Leuprolide encapsulation in poly(ethylcyanoacrylate) nanoparticles increased its absorption from a brushtail possum intestine (Kafka et al., 2011). Encapsulation in polyacrylic acid nanoparticles increased leuprolide oral bioavailability in rats from 0.26 to 0.55% when enterically coated (Iqbal et al., 2011), and calcitonin oral efficacy in rats 15 fold compared to a solution (Makhlof et al., 2011c). Encapsulation of salmon calcitonin in sustained release Eudragit RSPO nanoparticles increased its oral efficacy in rats compared to a drug solution (Cetin et al., 2012).

The intestinal absorption of polymeric particles has been shown to be restricted to small particles, <10µm, (Kompella and Lee, 2001). However, smaller particles may have a limited drug loading capacity and leave more drug vulnerable to intestinal degradation at, or near the surface of particles. Incompatibility of hydrophilic protein/peptide drugs with hydrophobic particles may result in low encapsulation efficiencies and rapid, uncontrolled drug release. Insulin and salmon calcitonin were complexed with fatty acids to increase their lipophilicity and encapsulation in PLGA nanoparticles (Yoo and Park, 2004, Sun et al., 2011b).

Production of polymeric particles often involves the use of solvents and high shear forces which need to be minimised to encapsulate fragile protein and peptide drugs.

1.11.2 Lipid particles

Lipid particles can provide protection and increased lipophilicity to increase protein/peptide drug oral bioavailabilities, table 1.13. Encapsulation of lipophilic cyclosporine A produced a 78% greater oral bioavailability than Neoral in dogs (Lai et al., 2010), and a similar bioavailability in humans (Bekerman et al., 2004). Solid lipid nanoparticles produced an insulin oral pharmacological availability of up to 5% in rats, relative to subcutaneous insulin, but these particles experienced high, immediate burst release *in vitro* (Yang et al., 2011, Sarmento et al., 2007, Trotta et al., 2005). As most proteins and peptides are hydrophilic they are less compatible with encapsulation within lipid particles reducing their encapsulation efficiency and causing uncontrolled and immediate burst release.

1.11.3 Micelles

Micelles can provide protection and enhanced permeation of loaded protein and peptide drugs, table 1.13. They increased *in vitro* permeation (Francis et al., 2005b) of cyclosporine A and its oral efficacy in rats (Zhang et al., 2010a, Zhang et al., 2010b). They also increased the oral activity of vasopressin in rats (Jones et al., 2008).

1.11.4 Liposomes

Liposomes are vesicles composed of a phospholipid bilayer with an aqueous core in which hydrophilic proteins and peptide drugs can be protected from enzymatic digestion, table 1.13. The lipid bilayer can fuse with the bilayer of cell membranes facilitating absorption of the drug into intestinal cells. They increased the *in vitro* stability of leuprolide (Guo et al., 2005), the oral bioavailability of octreotide in rats, 4.6 times that of a solution (Parmentier et al., 2011), and of cyclosporine A, 20% greater than Neoral (Guan et al., 2011). Oral efficacy of insulin in rodents was increased by encapsulation in chitosan coated liposomes (Wu et al., 2004).

However, liposomes have shown instability with bile acids (Anderson et al., 2001) and lipase, which could cause premature drug release *in vivo* leaving them vulnerable to degradation.

1.11.5 Microemulsions

Microemulsions are dispersions of two immiscible liquids such as oil and water stabilised by an interfacial film of surfactant molecules. They can improve drug solubilisation, provide protection from enzymes and enhance intestinal absorption. Hydrophilic protein and peptide drugs in an aqueous phase are dispersed in a protective oil phase. Lipophilic drugs in an oil phase are dispersed in an aqueous phase to increase their solubility in GI fluids.

Microemulsions have been used to deliver cyclosporine A due to its low water solubility and high lipid solubility (Odeberg et al., 2003, Sarciaux et al., 1995, Dunn et al., 1997, Ritschel et al., 1990, Lei et al., 2012). A microemulsion of cyclosporine A produced an oral bioavailability of 51.8% in rats compared to 41.6% for Neoral, relative to an intravenous dose (Gao et al., 1998). The success of oral microemulsions of cyclosporine A are due to its relatively small size and hydrophobicity.

Microemulsions increased the oral efficacy of salmon calcitonin in rats (Fan et al., 2011) and the absorption of vasopressin from rat small intestine (Ritschel, 1991). Emulsion and microemulsion formulations of insulin produced an oral glucose reduction in rats of 25-37.5% (Sharma et al., 2010, Toorisaka et al., 2003) and increased its pharmacological availability from undetectable to 7% (Elsayed et al., 2009). Emulsions of leuprolide increased its *in vitro* stability (Zheng et al., 1999a) and bioavailability from 0.08 to 6.8%, relative to an intravenous dose, when administered to the duodenum of rats (Adjei et al., 1993). A microemulsion of parathyroid hormone produced an oral bioavailability of 5.4% relative to a subcutaneous dose (Guo et al., 2011).

Incompatibility of hydrophilic protein and peptide drugs with the oil phase of an emulsion may have caused 60% calcitonin leakage in SGF from a microemulsion which would leave it vulnerable to degradation if administered orally (Fan et al., 2011).

Surfactant coating of proteins and peptides has been used to make them more lipophilic and compatible for loading in emulsions (Toorisaka et al., 2003).

1.11.6 Multiparticulates- potential for oral delivery

Encapsulation in multiparticulates alone can increase the oral bioavailability of protein and peptide drugs to more than 10% but bioavailabilities of more than 20% are only achievable in conjunction with other strategies. Attachment of targeting ligands to their surface, coating with mucoadhesive or pH sensitive polymers and the co-encapsulation of protease inhibitors and permeation enhancers are necessary for these greater oral bioavailabilities.

1.12 Commercial oral delivery strategies

In addition to academic research and development many biotechnology companies have also been pursuing oral protein and peptide formulations. This has been met with mixed success. Unfortunately this sector is littered with companies which have demonstrated promising results in preclinical or early stage clinical trials but have since gone into liquidation. This means the full potential of some delivery strategies have not been fully determined.

1.12.1 Discontinued/inactive oral delivery strategies

AutoImmune with Eli Lilly and Provalis with Cortecs Ltd developed oral insulin formulations which showed efficacy in phase II clinical trials but they have since been suspended. Oral insulin delivery strategies developed by Endorex, based on liposomes, by Apollo Lifesciences, based on vitamin B12 coated nanoparticles, and by Bow pharmaceuticals, based on encapsulation in a dextran matrix, have also been suspended. Diasome developed a nanosized oral insulin that was stable at low pH and in the bloodstream. This was tested in phase II and III clinical trials with type II diabetics in 2009 but there have been no further trials or information regarding this product. Oral formulations of insulin, developed by Diabetology, and calcitonin, by Bone Limited, demonstrated efficacy in Phase I and IIa clinical trials using Axcess delivery technology. This delivery technology involves encapsulation in enteric coated capsules with an absorption enhancer and bile acids.

These trials were conducted in 2004 and 2005, there have been no further trials but Diabetology recently announced a partnership with USV Limited to develop oral insulin for the Indian market.

1.12.2 Current oral delivery strategies in clinical trials

Despite the suspension of many projects some have completed successful clinical trials and are pursuing filings in the near future. These active programs are outlined in table 1.14.

Emisphere's Eligen technology utilises delivery agents, which interact non-covalently with protein and peptide drugs exposing their hydrophobic side chains, increasing their lipophilicity and absorption. This strategy was one of the most promising for protein/peptide delivery increasing the oral bioavailabilities of parathyroid hormone (Leone-Bay et al., 2001, Leone-Bay et al., 1996) , human growth hormone (Milstein et al., 1998), salmon calcitonin (Leone-Bay et al., 1995a, Leone-Bay et al., 1995b) and interferon-α (Milstein et al., 1998, Leone-Bay et al., 1995b) in rats and primates. However, a three year phase III clinical trial with salmon calcitonin, which ended in 2011, failed to meet primary and secondary endpoints in treating postmenopausal osteoporosis and its planned 2012 submission has been abandoned. Its oral programs for parathyroid hormone, human growth hormone and insulin for type I diabetes have all been terminated. Its only remaining oral peptide delivery program is with GLP-1 analogs and insulin for type 2 diabetes which entered phase I clinical trials in 2010.

Biocon have continued the development of an oral insulin formulation, IN-105, initiated originally by the Nobex Corporation. However, phase III clinical trials did not meet desired expectations, not thought necessarily to be due to a lack of efficacy but due to behavioural modifications of those taking the placebo. The project is still active though and a partnership with Bristol-Myers-Squibb for further development appears likely.

Table 1.14 *Currently active oral protein/peptide drug delivery programs in clinical trials*

Company	Protein/peptide drug	Oral delivery formulation/technology	Clinical trial status
Emisphere	Insulin/GLP-1 analogs	Eligen technology-Delivery agents inducing increased hydrophobicity	Phase I-2010
Biocon	Insulin	Conjugation to amphiphilic oligomers of PEG and alkyl groups/fatty acids (Soltero and Ekwuribe, 2001), with a fatty acid absorption enhancer	Phase III-failed to meet all endpoints but still active Phase II trials planned outside India 2013
Tarsa Therapeutics	Salmon Calcitonin (OSTORA)	Enteric tablet with citric acid, acylcarnitine, oral bioavailability increased from <1% to 20%	Phase III-successfully met endpoints 2012, New Drug Application (NDA) planned
Chiasma and Roche	Octreotide (Octreolin)	Transient permeation enhancer technology; solid, hydrophilic drug particles suspended in a hydrophobic medium with medium chain fatty acids	Phase III began Q4 2011
Oramed	Insulin/Exenatide (Eldor et al., 2010b)	Enteric coated tablet/capsule, protease inhibitor, omega-3 fatty acids and EDTA	Insulin-Phase II in US commence 2013 Exenatide- Phase Ib/IIa started Jan 2013
Unigene	Parathyroid hormone	Excipients inhibit digestion, enhance absorption, increase oral bioavailability <1% to 20%	Phase II completed Nov 2011 achieved primary endpoints
Merrion with Novo Nordisk	Insulin, GLP1	Gastrointestinal permeation enhancement technology: medium chain fatty acid matrix	Phase I for both compounds successfully completed 2013
Amarillo Biosciences	Interferon α	Orally dissolving lozenges targeting throat receptors to initiate immune response	Phase II completed 2012

The other oral protein/peptide delivery system that has completed phase III clinical trials is Tarsa Therapeutic's OSTORA, salmon calcitonin tablet. It successfully met all clinical endpoints and represents the most promising oral peptide strategy for future availability (Binkley et al., 2012). However an FDA Advisory Committee decided in 2013 that salmon calcitonin should no longer be broadly marketed as the risk of it causing cancer outweighs its benefits. This unfortunately may end the progress of this oral peptide which had progressed much farther than many others.

Chiasma's oral octreotide is currently undergoing phase III clinical trials and they are hopeful of filing an NDA in 2013. A new partnership with Roche was announced in February 2013 to develop and commercialise Octreolin. Unigene's oral formulation of parathyroid hormone, Amarillo Bioscience's oral formulation of interferon α and Oramed's oral insulin and exenatide have entered or completed phase II clinical trials and it remains to be seen if they can progress to further clinical trials.

The programs all involve proteins/peptides less than 6kDa, except for interferon α which is targeted to throat receptors and so doesn't have to overcome the stability and permeability issues of oral systemic delivery. The oral delivery of larger proteins to the systemic circulation does not look like becoming a reality soon. However the success of Tarsa Therapeutics phase III clinical trial with calcitonin suggests it may not be long before relatively large, hydrophilic peptides can be administered orally.

Emerging oral protein/peptide drug delivery strategies in preclinical or entering clinical trials seem mainly to be based on modified nanoparticles. Access Pharmaceuticals have formulated protein and peptide drugs in nanoparticles coupled to cobalamin, a vitamin B12 analog. NOD Pharmaceuticals are currently conducting phase I clinical and preclinical trials with oral insulin and exenatide mucoadhesive nanoparticles. NanoMega Corp have encapsulated insulin in chitosan shelled gamma γ-PGA nanoparticles and Oshadi drug administration have blended insulin with inert silica nanoparticles, a polysaccharide, suspended them in oil and loaded into enteric capsules.

1.13 Oral protein/peptide drug delivery success and future perspective

Numerous strategies for oral protein and peptide delivery have been attempted and shown some improvement in bioavailability compared to the drugs alone. Oral bioavailabilities of protein and peptide drugs are generally less than 5%, table 1.3, and most of the delivery strategies attempted have not raised them to more than 10%, relative to injected doses. The most successful strategies, which have raised oral bioavailabilities to ~20%, and those which have proved successful in clinical trials are combinations of many strategies. They combine encapsulation of the protein/peptide drug in multiparticulates, capsules or tablets with protease inhibitors and permeability enhancers, especially medium chain fatty acids. They may also be enteric coated or conjugated to targeting ligands.

As many of these delivery strategies may disrupt normal digestive processes and compromise the barrier function of the GI tract to incoming toxins implications of their long term use should be considered. Oral bioavailabilities should be reproducible and reliable to achieve a regulatory filing but incompatibility of hydrophilic protein and peptide drugs with hydrophobic delivery carriers can produce uncontrolled drug release (Yang et al., 2011, Fan et al., 2011, Anderson et al., 2001, Yang et al., 2012, Carino et al., 2000). Reproducible bioavailabilities may also be compromised by intra or inter subject variation in the GI environment as protein and peptide drug absorption is so highly dependent on its characteristics. Those drugs with a wide therapeutic window, such as the currently orally available desmopressin, may be more compatible for oral delivery due to this variability. Octreotide and calcitonin have large therapeutic windows and this may partly explain the progression of their oral formulations to phase III clinical trials.

Despite the failures encountered during clinical trials and the suspension of many projects oral protein/peptide drug formulations are closer than they ever have been. The success of Tarsa Therapeutics' oral salmon calcitonin in clinical trials increases the probability that other protein and peptide drugs will soon be orally available.

Oral bioavailabilities are still much lower than those of injected doses and therefore much higher doses must be administered orally to have the same effect. For more expensive protein and peptide drugs this could preclude their oral delivery. The toxicity implications of administering relatively large doses of protein and peptide drugs orally must also be considered.

Challenges for the future may be the scaling up for manufacture of the more complex particulate delivery formulations. Research and clinical trials have mainly focused on the delivery of oral peptides rather than proteins. Their larger size seems to multiply the challenges of oral delivery and this may have deterred development. While oral peptide delivery may soon be more widely available oral protein delivery to the systemic circulation may take longer and require strategies more specifically designed for them.

1.14 Aims of project

The aims of this project are to:

- Gain a greater understanding of the role of the GI tract in oral bioavailability limitations of protein and peptide drugs
    - Investigate the effects of protein/peptide size/structure on intestinal stability
    - Identify regions of the intestinal tract which orally delivered protein/peptide drugs need to be protected from
    - Identify the most favourable region/s of the intestinal tract for oral protein/peptide drug delivery
- Use this knowledge and research of previously attempted strategies to formulate rational oral protein/peptide delivery strategies
- Utilise formulation methods which provide the least threat to maintenance of fragile protein/peptide structure
- Produce formulations that cause minimal disruption to the GI tract in terms of digestion and cell membrane disruption
- Produce formulations with efficient encapsulation of protein and peptide drugs and which effectively control their release
- Test oral protein/peptide delivery strategies *in vitro* to determine their *in vivo* potential
- Investigate and remedy any limitations of these delivery strategies

Chapter 2

Investigation of the intestinal stability of lactase and development of its oral formulations

2.1 Introduction

While very few of the protein and peptide drugs listed in the BNF are orally available there are some proteins available as oral nutritional supplements. It is unknown how effective these are as they are not subject to the rigorous clinical testing of pharmaceutical drugs. However, further investigation into their delivery strategies may provide useful information about how to deliver protein and peptide drugs orally.

One such protein available as an oral nutritional supplement is the intestinal, digestive enzyme lactase. Lactase supplements are available to relieve the symptoms of lactose intolerance and are advised to be taken with dairy food, replacing the enzyme no longer produced in the small intestine.

As lactase supplements are easily obtainable an assessment of their formulations and ability to overcome the delivery obstacles described in chapter 1 should provide a good basis for development of oral protein/peptide drug delivery strategies. As lactase needs to be delivered to the small intestine and not to the systemic circulation to elicit its therapeutic effect only its intestinal instability rather than its permeability need be addressed. This also makes it a good candidate to begin development of oral protein/peptide delivery strategies

2.1.1 Lactase structure and function

Lactase is a large, multi-domain protein with quaternary structure and is part of the β-galactosidase family of enzymes found in Eukaryotes and Prokaryotes. The β-galactosidase of *E.coli* has been most extensively studied structurally (figure 2.1) and was first sequenced in 1970 (Fowler and Zabin, 1970). It is a 464kDa homotetramer of four 1024 amino acid polypeptide chains (Kalnins et al., 1983). Each of the four units consists of five domains. One of these contains the active site which is made up of elements from two subunits of the tetramer. The disassociation of the tetramer into dimers removes critical elements of the active site.

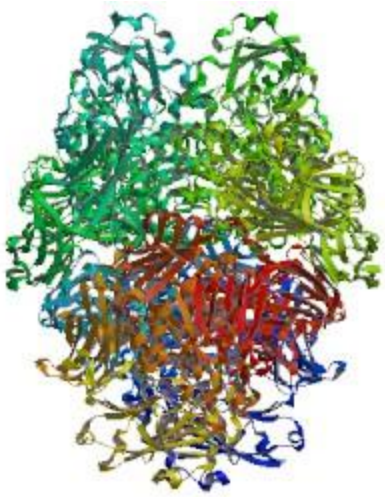


Figure 2.1 Computer simulation image based on X-ray diffraction of *E.coli* β-galactosidase (Juers et al., 2000)

In humans, lactase is a gastrointestinal enzyme found mainly along the brush border membrane of enterocytes that line the villi of the small intestine. Studies of human intestinal lactase showed a 145,000MW band for the polypeptide analysed by SDS-PAGE (Harvey et al., 1995). The lactase was also found to have an isoelectric point of 4.8 and optimum activity at pH 4.5-6.

The function of intestinal lactase is to hydrolyse lactose, the primary disaccharide in mammalian milk, to glucose and galactose (Montalto et al., 2006). It is secreted by neonatal mammals, including humans, to allow them to digest their mother's milk. Once weaning has been completed lactase production stops for the majority (Schulzke et al., 2009); 60% of human adults no longer produce lactase and are therefore unable to digest lactose (Itan et al., 2009). Lactase persistence evolved as milk digestion proved advantageous providing a relatively constant source of Vitamin D and calcium (Itan et al., 2009).

2.1.2 Lactose intolerance

Absence of lactase in lactose intolerance sufferers means any lactose consumed will not be digested but remain throughout the GI tract. Undigested lactose is fermented by colonic bacteria resulting in the formation of gas causing abdominal pain, bloating, flatulence and nausea (Schulzke et al., 2009).

Unabsorbed lactose and fermentation products raise the osmotic pressure of the colon causing water to enter resulting in diarrhoea. These symptoms arise approximately 30 minutes to two hours after the consumption of lactose.

Lactose intolerant individuals are often advised to avoid any lactose containing foods or drinks, however, these foods are a useful source of Vitamin D, calcium and phosphorus and reducing intake of them can result in decreased bone mineral density (Montalto et al., 2006).

To avoid the symptoms of lactose malabsorption and gain the benefits from the consumption of dairy foods lactose can be pre-hydrolysed by the addition of lactase (Montalto et al., 2006) or oral lactase supplements can be taken to digest consumed lactose.

2.1.3 Lactose pre-hydrolysis

Addition of lactase to milk significantly reduced hydrogen production by lactose intolerance sufferers (Montalto et al., 2005). Pre-hydrolysis of lactose avoids risking loss of lactase activity in the acidic stomach when orally administered. Consumption of lactase tablets (Lactaid) with whole milk resulted in more hydrogen production, detected in the breath, than pre-hydrolysed milk (Onwulata et al., 1989). The oral lactase may have been inactivated in the stomach before it could digest lactose, enabling its fermentation and hydrogen production in the gut.

The sugars produced by pre-hydrolysis, glucose and galactose, however are sweeter than lactose and unpalatable to many. To avoid this lactase can be microencapsulated before its addition to milk. This prevents lactose hydrolysis and increased sweetness by preventing contact between enzyme and substrate. Once the milk is consumed the microparticles release lactase and digestion can commence. Liposomes have been investigated as possible carriers but encapsulation efficiency is low, <30%, and long-term stability in milk is poor (Kim et al., 1999, Rao et al., 1995, Rodriguez-Nogales and Lopez, 2006). Encapsulation in fatty acid esters proved more promising with an encapsulation efficiency of 70% (Kwak et al., 2001). Their stability was also better; when added to milk there was no decrease in lactose or increase in sweetness detected during storage for 8 days.

However, it is unknown how this system will behave *in vivo* and if it will be able to release an active enzyme which can digest lactose. There was also an off taste to the milk possibly caused by the fatty acid esters.

2.1.4 Orally delivered lactase

2.1.4.1 Oral lactase stability

For orally delivered lactase to digest lactose in the small intestine it must first pass through the stomach and arrive in the small intestine in an active form. In chapter 1 gastric instability was frequently observed for large, three-dimensional proteins like lactase, such as ovomucoid (Zheng et al., 2010), chicken egg yolk immunoglobulin (Li et al., 2009) and other digestive enzymes (Scocca et al., 2007, Massicotte et al., 2008). This may be caused by changes in amino acid ionisation, due to the acidic pH, which result in disruption of bonds stabilising the secondary and tertiary structure of proteins. Lactase suffered more than 90% loss of enzymatic activity after two hours incubation at pH 1.2 (Alavi et al., 2002). Orally delivered lactase may also be susceptible to degradation by enzymes in the stomach and small intestine.

2.1.4.2 Alternative lactase origin

O'Connel & Walsh found a lactase from *Aspergillus niger* van Tiegh that had optimum activity at pH 2.5 and a 68% retention of activity after exposure to simulated gastric conditions (O'Connell, 2009). This may be a more suitable source of lactase for oral supplements, however it is only 10% active at pH 6.8. To overcome this a two segment capsule was developed combining the gastric active enzyme and a lactase active in the small intestine to allow continuous lactose hydrolysis in the proximal intestine.

2.1.4.3 Oral lactase supplements

Oral lactase supplements have a better palatability than pre-hydrolysed dairy food but have been shown to be less effective at preventing lactose malabsorption (Onwulata et al., 1989). Despite its acid sensitivity (Alavi et al., 2002) many lactase supplements don't include any enteric coating eg, Lactaid, Dairy Ease, Dairy Relief.

An assessment of the stability of lactase supplements revealed that those without enteric coating were almost completely inactivated after 2 hours incubation in acid, regardless of the presence of pepsin, table 2.1. This highlights the instability of lactase in acid and the necessity of enteric coating for oral delivery of active lactase to the small intestine. The supplements were however resistant to pancreatin digestion in simulated intestinal media, pH 6.8, (O'Connell and Walsh, 2006).

Table 2.1 *Active lactase release from supplements following 2 hours incubation in simulated gastric fluid (SGF) with and without pepsin*

Lactase supplement	**Active lactase remaining (%) in SGF**	**Active lactase remaining (%) in SGF + pepsin**
Lactaid (non-enteric)	3.57 [1]	-
Non-enteric product 1	6 [2]	2 [2]
Non-enteric product 2	0 [2]	0 [2]
Enteric product 3	100 [2]	100 [2]
Enteric product 4	90 [2]	90 [2]

[1] (Alavi et al., 2002), [2] (O'Connell and Walsh, 2006)

While these tests in simulated fluids provide an insight into the intestinal stability of lactase they may not give as accurate a reflection of *in vivo* behavior as incubation in animal/human intestinal fluids would.

Currently dosage instructions for oral lactase supplements advise taking >5000 lactase units per meal. A more protective oral lactase formulation may enable a reduction in the amount of lactase that needs to be administered to successfully control lactose intolerance, reducing production costs.

It can be argued that these tests are not completely representative as they only show what would happen in a fasted stomach. The dosage instructions for the supplements indicate they should be taken with food which would raise the stomach pH to about pH 5-6. At this pH lactase would not be denatured. However, the pH drops post ingestion so that after 30 minutes the gastric pH is between pH 2.5 and 3.5 and falls further to values of 1.5–2.2 (Dressman et al., 1990). At this pH the enzymatic activity of the non-enteric lactase would be lost.

2.1.5 Existing oral enzyme delivery strategies

Enzymes currently available as oral dosage forms are the pancreatic enzymes amylase, lipase and trypsin which comprise pancreatin used to treat pancreatic insufficiency. Like lactase they are intestinal enzymes necessary for the digestion of food in the small intestine. As with lactase they have demonstrated their instability in gastric conditions (Aloulou et al., 2008). Therefore to enable these enzymes to reach the small intestine in an active form they are mainly administered as gastric resistant formulations, table 2.2.

Table 2.2 *Pancreatin products available and their enteric excipients*

Pancreatin product	Dosage form	Enteric excipients
Creon 10000, 25000, 40000, micro	Capsules containing gastro resistant granules, minimicrospheres (0.7-1.6mm), gastro resistant granules alone	Hypromellose phthalate
Nutrizym 10, 22	Capsules containing enteric coated minitablets	Methacrylic acid copolymer, type C (Eudragit L30D)
Pancrex V granules, Forte tablets, powder, tablets	Granules, tablets, powder	Opaseal P17-0200 containing polyvinyl acetate phthalate
Pancrex V Capsules	Capsules	None
Pancrease HL Capsules	Capsules containing enterically coated minitablets	Methacrylic acid-ethyl acrylate copolymer (1:1)

Enteric coating of the pancreatin enzymes improved *in vivo* efficacy compared to uncoated products (Delchier et al., 1991, Dutta et al., 1988, Naikwade et al., 2009). In addition in one study all the subjects expressed a preference for the enteric coated product due to its better control of symptoms associated with pancreatic insufficiency (Naikwade et al., 2009).

Delayed enzyme release has been reported for some of the enteric coated tablets (Aloulou et al., 2008). To overcome this dosage form size has been reduced to enable mixing with the food contents of the stomach and accelerated enzyme release. Reduction of pancreatin dosage size from tablets to microspheres reduced GI symptoms (Beverley et al., 1987).

Pancreatin encapsulated in enteric Eudragit L100 microparticles preserved 99.78% of enzyme activity after acid incubation and more rapidly released pancreatin upon pH rise to pH 6.8 than pancreatin tablets (Naikwade et al., 2009). *In vivo* this should allow more opportunity for digestion as the enzymes are more rapidly released and will be available over more of the small intestine.

2.1.6 Lactase microparticles

Single unit, enteric coated dosage forms, such as tablets, have been observed to disintegrate 1.5-2 hours post-gastric emptying rather than immediately (Cole et al., 2002). This can result in reduced bioavailability and symptom control. Pancreatin microspheres were shown to be superior to tablets in terms of rapid release at pH 6.8 and alleviation of pancreatitis symptoms (Naikwade et al., 2009, Beverley et al., 1987). Microparticles are able to suspend in gastric fluids unlike larger dosage forms allowing reliable and fast gastric emptying. The increased surface area to volume ratio also enables rapid drug release. Lactase microparticles consisting of pH dependent and independent polymers have been formulated to take advantage of their beneficial release profile.

Non-enteric polymers used to produce microparticles encapsulating or adsorbing lactase include poly(lactic acid) (PLA) (Hayashi et al., 1994, Stivaktakis et al., 2005) and poly (lactic-co-glycolic acid) (PLGA) (Stivaktakis et al., 2004, Stivaktakis et al., 2005). Their applicability for oral lactase delivery is questionable however due to the lack of specific gastric protection. None of these formulations were tested *in vivo* or *in vitro* in SGF. Release was tested in phosphate buffered saline and lactase was immediately burst released, up to 41% in one hour (Hayashi et al., 1994). If this occurred *in vivo* upon dispersal in gastric fluids lactase activity would be destroyed. The homogenisation step used to prepare these particles was also shown to damage lactase reducing its antigenicity by 45%.

Coupling of wheat germ agglutinin (WGA) to PLGA microparticles was used to increase the residence time of lactase in the small intestine (Ratzinger et al., 2010). This increased mucoadhesion *in vitro* but there were no studies to investigate the stability of WGA attachment *in vitro* in SGF or *in vivo*. It is also not known if this system can prolong lactase residence time for long enough for it to fully digest consumed lactose.

2.1.7 Enteric microparticles

Existing non-enteric lactase supplements have proved unsatisfactory for protecting lactase in simulated gastric fluids. Researched oral lactase delivery strategies have either not been tested in or provided insufficient protection in simulated gastric conditions, or not shown satisfactory activity in small intestinal conditions. While enteric coated tablets/capsules can provide gastric protection they may suffer from a delayed enzyme release at small intestinal pH preventing complete lactose digestion. To combine the advantages of enteric protection and reduced dosage form size enteric microparticles of lactase have been produced.

The pH responsive polymer alginate (Dashevsky, 1998) and fatty acid esters (Kim et al., 2006) were used to produce lactase loaded microparticles. However neither were tested *in vivo* and only the fatty acid ester particles were tested in SGF. There was 15.2% lactase release in SGF in an hour, which would be denatured, and upon pH rise there was a slow lactase release. At pH 6 there was only 45% lactase release after 3 hours which rose to 80% at pH 7 in an hour. This delay could result in lactose persistence.

Alavi *et al* and Squillante *et al* produced pH responsive microparticles encapsulating lactase with Eudragit L100 (dissolves above pH 6) and Eudragit S100 (dissolves above pH 7) (Alavi et al., 2002, Squillante et al., 2003). The oil in oil emulsification solvent evaporation preparation method used produced particles with a mean diameter of 53-195µm which should enable rapid gastric emptying and lactase release. A high entrapment efficiency of >80% was achieved but there was a 32% loss of lactase activity during processing, possibly due to the use of high shear homogenisation (Squillante et al., 2003). Reducing temperature and homogenisation speed had a protective effect on activity.

The Eudragit L100 microparticles were able to prevent lactase release during dissolution studies in 0.1N HCl (Squillante et al., 2003). Upon pH rise to pH 6.8 there was only 25% released after 20 minutes and 65% after 100 minutes. This release rate was quite slow and could result in incomplete lactose digestion. Lactase release was determined not by its activity but by its UV absorbance and therefore it is unknown if the microparticles actually preserved lactase activity.

Both of these microparticle production methods used homogenisation and careful control of temperature. Homogenisation has been shown to have a detrimental effect on lactase activity (Stivaktakis et al., 2005). A simpler oil in oil emulsion solvent evaporation method of microparticle preparation has been used by Kendall *et al*. to produce prednisolone loaded microparticles composed of Eudragit L100 with the novel use of sorbitan sesquioleate as surfactant (Kendall et al., 2009). This method doesn't require temperature control, uses a slower emulsification speed, 1000rpm instead of 8000rpm (Alavi et al., 2002), and uses the less toxic ethanol instead of acetone or methanol to dissolve the polymer. Eudragit L100 microparticles produced were <40µm, uniform, spherical and had high encapsulation efficiency (>80%) and yield (>90%). They restricted release of the encapsulated drug in acid media and rapidly released the drug at pH 6.8. When administered orally to rats the drug was rapidly released and detected in the blood plasma. This method, previously just employed for the encapsulation of small molecules, has the potential to be used for the novel encapsulation of a large biological, lactase, in Eudragit L100 microparticles to provide protection in the stomach and target the small intestine for rapid, active enzyme release.

2.2 Aims

- To discover the stability of lactase throughout the gastrointestinal tract using simulated and porcine gastrointestinal fluids and human faecal fluids
    - To use this knowledge for rational design of oral lactase delivery vehicles
    - To investigate the oral stability of large molecular weight proteins with a tertiary structure
- To assess the stability of oral lactase supplements in the gastrointestinal fluids which were identified as damaging to lactase activity
    - To identify the formulation components that aid oral lactase stability
- To produce oral lactase formulations based on the results of the intestinal stability study, lactase supplements testing and previous oral delivery strategies for lactase and other enzymes
    - To characterise them in terms of physical characteristics (size, morphology), loading and encapsulation efficiency
    - To assess their *in vitro* lactase release and ability to preserve lactase activity in simulated gastrointestinal conditions
    - To analyse and investigate failure to retain lactase activity in simulated gastrointestinal conditions
    - To adapt formulations to overcome any failures in lactase protection/release

2.3 Materials

Lactase (β-Galactosidase), from *Aspergillus oryzae* (9.9units/mg solid) standardised with dextrin and pancreatin from porcine pancreas, activity at least 3x USP specifications were from Sigma Aldrich. Enzeco fungal lactase concentrate was from the Enzyme Development Corporation (81.51units/mg). Hydrochloric acid 37%, specific gravity 1.18 was from BDH. Pig gastric and intestinal fluids were collected from freshly slaughtered pigs and immediately frozen and stored at -80^{o}C. Human faecal fluids were from healthy individuals not taking antibiotics.

Faecal basal media materials: Bacteriological peptone and yeast extract were from Oxoid. Sodium chloride, L-cysteine hydrochloride, vitamin K, resazurin sodium salt and sodium hydroxide were from Fisher Scientific. Dipotassium hydrogen orthophosphate and magnesium sulphate 7-hydrate were from BDH. Calcium chloride dihydrate was from VWR. $NaHCO_3$ and haemin were from Sigma Aldrich. Bile salts were from Fluka Analytical. Tween 80 was from Fluka Chemika.

Lactase supplements analysed are listed and described in table 2.3.

Microcrystalline cellulose (Avicel) and croscarmellose sodium (Ac-Di-Sol) were from FMC Biopolymer. Magnesium stearate, sodium chloride and citric acid monohydrate were from Fisher Scientific. Triethyl citrate, sodium bicarbonate, soybean oil, monobasic potassium phosphate, sodium carbonate (anhydrous), o-Nitrophenyl-β-D-galactopyranoside (ONPG), ≥98%, fluorescein 5 (6)-isothiocyanate (FITC), magnesium hydroxide and phosphate buffered saline (PBS) tablets were from Sigma Aldrich. Glycerol monostearate was from Alfa Aesar. Polysorbate 80 was from Fluka Chemika. Trisodium citrate dehydrate, glacial acetic acid and ethanol 96% v/v were from BDH AnalaR.

Eudragit L100 was a gift from Degussa/ Evonik (Darmstadt, Germany), sorbitan sesquioleate (Alacel 83) was from Sigma Aldrich. Liquid paraffin BP was supplied by JM Loveridge Plc. Sodium phosphate, tribasic, anhydrous was from Alfa Aesar. n-hexane was from Fisher Scientific. Lipoid S100 phosphatidylcholine from soybean (soy lecithin) was from Lipoid GMBH. Lysosensor yellow/blue dextran 10,000 MW anionic, fixable was from Invitrogen Molecular Probes.

Table 2.3 *The ingredients, lactase content and dosage instructions for lactase supplements tested*

Supplement	Lactase units/weight, per capsule/tablet	Other ingredients	Dosage Instructions
Lactase 3500 (Solgar)	3500 units	Mannitol, microcrystalline cellulose, guar gum, magnesium stearate, vanilla sugar	With or just prior to dairy food
Say yes to dairy (Natural Organics Laboratories)	3000 units	Fructose, di-calcium phosphate, stearic acid, natural vanilla, magnesium stearate	Chew immediately before or after meal of dairy food
Lactaid Original (McNeil Nutritionals)	3000 units	Mannitol, Cellulose, Sodium Citrate, Magnesium Stearate	Swallow or chew with first bite of dairy foods
Lactase (Quest)	200mg	di-Calcium Phosphate, Microcrystalline Cellulose, Magnesium Stearate	With meals lactose containing food/drink
Dairy-Zyme (Countrylife)	3000 units	Cellulose (capsule shell), cellulose, magnesium stearate, silica	Immediately before or after consuming dairy products
Super lactase enzyme (Holland & Barrett)	1750 units	Soya Bean Oil, Capsule Shell, Emulsifier (Soya Lecithin), Thickener (Yellow beeswax)	Just before eating lactose containing food or drink
Up and Up	1750 units	Microcrystalline cellulose, polyalditol, mannitol, dicalcium phosphate, crospovidone, magnesium stearate, sodium citrate, silicon dioxide	Take with dairy food

2.4 Methods

2.4.1 Lactase activity-ONPG assay

o-nitrophenyl-β-D-galactopyranoside (ONPG) is a substrate for lactase and used in an assay to measure its activity (USP 35-NF 30). When lactase cleaves ONPG a yellow compound, o-nitrophenol (ONP), is released. This absorbs light at 420nm and the level of absorbance can be used to determine how much active lactase is present. One lactase activity unit is defined as the quantity of enzyme that will liberate 1µmol of ONP per minute at 37ºC at a pH of 4.5.

ONPG solution was prepared in a solution of glacial acetic acid, 4N sodium hydroxide and DI water adjusted to pH 4.50 ± 0.05. Lactase activity was determined by adding the sample (0.5ml) to be analysed to 3.7mg/ml ONPG solution (1ml) which had been incubated at 37°C for 10 minutes. This was incubated at 37°C, 100rpm in a Gallenkamp shaking incubator and removed after 15 minutes when 10% sodium carbonate solution (1.25ml) was added to stop the reaction. DI water (10ml) was added before measuring the absorbance at 420nm using a UV/Vis spectrophotometer (Cary 3E). Sample activity was measured with reference to a calibration curve of lactase standards prepared in the relevant media.

2.4.2 Intestinal stability

100µl of a 500µg/ml lactase solution was added to 9.9ml of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) with and without pancreatin (final lactase concentration of 5µg/ml). These were placed in a Gallenkamp shaking incubator at 37°C and agitated at 100rpm. Samples (0.5ml) were removed after 0, 5, 10, 20, 30, 60, 90 and 120 minutes and tested for lactase activity using the ONPG assay. Lactase stability was not tested in SGF with pepsin due to its complete activity loss in SGF alone rendering this test inexpedient.

SGF was prepared by dissolving 2g NaCl in DI water, adding 7ml of concentrated HCl and making up to 1 litre with DI water. The pH was adjusted to pH1.2 ± 0.5 with concentrated HCl and 5N NaOH. SIF was prepared by dissolving 6.8g of monobasic potassium phosphate in 250ml DI water and then adding 77ml of 0.2N NaOH before making up to 1 litre with DI water.

The pH was adjusted to pH 6.8 ± 0.5 with concentrated HCl and 5N NaOH before addition of pancreatin at a concentration of 10g/litre.

Pigs are omnivores like humans so assessing stability in pig intestinal fluids should provide information on its stability in humans. While possibly not completely reflecting what would happen in human intestinal fluids *in vivo* these results give a general picture of intestinal stability. 300µl of a 120µg/ml lactase solution was added to 900µl of gastric (pH 2.31), duodenal (pH 6.55), jejunal (pH 6.79), ileal (pH 6.86) and descending colonic fluids (pH 7.06) from a pig. The pig intestinal fluids were prepared by centrifuging at 10,000rpm for 10 minutes and the supernatant was used for testing. These mixtures were placed in a Gallenkamp shaking incubator at 37°C and agitated at 100rpm. 100µl samples were taken after 0, 5, 10, 20, 30, 60, 90 and 120 minutes and diluted by the addition of 500µl PBS to give a final lactase concentration of 5µg/ml and then analysed for active lactase using the ONPG assay.

300µl of a 120µg/ml lactase solution was added to 900µl of human faecal slurry and placed in a Gallenkamp shaking incubator using the parameters used in the previous tests. 100µl samples were taken at 0, 5, 10, 20, 30, 60, 90 and 120 minutes and diluted by the addition of 500µl PBS to give a final lactase concentration of 5µg/ml and analysed for active lactase using the ONPG assay.

The stability of lactase in these intestinal fluids was defined as the amount of active lactase recovered at each time point as a percentage of the total active lactase added initially. Changes in lactase concentration due to removal of samples were accounted for.

$$\text{Active lactase recovered (\%)} = \frac{Measured\ lactase\ concentration\ of\ sample\ (mg/ml)}{Initial\ lactase\ concentration\ (mg/ml)} \text{x100}$$

2.4.2.1 Human faecal slurry

Initially the basal medium was prepared as described here using the excipients listed in table 2.4. Peptone water and yeast extract were weighed into a glass bottle containing 1.3L of distilled water and autoclaved at 130°C for 20 minutes.

Sodium chloride, dipotassium hydrogen orthophosphate, magnesium sulphate 7-hydrate, calcium chloride dihydrate were weighed into a 200ml volumetric flask with approximately 150ml of distilled water and stirred to dissolve.

Polysorbate 80 was added and stirred completely until dissolved. The stopper was kept tightly closed to avoid dissolution of oxygen. L-cysteine and the bile salts were added and stirred until completely dissolved. Vitamin K, Haemin and the resazurin solution were added and stirred until completely dissolved. Sodium bicarbonate was added and stirred until dissolved and then the volume was made up to 200ml with distilled water. The solution was filtered using 0.45µm filters (Millex GP, Millipore, Ireland) into the autoclaved 1.3L solution containing the peptone water and yeast extract in a Laminar flow cabinet.

Table 2.4 *Composition of basal medium (Hughes et al., 2008)*

Ingredient	**Quantity per 1.5L**
Peptone Water	3.0g
Yeast Extract	3.0g
Sodium chloride	0.15g
Dipotassium hydrogen orthophosphate	0.06g
Magnesium sulphate 7-hydrate	0.015g
Calcium chloride dihydrate	0.01g
Sodium bicarbonate	3.0g
Haemin	0.0075g (Dissolved in 2 drops NaOH 1M)
L-cysteine HCl	0.75g
Bile salts	0.75g
Polysorbate 80	3.0ml
Vitamin K	15µl
Resazurin solution 0.025% (prepared in DI water)	6.0ml

Freshly voided human faeces from volunteers on no medication and who had not taken antibiotics in the previous 6 months were used to prepare the faecal slurry. In an anaerobic workstation, maintained at 37°C with a relative air humidity of 70%, the faecal material was diluted with phosphate buffered saline (PBS) pH 6.8±0.05 solution to obtain a 40% w/w slurry. The slurry was homogenised with an Ultra Turrax (IKA T18 Basic) at a speed of 18,000rpm/min until no large solid agglomerates could be seen.

The slurry was sieved through an open mesh fabric (Sefar Nitex^{TM}, pore size 350µm) to remove any unhomogenised fibrous material. The basal media was then added to the faecal slurry to achieve a 1:1 dilution. This slurry was then used to test the stability of lactase.

2.4.3 Lactase supplements

Lactase supplements, listed in table 2.3, were tested for residual lactase activity following exposure to simulated gastric and small intestinal environments using the USP (724) method for testing drug release from enteric coated articles. The supplements, in triplicate, were placed in 750ml 0.1N HCl, pH 1.2 ± 0.05, for 2 hours at 37±0.5°C, stirring at 50rpm to simulate gastric conditions in a USP II paddle apparatus dissolution bath. After 2 hours 250ml of pre warmed, to 37°C, 0.2M tribasic sodium phosphate was added and the pH adjusted to pH 6.8 ± 0.05 to simulate the transition into the small intestine. Stirring continued at 50rpm for a further 45 minutes and was increased to 250rpm for the final 30 seconds. Samples were removed, filtered using 0.45µm Millex filters and tested for lactase activity using the ONPG assay.

Each lactase supplement was also placed in 1 litre of pH 6.8 phosphate buffer for 2 hours and 45 minutes and stirred at 50rpm, increasing to 250rpm for the final 30 seconds. The temperature was 37°C throughout. This was done to determine the total active lactase released from each supplement in simulated small intestinal conditions and compare this to active lactase released when the supplements were first placed in acid. All tests were carried out in triplicate. Samples were filtered using 0.45µm Millex filters and tested for lactase activity using the ONPG assay.

Units of active lactase per tablet were calculated using a standard curve of measured lactase activity in pH 6.8 phosphate buffer. The difference in active lactase remaining following dissolution in 0.1N HCl shifting to pH 6.8 phosphate buffer compared to incubation in pH 6.8 phosphate buffer was calculated by subtracting the former from the latter. The percentage loss in lactase activity was calculated using the following equation:

$$\text{Lactase activity loss (\%)} = \frac{(Lactase\ in\ pH\ 6.8 - Lactase\ in\ pH 1.2-6.8)(units)}{Lactase\ in\ pH\ 6.8\ (units)} \text{ x100}$$

2.4.4 Self emulsifying dosage form with soy bean oil

Oil based dosage forms are frequently used for oral nutritional supplements including lactase. These are administered as a pre-concentrate consisting of a drug, oil, surfactant and co surfactant that form an oil in water (o/w) emulsion when dispersed in the GI tract. These formulations are known as self emulsifying drug delivery systems (SEDDS). The emulsion formed may offer protection to the drug by forming an oily barrier between it and the acidic pH of the stomach and GI enzymes. A water in oil microemulsion of insulin provided *in vitro* stability enhancement in simulated gastric fluid with pepsin (Toorisaka et al., 2005).

An oil based formulation of lactase was created by homogenising soy bean oil (2g), soy lecithin (400mg) and lactase (400mg) together at 24,000rpm for 1 minute using an Ultra Turrax T25 Ika-Werke small probe emulsifier to re-create an existing oral lactase supplement. During emulsification the mixture was placed on ice to prevent lactase denaturation caused by increasing heat. The appearance of the formulation upon mixing with aqueous media (pH 6.8 phosphate buffer) was visualised using a light microscope.

Active lactase content of the formulation was determined by placing 10mg of the liquid in 10ml pH 6.8 ± 0.05 phosphate buffer. Samples were placed in a Gallenkamp shaking incubator at 100rpm, 37±0.5°C for 1 hour. Samples were taken and diluted 25 times with pH 6.8 phosphate buffer and analysed for active lactase content using the ONPG test. Loading was determined by calculating the amount of active lactase (mg) per mg of SEDD formulation. The loading efficiency was calculated using the equation below. The total active lactase is the amount of lactase theoretically present if all added initially was incorporated into the SEDD formulation without any losses.

$$\text{Loading efficiency (\%)} = \frac{Measured\ active\ lactase}{Total\ initial\ lactase} \times 100$$

The ability of this formulation to protect lactase in acid was assessed by placing 25mg in 7.5ml of 0.1N HCl, pH 1.2 ± 0.05, for 2 hours. These samples were agitated in a shaking incubator at 100rpm and 37±0.5°C. After 2 hours 2.5ml of 0.2M tribasic sodium phosphate was added to raise the pH to 6.8 ± 0.05.

The samples were replaced in the Gallenkamp shaking incubator and agitated using the same parameters for a further 45 minutes. Samples were withdrawn and tested for active lactase release using the ONPG assay. Active lactase release was calculated using the equation below. The theoretical active lactase is all the lactase that could potentially be released from the formulation.

$$\text{Active lactase release (\%)} = \frac{Measured\ active\ lactase\ in\ sample}{Theoretical\ active\ lactase\ in\ sample} \times 100$$

The percentage of active lactase released from this formulation was compared to that from the Holland and Barrett lactase supplement using one way ANOVA to assess if there was a significant difference (Minitab 15). Results were considered significant if $p \leq 0.05$.

2.4.5 Enteric coated lactase tablets

Tablets were prepared by dry blending microcrystalline cellulose (Avicel), a filler and compression aid, (77%, 30.8g), croscarmellose sodium (AcDiSol), a disintegrant, (2%, 0.8g) and lactase (20%, 8g) for 10 minutes using a Pascall Engineering roller mixer. Magnesium stearate, a lubricant, (1%, 0.4g) was then added and blended for 1 minute. Placebo tablets were made by substituting lactase for microcrystalline cellulose (97%, 38.8g). The powder mix was fed into a single punch tabletting machine (Manesty, Speke, UK) fitted with a biconvex 8mm punch and die set (Holland, Nottingham, UK). The force used to make the tablets was 35kN for placebo tablets (without lactase) and 31.5kN for lactase tablets. The crushing strength of the tablets was measured using a Copley tablet hardness tester, acceptable limits were >80, <150N. Tablets were weighed and the height of the punches adjusted to gain the desired tablet weight of 200mg (40mg lactase).

Tablets (40g) were coated with the enteric polymer Eudragit L100 using a Strea-1 bottom spray fluidised bed spray coater (Aeromatic AG, Bubendorf, Switzerland). The coating solution, with a solids content of 10% w/w, consisted of Eudragit L100 (7g) dissolved in ethanol (77g) with a plasticizer, triethyl citrate (1.4g, 20% w/w of the dry polymer) and glidant, glyceryl monostearate (GMS) (0.35g, 5% w/w of the dry polymer) with polysorbate 80 (0.14g, 2% w/w of the dry polymer).

A 10% GMS dispersion was prepared by adding 7.5g GMS (10% w/w) and 3g polysorbate 80 (40% w/w based on GMS) to 64.5g water (86% w/w). This was stirred and heated to 70-80°C until the solution cleared. The dispersion was cooled to room temperature and added to the Eudragit L100 solution.

The coating solution was sprayed at a rate of 0.25g/min using an atomising pressure of 0.2 bar fed by a Minipuls 3 Gilson peristaltic pump. The fan capacity of the coating machine was 16.5 (air flow 165m^3/hour) and the drying temperature was 30°C. Coating commenced and at regular intervals tablets were removed and weighed to determine the coating extent. A weight gain of 8.89mg per tablet was required (5mg/cm^2) for effective enteric coating. Once this level of coating had been achieved coating was halted and the tablets further fluidised for 15 minutes in the coater to dry. The tablets were cured in a Gallenkamp incubator at 30°C overnight. The tablets were weighed again to determine the final weight gain and amount of polymer applied per cm^2.

2.4.5.1 Active lactase content of tablets

Lactase tablets (200mg), both coated and uncoated, were placed in 1 litre of pH 6.8 ±0.05 phosphate buffer in USP II paddle dissolution apparatus. Samples were stirred at 50rpm at 37°C for 2 hours and 45 minutes, increased to 250rpm for the final 30 seconds. Samples were taken at the end of this period and active lactase content of the tablets was determined using the ONPG assay. All tests were carried out in triplicate. The amount of active lactase (mg) per tablet was calculated using a calibration curve of lactase in pH 6.8 phosphate buffer. The loading efficiency was calculated using the equation below. The total active lactase is the amount of lactase which would be present in the tablets if all the lactase was successfully loaded into the tablets.

$$\text{Loading efficiency (\%)} = \frac{Measured\ active\ lactase\ (mg/tab)}{Total\ active\ lactase\ (mg/tab)} \times 100$$

2.4.5.2 *In vitro* release from enteric lactase tablets

The USP II paddle apparatus was employed to assess the enteric behaviour of the coated and uncoated tablets using the USP (724) method for testing drug release from enteric coated articles.

Coated and uncoated lactase tablets (200mg) were placed in vessels containing 750ml of 0.1N HCl, pH 1.2±0.05, for 2 hours, at 37±0.5°C, and stirred at 50rpm to simulate gastric conditions. After 2 hours 250ml of 0.2M tribasic sodium phosphate pre-equilibrated to 37±0.5°C was added to each vessel to raise the pH to 6.8±0.05 to simulate small intestinal conditions. Dissolution proceeded for a further 45 minutes and the paddle speed was increased to 250rpm for the final 30 seconds. Samples were taken after 60 and 120 minutes acid incubation and 0, 10, 20, 30 and 45 minutes pH 6.8 phosphate buffer incubation. Samples were diluted with pH 6.8 phosphate buffer if necessary prior to testing. Samples were tested for active lactase release using the ONPG assay. All tests were carried out in triplicate. Active lactase release was calculated using the equation below. The theoretical active lactase concentration is the concentration of lactase in the sample if all the loaded lactase was released from the tablets.

$$\text{Active lactase released (\%)} = \frac{Measured\ active\ lactase\ concentration\ in\ sample\ (mg/ml)}{Theoretical\ active\ lactase\ concentration\ (mg/ml)} \times 100$$

2.4.6 Preparation of enteric lactase microparticles

This method is based on that of Kendall *et al*. with the substitution of prednisolone for lactase. Eudragit L100 (3g) was dissolved in ethanol (30ml). Lactase (200mg) was suspended in the ethanol to prepare microparticles with a drug to polymer weight ratio of 1:15. This suspension of lactase in Eudragit L100 solution was emulsified into liquid paraffin (200ml) containing 1% (w/w) of sorbitan sesquioleate (Arlacel 83) as an emulsifying agent, using a Heidolph RZR1 stirrer (5cm diameter propeller) at 1000rpm. Stirring was carried out for 18 hours at room temperature to allow solvent evaporation and particle solidification. The microparticles formed were recovered by vacuum filtration through a Pyrex sintered glass filter (pore size 4; 5-15µm) and washed three times with *n*-hexane (50ml). Blank microparticles containing no lactase were also prepared using the same parameters. All microparticle formulations were prepared in triplicate.

2.4.6.1 Particle size and yield

The volume median diameter of the microparticles was measured using laser light scattering with a Malvern Mastersizer X with a 45mm lens (Malvern Instruments Ltd., Malvern, UK). The microparticles were suspended in 0.1N HCl and added dropwise into the magnetically stirred small volume diffraction chamber, containing 0.1N HCl until obscuration of 15-20% was achieved. Particle size analysis of each formulation was carried out in triplicate, and the polydispersity (span) was calculated as [D(v, 0.9)-D(v, 0.1)]/D(v, 0.5) which are the particle diameters at the 90^{th}, 50^{th} and 10^{th} percentile respectively of the particle size distribution curve.

The yield of particle production was calculated using the equation below. The theoretical mass of microparticles expected is the total mass of polymer and, if used, of lactase utilized to produce the microparticles.

$$\text{Yield (\%)} = \frac{observed\ mass\ of\ microparticles\ produced}{theoretical\ mass\ of\ microparticles\ expected} \times 100$$

2.4.6.2 Particle morphology

The morphology and size of the microparticles were examined by scanning electron microscopy (SEM) under a Philips Quanta 200F, Eindhoven, The Netherlands. Microparticles were fastened on a SEM stub using carbon adhesive pads and then coated with gold using an Emitech K550 sputter coater. Routine, high vacuum imaging at 5kV was used to reveal surface morphology of the microparticles.

The microparticles were visualized again using SEM at a lower voltage of 1kV and under a reduced vacuum. The particles were not sputter coated. These conditions were used to minimize the loss of any morphological details of the microparticles caused by sputter coating and using a higher voltage beam.

The morphology of the blank microparticles was visualized to an even greater degree of detail by employing an SEM with a new type of back scatter secondary electron detector (directional backscatter detector) that enabled the use of a low voltage beam, 500V and 300V, which revealed further surface detail not visible when using a high voltage beam (FEI, Eindhoven).

This new detector has a better efficiency at low voltage and more surface detail can be revealed than conventional low vacuum imaging using the large field detector or gaseous detection. The microparticles were imaged before and after being placed in 0.1N HCl.

2.4.6.3 Encapsulation efficiency and active lactase loading

300mg of lactase microparticles were filled into gelatin capsules and placed in 1 litre of pH 6.8±0.05 phosphate buffer for 45 minutes at 37±0.5°C using a USP II paddle apparatus dissolution bath. Paddle speed was 50 rpm and increased to 250rpm for the final 30 seconds. Samples were withdrawn and tested for lactase activity using the ONPG assay. The amount of active lactase (units) released was used to calculate the encapsulation efficiency of active lactase in the microparticles using the equation below. The total units of lactase added was the amount of lactase used to produce the microparticles. Drug loading was determined by measuring the units or mgs of active lactase per mg of microparticles.

Encapsulation efficiency (%) $= \frac{Measured\ units\ of\ lactase}{Total\ units\ of\ lactase\ added}$ x100

2.4.6.4 Differential scanning calorimetry (DSC)

A DSC 7 differential scanning calorimeter (Perkin Elmer Instruments) calibrated with indium was used to assess the thermal behaviour of lactase alone, blank Eudragit L100 microparticles and lactase loaded Eudragit L100 microparticles. Samples (2-4mg) were accurately weighed and placed in a non-hermetic aluminium pan. Pyris Thermal Analysis Software was used to record and analyse the data. Modulated DSC was conducted on the samples starting at -20°C and ending at 300°C at a heating rate of 3°C/minute, period of 60 seconds and an amplitude of ± 1°C.

2.4.6.5 Enteric protection- *in vitro* release of active lactase

USP II paddle apparatus was employed to determine if the microparticles could prevent lactase release and protect its activity in simulated gastric conditions. The microparticles were placed in 750ml 0.1N HCl, pH 1.2±0.05, for 2 hours at 37°C and 50rpm stirring speed to simulate gastric conditions.

The pH was raised to 6.8±0.05 to mimic the small intestine and dissolution proceeded for a further 45 minutes. This was done by adding 250ml of 0.2M tribasic sodium phosphate. The paddle speed was raised to 250rpm for the final 30 seconds. Samples were withdrawn and the amount of active lactase determined by the ONPG assay. All tests were carried out in triplicate. Active lactase release was calculated using the equation below. The theoretical active lactase is the amount of lactase expected if all the encapsulated lactase was released and was active.

$$\text{Active lactase release (\%)} = \frac{Measured\ active\ lactase\ in\ sample}{Theoretical\ active\ lactase\ in\ sample} \times 100$$

2.4.7 Spray drying lactase

To minimize uncontrolled release of lactase from Eudragit L100 microparticles in acid its particle size was reduced by spray drying. Drugs previously exhibited burst release, 30%+, from Eudragit L100 nanoparticles at pH 1.2 (Devarajan and Sonavane, 2007, Eerikainen et al., 2004, Raffin, 2006). Reduction of drug particle size increased encapsulation efficiency and reduced burst release (Thote and Gupta, 2005). Spray drying can be used to produce protein particles of a controlled size and shape. Despite the use of high temperatures the cooling effect of solvent evaporation during spray drying protects the protein. Spray drying the protein bovine serum albumin reduced its *in vitro* burst release from PLGA microspheres by 60% (Costantino et al., 2000). Lactase particle size has previously been successfully reduced to 2-4µm by spray drying (Broadhead et al., 1994).

Lactase was dissolved in DI water and spray dried using a Buchi mini spray dryer B-191 to reduce its particle size using these conditions:

Aspirator: 85%

Inlet Temperature: 190°C

Pump: 3ml/min (15%)

To determine the lactase activity of the spray dried samples they (10mg) were dissolved in DI water (200ml). The ONPG assay was used to assess lactase activity and this was compared to a calibration curve of lactase.

The following equation was used to determine the activity of spray dried lactase (units/mg) relative to non-spray dried lactase (9.9 units/mg).

$$\text{Units/mg spray dried lactase} = \frac{Absorbance\ spray\ dried\ lactase}{Absorbance\ of\ non\ spray\ dried\ lactase} \times 9.9\ \text{units/mg}$$

The morphology and size of the spray dried and non-spray dried lactase was visualized using SEM. Spray dried and non-spray dried lactase were dispersed in ethanol and then filtered by vacuum filtration through a Pyrex sintered glass filter (pore size 4; 5-15μm). Their morphology was then examined again. Spray dried lactase was encapsulated within Eudragit L100 microparticles using the method described above for non-spray dried lactase. Differences in encapsulation efficiency compared to non spray dried lactase were assessed by one-way ANOVA and deemed to be significant if $p \leq 0.05$, Minitab 15.

2.4.8 Fluorescent labeling and visualization of lactase

Lactase-FITC conjugation was carried out to visualise its location in the microparticles. 1mg/ml FITC was prepared in anhydrous DSMO (1ml) and 800μl of this was added to 10ml of 1mg/ml Enzeco lactase solution prepared in sodium bicarbonate buffer. Sodium bicarbonate buffer was prepared by dissolving 17.3g $NaHCO_3$ and 8.6g of Na_2CO_3 in 1 litre of DI water. The FITC lactase mixture was magnetically stirred wrapped in foil and in the dark for two hours. This was injected into a dialysis cassette which was placed in a large beaker of DI water and magnetically stirred in the dark. The water was changed regularly and dialysis proceeded overnight.

The dialysed FITC-lactase conjugation was freeze dried with a Virtis Advantage freeze drier. Initially the sample was frozen at -38ºC for 2 hours. It then was subjected to two drying phases; primary drying (sublimation) and secondary drying (desorption). Both drying phases were conducted under vacuum at a pressure of 200 mTorr. The primary drying phase proceeded at -30ºC for 2 hours and at -10ºC for 2 hours. The shelf temperature of the freeze dryer was at 0ºC for 20 hours during the secondary drying phase.

The freeze dried FITC-lactase conjugation was added to 3g of Enzeco lactase and dissolved in 200ml of DI water.

This was spray dried using an inlet temperature of 190°C, 85% Aspirator, 15% pump setting (3ml/min) using a Buchi mini spray dryer B-191. Eudragit L100 microparticles were prepared as before but in darkness.

The microparticles were visualised using CFLM, using a Zeiss LSM 510 microscope, to visualise the fluorescent protein in and on the microparticles . A Z-section was used to visualise FITC-lactase throughout the microparticles. Eudragit L100 alone was also visualised to ensure it produced no fluorescence.

2.4.9 Active lactase burst released from microparticles below pH 6

To quantify differences in burst release of non-spray dried and spray dried lactase from Eudragit L100 microparticles below the pH threshold of the polymer the particles were dispersed in pH 4.5 buffer. At this pH any released lactase will be active and therefore can be quantified using the ONPG method.

Gelatin capsules were filled with 30mg of microparticles encapsulating unmodified lactase or spray dried lactase. These were placed in 75ml of pH 4.5±0.05 buffer and stirred on a magnetic stirrer for 2 hours, 2 ml was withdrawn to detect active lactase release using the ONPG assay. 2ml of pH 4.5 buffer was added to the reaction mixture to replace the withdrawn sample and then 25ml of 0.2M tribasic sodium phosphate was added to raise the pH to 6.8±0.05. Stirring continued for 45 minutes then samples were withdrawn and analysed for active lactase release. Differences in lactase release from the two sets of microparticles were assessed for significance using one-way ANOVA and judged to be significant if $p \leq 0.05$, Minitab 15.

pH 4.5 buffer was prepared by mixing 470ml of 0.1M citric acid with 530ml of 0.1M trisodium citrate and adjusting the pH to pH 4.5±0.05 using concentrated HCl and 5N NaOH. 0.1M citric acid was prepared by dissolving 21.01g of citric acid in 1 litre of DI water. 0.1M trisodium citrate was prepared by dissolving 29.41g of trisodium citrate dihydrate in 1 litre of DI water.

2.4.10 Encapsulation of pH sensitive marker

The interior pH of microparticles has been monitored previously using pH sensitive markers.

Lysosensor yellow/blue® dextran (Ding and Schwendeman, 2008) was encapsulated in PLGA microspheres to detect increasing interior acidity from degrading PLGA. Confocal laser scanning microscopy was used to detect shifts in the marker's emission spectrum to different wavelengths depending on the pH.

Lysosensor yellow/blue dextran 10kDa was encapsulated within Eudragit L100 microparticles to monitor the interior pH. 5mg of lysosensor was suspended in the Eudragit L100 solution in ethanol (300mg in 3ml) prior to adding to 20ml liquid paraffin with 1% sorbitan sesquioleate. This oil in oil emulsion was stirred at 1500rpm for approximately 18 hours at room temperature to allow solvent evaporation and particle solidification. The microparticles formed were recovered by vacuum filtration as before and washed three times with *n*-hexane (50ml). The reaction mixtures were covered and kept in the dark throughout.

The microparticles were visualised with CFLM, using a Zeiss LSM 710 microscope, using a 10x lambda scan to detect the effect of surrounding pH on the encapsulated contents. The excitation wavelength used was 405nm. Microparticles were visualised dry, with 0.1N HCl and pH 6.8 phosphate buffer and their emission over wavelengths 430-720nm monitored.

2.4.11 Cryosectioning microparticles

Blank microparticles were glued to an AFM sample holder with cyanacrylate glue. These were trimmed with a cryotrim 45 diamond blade and then sectioned with an ultrasonic knife, feed 30nm, at a sectioning speed of 0.6mm/sec on water. The sections were picked up with a Perfect Loop and mounted on C-flat TEM grids. The carbon film of the grid contained 2µm holes. This was performed by Diatome Ltd, Switzerland. The sections were then examined by transmission electron microscopy. The sectioned microparticles were also examined by SEM.

2.4.12 Surface area analysis

The surface area of the Eudragit L100 microparticles without lactase (blank), with spray dried lactase and non-spray dried lactase was calculated using the mean dv0.5 size as the diameter of the microparticles.

This was divided by two to give the radius and this value used in the following equation to calculate the surface area of each sphere:

$A=4\pi r^2$

Where A is area of the sphere and r radius of each sphere.

BET was also used to determine the surface area of the microparticles. Surface area was measured using a Micromeritics TriStar 3000 by Agenda1 Analytical Services, Bradford. Molecules of an adsorbate gas were physically adsorbed onto the particle surfaces, including the internal surfaces of any pores, under controlled conditions within a vacuum chamber. An adsorption isotherm was obtained by measuring the pressure of the gas above the sample as a function of the volume of gas introduced into the chamber. The linear region of the adsorption isotherm was then used to determine the volume of gas required to form a monolayer across the available particle surface area, using BET theory, as described by the following equation

$$\frac{1}{v\left[\left({}^{P}/_{P_0}\right)-1\right]}=\frac{c-1}{v_m}\left(\frac{P}{P_0}\right)+\frac{1}{v_m c}$$

where v is the volume of gas, P is the pressure, P0 is the saturation pressure, vm is the volume of gas required to form a monolayer and c is the BET constant. Plotting relative pressure, φ (=P/P0), and volume allows the volume of a monolayer to be determined from the gradient and intercept of the line. The specific surface area can then be calculated using the cross sectional area of the gas molecules, the molecular volume of the gas and the weight of the sample.

The specific surface area can also be calculated from the results of a laser diffraction measurement using the following equation:

$$SSA=\frac{6\sum\frac{V_i}{d_i}}{\rho\sum V_i}=\frac{6}{\rho D[3,2]}$$

Vi is the relative volume in class i with a mean class diameter of d_i, ρ is the density of the material, and D[3,2] is the surface area weighted mean diameter. This was carried out automatically using the laser diffraction system software during microparticle size analysis using the Malvern Mastersizer. In carrying out this calculation, it is assumed that the particles are perfectly smooth, solid spheres.

2.4.13 Enteric microparticles with antacids

Antacids have previously been administered with acid labile drugs to raise gastric pH and maintain drug activity. Antacids co-administered with pancreatic enzyme supplements increased pH and enzyme activities *in vivo* (Graham, 1982). The acidic degradation products of PLGA nanoparticles can decrease the pH of the interior of the particles and destabilize acid labile peptides and proteins. To overcome this antacids were encapsulated in PLGA nanoparticles with insulin and this reduced its degradation in SGF and increased its oral bioavailability in rats (Sharma et al., 2012). The ability of co-encapsulated antacids to raise the interior pH of PLGA microspheres was detected by shifts in the emission spectrum of an encapsulated marker (Li and Schwendeman, 2005).

Co-encapsulation of antacids in enteric microparticles may provide additional protection to encapsulated lactase. Acid may encounter lactase near the surface of microparticles, antacids can provide local neutralization and increase lactase preservation during gastric transit.

Enteric microparticles with Eudragit L100 were prepared as described above with the addition of an antacid (1g) to the Eudragit L100 solution. The antacids used were magnesium hydroxide and sodium bicarbonate. They were suspended in the solution of the polymer prior to addition of lactase. The yield, size and morphology of the microparticles were evaluated as described before.

To determine the active lactase content of the microparticles, 10mg of lactase microparticles with co-encapsulated antacid were placed in 10ml of phosphate buffer, pH 6.8±0.05. Samples were placed in a Gallenkamp shaking incubator at 37±0.5°C and agitated at 100rpm for one hour.

At the end of this period samples were diluted 20 times with pH 6.8 phosphate buffer and the active lactase content determined by the ONPG assay. Loading and encapsulation efficiency were determined as in section 2.4.6.3.

The ability of these microparticles to protect encapsulated lactase in gastric conditions was assessed by placing 100mg of microparticles in 7.5ml of 0.1N HCl, pH 1.2±0.05, for 2 hours. These samples were agitated in a Gallenkamp shaking incubator at 100rpm and 37±0.5°C. After 2 hours 2.5ml of 0.2M tribasic sodium phosphate was added to raise the pH to 6.8 ± 0.05. The samples were replaced in the incubator and agitated for a further 45 minutes. Samples were withdrawn and tested for active lactase content using the ONPG assay. The samples from the microparticles containing magnesium hydroxide were diluted 10 times prior to testing. Active lactase release was calculated as in section 2.4.6.5.

Differences in particle size, span, encapsulation efficiency and active lactase release following dissolution of the lactase microparticles with antacids compared to those without were assessed by one-way ANOVA, Minitab 15. Results were considered to be significant if $p \leq 0.05$. Differences in active lactase release from the microparticles with antacids were also compared to the Holland and Barrett lactase supplement.

2.4.14 Self emulsifying dosage forms with soy bean oil and lactase microparticles

An oil based formulation of lactase Eudragit L100 microparticles was created by homogenising soy bean oil (2g), soy lecithin (400mg) and lactase microparticles (500mg) together at 24,000rpm for 1 minute using an Ultra Turrax T25 Ika-Werke small probe emulsifier. During emulsification the mixture was placed on ice.

Active lactase content was determined by placing 10mg of the liquid in 10ml pH 6.8±0.05 phosphate buffer. Samples were placed in a Gallenkamp shaking incubator at 100rpm and 37±0.5°C for 1 hour. Samples were taken, diluted 25 times with pH 6.8 phosphate buffer and analysed for active lactase content using the ONPG test. Loading and encapsulation efficiency were determined as in section 2.4.6.3.

The ability of this formulation to protect lactase in acid was assessed by placing150mg in 7.5ml of 0.1N HCl, pH 1.2±0.05, for 2 hours. These samples were agitated in a shaking incubator at 100rpm and 37±0.5°C. After 2 hours 2.5ml of 0.2M tribasic sodium phosphate was added to raise the pH to 6.8±0.05. The samples were replaced in the incubator and agitated for a further 45 minutes. Samples were withdrawn and tested for active lactase release using the ONPG assay. Active lactase release was calculated as in section 2.4.6.5. Active lactase release was compared to release from lactase in soy bean oil, lactase microparticles and lactase microparticles with co-encapsulated magnesium hydroxide after dissolution using one-way ANOVA, Minitab 15. Differences were judged to be significant if $p \leq 0.05$.

2.5 Results and Discussion

2.5.1 Intestinal stability

Lactase stability was assessed in simulated and pig gastrointestinal fluids and human faecal fluids. The results were used to design oral formulations of lactase.

2.5.1.1 Gastric fluids

Lactase was rapidly denatured in SGF, pH 1.2, without pepsin, table 2.5. After 5 minutes less than 1% of the initial lactase was active. Similarly lactase activity was quickly reduced in porcine gastric fluid, only 6% remaining after 5 minutes. Lactase was not tested in SGF with pepsin as it was completely denatured in SGF alone.

Table 2.5 *Active lactase recovery after incubation in SGF and porcine gastric fluids. Data represents means ± standard deviation (SD).*

Sample (minutes)	Active lactase recovered (%) SGF no pepsin	Active lactase recovered (%) pig gastric fluid
0	3.7 ± 5.2	38.9 ± 6.1
5	0.3 ± 0.1	6.2 ± 2.4
10	0.4 ± 0.1	5.6 ± 0.3
20	0.3 ± 0.4	7.5 ± 3.3
30	0.3 ± 0.3	4.9 ± 0.7
60	-0.5 ± 0.8	4.7 ± 0.5
90	-1.2 ± 0.8	3.6 ± 0.4
120	-1.4 ± 0.9	4.5 ± 2.5

2.5.1.2 Small intestinal fluids

Lactase incubation in simulated and porcine small intestinal fluids resulted in minimal lactase degradation, table 2.6.

Lactase stability with pancreatin has previously been shown (O'Connell and Walsh, 2006). Active lactase recovery was actually above 100% in many samples. This could be due to the method used to assess active lactase recovery. The ONPG reaction is based on the catalytic conversion of ONPG to yellow coloured ONP. Possibly the pancreatin and intestinal enzymes in the small intestinal samples were also able convert ONPG to ONP giving an inflated value for active lactase recovery. Lactase is a small intestinal enzyme so it may also have been present in the small intestinal fluids increasing the amount of active lactase measured in the sample. O'Connel and Walsh also detected similarly high (>100%) levels of lactase activity after incubation of lactase supplements in simulated intestinal fluid (O'Connell and Walsh, 2006).

Table 2.6 *Active lactase recovery after incubation in SIF and porcine small intestinal fluids. Data represents means ± SD.*

Sample (mins)	**Active lactase (%) SIF**	**Active lactase (%) SIF pancreatin**	**Active lactase (%) duodenal fluid**	**Active lactase (%) jejunal fluid**	**Active lactase (%) ileal fluid**
0	104.7 ± 4.8	125.2 ± 9.4	114.2 ± 1.4	109.8 ± 1.8	108.3 ± 2.7
5	106.8 ± 7.7	128.3 ± 1.3	109.4 ± 7.9	109.3 ± 1.5	102.6 ± 3.8
10	104.1 ± 3.0	133.4 ± 2.6	109.7 ± 1.4	104.4 ± 0.7	99.5 ± 7.7
20	110.3 ± 13.3	136.4 ± 4.2	105.1 ± 4.8	102.5 ± 1.1	101.2 ± 8.7
30	107.8 ± 3.0	138.6 ± 2.9	100.5 ± 9.2	105.3 ± 2.6	103.6 ± 5.1
60	106.8 ± 8.4	143.6 ± 2.5	88.1 ± 3.5	77.3 ± 2.6	106.3 ± 7.7
90	112.7 ± 5.7	146.1 ± 5.9	86.4 ± 7.1	74.3 ± 3.8	103.0 ± 8.1
120	96.1 ± 5.7	132.9 ± 23.6	98.8 ± 5.5	97.7 ± 1.7	96.0 ± 1.6

There was also greater than100% active lactase recovery following incubation in SIF without pancreatin. Possibly this fluid provides a more conducive environment for lactase activity than the pH 6.8 sodium phosphate buffer which the lactase standards were prepared in. Variations in lactase activity may have also arisen due to temperature variation on different days of testing, increasing or decreasing the rate of the enzymatic reaction, and the time between removing the sample and analysing its UV absorbance. Theoretically the reaction should be immediately halted by the addition of sodium carbonate but this may not have been immediately effective. Another variability factor could be the viscosity of the small intestinal fluids which may have caused uneven lactase distribution giving rise to varying results in sampled active lactase.

2.5.1.3 Colonic fluids

Lactase activity was not diminished by incubation in porcine colonic and human faecal fluids, table 2.7. As with the small intestinal fluids there was a greater than 100% recovery of active lactase. This again could be due to the enzymatic activity of these samples.

Table 2.7 *Active lactase recovery after incubation in porcine colonic and human faecal fluids. Data represents means ± SD.*

Sample (minutes)	**Active lactase recovered (%) pig colonic fluid**	**Active lactase recovered (%) human faecal slurry**
0	124.2 ± 4.8	98.3 ± 3.1
5	121.6 ± 8.6	121.9 ± 22.6
10	106.7 ± 5.6	111.1 ± 10.4
20	112.9 ± 13.7	111.2 ± 17.0
30	120.2 ± 8.0	144.0 ± 3.1
60	132.5 ± 5.4	119.3 ± 4.6
90	129.9 ± 15.0	133.0 ± 6.9
120	148.1 ± 5.0	132.8 ± 3.4

2.5.1.4 Overall intestinal stability

Lactase is completely denatured by the acidic pH of the gastric environment, figure 2.2. This result is in agreement with Alavi *et al* who found a greater than 90% loss of lactase activity after incubation at pH 1.2 (Alavi et al., 2002). In the small and large intestine it retains its activity, figure 2.2. As it is a small intestinal enzyme it is logical that it should be stable and active in the small and large intestinal environment.

The acidic pH of the gastric environment may alter the ionisation of its constituent amino acids and this would disrupt the bonds holding together its secondary, tertiary and quaternary structure. The disassociation of the lactase tetramer into dimers removes critical elements of the active site resulting in a lack of enzymatic activity. This loss of activity in gastric conditions has been observed previously with similarly large proteins such as ovomucoid (Zheng et al., 2010), chicken egg yolk immunoglobulin (Li et al., 2009) and other digestive enzymes (Scocca et al., 2007, Massicotte et al., 2008). Possibly their higher structures render them more susceptible to acidic pH than peptides with a less complex structure.

The stability of lactase in small and large intestinal fluids may be a result of the complexity of its structure. Its polypeptide chains are involved in secondary, tertiary and quaternary structures which may make individual peptide bonds less accessible to digestive enzymes. However if entering the small intestine after the stomach lactase may have been sufficiently unfolded by the gastric pH to expose its primary structure and peptide bonds to enzymatic digestion.

Figure 2.3 maps the overall lactase stability throughout the GI tract showing where it needs to be protected. Oral delivery strategies for lactase should protect it from the acidic pH of the stomach and release it in the favourable environment of the small intestine.

To digest lactose locally, in the intestine, it should be taken just before or with lactose containing food. The lactase should be released from the stomach into the small intestine with food in an active form so that it can begin digestion of lactose immediately and completely. Any delay may result in no or incomplete lactose digestion causing the symptoms associated with lactose intolerance.

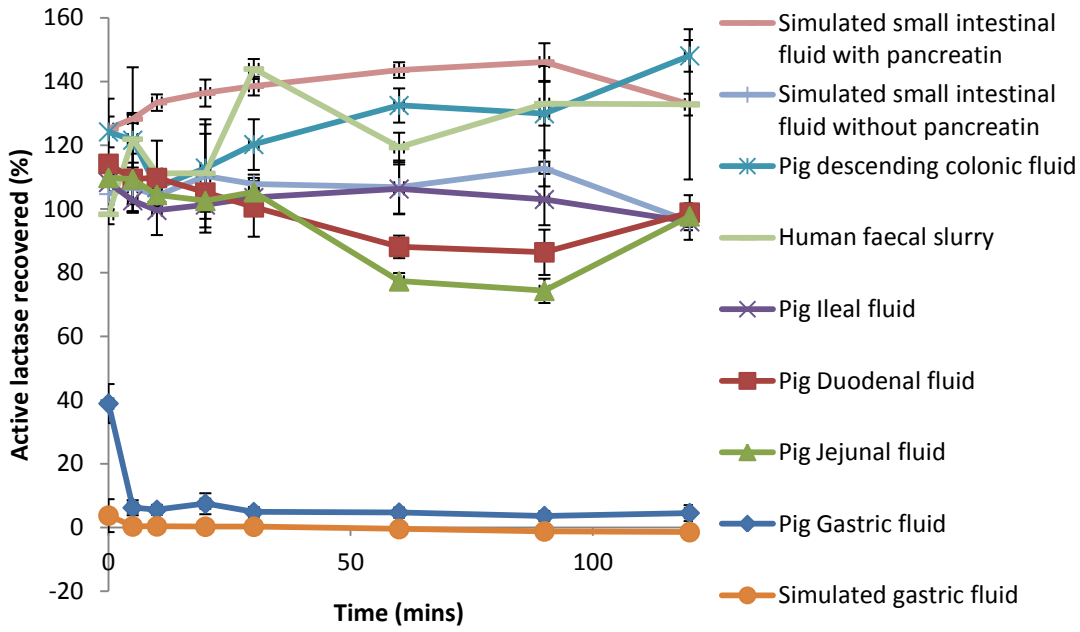


Figure 2.2 *Active lactase recovered after incubation in simulated, porcine and human intestinal fluids. Error bars show mean ± SD.*

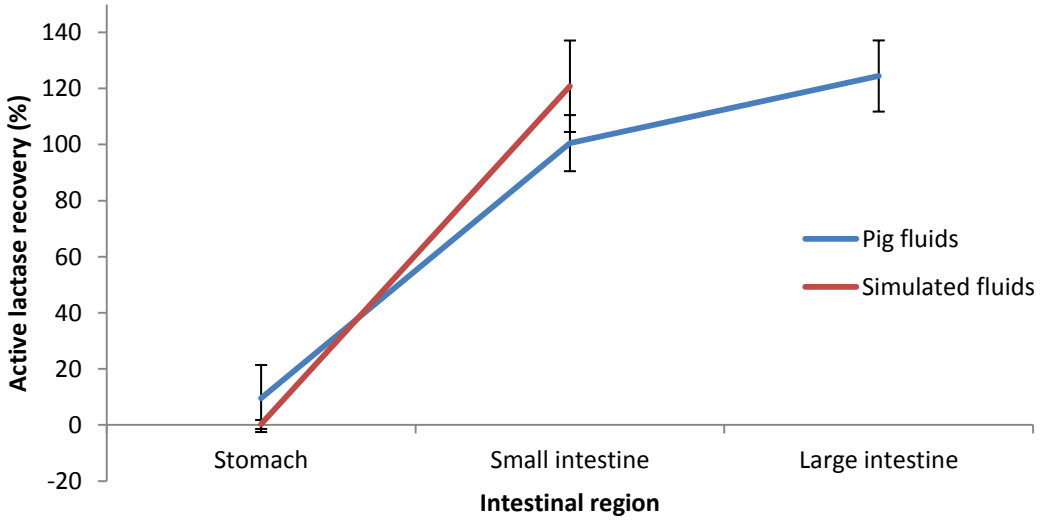


Figure 2.3 *Map of the stability of lactase throughout the gastrointestinal tract from studies in simulated and porcine intestinal fluids. Error bars show mean ± SD.*

2.5.2 Lactase supplements

After elucidating the stability of lactase in the intestinal fluids, and thus the requirements for an oral formulation of lactase, an investigation into currently available oral lactase supplements was conducted. The aim of this was to discover if these supplements were able to overcome the gastric stability barrier and what aspects of their formulations enable them to do this.

Oral lactase supplements for lactose intolerance sufferers were tested in conditions which simulate gastric pH and the transition to small intestinal pH to predict their *in vivo* behaviour. Supplements were only exposed to simulated gastric and intestinal media without enzymes as the stability studies demonstrated that it was the acidic pH alone which caused lactase denaturation.

For all oral lactase supplements there was a greater than 95% loss of active lactase after dissolution in 0.1N HCl prior to pH increase to pH 6.8, table 2.8 and figure 2.4. The Holland and Barrett supplement had the most active lactase recovered after acid incubation, 4.5% remained. However for all other supplements tested there was a greater than 99% loss in active lactase.

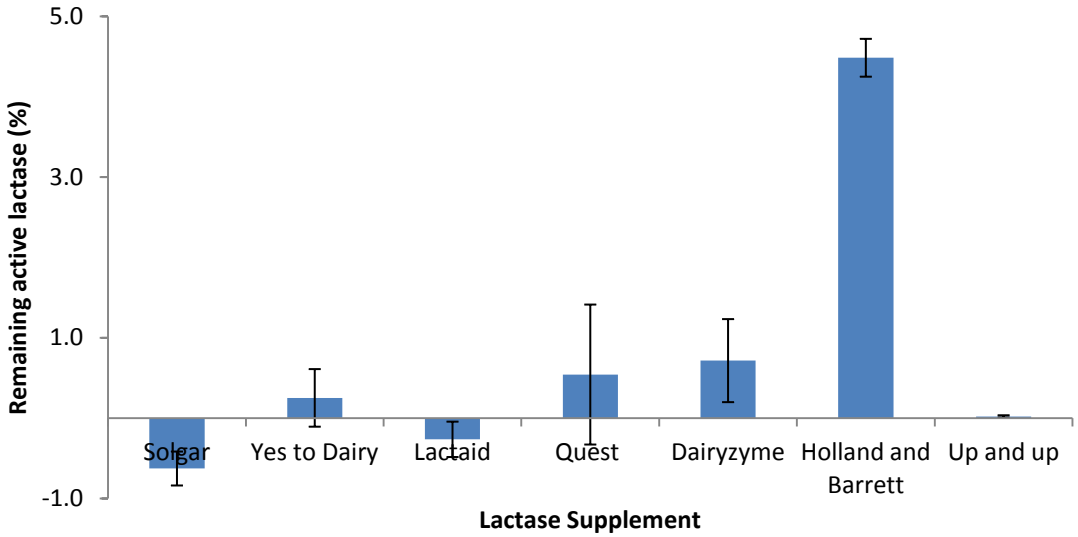


Figure 2.4 *Residual active lactase released from supplements after incubation in 0.1N HCl prior to pH rise to pH 6.8. Error bars show mean ± SD.*

Table 2.8 *Active lactase units detected per supplement after incubation in pH 6.8 phosphate buffer for 2 hours or in 0.1N HCl for 2 hours and 45 minutes in pH 6.8 phosphate buffer. Data represents means ± SD.*

Supplement	Units/dosage pH 6.8	Units/dosage pH 1.2 to 6.8	Difference (units)	Active lactase remaining pH 1.2 to 6.8 (%)
Solgar	2171.93 ± 110.11	-13.65 ± 4.59	2185.58 ± 4.59	-0.6 ± 0.2
Yes to dairy	1698.35 ± 58.19	4.26 ± 6.09	1694.09 ± 6.09	0.3 ± 0.4
Lactaid	1912.82 ± 66.46	-5.07 ± 4.21	1917.89 ± 4.21	-0.3 ± 0.2
Quest	1023.95 ± 74.49	5.54 ± 8.92	1018.41 ± 8.92	0.5 ± 0.9
Dairy Zyme	1073.86 ± 47.27	7.67 ± 5.55	1066.19 ± 5.55	0.7 ± 0.5
Holland & Barrett	1274.85 ± 28.65	57.16 ± 3.00	1217.69 ± 3.00	4.5 ± 0.2
Up and Up	1705.51 ± 50.76	0.33 ± 0.25	1705.18 ± 0.25	0.0 ± 0.0

All the supplements, except the Holland and Barrett supplement, were tablet or capsule powder based formulations without enteric protection. Therefore it is unsurprising they were unable to protect lactase in acid. Similar testing of lactase supplements in SGF also concluded enteric protection was vital for lactase activity survival (Alavi et al., 2002, O'Connell and Walsh, 2006). Only enteric supplements preserved lactase activity, >90%, after SGF incubation.

All of the supplements tested have dosage instructions advising them to be taken with or before eating lactose containing foods. The arrival of food may neutralise stomach acid and stop lactase inactivation. However, should the lactase supplement arrive in the stomach before food it is likely to be immediately denatured. Additionally the pH of the stomach will drop post food ingestion and return to values which would denature any non-enteric lactase.

The Holland and Barrett lactase supplement is a liquid formulation based on soy bean oil. Soy bean oil has been used as the oil phase of microemulsions and demonstrated its ability to enhance drug stability and absorption *in vivo* (Piao et al., 2006, Wu, 2009, Hauss, 1997, Yi, 2000). The higher active lactase recovery found with this supplement may be due to the formation of an oil in water emulsion when dispersed in SGF. This type of formulation tends to be used to increase the bioavailability of hydrophobic drugs which can dissolve in the oil phase such as cyclosporine A. However lactase is a hydrophilic protein and upon dispersion in gastric fluids it is more likely to partition into the acidic aqueous phase than remain in the protective oil phase. Therefore only 4.5% of the lactase in the supplement remained active after incubation in 0.1N HCl. This small proportion of lactase may have been protected from the acidic pH by the oily barrier. Water in oil microemulsions have previously enhanced the stability of peptide/protein drugs *in vitro* and *in vivo*; calcitonin (Fan et al., 2011) and insulin (Sharma et al., 2010, Toorisaka et al., 2003, Elsayed et al., 2009, Toorisaka et al., 2005).

2.5.3 Self emulsifying dosage forms with soy bean oil

The assessment of lactase supplements revealed only the Holland and Barrett supplement produced any measurable active lactase recovery after dissolution in 0.1N HCl and pH 6.8 phosphate buffer. It is thought that the oil phase of this formulation is able to provide a protective barrier against acid.

Therefore a recreation of this formulation was attempted using soy bean oil as the oil phase and soy lecithin as a stabiliser.

Small, spherical droplets of soy bean oil with lactase were formed upon dispersal of the formulation in pH 6.8 phosphate buffer, figure 2.5. This demonstrated what would happen when dispersed in the gastric and intestinal fluids. However it can't be seen if lactase remains in the oily phase or partitions into the external aqueous phase.

There was a high loading efficiency demonstrating that the soy bean oil and method of preparation did not have a detrimental effect on lactase activity, table 2.9. Dispersal of the oily formulation did not impair lactase activity. Exposure of lactase to the oil/water interface created upon dispersion could have changed it structurally and caused activity loss.

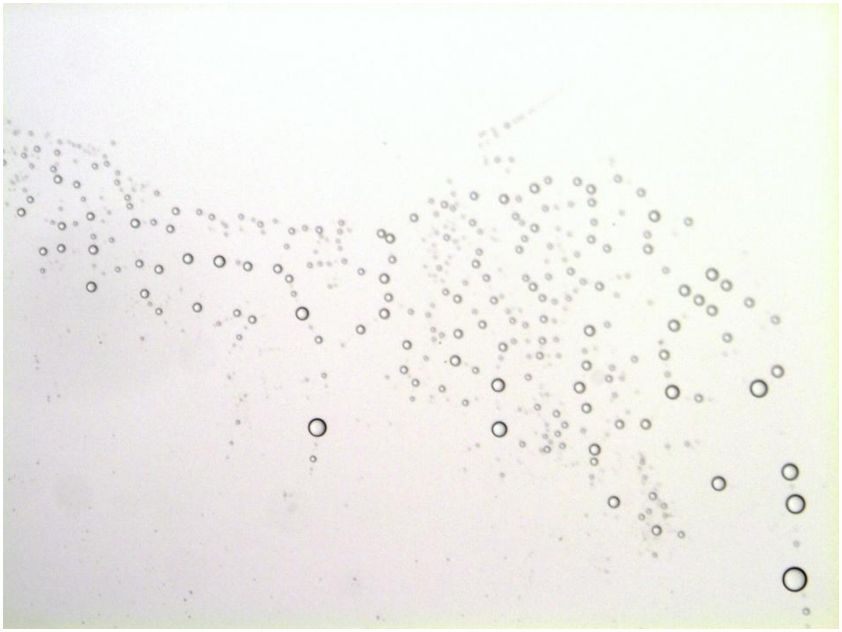


Figure 2.5 *Light microscope image of soy bean oil, lactase and soy lecithin formulation upon dispersion in pH 6.8 phosphate buffer*

Table 2.9 *Loading efficiency, loading of active lactase in soy bean oil and active lactase release after dissolution in 0.1N HCl followed by pH rise to 6.8. Data represents means ± SD.*

Formulation	Loading efficiency (%)	Active lactase loading (mg/mg)	Active lactase release (%)
Lactase, soy bean oil	106.7 ± 60.9	0.08 ± 0.05	0.7 ± 0.7

After dissolution in 0.1N HCl and pH 6.8 phosphate buffer there was a very small amount of active lactase released, 0.7%, from the lactase/soy bean oil formulation. Lactase is a hydrophilic enzyme and so upon dispersion in the aqueous 0.1N HCl it may move into this aqueous phase where it will be destroyed. A very small amount remains protected in the oil droplets. This formulation was significantly inferior ($p \leq 0.05$) to the Holland and Barrett formulation in terms of active lactase recovery after dissolution; 4.5% to 0.7%. One reason for this may be that the Holland and Barrett formulation was in a capsule and also contained beeswax. These may have provided additional barriers to acid denaturation of lactase.

The amounts and ratios of soybean oil, soy lecithin and lactase in the Holland and Barrett formulation were not known so could not be replicated exactly. Further testing to discover ideal ratios of the formulation components is needed to produce a more protective formulation. Possibly a water in oil emulsion, formed before administration, could provide protection to lactase in an inner aqueous phase within a protective external oil phase. Upon dispersion in gastric fluids more of the lactase may remain in the inner aqueous phase droplets within the protective oil rather than partitioning immediately into the denaturing gastric fluids.

2.5.4 Enteric lactase tablets

The intestinal stability study demonstrated lactase susceptibility at gastric pH. Analysis of stability of the non-enteric lactase supplements in 0.1N HCl revealed that none of them preserved more than 5% of their active lactase.

The most promising lactase formulation, soy bean oil based, was replicated but didn't manage to preserve even 1% of the formulated lactase in 0.1N HCl. These findings demonstrated that for lactase to be successfully orally delivered to the small intestine it requires enteric protection.

To provide gastric protection a lactase tablet formulation with enteric coating was developed and tested. Lactase supplements with enteric coating were able to protect enzyme activity during acid incubation (O'Connell and Walsh, 2006). Pancreatic enzymes had an increased efficacy when enteric coated (Delchier et al., 1991, Dutta et al., 1988, Naikwade et al., 2009).

Lactase tablets were coated with the enteric polymer Eudragit L100 giving a weight gain of 10.66mg per tablet and 8.39mg of polymer was applied per tablet giving a coating of 4.72mg/cm^2. There was a high loading efficiency of active lactase in the tablets demonstrating the efficiency of the process and its suitability, table 2.10. The greater than 100% loading efficiency of lactase could be due to non uniform blending of the tablet powder mixture.

Table 2.10 *Active lactase loading and loading efficiency in uncoated and Eudragit L100 coated lactase tablets. Data represents means ± SD.*

Sample	**Active lactase (mg/tab)**	**Loading efficiency (%)**
Uncoated lactase tablet	45.21 ± 2.85	113.0 ± 7.1
Eudragit L100 coated lactase tablet	43.22 ± 1.22	108.1 ± 3.1

2.5.4.1 *In vitro* release

There was no active lactase released from the uncoated tablets after dissolution in 0.1N HCl and then pH 6.8 phosphate buffer, table 2.11 and figure 2.6. Active lactase was released from enteric coated tablets after 10 to 20 minutes dissolution above the pH threshold of the enteric polymer, pH 6. There was complete active lactase release after 30 minutes dissolution in pH 6.8 phosphate buffer.

Table 2.11 *In vitro active lactase release from uncoated and enteric coated tablets after 2 hours in 0.1N HCl and 45 minutes in pH 6.8 phosphate buffer. Data represents means ± SD.*

Time (mins)	Coated tablet active lactase released (%)	Uncoated tablet active lactase released (%)
0	0.0 ± 0.0	0.0 ± 0.0
60	-0.7 ± 0.5	-1.0 ± 0.0
120 (pH1.2)	-0.6 ± 0.5	-1.0 ± 0.0
120 (pH6.8)	-0.8 ± 0.7	-1.3 ± 0.0
130	-0.8 ± 0.7	-0.8 ± 0.0
140	21.4 ± 14.3	-1.1 ± 0.3
150	102.6 ± 8.5	-1.3 ± 0.1
165	107.1 ± 8.5	-1.3 ± 0.0

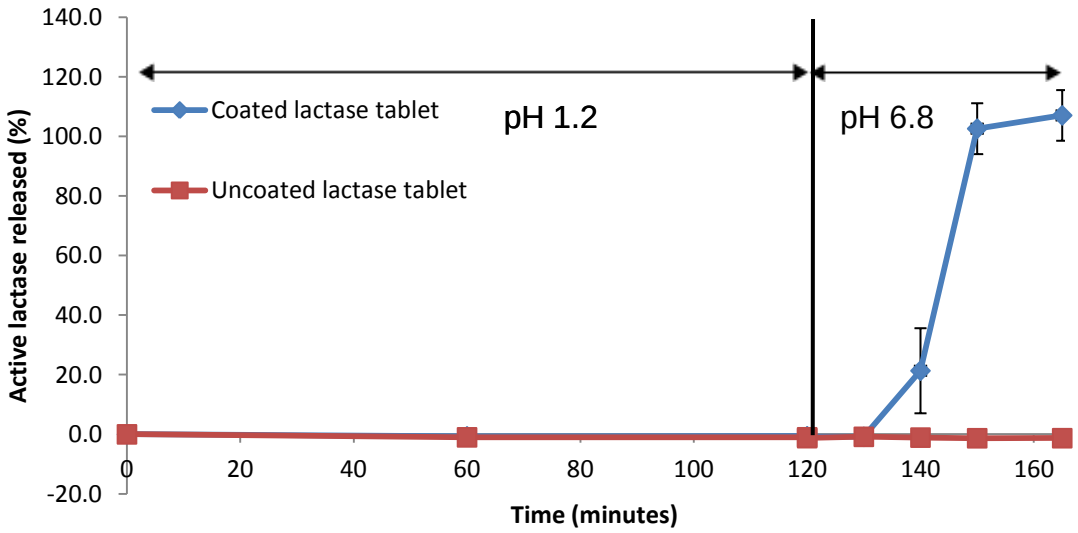


Figure 2.6 *Active lactase release from uncoated and enteric coated tablets in 0.1N HCl for 2 hours and pH 6.8 phosphate buffer for 45 minutes. Error bars show mean ± SD.*

2.5.5 Eudragit L100 microparticles

Enteric coated tablets successfully protected lactase from acid denaturation. However, during dissolution studies there was a delay in lactase release once the threshold pH of the polymer, pH 6, had been reached.

There was no release for the first 10 minutes at pH 6.8 and it took 30 minutes for there to be complete lactase release. Single unit, enteric coated dosage forms, such as capsules and tablets, have previously demonstrated their inability to disintegrate rapidly (Cole et al., 2002). This has implications for the digestion of lactose as it would allow lactose to persist undigested in the intestine. Lactase needs to be immediately available for lactose digestion as soon as it reaches the small intestine. Administering the tablets before food may achieve this but the emptying time of the tablets from the stomach and their dissolution are highly variable.

To overcome this delayed release, the dosage size can be reduced to increase the surface area and achieve faster lactase release. There was an increase in the rapidity of enzyme release and greater alleviation of symptoms when pancreatin microparticles were administered compared to tablets (Naikwade et al., 2009, Beverley et al., 1987).

Microparticles composed of non enteric polymers have been formulated to encapsulate lactase. However these have either prematurely released lactase in SGF or rapidly burst released their lactase making them unsuitable for oral lactase delivery (Kim et al., 2006, Hayashi et al., 1994). Additionally the preparation of PLGA and PLA microparticles involved homogenisation which can damage lactase integrity (Hayashi et al., 1994, Stivaktakis et al., 2004, Stivaktakis et al., 2005).

Enteric microparticles would be able to prevent lactase exposure to gastric pH, empty from the stomach with food and due to their large surface area should rapidly release lactase. The enteric polymer Eudragit L100, which was used to coat lactase tablets, has been used to produce pH sensitive microparticles encapsulating lactase. Squillante *et al* demonstrated their prevention of lactase release in acid but didn't show if the lactase retained its activity (Squillante et al., 2003). The release rate was also quite slow, only 25% of the encapsulated lactase was released after 20 minutes in pH 6.8 phosphate buffer and 65% was released in 100 minutes. Alavi *et al* also encapsulated lactase in Eudragit L100 microparticles employing an oil in oil emulsification solvent evaporation method (Alavi et al., 2002).

However these methods of microparticle preparation involved homogenisation which was shown to be damaging to lactase (Hayashi et al., 1994).

Eudragit L100 was used to produce microparticles encapsulating lactase (Kendall et al., 2009). This method has been used to encapsulate small molecular weight drugs. This method has advantages over the other methods used to produce Eudragit L100 lactase microparticles as it doesn't require temperature control, uses a slower emulsification speed (1000rpm instead of 8000rpm (Alavi et al., 2002)) and uses the less toxic ethanol instead of acetone or methanol to dissolve the polymer.

The blank and lactase loaded microparticles produced were less than 50µm and uniformly sized, figure 2.7 and table 2.12. SEM images confirmed this, figures 2.8 and 2.9. These are smaller than those produced previously to encapsulate lactase 53-57µm (Squillante et al., 2003) and 195µm (Alavi et al., 2002) and therefore they should empty more rapidly from the stomach with food. The yield was greater than 90% and there was complete active lactase encapsulation, table 2.12. This demonstrates the suitability of this method for producing microparticles with lactase as there was no enzyme destruction caused during preparation.

The mean encapsulation efficiency of active lactase was 80% for Squillante *et al* and only 60% for Alavi *et al*. In addition these methods were also damaging to lactase resulting in a 32% loss of activity during processing for Squillante *et al*. Alavi *et al* were able to preserve greater than 80% of lactase activity during processing but this was still not as compatible with lactase encapsulation as the Kendall *et al* method. The size, yield and encapsulation efficiency of lactase within Eudragit L100 microparticles was comparable to small molecular weight drugs encapsulated using the Kendall *et al* method. This demonstrates the suitability of this method to successfully encapsulate macromolecules, including proteins, within enteric microparticles.

Table 2.12 *Size, span, yield, encapsulation efficiency and lactase loading of blank and lactase loaded Eudragit L100 microparticles. Data represents means ± SD.*

Microparticles	Mean Size (μm)	Span	Yield (%)	Encapsulation efficiency (%)	Active lactase loading (units/mg)	Active lactase loading (mg/mg)
Blank	32.89 ± 1.51	1.02 ± 0.23	94.7 ± 4.9	-	-	-
Lactase	27.26 ± 0.68	0.94 ± 0.08	94.9 ± 2.5	102.5 ± 7.4	0.63 ± 0.05	0.064 ± 0.005

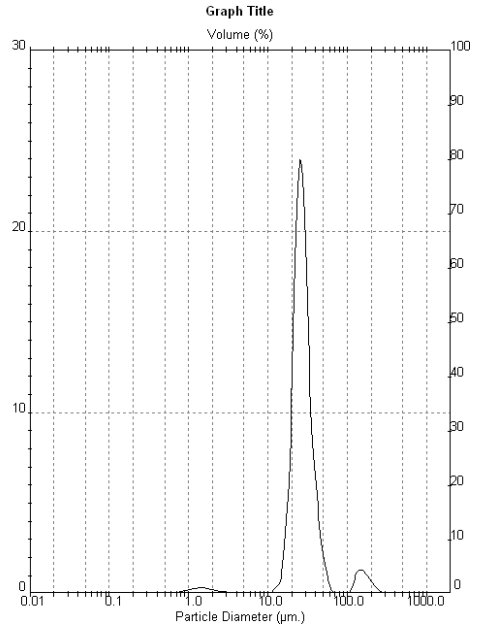


Figure 2.7 *Frequency curve of lactase microparticle size (image from Malvern Mastersizer)*

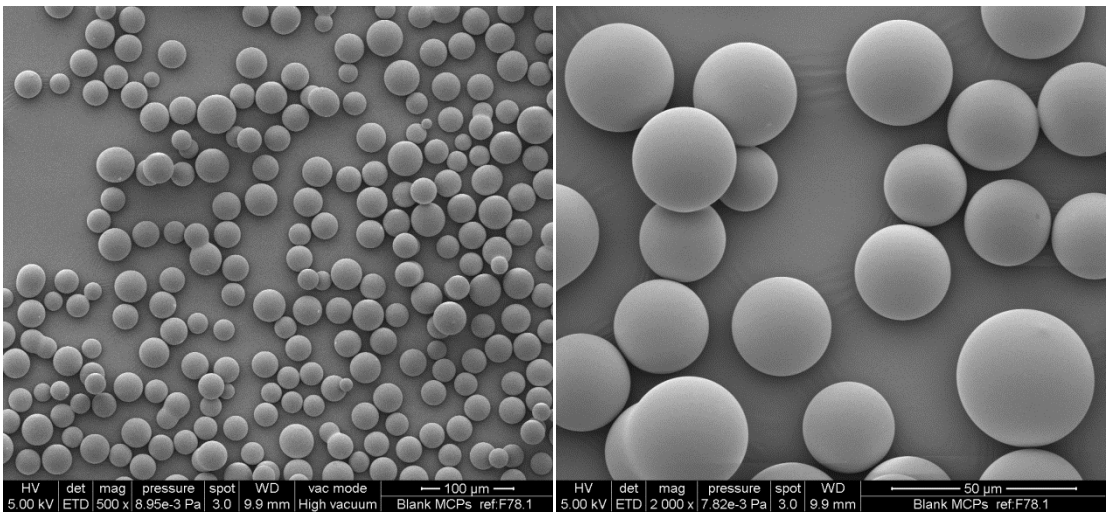


Figure 2.8 (a) and (b) *SEM images of blank Eudragit L100 microparticles, sputter coated, in a high vacuum and visualized with a 5kV beam*

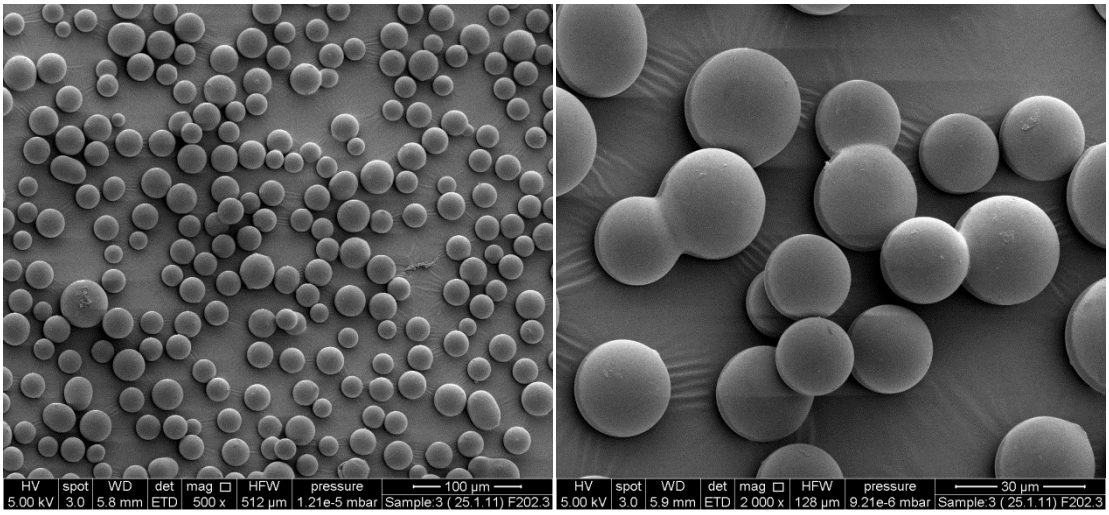


Figure 2.9 (a) and (b) *SEM images of microparticles encapsulating lactase, sputter coated, in a high vacuum and visualized with a 5kV beam*

DSC analysis of lactase, blank Eudragit L100 microparticles and lactase loaded microparticles revealed the encapsulated lactase was unchanged and crystalline, figures 2.10-12. Due to its insolubility in ethanol it has been encapsulated as a solid suspension. The DSC traces reveal that the thermal degradation profile of lactase did not change upon its encapsulation in Eudragit L100 microparticles but it is clearly associated with the particles.

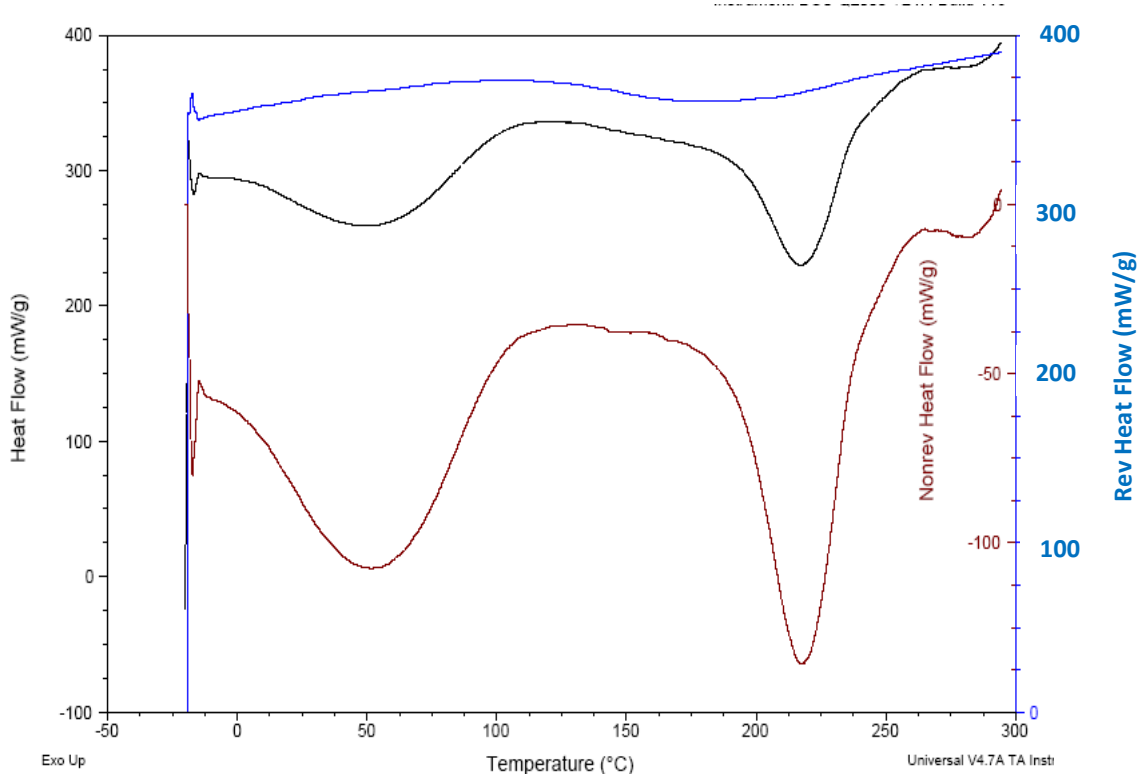


Figure 2.10 *DSC thermograph of blank Eudragit L100 microparticles*

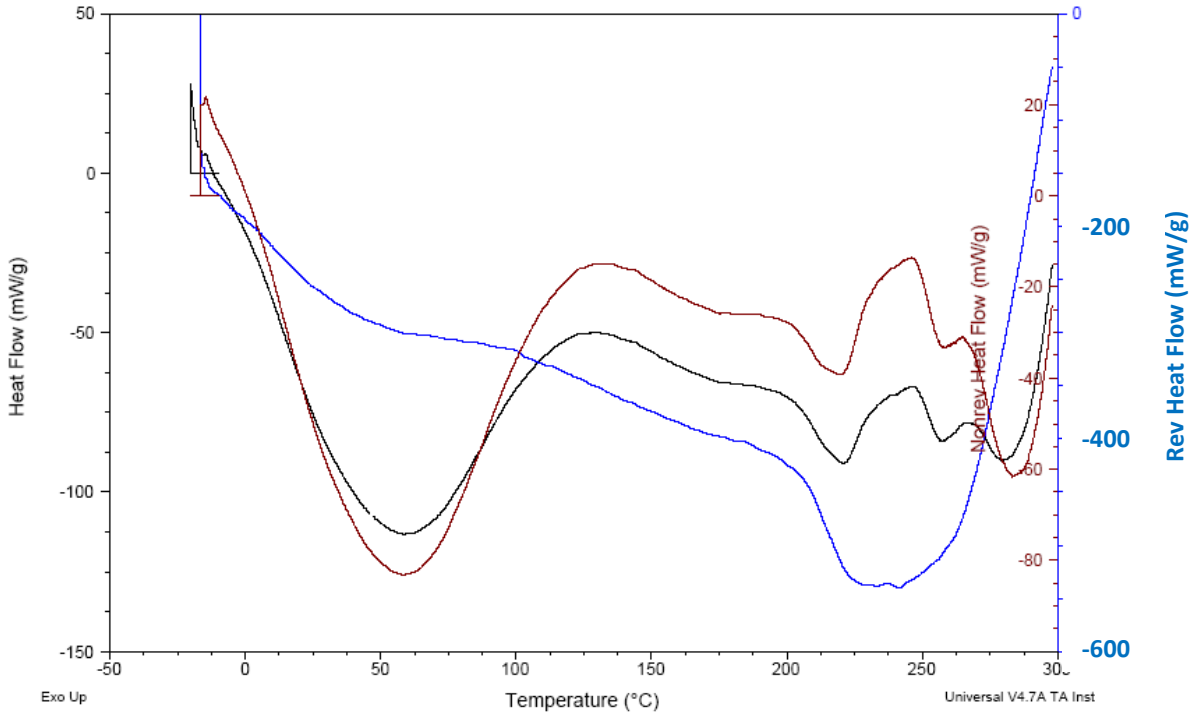


Figure 2.11 *DSC thermograph of lactase*

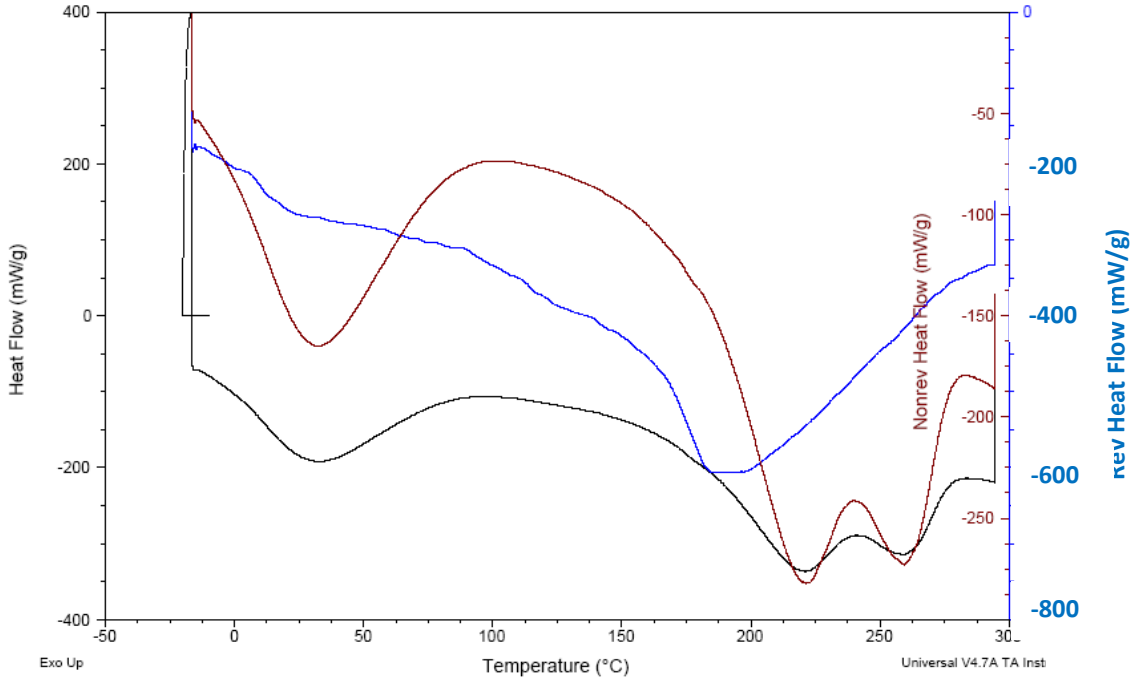


Figure 2.12 *DSC thermograph of lactase loaded Eudragit L100 microparticles*

There was no active *in vitro* lactase release after incubating the microparticles in 0.1N HCl, to simulate gastric conditions, and then raising the pH to 6.8, figure 2.13. Lactase was active and completely released in pH 6.8 buffer so it has not been denatured during its preparation and its release is not restricted at this pH. The microparticles were visibly intact at the end of the acid phase but possibly the lactase was burst released into the acid and immediately denatured or the acid has came into contact with encapsulated lactase.

Squillante *et al* demonstrated that their Eudragit L100 microparticles did prevent lactase release in 0.1N HCl and released it in pH 6.8 phosphate buffer. However, lactase release was determined by its UV absorption, not its activity. Therefore it was unknown whether the released lactase was active.

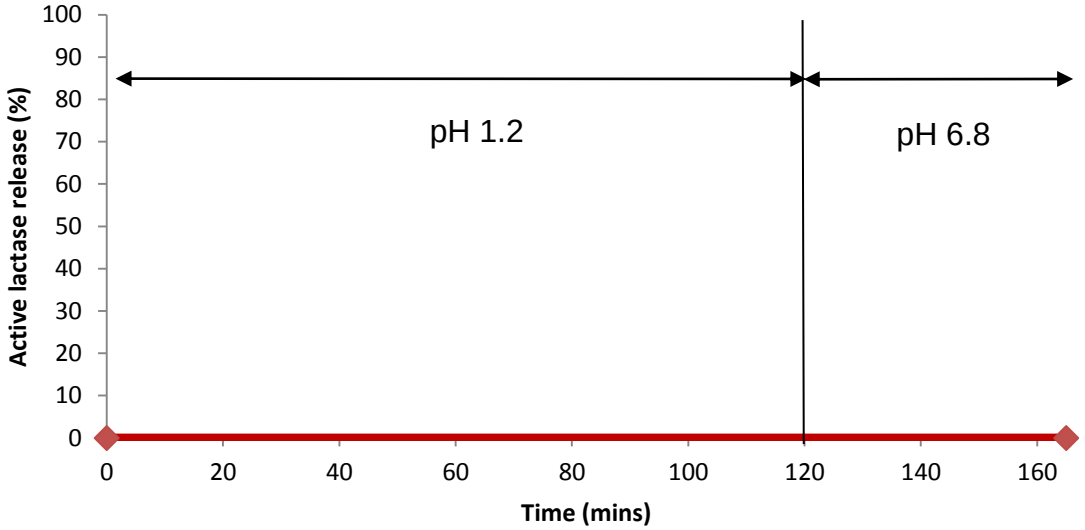


Figure 2.13 *Active lactase release from Eudragit L100 microparticles after 2 hours in 0.1N HCl and 45 minutes at pH 6.8. Error bars show the mean ± SD.*

2.5.6 Spray drying lactase

The failure of the microparticles to release active lactase after dissolution initially in acid led to the theory that the lactase may not be completely encapsulated within the microparticles and/or that it is prematurely burst released. This means that lactase would be in contact with 0.1N HCl and would be denatured before the pH rise when it should theoretically be released in an active form. As the lactase is suspended and not dissolved in the polymer solution prior to emulsification its particle size will determine the extent of its encapsulation. The lactase powder was visualised using SEM to determine its particle size, figure 2.14.

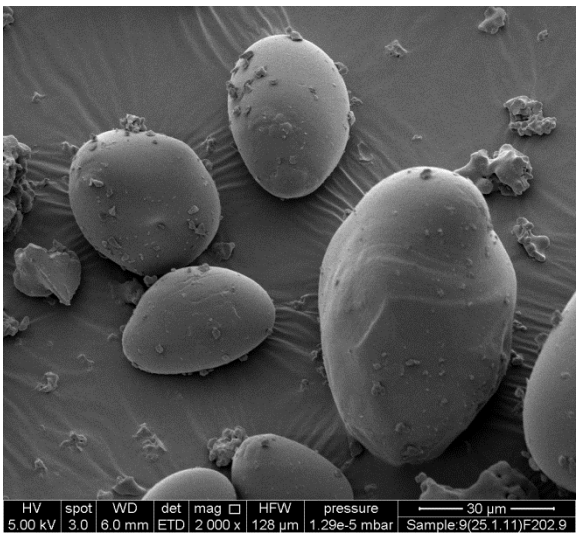


Figure 2.14 *SEM image of lactase powder*

SEM images revealed that some of the lactase particle sizes were greater than 30µm. As the mean size of the microparticles produced using the Kendall *et al* method was <50µm the lactase may not have been completely encapsulated and therefore was exposed at the microparticle surface and burst released.

Reducing drug particle size reduced their burst release (Prinn et al., 2002, Thote and Gupta, 2005). Spray drying has been used to successfully reduce the size of protein particles (Costantino et al., 2000) and lactase particle size has been reduced to 2-4µm by spray drying (Broadhead et al., 1994). The lactase powder was spray dried to reduce its size and increase its chances of retention in the microparticles in acid. Figure 2.15 shows the particle size was reduced by spray drying to less than 10µm. The lactase particles remained this size after dispersal in ethanol so they should remain small enough for successful encapsulation in the microparticles, figure 2.16. Ideally the particle size of lactase before and after spray drying would have been assessed using a Malvern Mastersizer, as for the microparticles. However there was not enough material available to do this. The spray dried lactase was tested for activity and it was shown that spray drying had only reduced its activity a little from 9.9 to 9.11units/mg.

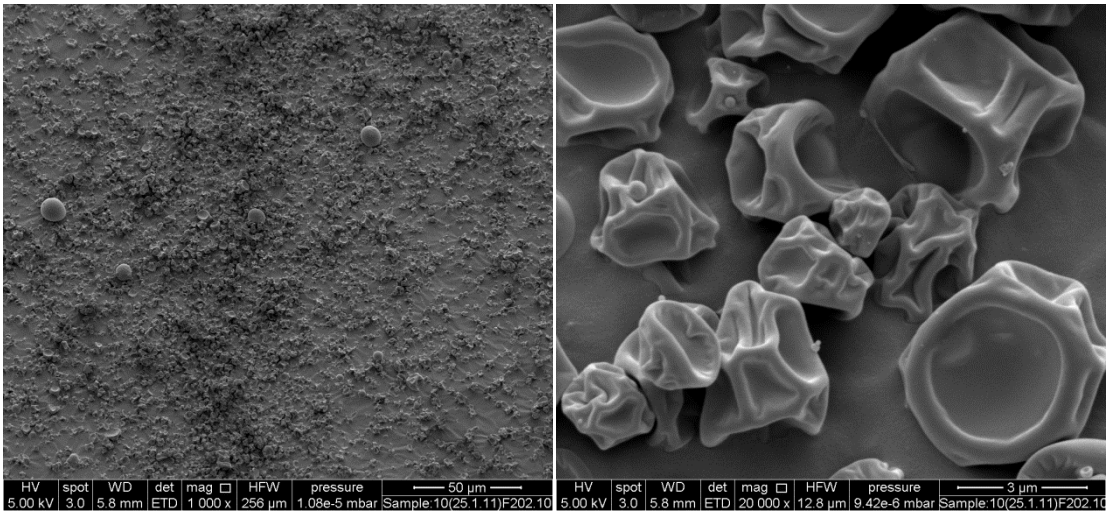


Figure 2.15 (a) and (b) *SEM images of spray dried lactase*

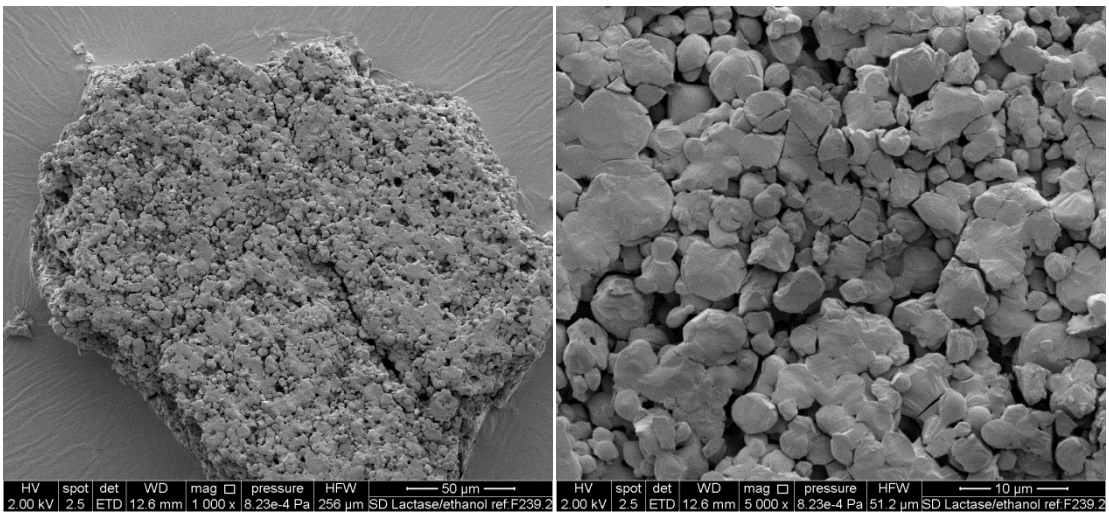


Figure 2.16 (a) and (b) *SEM images after spray dried lactase has been added to ethanol*

2.5.7 Eudragit L100 microparticles with spray dried lactase

The spray dried lactase with a reduced size was encapsulated in Eudragit L100 microparticles. The microparticles produced were uniformly sized and less than 50µm, table 2.13 and figure 2.17 and the SEM images confirmed this, figure 2.18. The yield was 99% but encapsulation efficiency of active lactase was significantly ($p \leq 0.05$) reduced to 84% compared to non-spray dried lactase, table 2.13. Possibly the smaller size of the lactase meant it escaped encapsulation.

Table 2.13 *Size, span, yield, encapsulation efficiency and spray dried lactase loading of Eudragit L100 microparticles. Data represents means ± SD.*

Microparticles	Mean size (μm)	Span	Yield (%)	Encapsulation efficiency (%)	Active lactase loading (units/mg)	Active lactase loading (mg/mg)
Spray dried lactase	30.25 ± 2.07	0.83 ± 0.31	99.4 ± 1.3	84.1 ± 3.1	0.48 ± 0.02	0.048 ± 0.00

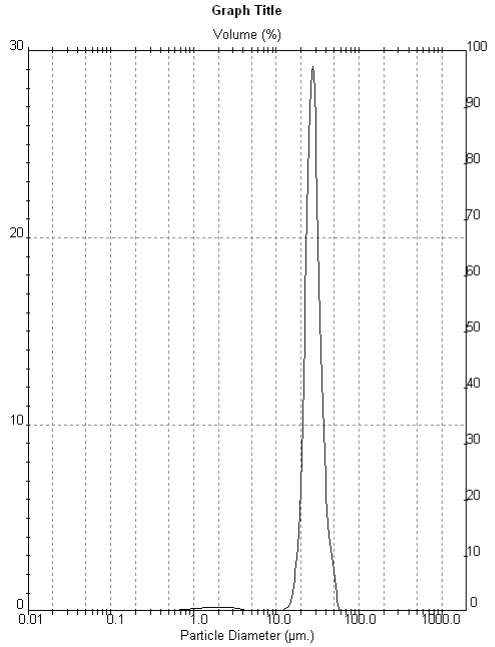


Figure 2.17 *Frequency curve of microparticle size with spray dried lactase (image from Malvern Mastersizer)*

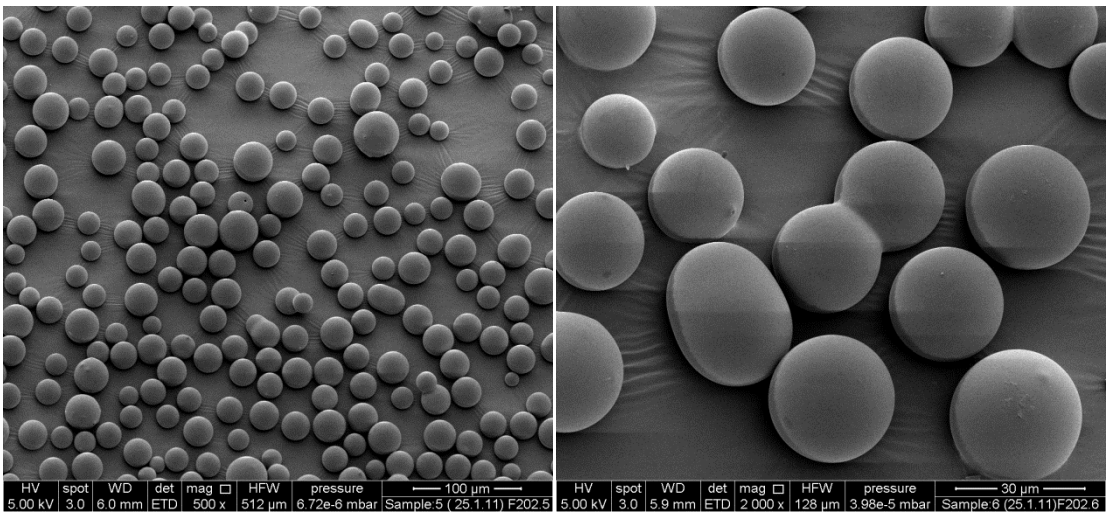


Figure 2.18 (a) and (b) *SEM images of Eudragit L100 microparticles with spray dried lactase, sputter coated and imaged in a high vacuum with a 5kV beam.*

Lactase was fluorescently labeled and spray dried to visualize its encapsulation within Eudragit L100 microparticles. The Eudragit L100 polymer was viewed alone using CFLM and shown not to fluoresce. Fluorescently labeled lactase was visible within the microparticles demonstrating its encapsulation within the particles, figure 2.19. Some fluorescent lactase is visible at the surface of the microparticles which could be susceptible to denaturation in acid. Lactase at or near the surface may also be burst released from the microparticles upon dispersal in 0.1N HCl.

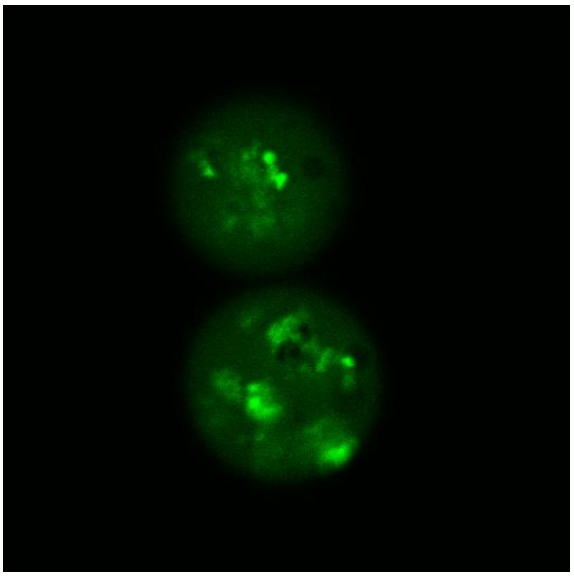


Figure 2.19 *CFLM image of encapsulated, spray dried FITC labelled lactase in Eudragit L100 microparticles*

2.5.7.1 *In vitro* release

Despite lactase size reduction there was no active lactase released from the microparticles during dissolution studies in 0.1N HCl (pH 1.2) and then pH 6.8 phosphate buffer, despite the particles remaining visibly intact in acid. This lack of active lactase may be caused by immediate lactase release from the microparticles in 0.1N HCl. Premature burst release of encapsulated drug from Eudragit L100 microparticles at pH 1.2 has been observed previously (Devarajan and Sonavane, 2007, Eerikainen et al., 2004, Raffin, 2006).

To determine if the lactase was burst released the microparticles were initially placed in pH 4.5 buffer, a pH below the pH 6 threshold of the polymer. At pH 4.5 lactase is active so any lactase released can be quantified and an estimate of lactase released in 0.1N HCl can be made.

There was a burst release of 64% of non-spray dried lactase from the microparticles at pH 4.5, table 2.14 and figure 2.20. Spray drying the lactase significantly ($p \leq 0.05$) reduced this to 8%. Lactase released prematurely into 0.1N HCl would have been instantly denatured. These results demonstrated that spray drying lactase, reducing its size, allowed a more complete encapsulation of lactase and reduced its burst release. After pH rise to pH 6.8 there was an almost complete release of active lactase from the microparticles with non-spray dried lactase, 85%, and spray dried lactase, 80%, in 45 minutes. This was a faster and more complete release of encapsulated lactase than from the microparticles produced by Squillante *et al* which only released 65% of encapsulated lactase after 100 minutes in pH 6.8 phosphate buffer (Squillante et al., 2003).

Table 2.14 *Active lactase release from Eudragit L100 microparticles with non-spray dried and spray dried lactase in either 0.1N HCl or pH 4.5 buffer for 2 hours and 45 minutes in pH 6.8 phosphate buffer. Data represent means ± SD.*

Microparticles	Active lactase release (%) pH 1.2 to 6.8	Active lactase release (%) pH 4.5	Active lactase release (%) pH 4.5 to 6.8
Non-spray dried lactase	-0.2 ± 0.8	64.1 ± 5.6	84.5 ± 7.7
Spray dried lactase	-0.3 ± 0.7	8.5 ± 2.6	80.1 ± 3.8

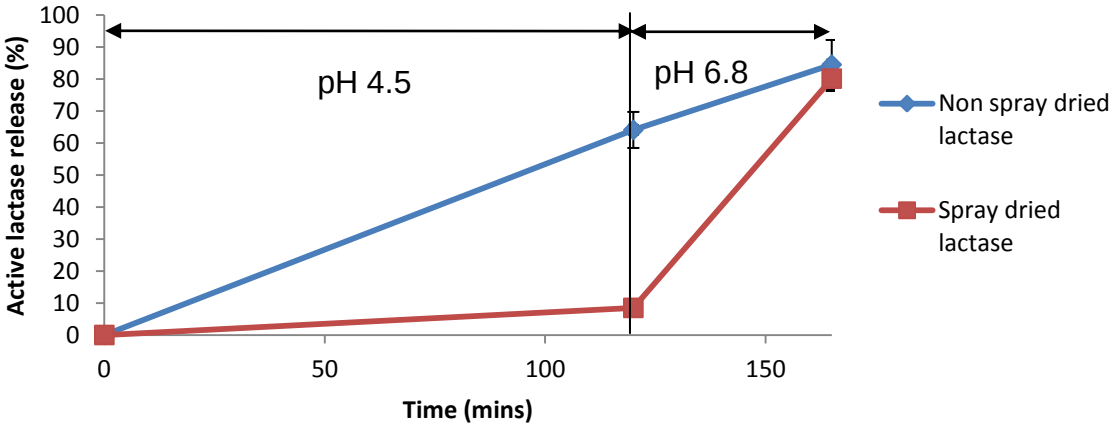


Figure 2.20 *Active lactase release from Eudragit L100 microparticles with non-spray dried and spray dried lactase in pH 4.5 buffer for 2 hours and 45 minutes in pH 6.8 phosphate buffer. Error bars show mean ± SD.*

Spray drying lactase prevented over 90% of the lactase being prematurely released from the microparticles. However, despite lactase not being released in 0.1N HCl when the pH rose above pH 6 there was still no active lactase released. Possibly acid is able to penetrate the microparticles and denature the lactase within. When the pH is raised and the microparticles break down the lactase that is released may already have been denatured. A similar situation occurred for the enzymes lactate dehydrogenase (LDH) and alpha amylase (AMY) encapsulated in Eudragit S100 microparticles (Scocca et al., 2007). Despite the microparticles remaining visually intact after acid incubation there was a 46% loss of LDH activity and 74% loss of AMY activity suggesting acid is able to come into contact with these encapsulated enzymes.

It was found that release of drugs with a molecular weight less than 300Da from Eudragit L100 microparticles was not controlled in acid (Alhnan, 2010). This suggests that it is possible for these smaller drug molecules to move out of the microparticles and therefore it seems logical that the surrounding media is able to move into the microparticles. This would enable acid to enter the microparticles and denature encapsulated lactase.

2.5.8 Exploration of Eudragit L100 microparticle porosity

To ascertain if acid was able to enter the microparticles a marker, whose emission wavelength varied depending on the pH of its environment, was encapsulated in the Eudragit L100 microparticles. The marker Lysosensor yellow/blue® dextran had previously shown its ability to detect the pH of the interior of PLGA microspheres (Ding and Schwendeman, 2008). Figure 2.21 shows that the peak emission wavelength of the encapsulated marker in dry conditions or pH 6.8 buffer was about 490nm. When the microparticles were placed in acid the peak emission wavelength shifted to approximately 515nm. The shift in emission to a longer wavelength indicated a decrease in the interior pH of the microparticles. This showed acid was able to permeate the microparticles and encounter the encapsulated marker.

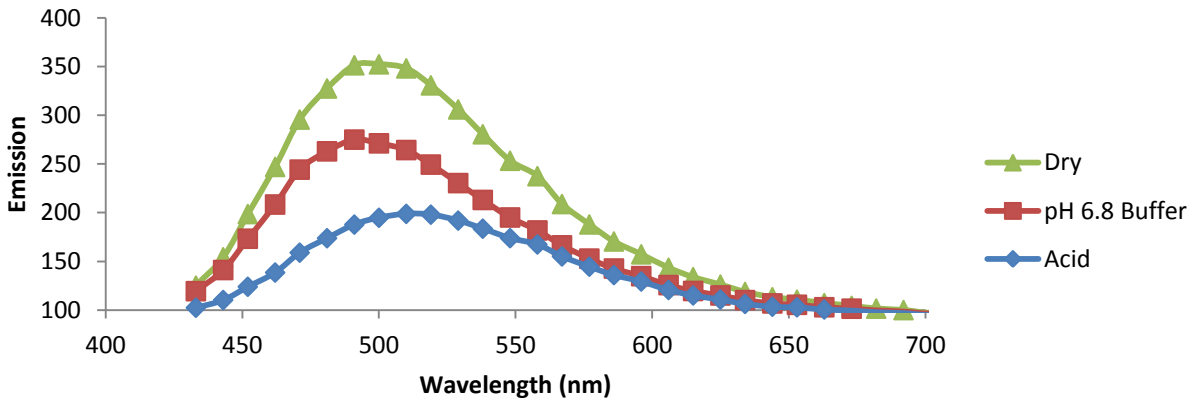


Figure 2.21 *Emission from lysosensor yellow/blue dextran marker in Eudragit L100 microparticles in dry conditions, pH 6.8 phosphate buffer and 0.1N HCl*

2.5.9 Surface morphological investigation

SEM images produced in a low vacuum and using low accelerating voltage on non-coated microparticles revealed that some had visible pores figures 2.22 and 2.23.

These pores were found in particles with encapsulated non-spray dried and spray dried lactase and appeared to be 1µm in diameter or less. Possibly sputter coating was able to cover or melt some of these pores so they were not visible previously. These images may explain why lactase is released from microparticles below pH 6. The pores would also allow acid to enter the particles and denature lactase before it is released. However, these pores were not visible in all the microparticles so don't fully explain how all the encapsulated lactase is denatured in 0.1N HCl.

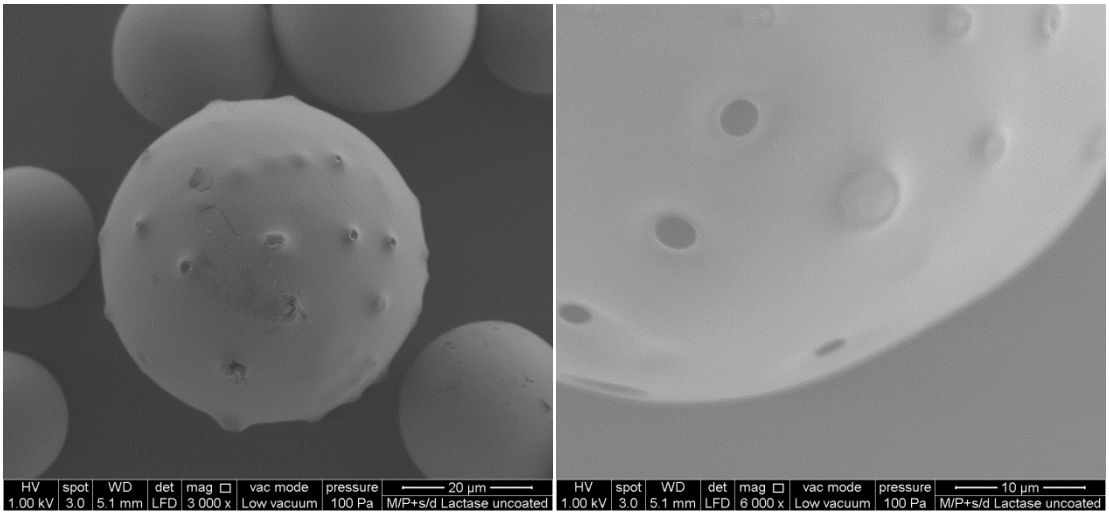


Figure 2.22 (a) and (b) *SEM images of microparticles with non-spray dried lactase, they were not coated and visualised in a low vacuum with a 1kV beam*

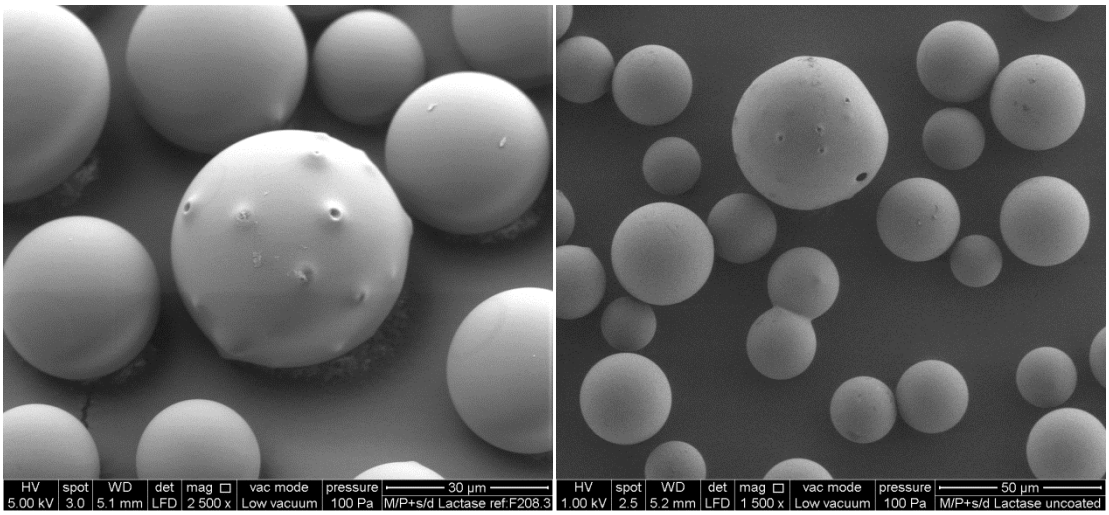


Figure 2.23 (a) and (b) *SEM images of microparticles with spray dried lactase, they were not coated and visualised in a low vacuum with a 5 and 1kV beam*

To further investigate the surface morphology of the microparticles, and elucidate how acid is able to penetrate them, blank microparticles were visualised under a Quanta 250F SEM with a new type of back scatter electron detector that performs well at low accelerating voltages. This configuration revealed surface structures not seen when using the conventional higher voltage beam. There appeared to be small 'lumps' and crystals on the surface of the microparticles, figure 2.24.

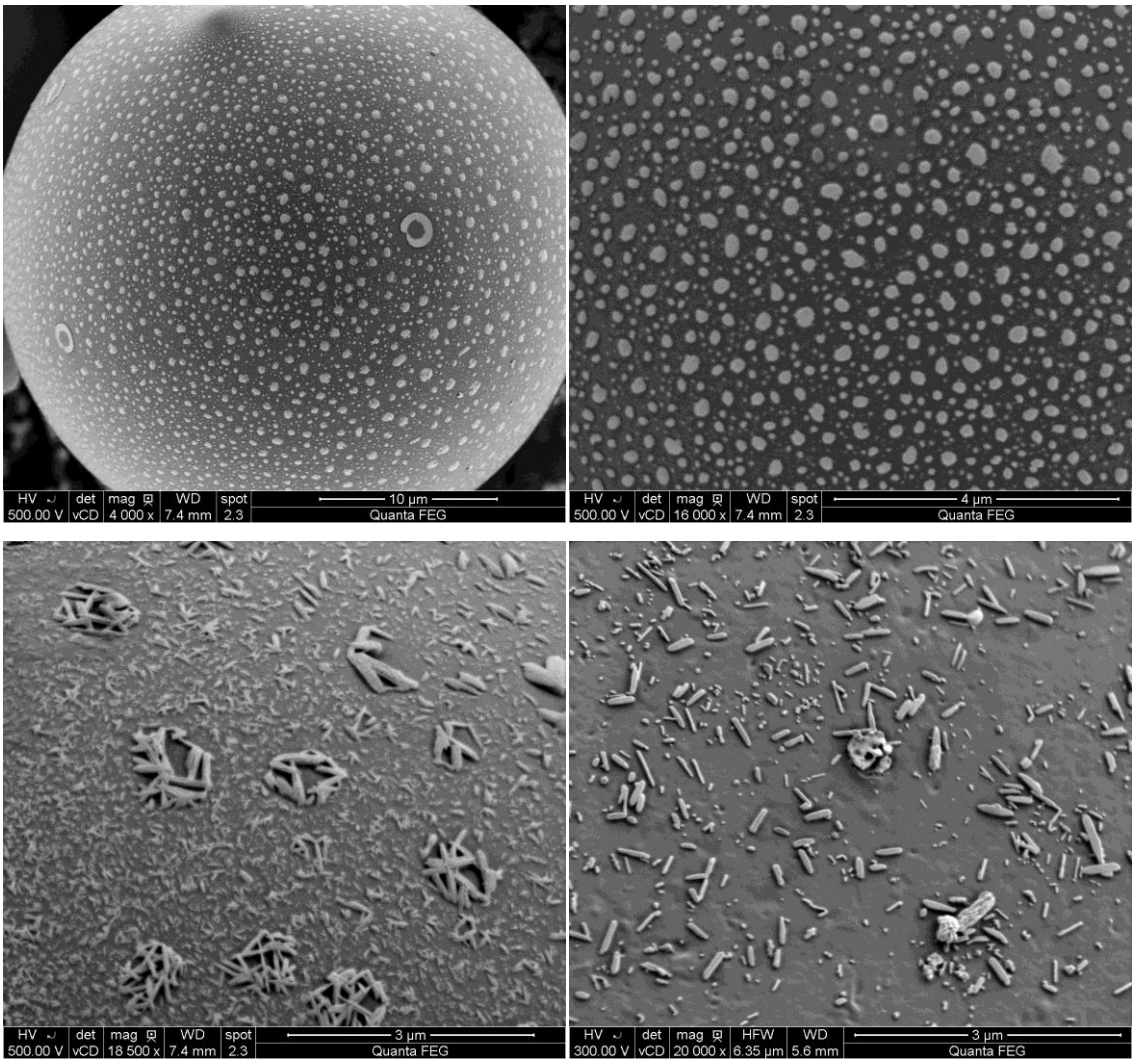


Figure 2.24 (a), (b), (c) and (d) *SEM images of blank Eudragit L100 microparticles generated using an SEM with a new back scatter detector allowing the use of a 500 volt beam*

After the microparticles had been placed in 0.1N HCl and visualised again these surface structures disappeared, figure 2.25. As these crystals appear on the surface of the blank microparticles they cannot be lactase.

They are most likely either to be Eudragit L100 or the surfactant. As they disappear in acid they are unlikely to be the acid resistant polymer and more likely to be residual sorbitan sesquioleate surfactant not removed during washing.

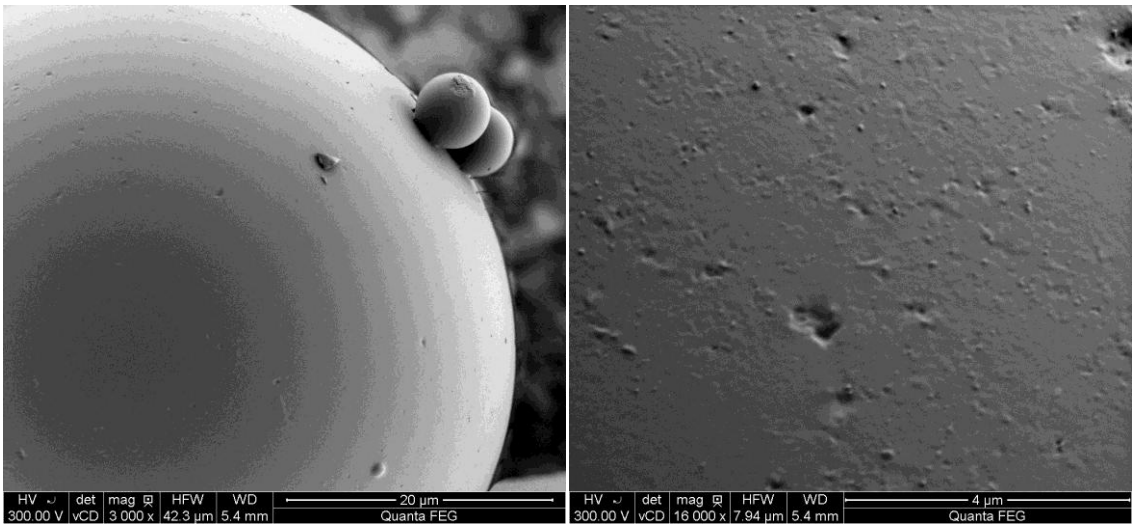


Figure 2.25 (a) and (b) *SEM images of blank Eudragit L100 microparticles after acid incubation generated using an SEM with a new back scatter detector enabling the use of a 500 volt beam*

Currently instruments which could be used to determine the chemical nature of these surface structures use a beam with a voltage higher than 500V. As they appear to be sensitive to high voltages they would be burnt off and unable to be identified.

Possibly removal of these surface structures during acid incubation may be linked to acid entry into the particles. Their removal may damage the surface of the microparticles allowing acid entry. It may show that the microparticle surface is not made up purely of acid resistant Eudragit L100 and this other component, which is not acid resistant, could allow acid ingress into the interior of the microparticles.

2.5.10 Interior morphological investigation

The interior of the microparticles was investigated by sectioning them. Cryosections of particles on a sample block show that there are visible pores within the microparticles, figure 2.26.

When the sections were placed on a grid it appeared that there were pores with a diameter of 2µm and smaller ones with a diameter of about 500nm, figure 2.27.

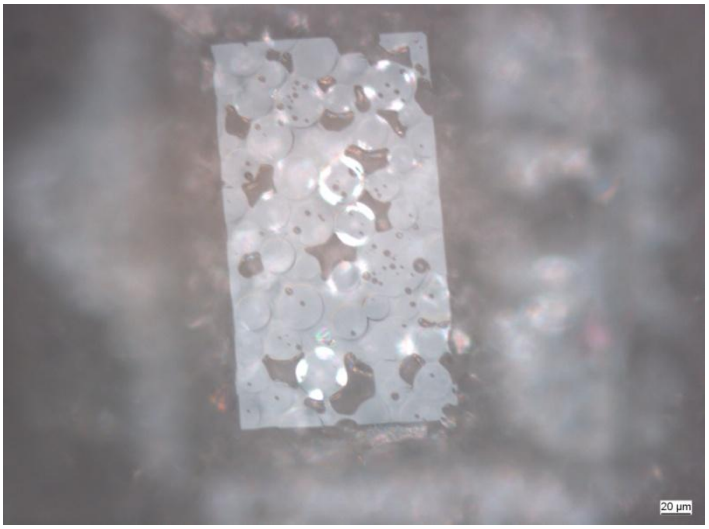


Figure 2.26 *Cryosections of blank Eudragit L100 microparticles on sample block*

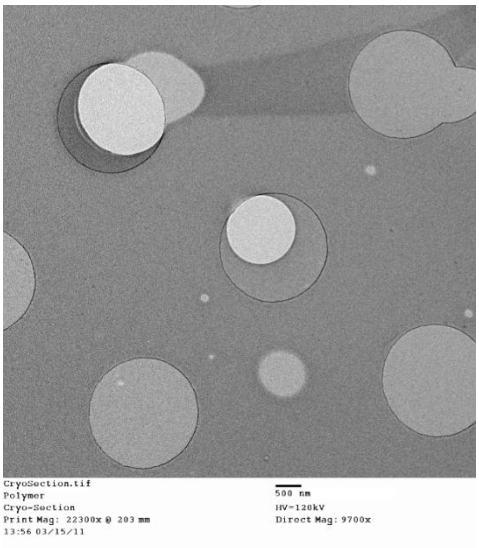
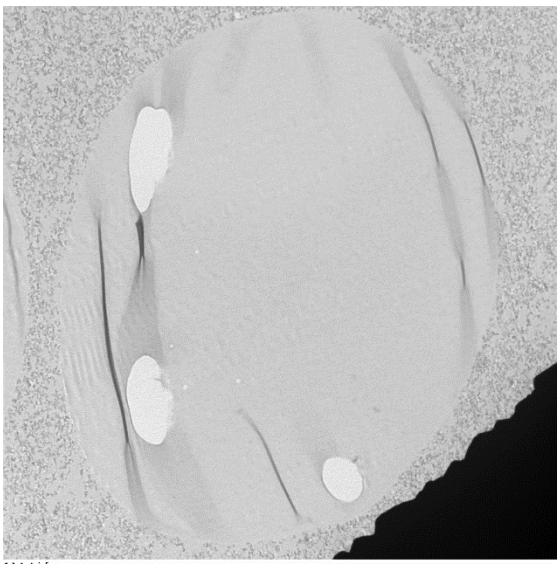
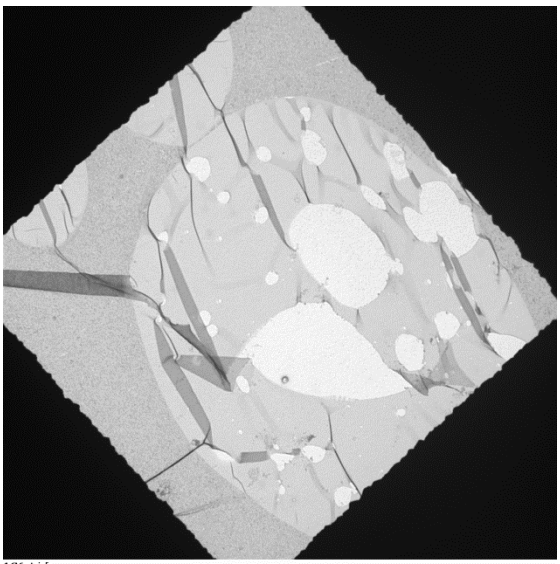


Figure 2.27 *TEM image of sectioned blank Eudragit L100 microparticles mounted on a C flat grid with 2µm holes*

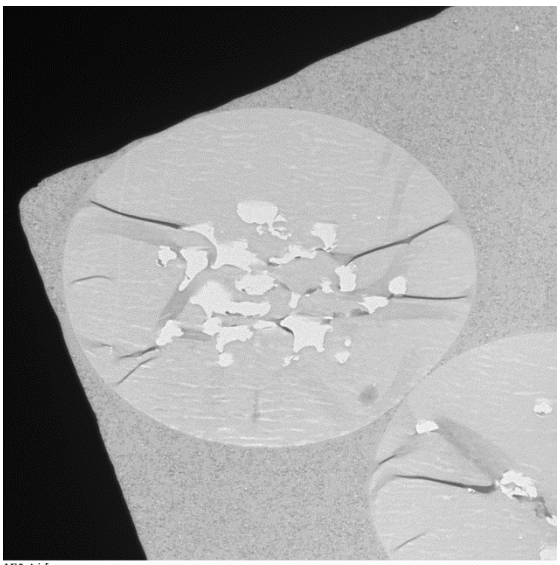
TEM images of sections from blank, unmodified lactase and spray dried lactase loaded microparticles revealed there were visible pores inside all of them, figure 2.28.



2.28 (a)



2.28 (b)



2.28 (c)

Figure 2.28 (a), (b) and (c) *TEM images of cryosections of (a) blank Eudragit L100 microparticles, (b) non-spray dried lactase loaded microparticles and (c) spray dried lactase loaded microparticles*

The pore shape is slightly deformed in the TEM images, this is due to the preparation procedure and doesn't exactly reflect their shape within the microparticles.

As the microparticles were sectioned on water some of the pores seen in the lactase loaded microparticles probably resulted from the water soluble lactase dissolving. However, there were pores visible in the blank microparticles so not all the pores in the lactase loaded particles are due to the lactase dissolving.

Pores visible in the sections from non-spray dried lactase loaded microparticles were round, relatively large and spread throughout the microparticles. The pores visible in the sections from spray dried lactase loaded microparticles were smaller, angular shaped and more concentrated in the centre of the section. These differing images reveal variations in lactase encapsulation. When lactase is not spray dried the encapsulated lactase is large and round and found throughout the particle. Spray drying lactase resulted in smaller and more angular lactase particles and this is reflected in the pores visible in the TEM of the microparticle section. The smaller lactase particles were also more deeply embedded within the microparticles. These images provide further evidence as to why more non-spray dried lactase was burst released from the microparticles than spray dried lactase as more of it was present towards the surface.

SEM images of blank, sectioned microparticles also revealed the presence of pores, figure 2.29. Pores within the microparticles may provide channels for the acid to permeate the particles and encounter encapsulated lactase. This will result in the lactase being denatured before it is released from the microparticles.

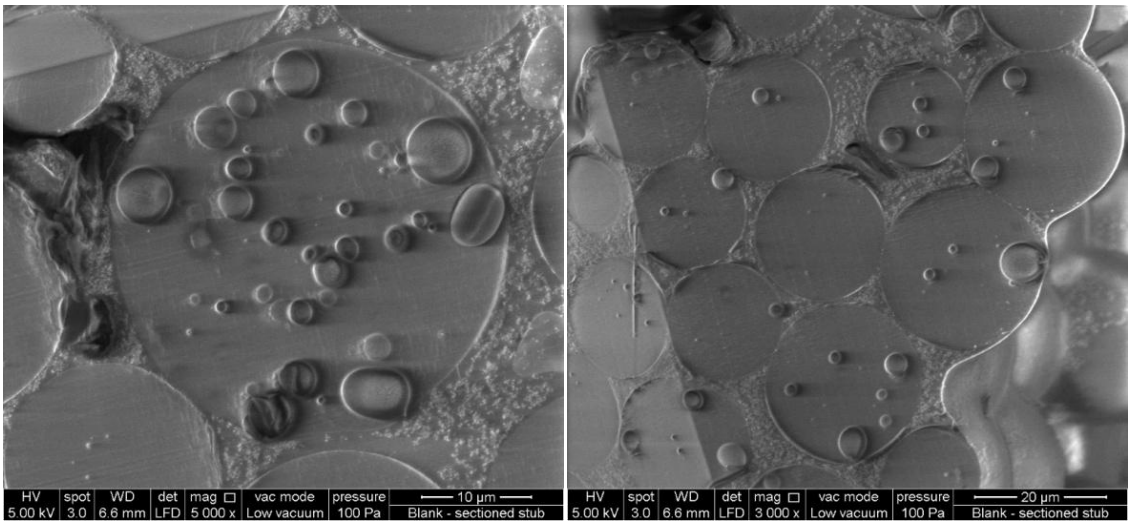


Figure 2.29 (a) and (b) *SEM images of cryosectioned blank Eudragit L100 microparticles*

2.5.11 Eudragit L100 microparticle surface area

The surface area of the microparticles was assessed using three different methods. The first method calculated the surface area of an individual microparticle using the mean dv 0.5 measured by the Malvern Mastersizer using an equation to determine sphere surface area. This calculation assumes a perfectly spherical and smooth microparticle.

Specific surface area was calculated by the Malvern Mastersizer during size analysis by dividing the total area of the microparticles by the total weight. This method also assumes the particles are perfectly smooth and solid spheres. Surface area was also determined using BET. This utilizes adsorption of gas to the surface of the particles to determine surface area. Unlike the other two methods this does not assume the microparticles are smooth and perfectly spherical. It will be affected by particle porosity and roughness. If the microparticles do have pores, which could allow acid to enter, the BET surface area will increase relative to the surface area calculated using the other two methods.

The surface area calculated from the sphere surface area equation was directly linked to the mean size of the microparticles and increased with increasing size, table 2.15. The specific surface area calculated by the Malvern Mastersizer increases with finer particles. The total area of the particles will increase as their size decreases for a given weight of particles. The results in table 2.15 correlate with that theory as the microparticles with non-spray dried lactase are the smallest (largest surface area) and the blank microparticles are largest (smallest surface area).

The surface areas determined by BET showed the same rank order of surface area however there was a greater difference between the surface areas of the different microparticles. The BET results imply pores were introduced into the microparticle surface by encapsulation of lactase and possibly were responsible for acid influx into the particles. When the larger, non-spray dried lactase was encapsulated more porous particles were formed than with the spray dried lactase. This could explain why more non-spray dried lactase was released in 0.1N HCl.

Table 2.15 *Surface areas of blank, non-spray dried lactase and spray dried lactase loaded Eudragit L100 microparticles. Data represents means ± SD.*

Microparticles	Calculated surface area (μm²)	Specific surface area from Mastersizer (m²/ml)	BET Surface Area (m²/g)
Blank	3405.24 ± 315.83	0.29 ± 0.00	0.25
Non-spray dried lactase	2335.45 ± 117.18	0.32 ± 0.01	0.63
Spray dried lactase	2887.31 ± 390.42	0.31 ± 0.01	0.37

2.5.12 Eudragit L100 microparticles with antacids

Eudragit L100 microparticles with spray dried lactase restricted its release in acid. However, they did not prevent the entry of the acid into the particles. This was possibly due to pores within the microparticles.

To counteract degradation in the stomach additional excipients can be added to raise gastric pH. Antacids co-administered with pancreatic enzymes were able to increase their *in vivo* activity (Graham, 1982). However lowering the pH of the stomach could impair its ability to destroy ingested toxins. Alternatively rather than using antacids to increase the pH of the stomach, if they are encapsulated within enteric microparticles only their interior pH is raised. Antacids were co-encapsulated with insulin in PLGA nanoparticles to neutralise acidity produced by PLGA degradation (Sharma et al., 2012, Li and Schwendeman, 2005). The antacids, magnesium hydroxide and sodium bicarbonate, were encapsulated in Eudragit L100 microparticles with spray dried lactase to neutralise acid influx in the stomach preventing lactase denaturation.

Eudragit L100 microparticles with magnesium hydroxide were significantly ($p \leq 0.05$) larger, greater than 200μm, and had a significantly ($p \leq 0.05$) greater span than those without, table 2.16 and figure 2.30. This suggests magnesium hydroxide may have affected the formation of the microparticles resulting in larger particles or aggregates of particles.

The encapsulation efficiency was also significantly (p≤0.05) reduced compared to when lactase alone was encapsulated, 55%. Possibly there is some denaturation of the lactase caused by the inclusion of magnesium hydroxide and therefore active lactase encapsulation is reduced. The effect of magnesium hydroxide on microparticle formation may also have reduced lactase encapsulation. Further work to find an optimal ratio of magnesium hydroxide to polymer for formation of discrete microparticles should be carried out.

Eudragit L100 microparticles with sodium bicarbonate were significantly (p≤0.05) larger than the particles without but were still less than 100μm suggesting the addition of sodium bicarbonate caused less aggregation than magnesium hydroxide, table 2.16 and figure 2.31. There was also complete encapsulation of active lactase suggesting there was no detrimental effect of sodium bicarbonate on lactase activity or encapsulation.

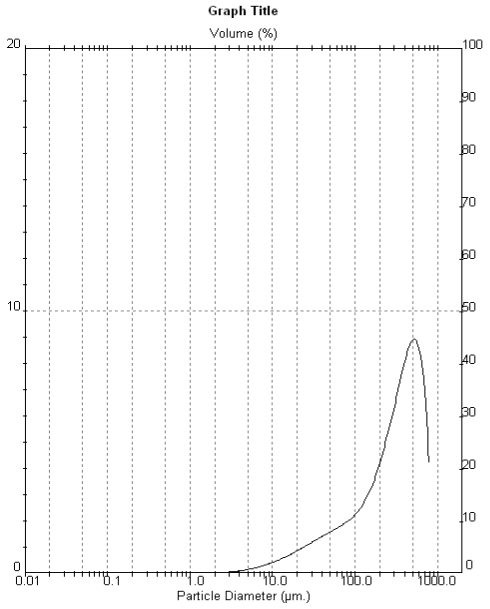


Figure 2.30 *Frequency curve of the size of lactase microparticles with encapsulated magnesium hydroxide (image from Malvern Mastersizer)*

Table 2.16 *Size, span, yield, encapsulation efficiency and lactase loading of blank and lactase loaded Eudragit L100 microparticles with encapsulated magnesium hydroxide or sodium bicarbonate. Data represents means ± SD.*

Microparticles	Mean Size (μm)	Span	Yield (%)	Encapsulation efficiency (%)	Active lactase loading (mg/mg)
Blank magnesium hydroxide	285.09 ± 89.83	2.03 ± 0.45	91.9 ± 5.4		
Lactase magnesium hydroxide	243.74 ± 51.79	2.31 ± 0.25	91.0 ± 4.1	55.2 ± 26.9	0.026 ± 0.01
Blank sodium bicarbonate	98.66 ± 44.08	2.38 ± 0.65	92.3 ± 5.7		
Lactase sodium bicarbonate	88.57 ± 42.49	1.93 ± 0.48	91.9 ± 7.3	108.7 ± 34.9	0.052 ± 0.02

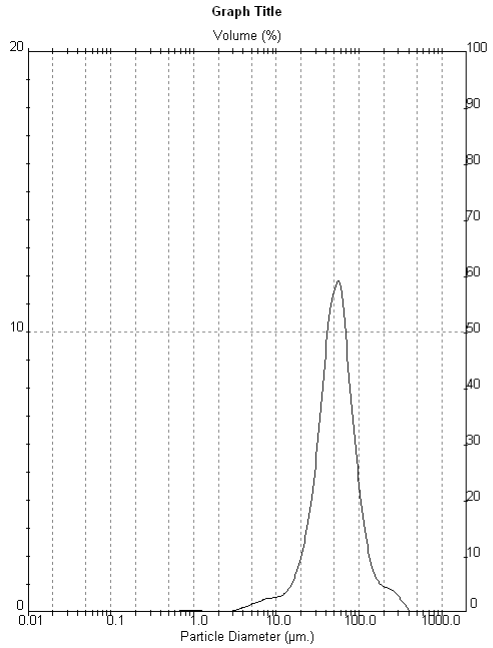


Figure 2.31 *Frequency curve of the size of lactase microparticles with encapsulated sodium bicarbonate (image from Malvern Mastersizer)*

Morphological analysis using SEM showed incorporation of magnesium hydroxide into the microparticles seemed to cause the microparticles to aggregate or not form separate particles, figure 2.32 and 2.33. There are some individual microparticles of less than 100µm but most are joined together in aggregates.

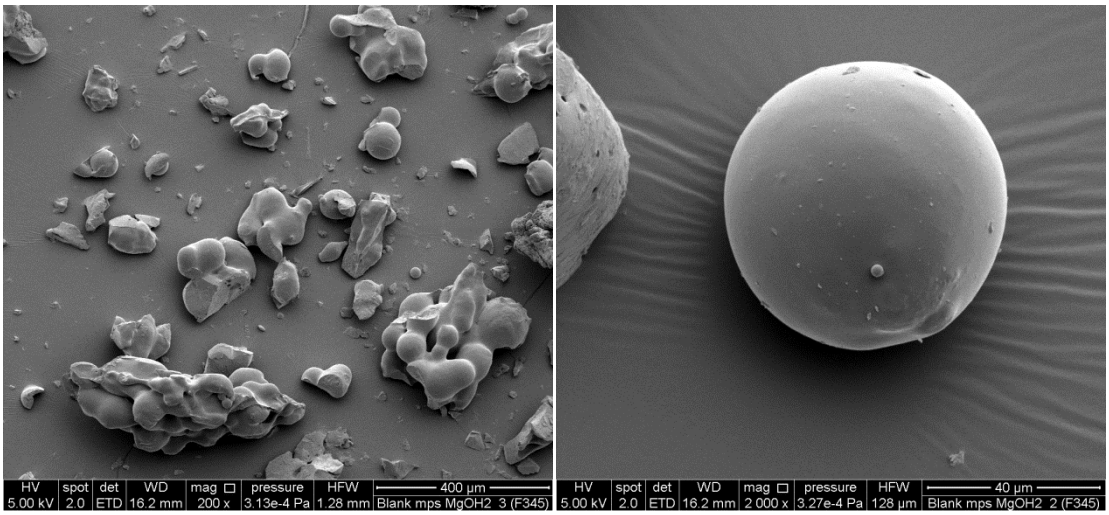


Figure 2.32 (a) and (b) *SEM images of blank Eudragit L100 microparticles with encapsulated magnesium hydroxide*

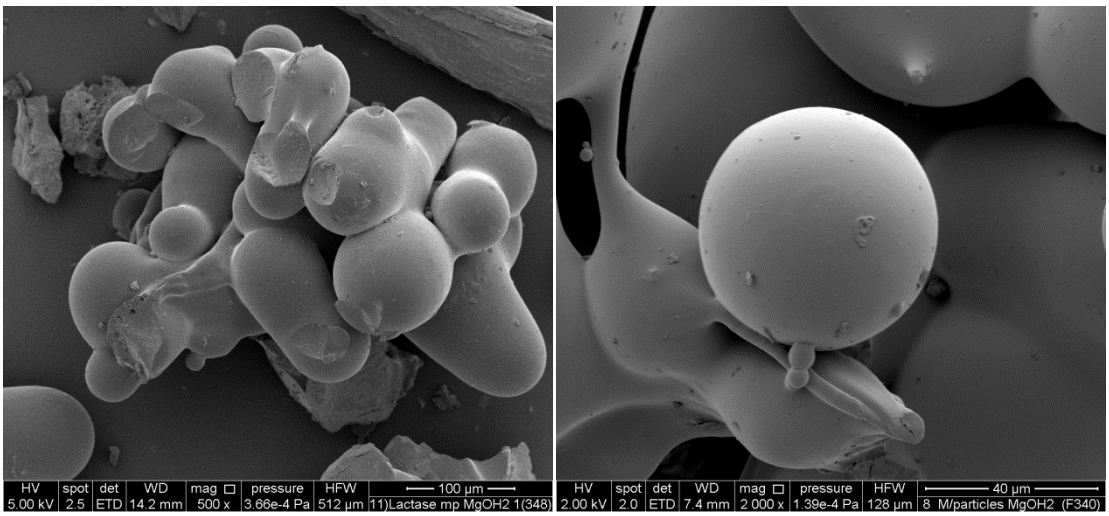


Figure 2.33 (a) and (b) *SEM images of Eudragit L100 microparticles with encapsulated lactase and magnesium hydroxide*

The incorporation of sodium bicarbonate into Eudragit L100 microparticles also caused some aggregation but these were smaller than those with magnesium hydroxide. The particles formed were spherical and less than 100µm even when joined into aggregates, figures 2.34 and 2.35.

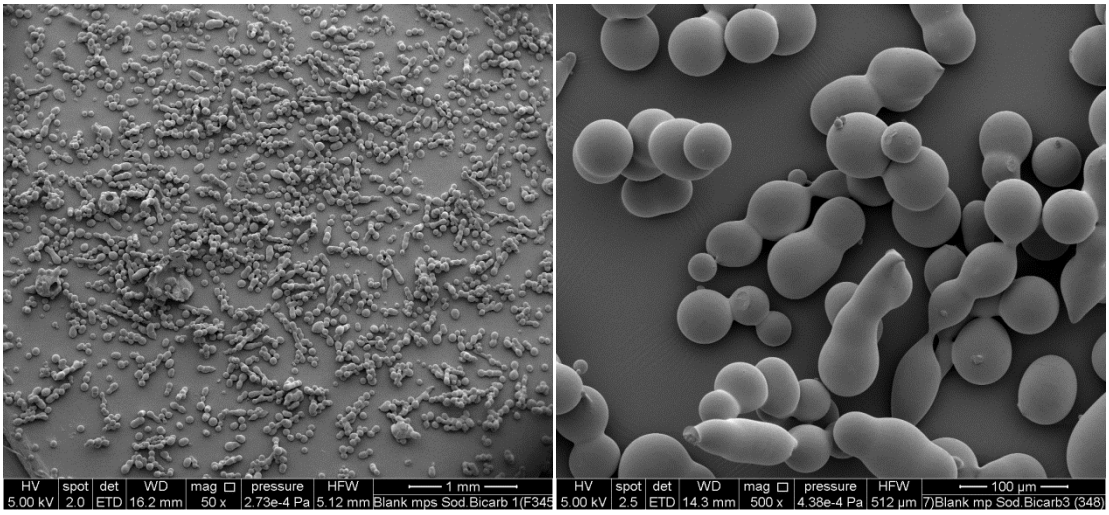


Figure 2.34 (a) and (b) *SEM images of blank Eudragit L100 microparticles with encapsulated sodium bicarbonate*

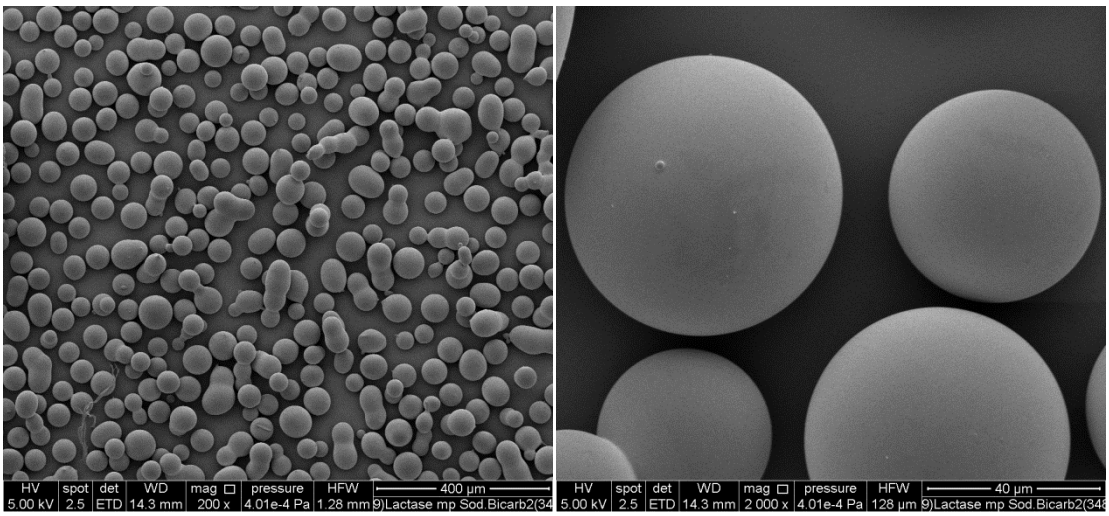


Figure 2.35 (a) and (b) *SEM images of Eudragit L100 microparticles with encapsulated lactase and sodium bicarbonate*

2.5.12.1 *In vitro* release

There was 9% of encapsulated active lactase released from the microparticles with magnesium hydroxide but only 0.1% from the microparticles with sodium bicarbonate after dissolution in 0.1N HCl and pH 6.8 phosphate buffer, table 2.17. The pH of 0.1N HCl after the microparticle dispersal for two hours showed the buffers had not been released from the microparticles to an extent that they neutralised the acid, table 2.17.

Table 2.17 *Active lactase release after 2 hours in 0.1N HCl and 45 minutes in pH 6.8 phosphate buffer from Eudragit L100 microparticles with magnesium hydroxide or sodium bicarbonate and the pH of 0.1N HCl after 2 hours with microparticles. Data represents means ± SD.*

Lactase microparticles	**Active lactase recovery (%) pH 1.2 to 6.8**	**pH of 0.1N HCl after 2 hours with particles**
Magnesium hydroxide	9.2 ± 2.5	1.25 ± 0.28
Sodium bicarbonate	0.1 ± 0.2	1.08 ± 0.06

Encapsulated magnesium hydroxide was able to neutralise some of the incoming acid and protect almost 10% of the encapsulated lactase. It didn't neutralise the external media so should have none of the detrimental effects of raising gastric pH.

Protection may also have occurred due to the larger size of the aggregated particles. The proportion of active lactase released after dissolution was significantly higher from the microparticles with magnesium hydroxide than from those without it and from the Holland and Barrett lactase supplement. Sodium bicarbonate was unable to protect the encapsulated lactase. Possibly a greater amount of sodium bicarbonate is needed to do this or its location within the microparticles doesn't allow lactase protection.

It would be useful to encapsulate a pH sensitive marker within these microparticles to determine if the encapsulated antacids are able to neutralise acid entering the microparticles.

2.5.13 Eudragit L100 microparticles and soy bean oil

Eudragit L100 microparticles with encapsulated spray dried lactase were also formulated in soy bean oil with soy lecithin to provide a barrier to acid influx. Soy bean oil was able to preserve 4.5% of lactase activity after acid incubation of the Holland and Barrett lactase supplement. Replication of this supplement only resulted in 0.65% activity preservation. This may have been due to the hydrophilic lactase partitioning into the aqueous 0.1N HCl. However this would not be an issue for lactase encapsulated within Eudragit L100 microparticles. Eudragit L100 microparticles are more lipophilic and so would be more likely to remain in the protective oily droplets.

There was a significantly ($p \leq 0.05$) higher active lactase release, 3.6%, from the lactase microparticles in soy bean oil, table 2.18 , than from lactase alone in soy bean oil, table 2.9. The microparticles may provide a further barrier to acid than the soy bean oil droplets alone. Lactase alone is also more able to partition into the acidic aqueous phase. Soy bean oil limited acid influx into the microparticles and significantly ($p \leq 0.05$) increased active lactase release following incubation in acid compared to lactase microparticles alone. However, co-encapsulation of magnesium hydroxide in the microparticles was significantly ($p \leq 0.05$) better at preserving lactase activity in acid.

Table 2.18 *Encapsulation efficiency, loading of active lactase in a soy bean oil formulation of lactase microparticles and active lactase release after dissolution in 0.1N HCl and pH 6.8 phosphate buffer. Data represents means ± SD.*

Formulation	Encapsulation efficiency (%)	Active lactase loading (mg/mg)	Active lactase release (%)
Lactase microparticles and soy bean oil	101.6 ± 29.7	0.002 ± 0.00	3.6 ± 3.9
Lactase microparticles	102.5 ± 7.4	0.064 ± 0.01	-0.2 ± 0.8

2.5.14 Overall assessment of oral lactase delivery strategies

The lactase Eudragit L100 microparticles with co-encapsulated magnesium hydroxide were superior in terms of active lactase preservation in simulated gastric conditions to the currently available Holland and Barrett lactase supplement, a lactase soy bean oil formulation, Eudragit L100 microparticles, Eudragit L100 microparticles with co-encapsulated sodium bicarbonate and the microparticles formulated in soy bean oil, figure 2.36.

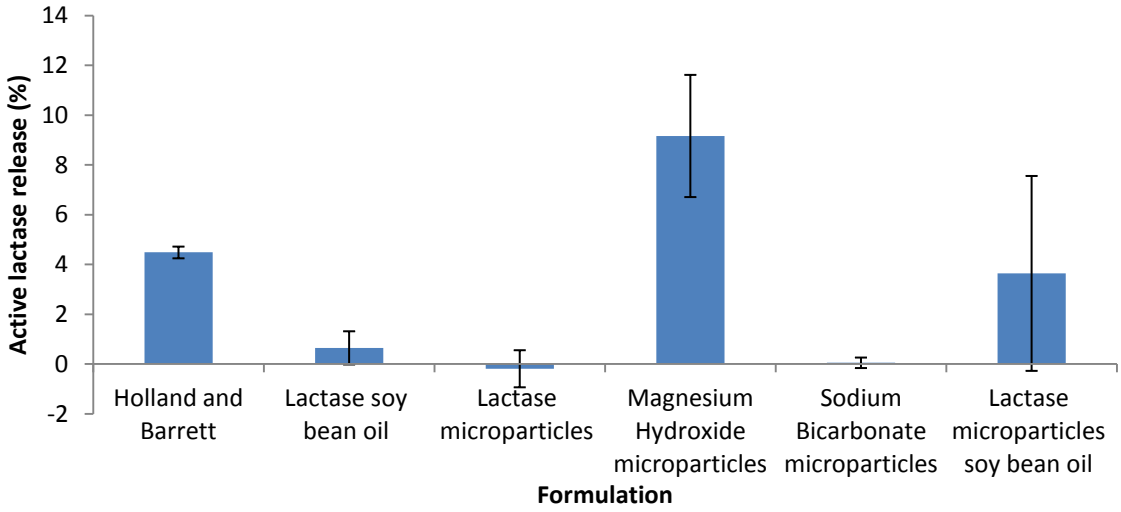


Figure 2.36 *Active lactase release from existing and developed oral lactase formulations after dissolution in acid and pH 6.8 phosphate buffer. Error bars show mean ± SD.*

2.6 Conclusion

Investigations into the stability of lactase in simulated and porcine gastrointestinal fluids and human faecal fluids demonstrated its instability in acidic conditions and stability in small and large intestinal fluids. These results confirmed protection from acid is essential for active lactase delivery to the small intestine.

All the oral lactase supplements subjected to dissolution testing initially in acid and then pH 6.8 phosphate buffer lost more than 95% of their initial lactase activity. The only supplement that was able to preserve more than 1% of the initial lactase activity after acid incubation was the Holland and Barrett soy bean oil based formulation. The others were non-enteric, powder based formulations. The oil may have acted as a barrier between lactase and acid preventing its degradation. The Holland and Barrett supplement still only protected 4.5% of its lactase. This may be due to hydrophilic lactase partitioning into the damaging acidic aqueous fluids and not remaining in the protective soy bean oil droplets. An attempt to recreate this formulation resulted in inferior lactase protection to the original supplement. This may have been due to a lack of some elements of the original formulation and non-optimised excipient ratios.

Due to the acid sensitivity of lactase and the low protection offered by the oil based formulation enteric formulations were investigated. Enteric coated lactase tablets completely preserved lactase activity during two hours incubation at pH 1.2. Active lactase release upon pH rise to pH 6.8 was not immediate, no lactase was released during the first 10 minutes and was only completely released after 30 minutes. This release rate may not be rapid enough to prevent lactose persistence and fermentation. A slow release rate has previously been reported for enteric coated pancreatin tablets and a reduction in dosage form size was found to be beneficial for faster release and greater alleviation of symptoms (Aloulou et al., 2008, Beverley et al., 1987, Naikwade et al., 2009).

Eudragit L100 microparticles encapsulating lactase were produced using a slightly different method (Kendall et al., 2009) to those previously used to formulate lactase (Alavi et al., 2002, Squillante et al., 2003).

Smaller microparticles with an increased encapsulation efficiency and no loss of lactase activity were produced. This method, previously used to encapsulate only low molecular weight drugs, demonstrated its suitability and superiority over previously used methods to encapsulate macromolecules, such as protein and peptide drugs.

However, following pH shift dissolution from pH 1.2 to pH 6.8 there was no active lactase release. It was thought this occurred due to lactase being burst released prematurely into the acid and destroyed. At pH 4.5, below the pH 6 threshold of the polymer, there was a burst release of 64% of the lactase. To overcome this lactase was spray dried to reduce its particle size. This reduced the burst release of lactase to 8% at pH 4.5 and fluorescently labelled spray dried lactase was shown to be encapsulated within the microparticles and not just at the surface. However after dissolution initially in 0.1N HCl there was still no active lactase released. More than 90% of the encapsulated lactase is not released below pH 6 so therefore the acid may be able to enter the microparticles.

A marker encapsulated in the microparticles showed acid influx into the particles by a shift in its emission wavelength. SEM images showed pores in some of the particles, which had not been sputter coated, which could explain the burst release of some of the encapsulated lactase. However this doesn't explain the complete loss of lactase activity. Visualisation using an SEM with a new type of back scatter secondary electron detector and low voltage beam revealed small surface structures on the microparticles which disappeared upon acid incubation. Currently it is not possible to determine the identity of these structures. Possibly their disappearance in acid is linked to acid ingress. Visualisation of cryosections of the microparticles showed a porous interior which may provide a route by which acid can come into contact with encapsulated lactase.

Surface area analysis of the microparticles suggested encapsulation of lactase increased their porosity. The particles with non-spray dried lactase were more porous than those with spray dried lactase which may explain why more of this lactase was burst released in acid.

To neutralise acid entering the microparticles the antacids sodium bicarbonate and magnesium hydroxide were co-encapsulated with lactase in Eudragit L100 microparticles. This increased the size of the microparticles and, with magnesium hydroxide especially, caused aggregation of the microparticles. Sodium bicarbonate was only able to preserve 0.1% of the encapsulated lactase after dissolution in acid initially. Magnesium hydroxide however was able to protect 9.2% of the encapsulated lactase. It achieved this without neutralising the external acid demonstrating its potential as an oral delivery strategy that would not impair the acidic barrier of the stomach.

The microparticles were also formulated in soy bean oil to limit acid influx. There was a 3.6% recovery of active lactase after dissolution from pH 1.2 to 6.8. This is more than for lactase alone in soybean oil. Possibly this is because there are now two barriers between lactase and acid. Additionally lactase is more hydrophilic than the Eudragit L100 microparticles so is more likely to partition from the protective oily phase into the acidic aqueous phase.

Eudragit L100 microparticles with co-encapsulated magnesium hydroxide were superior in terms of active lactase preservation in simulated gastric conditions to the currently available Holland and Barrett lactase supplement and the other oral formulations tested. However even this formulation only preserved approximately 9% of the activity of encapsulated lactase after dissolution in acid. While this is significantly inferior to the protection offered by enteric tablets the benefits these microparticles offer in terms of rapid gastric emptying and enzyme release make them worth further development. Future success of this Eudragit L100 microparticle formulation is dependent on overcoming or eliminating permeability to acid.

Chapter 3

Investigation of the intestinal stability of insulin and development of is oral formulations

3.1 Introduction

Chapter 2 investigated the intestinal stability and oral formulation strategies for a large protein, lactase. This chapter will focus on the oral delivery of a small protein, insulin, that requires not only stabilisation in the intestinal tract but also permeation enhancement to elicit its therapeutic action. As the timing and duration of insulin availability is vital to control glycemic excursions in diabetic patients following food ingestion oral delivery strategies will also have to provide a consistent and predictable insulin absorption. This chapter will seek to analyse its intestinal stability comprehensively and use this information to produce an oral insulin formulation.

Chapter 2 demonstrated that the release of a large protein, lactase, could be controlled in the gastrointestinal tract by encapsulation in enteric Eudragit L100 microparticles. The method used to produce these microparticles enabled a high encapsulation efficiency and did not impair lactase functionality. These particles were not impermeable to the influx of acid so will need further development to protect acid labile protein and peptide drugs. They may still be able to provide protection from intestinal enzymes. Smaller proteins or peptides which lack the structural complexity of lactase may be more compatible with encapsulation in these microparticles as they may not be so affected by acid influx.

3.1.1 Insulin structure and function

Insulin is a peptide hormone of 5.8kDa consisting of 51 amino acids in two polypeptide chains, an A chain with 21 amino acids and B chain with 30 amino acids, figure 3.1. Insulin was the first pure protein therapeutic molecule discovered, in 1922 (Khafagy el et al., 2007), and the first protein to have its sequence determined, in 1951.

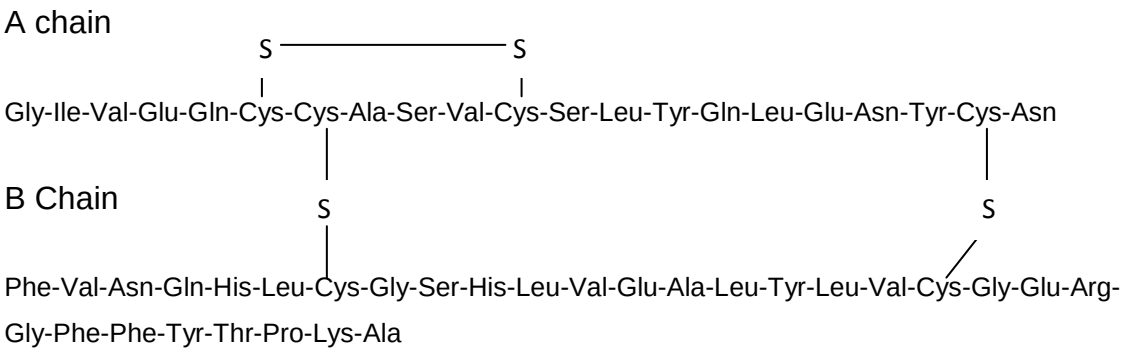


Figure 3.1 *Insulin sequence and structure*

The two polypeptide chains of insulin have alpha helical and beta pleated sheet secondary structures, visualized by x-ray diffraction, figure 3.2. These are stabilized into a three dimensional, tertiary structure by three disulphide bridges and other interactions including Van der Waals forces. Insulin is stored as an inactive hexamer held together by zinc ions and hydrophobic interactions. It is broken down to its active monomeric form to exert its action.

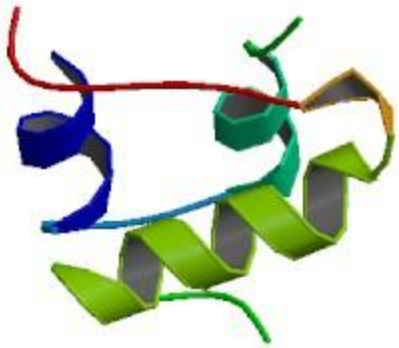


Figure 3.2 *Computer simulation image based on X-ray diffraction of insulin (Timofeev et al., 2010)*

Insulin is produced in the pancreas and regulates carbohydrate and fat metabolism. The binding of insulin to receptors causes cells in the liver, muscle and fat tissue to take up glucose from the blood and store it as glycogen maintaining blood glucose levels within very narrow limits, 70-110mg/dL for a fasting adult. Higher than normal levels of blood glucose is known as hyperglycemia, lower than normal levels is known as hypoglycemia.

When the control of insulin on blood glucose levels fails it results in diabetes. This is characterized by persistent hyperglycemia and is life threatening. Type 1 diabetics no longer produce insulin and type 2 diabetes results from resistance of cells to insulin and insufficient insulin production by the pancreas.

Type 1 diabetics rely on daily administration of exogenous insulin to reduce dangerously high blood glucose levels and stay alive. It is estimated more than 30 million people globally have type 1 diabetes. Type 2 diabetics are not dependent on insulin but more than 40% use it to maintain blood glucose levels. The global insulin market was estimated to be worth approximately $12 billion in 2011.

3.1.2 Current administration of insulin

Insulin is currently available only as an injectable in the US and Europe. Generally insulin is self administered subcutaneously, rarely intramuscularly or intravenously. An insulin dosage regimen is tailored individually to suit particular diets and lifestyles. There are various forms of insulin available with rapid, intermediate and long lasting action. These forms can be used in combination to provide the best maintenance of blood glucose levels.

The parenteral route is not ideal due to the stress, pain, non-compliance, risks and cost issues of injections. The chronic nature of insulin injections for the treatment of diabetes can cause local hypertrophy and fat deposition at the injection site. Insulin injections can also cause hyperinsulinemia, hypoglycemia and weight gain.

Reformulation of insulin to provide alternative, non-parenteral medicines has been and continues to be a popular research topic due to the negatives associated with injections and the large number of users. Recombinant DNA has aided this process as large amounts of insulin can now be made. Pfizer, in collaboration with Nektar Therapeutics, delivered the first non parenteral insulin formulation to the EU and US market in 2006, Exubera, an inhaled form of insulin for pulmonary delivery. However there was insufficient market uptake and withdrawal by Pfizer in 2007. Its lack of acceptance may have been due to difficulties with accurate dosing and the need to still inject insulin to maintain a basal insulin level due to Exubera's short action.

A buccally delivered insulin, Oral-lyn, which is administered into the mouth by a spray (RapidMist device) has been developed by Generex and is available for treatment of type 1 and 2 diabetes in some countries, including India, but not the US or Europe. Insulin delivered by this system is absorbed from the buccal mucosa so does not have to overcome the delivery challenges of the GI tract.

3.1.3 Oral insulin

As well as the benefits of oral delivery over parenteral in terms of compliance, ease of use and costs oral delivery of insulin is advantageous as it is a more physiological delivery route than that by subcutaneous injection. Orally delivered insulin would be absorbed from the GI tract into the portal vein, its delivery to the liver and binding to receptors there mimicking the normal physiologic route of insulin. Orally delivered insulin may also reduce the risks of hypoglycemia seen with subcutaneously delivered insulin due to a reduced systemic exposure.

Attempts at oral insulin delivery initiated by Dr Joslin soon after its discovery in 1922 have been reported in oral insulin reviews (Heinemann and Jacques, 2009, Kalra et al., 2010). These trials had to be halted due to lack of metabolic control, possibly due to its low oral bioavailability. Oral delivery of insulin may also pose some risks as it is a known mitogen. Due to the frequency of insulin administration the effects of excipients of its oral formulations would also have to be considered.

3.1.4 Barriers to oral delivery

The oral bioavailability of insulin in rats was found to be less than 1% (Takeuchi et al., 1996, Tozaki et al., 2001). This is a result of poor intestinal stability and permeability.

Insulin was degraded in gastric conditions, only 0-10% remained intact after 5 minutes incubation in simulated gastric fluid (SGF) with pepsin (Jain et al., 2012, Han et al., 2012, Qi and Ping, 2004, Makhlof et al., 2011b) and was completely degraded in 3 minutes in gastric fluid from a pig (Werle et al., 2008). Insulin is soluble and stable in weak acids so it may not be the acidic pH of these fluids causing its degradation but pepsin as insulin contains several peptide bonds which would be vulnerable to pepsin digestion.

Insulin was also vulnerable to small intestinal enzymes. Only 10% remained intact after two hours incubation in simulated intestinal fluid with pancreatin (Jain et al., 2012). Of the three main luminally secreted intestinal enzymes insulin was most vulnerable to chymotrypsin and was completely degraded by it within 15 minutes (Werle et al., 2008). Insulin was more stable with trypsin and elastase. After three hours 92.5% of insulin remained intact with trypsin (Schilling and Mitra, 1991) and 35% remained intact with elastase (Werle et al., 2008). In porcine small intestinal fluids insulin was completely degraded within a few minutes due to the combined digestive effects of these enzymes (Werle et al., 2008).

Despite the reduced proteolytic activity of the large intestine insulin was found to be vulnerable to degradation here. It was degraded in rat cecal contents with a degradation half life of 34 minutes (Tozaki et al., 1997, Tozaki et al., 1995). Its degradation is due to microbial enzymes and fermentation. Any oral insulin targeted to the colon would have to overcome this.

The intestinal permeability of insulin is limited by its large size, limiting its paracellular uptake, and its hydrophilicity limiting its passive, transcellular transport. Insulin absorption from the intestinal segments of a rat was found to be greatest from the ileum, then the duodenum and least from the jejunum (Han et al., 2012). These differences in absorption may be due to reduced proteolytic activity in the ileum and the presence of M cells in Peyer's patches which are a portal for macromolecule uptake. The thick mucus covering the cells in the jejunum may have reduced insulin absorption.

3.1.5 Oral insulin delivery strategies

The oral delivery strategies described in chapter 1 have been widely explored for the oral delivery of insulin. Representative examples of these strategies and their in *vitro/in vivo* effects are presented in table 3.1.

While these strategies increased oral insulin pharmacological availability and efficacy compared to an insulin solution from negligible levels, their relative availabilities compared to subcutaneous insulin were less than 15% if used exclusively.

When strategies were combined, as with ligand attachment to insulin-loaded nanoparticles, oral insulin availability increased above 20% (Chalasani et al., 2007, Petrus et al., 2007, Jain et al., 2012). These strategies can disrupt normal digestive processes and possibly enable absorption of potentially harmful molecules so the long term effects of administering them chronically needs to be considered.

3.1.6 Commercial oral insulin delivery

Despite extensive research very few strategies have been tested in humans. Clinical data may not have been published due to the competitive nature of oral insulin formulation development. The strategies that have progressed furthest clinically are those that have been or are being pursued by companies, table 3.2.

The oral insulin formulations that have progressed furthest clinically are those of Diabetology (Kalra et al., 2010, Luzio et al., 2010), Emisphere (Heise et al., 2004), Biocon (Kalra et al., 2010, Khedkar et al., 2010), Oramed (Kidron et al., 2008, Eldor et al., 2010a) and Diasome (Schwartz et al., 2008). These strategies have demonstrated safety and tolerability in phase I and II clinical trials and increased insulin levels and reduced glucose levels in healthy, type 1 or type 2 diabetic volunteers. They produced relative bioavailabilities of between 5 and 10% relative to subcutaneous insulin and glucose reductions of 7-55% (Kidron et al., 2008, Eldor et al., 2010a, Kalra et al., 2010, Khedkar et al., 2010, Luzio et al., 2010, Heise et al., 2004). The high number of discontinued projects is usually the result of poor metabolic control.

Table 3.1 *Examples of delivery strategies tested in vitro and in vivo for the oral delivery of insulin*

Enzyme inhibition	**Colon targeted delivery**	**Mucoadhesion**	**Permeation enhancers**	**Ligand attachment**	**Colloidal carriers**
Increased *in vitro* protection[1-4]		Increased *in vitro* mucoadhesion[7-12]			Increased *in vitro* protection and *in vivo* oral efficacy Lipsomes[18], PLGA nanoparticles 7.6-12.7% oral PA, rats [8, 17, 19-20] , poly (alkyl cyanoacrylate) nancapsules[21], emulsions[22] , microemulsions[23]
6.15% oral PA, rats[5]	3.38-5.02% oral PA rats/dogs[5-6]	Chitosan- 10.5% oral PA rats[8]	Bile salts with liposomes[13]/ PLGA nanoparticles[14] ~11% oral PA rats	Vitamin B12[15-16], folate[17] attached to nanoparticles >20% oral PA rats	

Pharmacological availability (PA), relative to subcutaneous insulin

[1] (Agarwal et al., 2001), [2] (Tozaki et al., 1997), [3] (Morishita et al., 1993), [4] (Bai et al., 1996), [5] (Kraeling and Ritschel, 1992), [6] (Tozaki et al., 2001), [7] (Takeuchi et al., 1996), [8] (Zhang et al., 2012a), [9] (Wood et al., 2006), [10] (Wood et al., 2010), [11] (Zhang et al., 2012b), [12] (Toorisaka et al., 2012), [13] (Niu et al., 2012), [14] (Sun et al., 2011a), [15] (Chalasani et al., 2007), [16] (Petrus et al., 2007), [17](Jain et al., 2012), [18] (Niu et al., 2011), [19] (Cui et al., 2006b), [20] (Yang et al., 2012), [21] (Damge et al., 1997), [22] (Toorisaka et al., 2003), [23] (Sharma et al., 2010)

Table 3.2 *Commercial insulin strategies past and present and their clinical testing status*

Company- oral insulin	**Delivery strategy**	**Clinical stage**
Biocon- IN105 (continuing work of Nobex Corporation)	Insulin conjugation to amphiphilic oligomer alkylated-PEG provides enzymatic protection, increased permeation. Permeation enhancer sodium caprate included in formulation	Phase III –type 2 diabetics, failed to meet primary endpoint, 2011 Phase I/II- type 2 diabetics Phase I- type 1 diabetics, 2011
Oramed ORMD-0801	Protease inhibitor (eg aprotinin), omega-3 fatty acids for insulin preservation, EDTA absorption enhancer, tablet enteric coated with Eudragit L100	Phase I/II- type 2 diabetics Phase I/II- type 1 diabetics, 2008
Diabetology-Capsulin July 2012- partnership with USV Limited announced to develop for Indian market	Axcess technology- absorption enhancer and solubiliser in enteric coated capsules	Phase I/II- type 1 and 2 diabetics, 2005
Emisphere	Eligen technology- synthetic, non acylated amino acid carrier 4-CNAB used to expose hydrophobic residues of insulin enabling transcellular uptake	Phase I/IIa- type 2 diabetics, discontinued with type 1 diabetics

Novo Nordisk and Merrion-NN1953	Gastrointestinal permeation technology (GIPET), matrices of medium chain fatty acids to open paracellular channels loaded in enteric capsule	Phase I-completed 2012, no results yet
NOD Pharmaceuticals-Nodlin	Bioadhesive nanoparticles	Phase I- completed 2012, significant glucose response
Oshadi Drug Administration	Blend with inert silica nanoparticles and polysaccharide suspended in oil and loaded in enteric capsules	Phase I- type 1 diabetics 2011
Access Pharmaceuticals	Insulin/insulin nanoparticles coupled to vitamin B12 analog cobalamin	Proof of principle in animal models
NanoMega Medical Corporation	Chitosan-γ-PGA nanoparticles	Blood glucose reductions in rats
Diasome	Liposomes with hepatocyte targeting molecule	Phase II/III-type 2 diabetics commenced 2008- discontinued
Provalis, Cortecs Ltd- Macrulin	Water in oil microemulsion	Phase II- type 2 diabetics- efficacy shown but discontinued
Eli Lilly and AutoImmune-AI 401		Phase II- type 1 diabetics- delay disease onset, but no effect on those less than 20 years old, discontinued
Apollo Life Sciences- Oradel	Nanoparticles attached to vitamin B12	Phase I- discontinued
Endorex	Liposomes- orasomes	Stable *in vitro*, discontinued

Only the formulations of Diasome and Biocon have reached phase III clinical trials. The results of Diasome's trials have not been reported and there has been no news of this project since 2009. Biocon's recent phase III trial with type 2 diabetics failed to meet its primary goal of lowering HbA1c levels by 0.7% relative to a placebo. This is a marker of average plasma glucose levels over time. It is thought this may not necessarily be due to a lack of efficacy but behavioural modifications of those taking the placebo. It did however produce a statistical reduction in post prandial glucose levels and this project is still being pursued.

High inter subject variability of insulin absorption has been commonly observed during clinical trials and been seen with the formulations of Biocon (Kalra et al., 2010, Khedkar et al., 2010), Emisphere (Heise et al., 2004) and Diabetology (Whitelow et al., 2005). This could result in inconsistent blood glucose control. This may be due to the inter-individual GI variability, intra individual variation in insulin absorption has not been explored.

Necessarily to fulfil its function insulin must be administered usually before, rarely after, food. Food ingestion hampered the oral efficacy of the formulations of Biocon (Khedkar et al., 2010), Emisphere and Diasome (Blair Geho et al., 2009) increasing glycemic excursions. Possibly they are delayed in the stomach where degradation may occur, if not enteric coated, and arrival in the small intestine is postponed. Timing of administration, with regards food ingestion, should be optimised to minimise absorption delays but also to ensure it is present at therapeutic levels following a meal.

Onset of insulin absorption and subsequent blood glucose reduction was affected by enteric coating. Enteric coated formulations of Oramed and Diabetology had a much slower and prolonged insulin absorption and glucose reduction than the non-enteric formulations of Biocon and Emisphere. This could be due to a gradual rather than immediate dissolution of the coating. Insulin levels increased following administration of the enteric formulations 80 to 250 minutes post dosing and glucose levels were still reducing after seven hours (Kidron et al., 2008, Eldor et al., 2010a, Kidron et al., 2009, Whitelow et al., 2005).

In contrast insulin absorption and glucose reduction for the non-enteric formulations was rapid reaching maximum levels 20 to 50 minutes post dosing and only lasting for 80 minutes, possibly due to their lack of enzyme protection (Khedkar et al., 2010, Kidron et al., 2004, Kapitza et al., 2010).

Enteric coated formulations will be able to maintain a basal level of insulin over a longer period but prandial glucose control may be hampered due to its slow absorption. Additionally its sustained effects may cause late post prandial hypoglycaemia. Non-enteric formulations may provide better, faster control of post prandial glycemia but their short duration may necessitate the use of a longer acting insulin to maintain basal levels.

Despite the progress which has been made with oral insulin delivery strategies, on average, their relative biopotency compared to subcutaneous insulin is approximately 10%. Therefore 10 times as much insulin would have to be administered orally to have the same effect as a subcutaneous dose, incurring higher costs.

3.1.7 Enteric multiparticulates

Insulin encapsulation in enteric micro or nanoparticles can provide protection from pepsin and a more prolonged glucose reduction than non-enteric formulations reducing the need for additional insulin. Their small size will allow them to be suspended in gastric fluids, unlike larger dosage forms, enabling reliable, fast and uniform gastric emptying. Enteric tablets/capsules are more affected by the presence of food than smaller dosage forms (Al-Habet and Rogers, 1989, Adair et al., 1992). Their increased surface area also enables rapid drug release. This could reduce the delay and variability in insulin absorption found with enteric capsules or tablets and lessen the risk of late post prandial hypoglycaemia.

3.1.7.1 *In vitro* testing

Enteric polymers, used to coat or form micro and nanoparticulates, have restricted insulin release to less than 25%, *in vitro*, in acidic conditions, table 3.3, however their ability to protect insulin from pepsin digestion was only tested in a few cases.

Table 3.3 *Insulin release from enteric micro/nanoparticulates in acid and at small intestinal pH, and its protection from pepsin*

Enteric formulation	**Acid release (%)**	**Pepsin protection (% degraded)**	**Release at small intestinal pH (%)**
HPMCP/HP55 (nanoparticles)	<20% 2 hours[1-5]	70% 2 hours[1]	60-85% 2 hours[4-5]
Eudragit L100/S100/L30D (nanoparticles, microparticles)	<20% 2 hours[6-11]		Up to 55-90% 2 hours[6-7, 9-12]
Hyaluronic acid nanoparticles	<10% 2 hours[13]	55% 2 hours[13]	80% 2 hours[13]
Poly (methacrylic acid) complexation hydrogel microparticles	<20% 2 hours[14-17]		60-90% 2 hours[14-15, 17]
Chitosan-poly(γ-glutamic acid) (γ-PGA) nanoparticles	<25% 2 hours[3, 18-20]		75-85% 2 hours[18-20]
Chitosan phthalate microspheres	20%, 2 hours[21-22]	12% 2 hours[21-22]	Up to 55% 2 hours[21-22]

HPMCP/HP55= hydroxypropylmethylcellulose phthalate

[1] (Makhlof et al., 2011b), [2] (Wu et al., 2012b), [3] (Sonaje et al., 2010a), [4] (Cui et al., 2007), [5] (Wu et al., 2012a), [6] (Zhang et al., 2012b), [7] (Naha et al., 2008), [8] (Sonia and Sharma, 2012), [9] (Mundargi et al., 2011a), [10] (Jain et al., 2006), [11] (Jain et al., 2005), [12] (Agarwal et al., 2008), [13] (Han et al., 2012), [14] (Sajeesh and Sharma, 2010), [15] (Sajeesh et al., 2010), [16] (Carr and Peppas, 2010), [17] (Wood et al., 2006), [18] (Sonaje et al., 2010b), [19] (Lin et al., 2007), [20] (Lin et al., 2008), [21] (Ubaidulla et al., 2007a), [22] (Ubaidulla et al., 2007b)

Results showed a disparity between the proportion of encapsulated insulin released in acid and the proportion protected from pepsin. HPMCP cross-linked chitosan nanoparticles prevented 85% of insulin release at pH 1.2 in two hours, however only 30% remained intact after the same period with pepsin (Makhlof et al., 2011b). Similar results were found with hyaluronic acid nanoparticles which prevented >90% of insulin release at pH 1.2 in two hours but only protected 45% with pepsin (Han et al., 2012). Although insulin was not released from the particles pepsin was still able to digest it. It seems unlikely that pepsin was able to penetrate into the particles as it is a large protein, 35kDa, and the release of the smaller insulin, 5.8kDa, was restricted. Insulin may have been present at or near the surface of the particles and therefore pepsin was able to digest it.

The only formulation that provided comparable insulin release in acid and protection from pepsin were the chitosan phthalate microspheres (Ubaidulla et al., 2007a, Ubaidulla et al., 2007b). Approximately 20% of encapsulated insulin was released at pH 2 and 88% was protected from pepsin digestion. In this study only 47% of unprotected insulin was degraded with pepsin in two hours which is a surprisingly high figure compared to other studies where it was rapidly degraded (Jain et al., 2012, Han et al., 2012, Makhlof et al., 2011b, Qi and Ping, 2004). The source of pepsin, its concentration or experimental conditions may not comparable to other studies and the protection of insulin should be viewed considering this.

Release at the threshold pH of the enteric formulations was greater than 55% within two hours in all cases. Insulin release from nanoparticles loaded in enteric coated capsules was slower, 50-68% released over four hours (Wu et al., 2012b, Sonaje et al., 2010a). This implies insulin absorption may be faster *in vivo* from enteric micro and nanoparticles than enteric capsules or tablets.

3.1.7.2 *In vivo* testing

When orally administered to rats or rabbits enteric micro/nanoparticles enabled insulin absorption and blood glucose reduction, table 3.4, insulin solutions had a negligible effect.

Table 3.4 *In vivo glucose reduction and pharmacological availability, relative to subcutaneous (sc) insulin, of orally administered insulin loaded enteric micro/nanoparticles in rats/rabbits*

Enteric formulation	**T max for glucose reduction**	**Duration of glucose reduction**	**Pharmacological availability (%)**
HPMCP/HP55 nanoparticles	3-12 hours[1-3]	24 hours[1-2]	6.27-11.3%[1-4]
Eudragit L100/S100/L30D (nanoparticles, microparticles)	2-4 hours[5-8]	3-8 hours[6-8]	2.65%[7]
Hyaluronic acid nanoparticles	7 hours[9]	8 hours[9]	
Poly(methacrylic acid) complexation hydrogel microparticles	2 hours[10]	10 hours[10]	2.45%[10]
Chitosan-γ-PGA nanoparticles	5-6 hours[11-12]	10-24hours[11-12]	16%[12]
Chitosan phthalate microspheres	5 hours[13]	20 hours[13]	18.66%[13]

[1] (Wu et al., 2012a), [2] (Makhlof et al., 2011b), [3] (Cui et al., 2007), [4] (Wu et al., 2012b), [5] (Naha et al., 2008), [6] (Mundargi et al., 2011a), [7] (Zhang et al., 2012b), [8] (Jain et al., 2005), [9] (Han et al., 2012), [10] (Sajeesh et al., 2010), [11] (Lin et al., 2007), [12] (Sonaje et al., 2010b), [13] (Ubaidulla et al., 2007a)

They may have increased insulin availability by limiting its release in the stomach, minimising degradation. Duration of glucose reduction and insulin pharmacological availability may be underestimated as some of these experiments were halted before glucose levels returned to basal levels. The anticipated faster glucose response and shortened period of glucose lowering by administering enteric multiparticulates rather than larger capsules or tablets was not observed, table 3.4. The fastest glucose response was produced by the poly(methacrylic) acid hydrogel and Eudragit particles. Maximum glucose reductions occurred two to four hours post dosing which is comparable to the enteric capsules and tablets of Oramed and Diabetology (Kidron et al., 2008, Eldor et al., 2010a, Kidron et al., 2009, Whitelow et al., 2005). Possibly the particulates aggregated delaying dissolution.

Eudragit nano/microparticles restricted insulin release *in vitro* in acid and released up to 90% encapsulated insulin upon pH rise. Of the enteric multiparticulate formulations tested they offer the best potential for protection, faster glucose reduction and shortened period of glucose reduction. However, these particles had lower insulin pharmacological availabilities than some of the other formulations, particularly those with chitosan (Makhlof et al., 2011b, Sonaje et al., 2010b, Ubaidulla et al., 2007a)**.** Chitosan can increase mucoadhesion and paracellular absorption. Enteric protection alone may not be sufficient for acceptable oral insulin pharmacological availability but additional excipients can increase this.

3.1.7.3 Methods of production

As the methods used to produce enteric multiparticulates often entail the use of solvents and high shear forces there is a risk of denaturing insulin during its encapsulation. Their more complex formation, compared to enteric coating of capsules or tablets, may explain why they have not been popular so far with companies. The ideal method should be simple and use the least damaging parameters possible.

The majority of enteric nano/microparticles are produced using a water in oil in water (w/o/w) double emulsion solvent evaporation method.

This involves potentially damaging solvents such as dichloromethane (DCM), and using sonication, homogenisation, centrifugation and lyophilisation (Wu et al., 2012a, Cui et al., 2007).

Eudragit L100 and S100 particles encapsulating insulin have been formed by double emulsion solvent evaporation (Mundargi et al., 2011a, Jain et al., 2005, Jain et al., 2006) and oil in oil (o/o) emulsion solvent evaporation (Morishita et al., 1993). The advantages of the o/o method over the double emulsion method were the use of less toxic ethanol to dissolve the polymer and no homogenisation. Insulin encapsulation in HPMCP microspheres was increased from 30 to 70% by the o/o method (Qi and Ping, 2004). Hydrophilic insulin may have escaped encapsulation by migration to the outer aqueous phase in the w/o/w method whereas this is not possible for the o/o method. *In vitro* insulin release in acid was reduced from 20% to nothing and insulin recovery increased from 40% to 80% with pepsin when encapsulated by the o/o method. More insulin may be present at the surface of the w/o/w produced microparticles due to its localisation at the oil water interface. Insulin loaded particles produced by the o/o method significantly reduced glucose levels when orally administered to rats, those produced by the w/o/w method had a weak effect.

In chapter 2 the o/o emulsion solvent evaporation method of Kendall *et al*. was used to encapsulate the large protein, lactase, in enteric Eudragit L100 microparticles (Kendall et al., 2009). Uniformly sized particles of <100µm with high yields, >90%, and encapsulation efficiencies, >80%, were formed. Lactase activity was not impaired using this method demonstrating its suitability for protein encapsulation. Spray dried lactase release in acid was restricted by the particles although they were possibly permeable to acid. Insulin needs protection from pepsin, which these particles could provide, and as it is soluble and active in weak acids may not be as vulnerable to any incoming acid. This method is simpler than a w/o/w method and avoids the use of potentially damaging homogenization, centrifugation or lyophilisation. These microparticles may be able to provide a more rapid and uniform small intestinal release and faster insulin absorption than larger enteric coated dosage forms. They also offer the potential to minimize the variability and food effects on insulin absorption.

3.2 Aims

- To model the stability of insulin throughout the gastrointestinal tract using simulated and porcine gastrointestinal fluids and human faecal fluids;
    - To use this knowledge for rational design of oral insulin delivery vehicles
    - To gain an insight into the oral stability of small proteins
- To produce oral insulin formulations which have the potential ;
    - To protect insulin from identified intestinal stability challenges
    - To minimise insulin absorption variability and vulnerability to food effects seen with clinically trialled insulin formulations
    - To provide sufficiently rapid post prandial glucose control
    - To prolong insulin absorption enough to not require administration of additional insulin
    - To not prolong glucose reduction for so long post prandially that hypoglycaemia occurs
- To formulate insulin using parameters which are least potentially damaging to insulin integrity and most conducive for the desired release characteristics
- Assessment of oral insulin formulations
    - To characterise them in terms of physical characteristics (size, polydispersity, morphology), loading and encapsulation efficiency
    - To assess their *in vitro* insulin release and ability to protect insulin

3.3 Materials

Human recombinant insulin, pepsin from porcine gastric mucosa, 469 units/mg solid, 924 units/mg protein, pancreatin from porcine pancreas, activity at least 3x USP specifications and trifluoroacetic acid were from Sigma Aldrich. Hydrochloric Acid 37%, specific gravity 1.18, was from BDH. Acetonitrile HPLC grade and sodium hydroxide were from Fisher Scientific. Pig gastric and intestinal fluids were from freshly slaughtered pigs and were immediately frozen and stored at -80^{o}C. Human faecal fluids were from healthy individuals not taking antibiotics.

Faecal basal media materials: Bacteriological peptone and yeast extract were from Oxoid. Sodium chloride, L-cysteine hydrochloride, vitamin K, resazurin sodium salt and sodium hydroxide were from Fisher Scientific. Dipotassium hydrogen orthophosphate and magnesium sulphate 7-hydrate were from BDH. Calcium chloride dihydrate was from VWR. $NaHCO_3$ and haemin were from Sigma Aldrich. Bile salts were from Fluka Analytical. Tween 80 was from Fluka Chemika.

Eudragit L100 was a gift from Degussa/ Evonik (Darmstadt, Germany), sorbitan sesquioleate (Alacel 83), monobasic potassium phosphate and phosphate buffered saline tablets (PBS) were from Sigma Aldrich. Liquid paraffin BP was supplied by JM Loveridge Plc. Sodium phosphate, tribasic, anhydrous was from Alfa Aesar. n-hexane and citric acid were from Fisher Scientific. Ethanol, 96% v/v was from BDH.

3.4 Methods

3.4.1 Insulin intestinal stability

400µl of a 2mg/ml insulin solution (prepared in 0.001N HCl) was added to 1.6ml of simulated gastric fluid (SGF) with and without pepsin and simulated intestinal fluid (SIF) with and without pancreatin (final insulin concentration of 0.4mg/ml). These were placed in a Gallenkamp shaking incubator at 37°C and agitated at 100rpm. Samples (0.15ml) were removed after 0, 15, 30, 60, 90 and 120 minutes and added to either 0.45ml 0.002M NaOH to raise the pH of acidic fluids or 0.01N HCl to lower the pH of small intestinal fluids to halt the reaction (final insulin concentration 0.1mg/ml). These samples were analysed for insulin content using HPLC. SGF and SIF were prepared as described in chapter 2, section 2.4.2.

400µl of a 2mg/ml insulin solution was added to 1.6ml of gastric (pH 3.62), duodenal (pH 6.77), jejunal (pH 6.94), ileal (pH 7.48) and descending colonic fluids (pH 7.4) from a pig, giving a final insulin concentration of 0.4mg/ml. The intestinal contents were centrifuged at 10,000rpm for 10 minutes and the supernatants used to test stability. These mixtures were placed in a Gallenkamp shaking incubator using the parameters used for the simulated intestinal fluids.

Samples (0.15ml) were removed after 0, 15, 30, 60, 90 and 120 minutes and added to either 0.45ml 0.002M NaOH to raise the pH of acidic fluids or 0.01N HCl to lower the pH of small and large intestinal fluids to halt the reaction (final insulin concentration 0.1mg/ml). These samples were then filtered and analysed for insulin content using HPLC.

400µl of a 2mg/ml insulin solution was added to 1.6ml of human faecal slurry and placed in a Gallenkamp shaking incubator using the parameters used in the previous tests (final insulin concentration 0.4mg/ml). Samples (0.15ml) were removed after 0, 15, 30, 60, 90 and 120 minutes and added to 0.45ml 0.01N HCl to lower the pH to halt the reaction (final insulin concentration 0.1mg/ml). These samples were centrifuged at 10,000rpm for 10 minutes and the supernatants analysed for insulin content using HPLC. The human faecal slurry was prepared as described in chapter 2, section 2.4.2.1.

Insulin recovery in the withdrawn samples was calculated using the equation below. The theoretical concentration of the sample is the concentration of insulin assuming 100% recovery. This value was calculated with respect to changes in the sample volume during the course of the experiments.

$$\text{Insulin recovered (\%)} = \frac{Measured\ insulin\ concentration\ (\mu g/ml)}{Theoretical\ insulin\ concentration\ (\mu g/ml)} \text{x100}$$

3.4.2 HPLC method

Samples were run in a mobile phase of 0.1% (v/v) trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile (30-60%) at 40°C over a run time of 23 minutes at a flow rate of 1ml/min. 20µl samples were injected onto and separated with a Discovery 300°A C18 column and detected at 210nm.

3.4.3 Eudragit L100 microparticle preparation

This method is based on that of Kendall *et al*. with the substitution of prednisolone for insulin. Eudragit L100 (300mg) was dissolved in ethanol (3ml). Recombinant human insulin (5mg) was suspended in the ethanol to prepare microparticles with a drug to polymer weight ratio of 1:60 and stirred for at least 30 minutes. This suspension of insulin in Eudragit L100 solution was emulsified into liquid paraffin (20ml) containing 1% (w/w) of sorbitan sesquioleate (Arlacel 83) as an emulsifying agent, using a Heidolph RZR1 stirrer at 1500rpm.

Stirring was carried out for 18 hours at room temperature to allow solvent evaporation and particle solidification. The microparticles formed were recovered by vacuum filtration through a Pyrex sintered glass filter (pore size 4; 5-15μm) and washed three times with *n*-hexane (50ml). Blank microparticles containing no insulin were also prepared using the same parameters. All microparticle formulations were prepared in triplicate.

The particle size and yield were determined as described in chapter 2, section 2.4.6.1. The morphology and size of the microparticles were examined by scanning electron microscopy (SEM) (Philips XL30, Eindhoven, Holland) with routine, high vacuum imaging at 5kV as described in chapter 2, section 2.4.6.2.

3.4.4 Encapsulation efficiency and insulin loading

Microparticles with encapsulated insulin (20mg) were added to 1ml pH 6.8±0.05 phosphate buffer, in triplicate, and placed in a Gallenkamp shaking incubator at 100rpm, 37°C for 45 minutes to determine insulin encapsulation efficiency and loading. After 45 minutes samples were centrifuged at 10,000rpm for 10 minutes and 0.15ml of the supernatant added to 0.45ml 0.01N HCl to solubilise insulin. This was analysed for insulin content using HPLC. Drug loading was determined by measuring the μgs of insulin per mg of microparticles. Encapsulation efficiency was calculated using the equation below. The total amount of insulin added was the initial insulin added during formation of the microparticles.

$$\text{Encapsulation efficiency (\%)} = \frac{Measured\ amount\ of\ insulin\ in\ microparticles(mg)}{Total\ amount\ of\ insulin\ added\ (mg)} \text{ x100}$$

3.4.5 *In vitro insulin release*

To determine if the microparticles were able to protect insulin in gastric conditions they (20mg) were added to 0.75ml SGF, pH 1.2±0.05, with and without pepsin and placed in a Gallenkamp shaking incubator at 100rpm, 37°C for 2 hours. Samples with SGF alone were centrifuged and 0.5ml of supernatant removed and analysed for burst released insulin by HPLC. 0.5ml of SGF without pepsin was added to replace the withdrawn sample. After 2 hours 0.25ml of tribasic sodium phosphate was added to all samples to increase the pH to 6.8±0.05.

Samples were replaced in the shaking incubator and the experiment continued for a further 45 minutes. Samples were centrifuged for 10 minutes at 10,000rpm and 0.15ml of the supernatants added to 0.45ml 0.01N HCl to stop the reaction. The samples were analysed for insulin release using HPLC. All tests were carried out in triplicate.

Insulin microparticles (20mg) were added to 1ml of pig gastric fluid and placed in a Gallenkamp shaking incubator for 2 hours at 100rpm, 37°C. After this period samples were centrifuged at 10,000rpm for 10 minutes and the supernatants discarded. The microparticles were resuspended in pH 6.8±0.05 phosphate buffer and replaced in the shaking incubator for a further 45 minutes. Samples were again centrifuged using the same parameters. 0.15ml of the supernatant was added to 0.45ml 0.01N HCl to halt the reaction and this was analysed for insulin content by HPLC.

Insulin release was calculated using the equation below. The theoretical insulin concentration is the insulin concentration expected if it was all released.

$$\text{Insulin release (\%)} = \frac{Measured\ insulin\ concentration\ (\mu g/ml)}{Theoretical\ insulin\ concentration\ (\mu g/ml)} \text{x100}$$

3.4.6 Stability testing of insulin with citric acid

0.2ml of insulin solution (2mg/ml in 0.001N HCl) and 2mg of citric acid were added to 1ml of SIF with pancreatin. This was placed in a Gallenkamp shaking incubator at 100rpm, 37°C for 30 minutes. The pH was measured (pH 4) and the sample analysed for insulin content by HPLC. Insulin recovery was calculated using the equation below. The theoretical insulin concentration is the concentration of insulin expected if all was recovered.

$$\text{Insulin recovered (\%)} = \frac{Measured\ insulin\ concentration\ (\mu g/ml)}{Theoretical\ insulin\ concentration\ (\mu g/ml)} \text{x100}$$

3.4.7 Eudragit L100 microparticles with citric acid

This method is based on that of Kendall *et al*. with the addition of citric acid to the polymer and insulin suspension. Eudragit L100 (300mg) was dissolved in ethanol (3ml). Citric acid (100mg or 30mg) was added to this solution. Recombinant human insulin (5mg) was suspended in this and stirred for at least 30 minutes. Microparticle production and harvest were carried out as before.

Blank microparticles containing no insulin were also prepared using the same parameters. All microparticle formulations were prepared in triplicate. The yield was calculated as described in chapter 2, section 2.4.6.1, and morphology of the microparticles was visualized using SEM as before.

The microparticles (20mg) containing 30mg citric acid, in triplicate, were placed in 1ml SIF without pancreatin or 1ml SGF with pepsin for 2 hours in a Gallenkamp shaking incubator at 37°C, 100rpm. The SGF samples were centrifuged at 10,000rpm for 10 minutes and the supernatants discarded. Microparticles were resuspended in either SIF without pancreatin or SIF with pancreatin and replaced in the shaking incubator using the same parameters for a further 45 minutes. Samples were centrifuged again using the same parameters and 0.15ml of the supernatants added to 0.45ml 0.01N HCl to halt the reaction. These samples were analysed for insulin content using HPLC. Insulin release was calculated using the equation in section 3.4.6. The pH of the samples was measured at the completion of the experiment.

3.5 Results

3.5.1 Insulin intestinal stability

Incubation of insulin in SGF and SIF without enzymes for two hours resulted in almost complete intact insulin recovery, table 3.5, figure 3.3. This suggests insulin primary structure is stable at gastric pH, pH 1.2, and small intestinal pH, pH 6.8. The disulphide bonds which hold the A and B chains of insulin together appear not to break at this pH and therefore the structure remains intact. This stability at low pH has previously been demonstrated during a study assessing the crystalline structure of insulin (Whittingham et al., 2002). There were some conformational changes at low pH compared to neutral pH but the most important dimer forming contacts were preserved. This may be due to the stability of the disulphide bridges and the lack of critical salt bridges which would be weakened by protonation. However with enzymes, pepsin and pancreatin, insulin was immediately and completely degraded, figure 3.3. Previously insulin was completely degraded within 5 minutes with pepsin (Makhlof et al., 2011b) and 90% degraded with pancreatin (Jain et al., 2012). Insulin contains peptide bonds susceptible to pepsin and small intestinal enzymes resulting in this rapid digestion.

Table 3.5 *Insulin recovery in SGF, without pepsin, and SIF, without pancreatin. Data represents means ± SD.*

Sample (min)	Insulin recovered (%) SGF	Insulin recovered (%) SIF
0	95.0 ± 4.9	87.2 ± 9.9
15	96.4 ± 9.4	99.1 ± 5.3
30	91.5 ± 4.2	94.0 ± 2.8
60	94.6 ± 10.2	94.7 ± 3.3
90	93.5 ± 7.9	96.6 ± 1.8
120	88.4 ± 7.7	90.9 ± 1.3

Insulin susceptibility to gastric and small intestinal enzymes was also shown in porcine GI fluids. Incubation in porcine gastric and small intestinal fluids, duodenal, jejunal and ileal, resulted in an immediate and complete degradation of insulin, figure 3.3. These results are in agreement with previous studies which reported complete insulin digestion upon addition to porcine gastric and small intestinal fluids within 3 minutes (Werle et al., 2008).

Immediate and complete degradation of insulin also occurred when it was incubated in porcine colonic and human faecal fluids, figure 3.3. Insulin was more stable in rat cecal contents with a half life of 34 minutes (Tozaki et al., 1997, Tozaki et al., 1995). The cecum is at the beginning of the large intestine whereas the porcine colonic fluids were from the descending colon and the human faecal fluids have been expelled from the colon. These fluids may therefore contain different or a higher concentration of bacteria which can mediate insulin degradation.

This investigation of intestinal stability has shown that insulin is vulnerable to enzymatic degradation throughout the GI tract. Further testing in human intestinal fluids may give a more accurate prediction of oral insulin degradation *in vivo*.

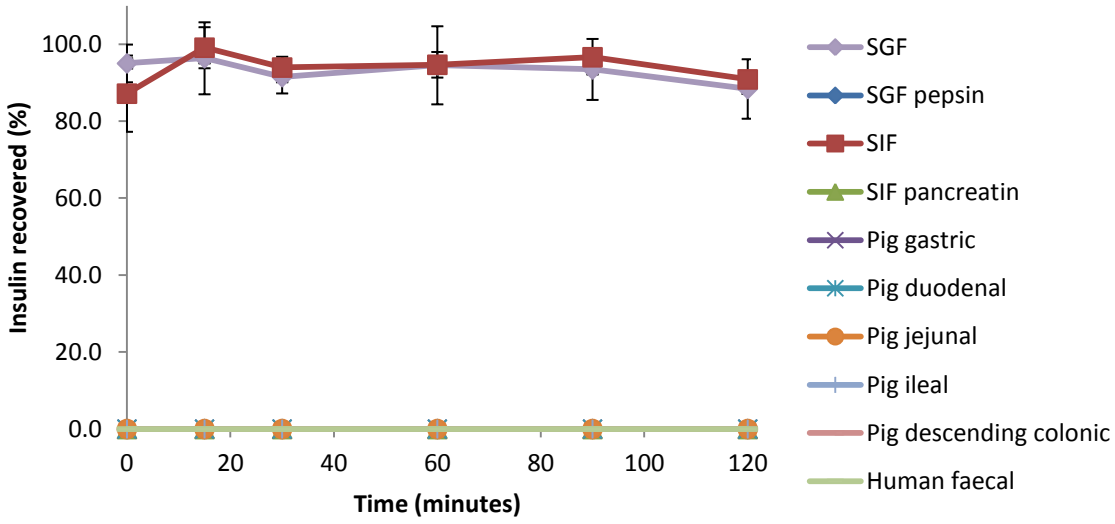


Figure 3.3 *Insulin recovery following incubation in simulated gastric and intestinal fluids with and without enzymes, porcine small intestinal and colonic fluids and human faecal fluids. Error bars show mean ± SD.*

3.5.2 Eudragit L100 microparticles

For successful oral delivery of insulin, based on the intestinal stability results, protection from gastric and intestinal enzymes is essential. The first enzymatic barrier to overcome is pepsin in the stomach. Enteric strategies have therefore been selected for many oral insulin formulations.

Some of the commercial oral insulin formulations clinically trialled have displayed variability (Kalra et al., 2010, Khedkar et al., 2010, Heise et al., 2004, Whitelow et al., 2005) in insulin absorption and vulnerability to disruption of absorption by food (Khedkar et al., 2010, Blair Geho et al., 2009). The non-enteric formulations of Biocon and Emsiphere (Khedkar et al., 2010, Kidron et al., 2004, Kapitza et al., 2010) have demonstrated rapid but short lasting glucose control, possibly due to lack of enteric protection which may necessitate the use of additional basal insulin. The enteric coated capsule and tablet formulations of Diabetology and Oramed exhibited a delayed and prolonged glucose reduction in clinical trials which may risk late post prandial hypoglycaemia (Kidron et al., 2008, Eldor et al., 2010a, Kidron et al., 2009, Whitelow et al., 2005).

Enteric micro/nanoparticles may provide solutions to these problems. Due to their small size they offer a faster and more uniform gastric release than larger dosage forms (Naikwade et al., 2009, Beverley et al., 1987) potentially enabling insulin formulations to overcome the variability in absorption previously seen in clinical trials. Their small size may also reduce the effects of food on insulin absorption which can affect larger dosage forms. They could offer a better prandial glucose control than enteric capsules and tablets, while still providing gastric protection, and a more prolonged control of glucose than the non-enteric forms. Their more immediate insulin release, due to increased surface area, should lessen the risk of late post prandial hypoglycaemia compared to larger enteric dosages.

Insulin loaded, enteric, Eudragit micro and nanoparticles were able, in previous studies, to restrict insulin release in acid, therefore offering protection from pepsin digestion (Zhang et al., 2012b, Naha et al., 2008, Sonia and Sharma, 2012, Mundargi et al., 2011a, Jain et al., 2006, Jain et al., 2005). I*n vivo* they generally caused a faster maximum glucose reduction than other enteric micro/nanoparticles offering a better opportunity for controlling prandial glycemic excursions (Naha et al., 2008, Mundargi et al., 2011a, Zhang et al., 2012b, Jain et al., 2005). While the glucose response was no faster than that produced by larger, enteric coated capsules and tablets in clinical trials these particles offer the best potential for reducing the delay in insulin absorption. They were therefore selected for formulating insulin.

Chapter 2 showed Eudragit L100 microparticles can prevent the release of an encapsulated protein but are not impermeable to acid. As the primary structure of insulin is stable in acid, demonstrated in the intestinal stability studies, this may not be a problem. Pepsin is a large enzyme, 35kDa, and therefore is unlikely to be able to permeate the microparticles and destroy the encapsulated insulin. Therefore they were selected as a delivery strategy to protect insulin from pepsin.

The method selected to produce insulin loaded Eudragit L100 microparticles was based on that of Kendall *et al*.

This o/o emulsion solvent evaporation method was selected due to its lack of potentially harmful solvents, such as DCM, and high shear processes such as homogenisation or sonication which could damage insulin. Other advantages of using an o/o method rather than w/o/w include a reduced risk of hydrophilic insulin migrating to an outer aqueous phase, reducing its encapsulation and leaving it vulnerable on the surface of the particles to premature gastric release and pepsin digestion (Qi and Ping, 2004).

The microparticles had a dv,0.5 less than 100μm and a span less than 2.5, table 3.6. SEM images show that most of the microparticles are actually smaller than 40μm, spherical and uniformly sized, figure 3.4. The dv,0.5 determined by the Mastersizer is larger than this possibly due to being skewed by a few much larger particles, as seen in figure 3.4 (a). The particles are much smaller than those previously produced using an o/o method which were 300-500μm, possibly due to the use of the sorbitan sesquioleate surfactant (Morishita et al., 1993). The particle size of insulin loaded Eudragit L100/S100 microparticles produced using high speed homogenisation were between 4-70μm. This method has managed to produce particles of a comparable size but without potentially damaging high shear homogenisation (Mundargi et al., 2011a, Jain et al., 2005, Jain et al., 2006).

Table 3.6 *Size, span, insulin loading, encapsulation efficiency and yield of Eudragit L100 microparticles with encapsulated insulin. Data represents means ± SD.*

Microparticles	**Size (μm)**	**Span**	**Insulin loading (μg/mg)**	**Encapsulation efficiency (%)**	**Yield (%)**
Insulin: Eudragit L100	90.85 ± 17.55	2.09 ± 0.21	11.83 ± 2.07	72.2 ± 12.6	78.4 ± 5.6

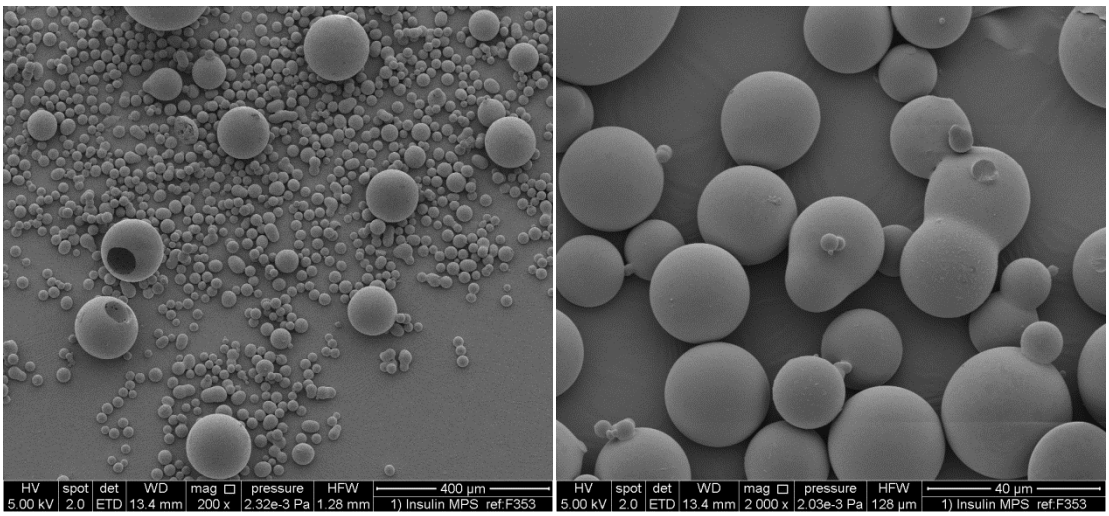


Figure 3.4 (a) & (b) *SEM images of Eudragit L100 microparticles with encapsulated insulin*

There was a high yield, >75%, and encapsulation efficiency, >70%, table 3.6. Insulin encapsulation efficiency was slightly lower than with the w/o/w double emulsion method, 82-85% (Jain et al., 2005, Jain et al., 2006), and the previously used o/o method, 80% (Morishita et al., 1993). The encapsulation efficiency had a high standard deviation and some of the batches actually had an insulin encapsulation efficiency >80%. The small batch size may be responsible for this variability and higher proportional insulin losses by attachment to glassware and stirrers. The yield and encapsulation efficiency were also lower than those produced by Kendall *et al.* with prednisolone, possibly due to scaling down the amounts of excipients.

These results show this is a suitable method for encapsulating insulin within enteric microparticles. The microparticles produced are micron sized, spherical and mainly uniform which should enable their rapid and uniform gastric emptying, small intestinal dissolution and insulin release. There is a high yield which is beneficial when working with expensive and small amounts of peptide and protein drugs. The encapsulation efficiency is high demonstrating insulin has not been degraded by the microparticle production process.

3.5.2.1 *In vitro* insulin release

The ability of the microparticles to prevent insulin release in gastric conditions and protect insulin from pepsin degradation was investigated.

The microparticles were immersed in SGF without pepsin, SGF with pepsin and porcine gastric fluid for two hours. Insulin release during this period could only be assessed in the SGF without pepsin as in the other media any insulin released would be degraded and not quantifiable. There was a burst release of 28% of the insulin during this period, table 3.7, figure 3.5. This may be due to instant release of insulin located near the surface of the microparticles. This does not fulfil the USP (724) requirements for an enteric dosage form which state, on average, no more than 10% of the drug load should be released in acid. The release of insulin from these particles in acid is also greater than from previously tested Eudragit nano/microparticles, which released <20% (Zhang et al., 2012b, Naha et al., 2008, Sonia and Sharma, 2012, Mundargi et al., 2011a, Jain et al., 2006, Jain et al., 2005). This may be due to a sub-optimal ratio of enteric polymer to insulin therefore not preventing all insulin release at low pH. To increase the enteric protection the amount of Eudragit L100 could be increased, or a reduction in stirring speed or sorbitan sesquioleate may allow larger particles to form which would provide more protection.

Table 3.7 *Intact insulin release from Eudragit L100 microparticles after 2 hours in SGF with and without pepsin and porcine gastric fluid followed by pH rise to pH 6.8. Data represent means ± SD.*

Incubation media	**Insulin release 120 minutes (%)**	**Insulin release 165 minutes (%)**
SGF to pH 6.8	28.3 ± 3.0	114.1 ± 29.4
SGF and pepsin to pH 6.8	-	79.7 ± 12.5
Pig gastric fluid to pH 6.8	-	48.8 ± 9.3

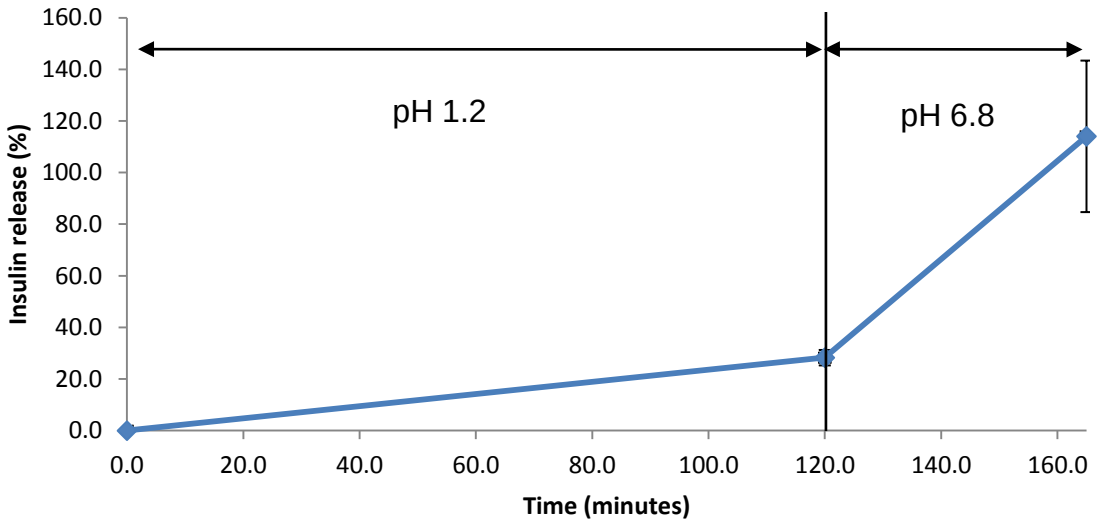


Figure 3.5 *Insulin release from Eudragit L100 microparticles after incubation for 2 hours in SGF without pepsin followed by a pH rise to pH 6.8. Error bars show mean ± SD.*

After two hours in gastric media the pH was raised to pH 6.8 to simulate transition into the small intestine. After incubation in SGF without pepsin there was complete and rapid release of intact insulin within 45 minutes, table 3.7, figures 3.5 & 3.6. This fulfils the USP (724) requirements for drug release from an enteric formulation during the buffer stage of dissolution testing.

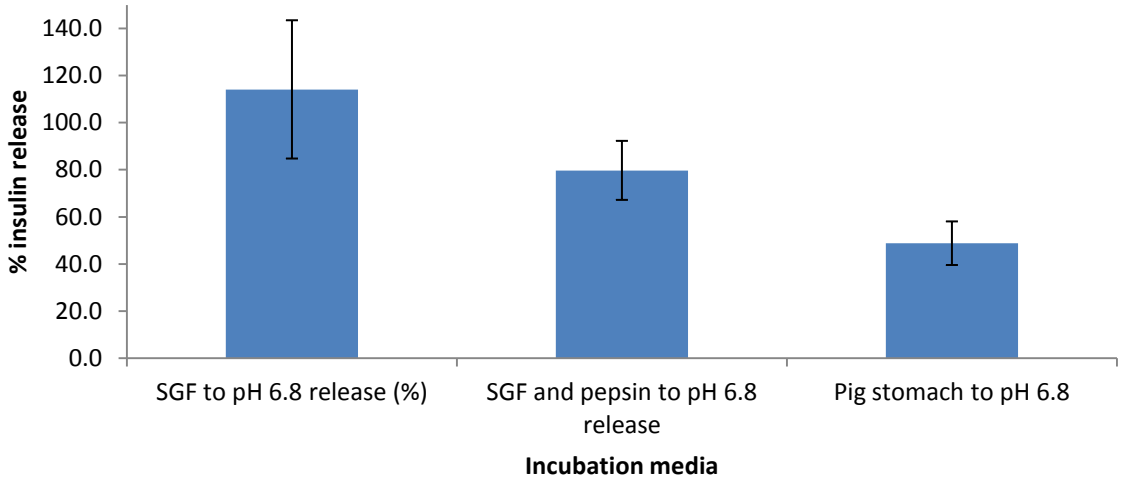


Figure 3.6 *Insulin release from Eudragit L100 microparticles after incubation in either SGF with or without pepsin, or porcine gastric fluid for 2 hours and 45 minutes in pH 6.8 phosphate buffer. Error bars show mean ± SD.*

The release of insulin from these particles at small intestinal pH was faster than from Eudragit L100/S100 particles prepared by dissolving the polymer in DCM and using an outer aqueous phase. After 45 minutes these particles released less than 40% of their encapsulated insulin (Jain et al., 2006, Jain et al., 2005). The rapid release of insulin was comparable to other o/o produced insulin loaded Eudragit microparticles, 60-90% in 45 minutes (Morishita et al., 1993, Mundargi et al., 2011a). The slower evaporation of ethanol may have allowed the formation of a more porous microparticle interior. This would allow faster dissolution of the particles and insulin release at small intestinal pH. A faster solvent evaporation rate forming polylactide microspheres resulted in a less porous microstructure (Hong et al., 2005). This may be due to a reduced opportunity for solvent droplet coalescence within the particles preventing the formation of larger internal pores. Further investigation of the morphology of microparticles produced using DCM or ethanol and an outer oil or aqueous phase would be useful to fully elucidate their influence on drug release.

The faster release in small intestinal conditions *in vivo* may allow faster insulin absorption and blood glucose reduction providing better immediate post prandial glycemic control. The complete release may also prevent glucose reduction occurring for so long that late post prandial hypoglycaemia may arise.

After incubation in SGF with pepsin 80% of encapsulated insulin released in pH 6.8 phosphate buffer was intact, table 3.7, figure 3.6. The remaining 20% was possibly burst released in the gastric media and degraded by pepsin. After incubation in porcine gastric fluid there was 49% intact insulin release upon pH rise, table 3.7, figure 3.6. The remaining 51% was either burst released in the stomach fluid and degraded by the enzymes or insulin exposed at the particle surface was digested. There was a greater loss of insulin after incubation of the microparticles in porcine stomach fluid than in SGF with pepsin. Possibly the activity or concentration of pepsin in this fluid was greater than in the simulated fluid. Some of the enzymes in the stomach sample may have remained after the pH change and were active at the higher pH allowing insulin degradation. They may also have been re-activated by the pH decrease at the end of the experiment.

Insulin released from the microparticles after incubation in SGF with pepsin or porcine gastric fluid had a comparable HPLC retention time to insulin alone, Figure 3.7. This suggests its ionisation and tertiary structure was not altered in the microparticles and was successfully protected from gastric conditions.

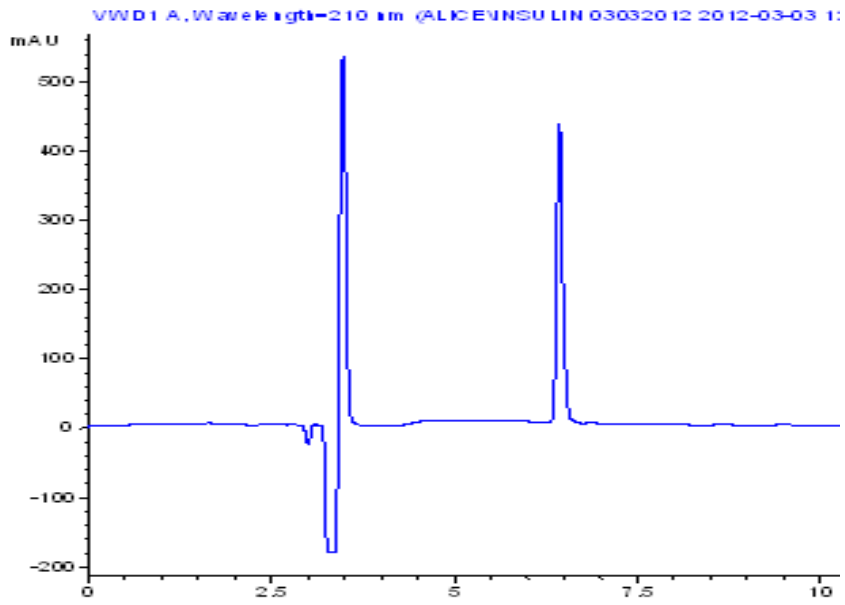


Figure 3.7 (a)

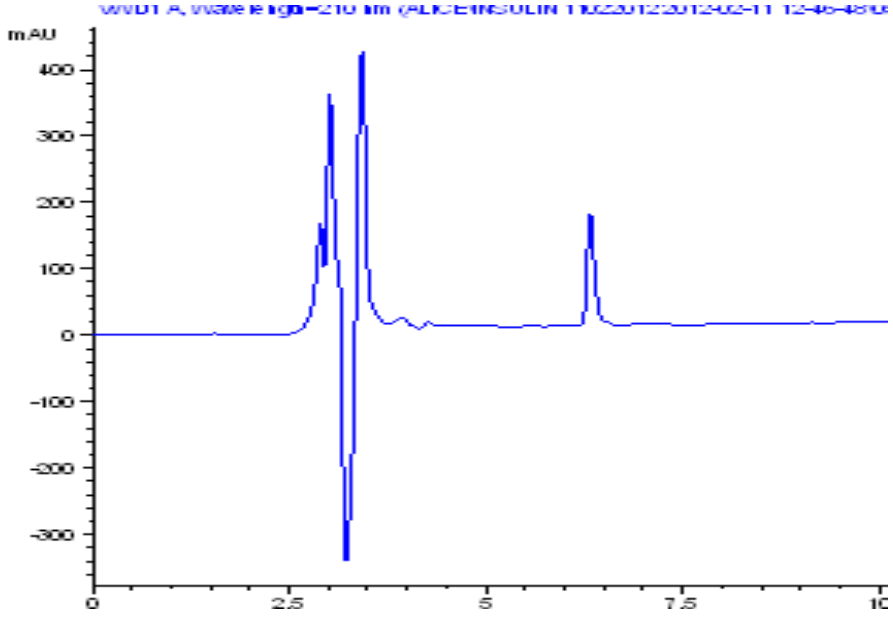


Figure 3.7(b)

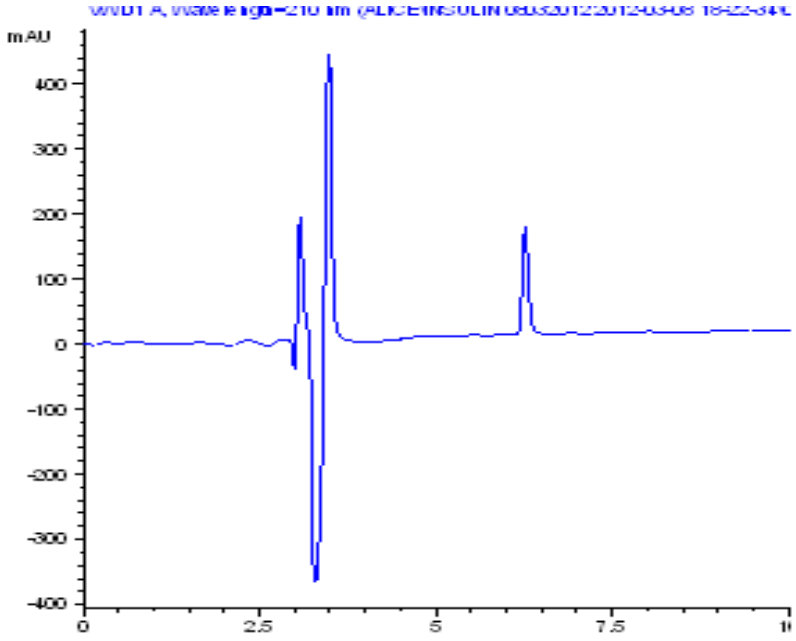


Figure 3.7 (c)

Figure 3.7 *HPLC chromatograms of (a) insulin (b) insulin released from Eudragit L100 microparticles after incubation in SGF with pepsin then pH 6.8 phosphate buffer (c) insulin released from Eudragit L100 microparticles after incubation in pig gastric fluid, then pH 6.8 phosphate buffer*

These particles provided more protection from pepsin digestion than enteric HPMCP cross linked chitosan nanoparticles (Makhlof et al., 2011b) and hyaluronic acid nanoparticles (Han et al., 2012) which only protected 30% and 45% of their encapsulated insulin respectively. This suggests insulin may have been exposed at the surface of particles and digested when exposed to pepsin. This demonstrates the success of this method for successfully encapsulating insulin within the particles and leaving only a small proportion vulnerable to pepsin digestion. None of the other particles were tested with actual gastric fluids.

In vivo testing would be useful to determine if insulin absorption can be increased by encapsulation in these microparticles and if glucose lowering is faster than with enteric capsules/tablets. It would also be useful to determine if insulin absorbance from these particles is affected by the timing of food ingestion post dosing and if they are less affected than enteric capsules/tablets.

3.5.3 Eudragit L100 microparticles with citric acid

Insulin released from Eudragit L100 microparticles in the small intestine would be vulnerable to enzyme digestion. To overcome this obstacle citric acid was also encapsulated within the microparticles. The aim of this strategy was to reduce the pH around the released insulin inactivating any intestinal enzymes in the vicinity. Citric acid has been used in oral formulations of protein and peptide drugs and demonstrated its ability to minimise enzymatic degradation (Fjellestad-Paulsen et al., 1995, Fredholt et al., 1999, Lee et al., 2000, Lee et al., 1999, Wu et al., 2010, Ushirogawa et al., 1992). By encapsulating citric acid within the enteric microparticles it ensures it is only released in the small intestine simultaneously with the insulin so it has the maximum potential for protection and minimal disruption of digestion. This should also minimise the loss of protection seen previously due to its intestinal spreading (Lee et al., 2000).

To test the hypothesis that citric acid can lower the pH and disable intestinal enzymes it was added to SIF with pancreatin and insulin. After 30 minutes incubation the pH of the mixture was pH 4 showing citric acid had lowered the pH. In stability studies of insulin with SIF and pancreatin insulin was instantly degraded, figure 3.3, with citric acid 14% of the insulin was intact after 30 minutes incubation. This shows lowering the pH of intestinal fluids can inactivate enzymes and protect insulin.

All the microparticles with co-encapsulated citric acid had a yield of >50%, table 3.8. The addition of citric acid prevented the formation of discrete microparticles. When 100mg of citric acid was added there were no microparticles formed just large aggregates, figure 3.8 (a) and (b). Reducing the amount of citric acid to 30mg allowed the partial formation of microparticles but they were still aggregated figure 3.9 (a) and (b). As these large aggregates were formed it was not possible to use the Mastersizer to measure their size and dispersity. Citric acid appears to have affected the polymer preventing it forming microparticles. This may be due to the plasticizing effect of citric acid on these polymers (Andrews et al., 2008, Schilling et al., 2007). The increased pliability of the polymer may have prevented the formation of discrete particles and enabled the formation of large aggregates.

Further testing is needed to discover the influence of citric acid on the glass transition temperature of Eudragit L100 and this can be used to find the optimal ratio of citric acid to Eudragit L100.

Table 3.8 *Yields of blank and insulin loaded Eudragit L100 microparticles with either 100mg or 30mg of citric acid. Data represents means ± SD.*

Microparticles	**Yield (%)**
Insulin with 100mg citric acid	81.5 ± 42.6
Blank with 100mg citric acid	87.4 ± 74.8
Insulin with 30mg citric acid	53.4 ± 28.2
Blank with 30mg citric acid	59.0

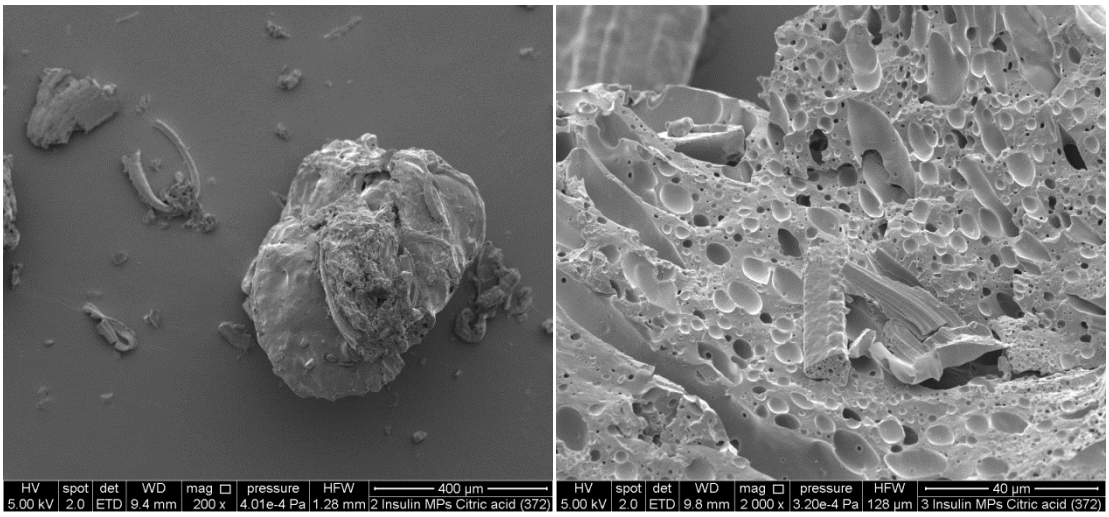


Figure 3.8 (a) and (b) *SEM images of insulin loaded Eudragit L100 microparticles with 100mg of co-encapsulated citric acid*

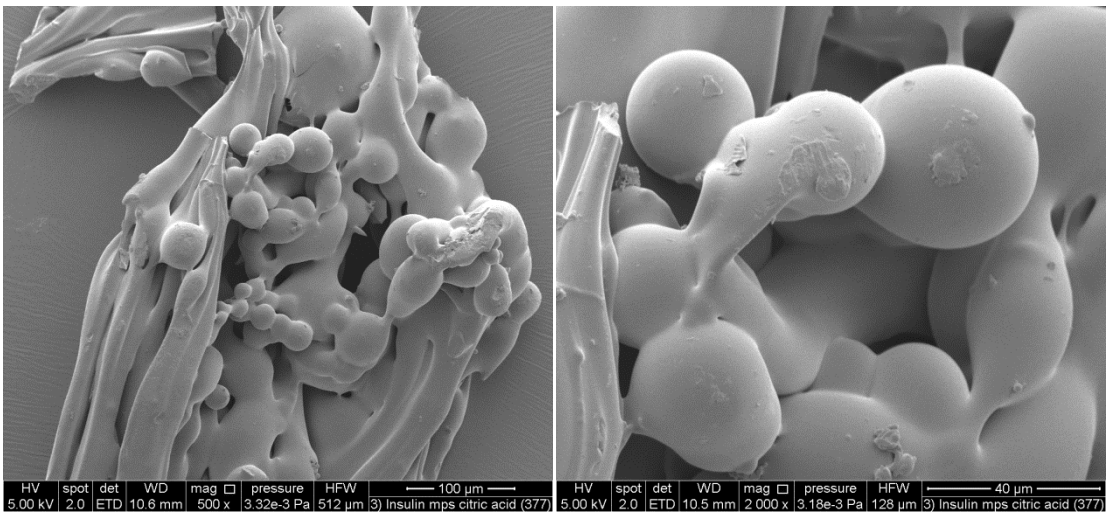


Figure 3.9 (a) and (b) *SEM images of insulin loaded Eudragit L100 microparticles with 30mg of co-encapsulated citric acid*

The release of insulin from the aggregates of Eudragit L100, insulin and citric acid was tested in SIF without pancreatin. There was no released insulin detected after two hours. The average pH of the samples at the end of the experiment was 5.13. Citric acid may not have been encapsulated within the microparticles so was free to lower the pH below the pH threshold of the Eudragit L100 polymer. Therefore the aggregates did not break down and did not release insulin. Large aggregates were visible at the end of the incubation period.

There was also no insulin detected after incubation of the particles in SGF and pepsin for two hours and then 45 minutes in either SIF with pancreatin or SIF without pancreatin. This may be due to pepsin and/or pancreatin degradation of any released insulin. However the pH of the samples at the end of the experiment were all below the pH threshold of Eudragit L100, pH 6, potentially restricting its dissolution and insulin release. The average pH of the samples after incubation in SGF with pepsin and SIF with or without pancreatin was 5.25. Aggregates were also visibly intact at the end of the experiment.

The failure to form microparticles may be a cause of the failure to detect any insulin release. The aggregates may be just mixtures of Eudragit L100, citric acid and insulin. Therefore there would be no protection of insulin from pepsin and the citric acid would be released without control.

The insulin may also be so deeply embedded within the large aggregates that its release may be delayed.

To determine if burst released citric acid is preventing insulin release by lowering the pH below the threshold for Eudragit L100 the pH of the samples should be raised above pH 6. It can then be determined if insulin has been protected from pepsin and if citric acid released simultaneously with insulin can inactivate pancreatin and preserve insulin.

3.6 Conclusion

Despite numerous attempts to develop an oral formulation of insulin that is capable of controlling the symptoms of diabetes no such formulation is yet available. The largest hurdles to oral insulin delivery are its intestinal instability and incompatibility with permeation from the GI tract but additional challenges arising during clinical trials are a need to eliminate variability in insulin absorption and minimise variability in insulin availability caused by food consumption.

Investigations into the intestinal stability of insulin found that its primary structure is stable at gastric and small intestinal pH but it was rapidly degraded by enzymes found throughout the GI tract. The structure of this small protein is stabilised at low pH by disulphide bonds, however the enzymes of the GI fluids can easily access susceptible peptide bonds and break them.

Encapsulation of insulin in Eudragit L100 microparticles was selected as an oral delivery strategy for their ability to protect insulin from pepsin digestion and their potential, due to their small size, to produce faster post prandial glycemic control than larger enteric dosage forms. Their small size may also be able to minimise variability in insulin absorption and susceptibility to absorption disruption by the presence of food experienced by oral insulin formulations in clinical trials.

An o/o emulsion solvent evaporation method, based on that developed by Kendall *et al*. was selected to encapsulate insulin in Eudragit L100 microparticles. In chapter 2 this method successfully encapsulated a large protein, lactase, and restricted its release in acid.

This method was also selected for its lack of potentially damaging parameters which could endanger the integrity of insulin and benefits of increased encapsulation, reduced burst release and increased pepsin protection than those produced by a w/o/w method.

The microparticles had a high insulin encapsulation efficiency, >72%, and a mean particle size of <100µm. This was comparable to insulin loaded Eudragit microparticles produced using homogenisation but didn't involve exposure to high shear forces. The Eudragit L100 microparticles prevented 72% of insulin release in acid and protected 80% of encapsulated insulin from pepsin digestion. This implies the vast majority of insulin was successfully fully encapsulated within the particles and not left exposed on their surface. Insulin release from the Eudragit L100 microparticles was rapid and complete within 45 minutes at small intestinal pH. This rapid release makes these particles more suitable to promote a faster insulin absorption and control prandial glycemic excursions than larger dosage forms. The complete release may also prevent glucose reduction occurring for so long that late post prandial hypoglycaemia may arise.

To provide enzymatic protection in the small intestine citric acid was co-encapsulated in the microparticles. Citric acid released simultaneously with insulin would be able to lower local pH and inactivate enzymes reducing insulin degradation. However, the plasticizing effect of citric acid on Eudragit L100 hampered the formation of microparticles instead forming large aggregates. This would restrict rapid and uniform gastric emptying. Their large size and release of citric acid appeared to prevent insulin release in any media *in vitro*. However, *in vivo*, the higher buffer capacity of the small intestinal fluids may prevent released citric acid lowering the pH below the threshold pH of Eudragit L100. Therefore they may still offer a mode of successful oral insulin delivery.

Insulin loaded Eudragit L100 microparticles offer a promising oral delivery strategy. Their protection of insulin from pepsin digestion and rapid release at small intestinal pH could enable insulin absorption and glucose reduction sufficient and fast enough to control post prandial glycemia. Additional excipients may further increase their potential to successfully deliver insulin orally.

Chapter 4

Investigation of the intestinal stability of Peptide 1 and development of its oral formulations

4.1 Introduction

The intestinal stability and oral delivery of a recently discovered small (10 amino acids), chemotherapeutic peptide, Peptide 1, was investigated in this chapter. While this peptide lacks the more complex structure of lactase and insulin it is still larger than commonly orally delivered low molecular weight drugs and still has to contend with the issues of intestinal instability and impermeability outlined in chapter 1. Investigation into its intestinal stability, combined with the results of the previous chapters, should provide an insight into the relationship between the size and structure of protein and peptide drugs and their stability.

Gonadotrophin releasing hormone (GnRH) agonists are peptides of a similar size to peptide 1 and are used to treat prostate and breast cancers. They are available as injectable, depot formulations of polymeric particles of poly (D,L-lactide-co-glycolide) (PLGA) which provide protection to the peptide and sustain their release to reduce the frequency of injections. Therefore they have been used for oral delivery of peptides and increased their gastrointestinal stability and permeability but they have experienced problems of immediate, uncontrolled peptide release. If administered orally the encapsulated peptide could be burst released in the stomach where it may be degraded by acid or pepsin. To prevent gastric degradation of burst released peptide the encapsulation of peptide loaded PLGA nanoparticles in enteric Eudragit L100 microparticles, which successfully encapsulated insulin and lactase in the previous chapters, has been investigated here.

4.1.1 Peptide 1, function and structure

Peptide 1 is a 10 amino acid peptide, 1190.4 MW, which can halt the growth of human mammary and prostate cancer cells (Migliaccio et al., 2007). Peptide 1 inhibits the interaction of androgen receptor (AR)-estradiol receptor (ER) complex with Src and stops its activation by mimicking the sequence that interacts with Src. This prevents the progression from G1 to S (synthesis) in the cell cycle of cancer cells, stopping their growth.

Peptide 1 was used to treat human prostate and mammary cancer cells (LNCaP and MCF-7) *in vitro* and inhibited androgen or estradiol induced association between the AR/ER and Src, Src activation, DNA synthesis and S phase entry.

When administered intraperitoneally to mice the peptide was taken up by and strongly inhibited the growth of LNCaP xenografts.

4.1.2 Chemotherapeutic peptides

The GnRH agonists, buserelin, goserelin, histrelin, leuprorelin and triptorelin, are of a similar size and structure to peptide 1 and are used to treat prostate and breast cancers, table 4.1. These synthetic peptides are modelled on GnRH and bind to the GnRH receptor for a prolonged period reducing sex hormones and enabling treatment of hormonally sensitive cancers.

All the GnRH agonists available are solely delivered by injection, except for a nasal spray of buserelin, and none are available orally, table 4.1. Except for buserelin the injectable GnRH agonist formulations provide a sustained release of the drug over 1, 3, 6 or 12 months, dependent on the properties of their polymeric carrier. The sustained release capacity of goserelin, leuprorelin and triptorelin formulations is conferred by the biocompatible and biodegradable polymers polylactic acid (PLA) and PLGA. They have been used to form microcapsules for leuprorelin and triptorelin delivery. The peptide is released initially by diffusing through pores in the spheres followed by slower, sustained release as the microspheres are eroded and the pores enlarged (Crotts and Park, 1998). The microspheres are able to protect the encapsulated peptide and by providing a sustained release the inconvenience and pain of frequent injections is avoided (Dai et al., 2005).

None of the protein or peptide drugs currently used in cancer treatment are available as oral formulations. This is probably due to intestinal instability and limited absorption, due to their relatively large size and hydrophilicity, as described in chapter 1. Despite these obstacles oral delivery would be preferable to injections because of increased convenience, patient compliance, ease of administration and reduced production costs.

Table 4.1 *Molecular weight (MW) of GnRH agonists, GnRH pharmaceutical products available and their sustained release component*

GnRH agonist	**MW**	**Amino acids**	**Pharmaceutical product**	**Sustained release polymer**
Buserelin	1299	9	Suprefact (Sanofi-Aventis) subcutaneous (sc) injection, nasal spray	-
Goserelin	1269	10	Novgos (Genus) implant , monthly sc injection Zoladex (Astrazeneca) implant, monthly sc injection, Zoladex LA implant, 3 monthly sc injection	PLGA
Histrelin	1324	9	Vantas (Orion Pharma), 12 monthly sc implant	Hydrogel reservoir, acrylic copolymer shell
Leuprorelin	1209	9	Prostap 3, SR (Takeda) 1 and 3 monthly microcapsule depot by sc injection	PLA, PLGA
Triptorelin	1312	10	Decapeptyl SR (Ipsen), Gonapeptyl depot (Ferring), intramuscular or sc injection 1, 3 or 6 monthly depots of sustained release microcapsules	PLGA

Subcutaneous (sc)

4.1.3 Barriers to oral delivery of peptides for cancer treatment

Intestinal stability studies revealed GnRH and leuprorelin are vulnerable to degradation by small intestinal enzymes. GnRH was degraded by the intestinal enzymes chymotrypsin (Wen et al., 2002c, Walker et al., 2001) and elastase (Walker et al., 2001) but was not degraded by trypsin. It was also rapidly degraded in small intestinal extracts from possum and rat intestines and had a half life of only 22 minutes in the possum jejunal extract (Wen et al., 2002c, Wen et al., 2002b, Wen et al., 2002a, Zheng et al., 1999a). Rates of GnRH degradation in the brushtail possum corresponded to levels of proteolytic activity in the intestinal segments; greatest in the jejunum and ileum and least in the duodenum and colon (Wen et al., 2002b, Wen et al., 2002c).

The intestinal permeability of the GnRH agonist leuprorelin has been assessed *in vitro* with rabbit intestinal segments and *in vivo* in rats by site directed delivery (Zheng et al., 1999b). Permeability in rabbit intestinal segments was very low but was greater in the ileum and colon than the jejunum. In anesthetized rats the bioavailability of leuprorelin was only 1.28% when administered to the jejunum increasing to 5.62% in the ileum and 9.59% in the colon (Zheng et al., 1999b). Bioavailability was reduced in conscious rats to only 0.23% when administered to the duodenum (Adjei et al., 1993), 0.58% in the ileum and 0.41% in the colon (Zheng et al., 1999b). These differences may be due to greater enzymatic activity and thicker mucus covering in the jejunum or the presence of macromolecule sampling M cells in the ileum. Intact peptide was recovered from these intestinal regions at the end of the experiment demonstrating that even when the peptide was not degraded it was not absorbed due to its low permeability.

These experiments show the vulnerability of these peptides to degradation in the small intestine and their low permeability demonstrating why they are not delivered orally. The permeability and stability studies conducted did not take into account any degradation that may have occurred in the stomach so oral bioavailability figures may actually be lower than indicated. As these peptides are used to treat cancer it is absolutely vital that the dose delivered is therapeutic and reliable.

Peptide 1 has a similar molecular weight and structure to the GnRH agonists and therefore may also have a low oral bioavailability due to similar intestinal instability and impermeability.

4.1.4 Oral delivery strategies investigated for peptide chemotherapeutics

To overcome the instability of GnRH agonists protease inhibitors have been used in oral formulations. Protease inhibitors inhibited enzymatic degradation of buserelin (Kotze et al., 1997a) and leuprorelin (Zheng et al., 1999a) *in vitro* with rat intestinal homogenates and increased leuprorelin permeation in a rabbit intestinal sac (Guo et al., 2004). However this strategy may not be ideal for oral delivery as it could disrupt the digestion of dietary proteins.

To increase the permeability of GnRH agonists mucoadhesive polymers, chitosan and carbomer, have been investigated. They increased *in vitro* transport of buserelin and leuprorelin across Caco-2 cells and rat intestinal mucosa (Kotze et al., 1997a, Thanou et al., 2000a, Kotze et al., 1997b, Guo et al., 2004, Iqbal et al., 2012). As well as increasing intestinal residence time due to their adhesive properties they appeared to open tight junctions between the cells increasing paracellular absorption. N-trimethyl chitosan chloride reduced the transepithelial electrical resistance (TEER) of Caco-2 cells and fluorescent markers were seen in the intercellular spaces indicating an opening of the tight junctions (Kotze et al., 1997b, Guo et al., 2004). *In vivo*, carbomer and chitosan increased buserelin and leuprorelin bioavailability from less than 1% for the drug solutions to up to13% when administered intraduodenally and 4.52% when administered orally to rats (Thanou et al., 2000a, Luessen et al., 1996, Iqbal et al., 2012).

Other delivery strategies for GnRH agonists include encapsulation in polymeric nanoparticles (Iqbal et al., 2011) , solid lipid nanoparticles (Yuan et al., 2009) and liposomes (Carafa et al., 2006). These formulations can provide protection to the encapsulated peptide and may be able to enhance permeability. However, these systems suffered from an immediate burst release *in vitro* of up to 60% in 10 minutes which would leave the released drug vulnerable to degradation (Iqbal et al., 2011).

Only one of these systems was tested *in vivo* and produced an oral bioavailability of 0.55% in rats, a small improvement compared to the 0.26% bioavailability of an oral leuprorelin solution (Iqbal et al., 2011).

Of the oral delivery strategies investigated for the GnRH agonists here none produced an oral bioavailability of more than 5% compared to an intravenous dose so more work is needed to enable oral delivery.

4.1.5 PLGA nanoparticles for oral delivery of proteins/peptides

Injectable microspheres of PLGA and PLA encapsulating GnRH agonists prevent the peptide being rapidly degraded and provide a prolonged release. Another advantage of PLGA is its lack of toxicity as it degrades to lactic and glycolic acids which are metabolised by the Krebs cycle (Singh et al., 2008). Compared to liposomes polymeric particles have greater stability in biological fluids and during storage (Pinto Reis et al., 2006). Due to their proven benefits they have been explored for oral protein and peptide drug delivery too.

PLGA particles have been used to encapsulate peptide and protein drugs; salmon calcitonin (Yoo and Park, 2004), cyclosporine A (Italia et al., 2007), ovalbumin (Uchida et al., 1994, Challacombe et al., 1992, Garinot et al., 2007), HBsAg antigen (Gupta et al., 2007), helodermin (des Rieux et al., 2007), BSA (Blanco and Alonso, 1998, Panyam et al., 2003), lysozyme (Blanco and Alonso, 1998, Nam et al., 2000), leuprorelin acetate (Luan et al., 2006), thymopentin (Yin et al., 2007) and erythropoietin (Geng et al., 2008). They have increased their oral bioavailability and induced immune responses in rodents (Yoo and Park, 2004, Gupta et al., 2007, Uchida et al., 1994, Challacombe et al., 1992).

The most frequently investigated protein/peptide drug for oral delivery in PLGA particles is insulin (Sun et al., 2011b, Yang et al., 2012, Cui et al., 2006b, Carino et al., 2000, Sun et al., 2011a, Teply et al., 2008, Cheng et al., 2006, Pan et al., 2002, Li et al., 2004, Wu et al., 2012b). *In vivo* they increased its oral bioavailability compared to an orally administered insulin solution. Oral bioavailabilities, compared to an injected dose of insulin, ranged between 5.11 and 15.9% in rodents (Sun et al., 2011b, Cui et al., 2006b, Carino et al., 2000, Sun et al., 2011a, Teply et al., 2008, Pan et al., 2002, Li et al., 2004, Wu et al., 2012b).

Non-peptide cancer drugs have also been encapsulated in PLGA particles; doxorubicin (Wang et al., 2009), cisplatin (Dhar et al., 2008) and paclitaxel (Danhier et al., 2009). They induced apoptosis of cancer cells *in vitro* (Wang et al., 2009) and produced a greater effect than an unencapsulated drug (Danhier et al., 2009).

4.1.5.1 PLGA particles- stability and permeability enhancement

PLGA particles provide a physical barrier between an encapsulated drug and the acidic pH of the stomach and enzymes of the gastrointestinal tract thereby increasing their oral bioavailability.

PLGA particles have shown their ability to increase the *in vitro* permeability of a hydrophilic marker (Cohen-Sela et al., 2009), paclitaxel (Westedt et al., 2007), salmon calcitonin (Yoo and Park, 2004) and BSA (Panyam et al., 2003) compared to an unencapsulated drug in cell culture models (RAW 264 cells, rabbit vascular smooth muscle cells, Caco-2 cells, rabbit conjunctival epithelial cells). After uptake the particles could be seen within the cells (Westedt et al., 2007)

As the PLGA particles are more lipophilic than the encapsulated hydrophilic protein or peptide their transport across the lipid membrane of cells can be increased and their transcellular transport has been observed (des Rieux et al., 2007). Studies with rabbit conjunctival epithelial cells and Caco-2 cells demonstrated the TEER was unchanged with PLGA particles indicating the junctions between cells were not opened and their transport was transcellular not paracellular (Qaddoumi et al., 2004, Yoo and Park, 2004).

The M cells of Peyer's patches, lymphoid nodules found in the small intestine, are a potential portal for PLGA particle uptake. M cells sample and transport intact antigens from the GI tract to underlying lymphoid tissues and have high transcytosis capabilities. This route of nanoparticle absorption appears to be preferred to that via the enterocytes (Kim et al., 2002). A human M cell culture model increased PLGA particle uptake 600 fold compared to a Caco-2 cell model (des Rieux et al., 2007). There was also greater uptake of PLGA particles in a rat in situ intestinal loop model in areas with Peyer's patches than in areas without (Desai et al., 1996).

However it is not known how relevant these studies are to indicate human *in vivo* behaviour as rodents have a higher percentage of M cells, 10-50%, in their Peyer's patches compared to humans, with only 5%.

The size of the PLGA particles appears to be crucial to their permeation. PLGA particle uptake was found to be size dependent in Caco-2 cells (Gaumet et al., 2009), rat in situ intestinal loop (Desai et al., 1996) and rabbit conjunctival epithelial cells (Qaddoumi et al., 2004). In all cases the smallest 100nm particles showed the highest uptake compared to larger particles (Gaumet et al., 2009, Desai et al., 1996, Qaddoumi et al., 2004). PLGA particles of 100nm and 300nm were seen intracellularly in Caco-2 cells whereas those greater than 300nm were not but were observed on the apical membrane of Caco-2 cells (Gaumet et al., 2009). Particles of 100nm diffused through submucosal layers of a rat intestinal loop but particles of 500nm or greater were localised in the epithelial lining (Desai et al., 1996). Ideally PLGA particles for oral delivery of proteins and peptides should be less than 300nm for successful absorption.

Oral bioavailabilities of protein/peptide drugs encapsulated in PLGA particles can be further increased by chitosan coating. Its mucoadhesive properties, positive charge, enhancing interaction with negatively charged GI tract cells, and ability to open paracellular channels have increased the *in vitro* absorption and *in vivo* bioavailability in rats of buserelin (Kotze et al., 1997a, Thanou et al., 2000a, Kotze et al., 1997b), insulin (Zhang et al., 2012a), cyclosporine A (Malaekeh-Nikouei et al., 2008) and elcatonin (Kawashima et al., 2000). Insulin oral bioavailability in rats was increased from 7.6% to 10.5% when PLGA particles were coated with chitosan (Zhang et al., 2012a).

4.1.5.2 Formation of PLGA particles

PLGA particles can be formed by many methods, a single emulsion method can be used for hydrophobic molecules but for hydrophilic proteins and peptides the most frequently used method is the water in oil in water (w/o/w) double emulsion solvent evaporation method. Commercial, injectable PLGA microspheres of leuprorelin were produced using this method (Okada, 1997).

Nanoparticles encapsulating protein and peptide drugs with a mean size less than 300nm were produced (Yang et al., 2012, Pan et al., 2002, Wu et al., 2012b, Garinot et al., 2007) with protein/peptide encapsulation efficiencies from 30% to 80% (Yang et al., 2012, Teply et al., 2008, Cheng et al., 2006, Pan et al., 2002, Li et al., 2004, Wu et al., 2012b, Garinot et al., 2007, Gupta et al., 2007) demonstrating the suitability of this method for their production. Varying the parameters of the w/o/w method can affect the physicochemical characteristics of the PLGA particles and these in turn affect their behaviour *in vitro* and *in vivo*.

4.1.5.3 Overcoming drug burst release from PLGA particles

One of the common problems encountered with protein/peptide drug loaded PLGA particles is their uncontrolled initial burst release (Yang et al., 2012, Cui et al., 2006b, Carino et al., 2000, Pan et al., 2002, Wu et al., 2012b, Yoo and Park, 2004, Blanco and Alonso, 1998, Panyam et al., 2003, Luan et al., 2006). This initial burst could be detrimental to the oral bioavailability of an encapsulated peptide drug as it would be released and potentially destroyed in the GI tract before being absorbed into the bloodstream. This initial burst could be due to the peptide being present at the surface of the particles or released from pores in the particle. To prevent burst release in the stomach enteric polymers have been used in conjunction with or to coat polymeric particles. Encapsulation of protein/peptide loaded nanoparticles in microparticles has also reduced burst release. Reduction of burst release can reduce degradation and increase the amount of intact drug which can be absorbed.

Enteric coatings reduced *in vitro* drug release in acid and reduced pepsin digestion of insulin and ovalbumin loaded in PLGA particles (Naha et al., 2008, Cui et al., 2006a, Delgado et al., 1999, Wu et al., 2012b). Enteric coating of PLGA/PLA particles also increased bioavailability and produced a sustained effect *in vivo*, compared to uncoated particles, of insulin (Naha et al., 2008, Cui et al., 2006a), ovalbumin (Delgado et al., 1999), leuprorelin (Iqbal et al., 2011) and calcitonin (Cetin et al., 2012). Oral bioavailability of encapsulated insulin was increased from 3.68% to 6.27% by enteric coating (Cui et al., 2006a). Oral leuprorelin bioavailability increased 4.2 fold following enteric coating of tablets of drug loaded polyacrylic acid nanoparticles (Cetin et al., 2012).

Microencapsulation of poly ε caprolactone nanoparticles in microparticles of ethylcellulose or Eudragit RS or a blend of these polymers reduced *in vitro* burst release of triptorelin from 71% from the nanoparticles to only 5.4% from the ethylcellulose/Eudragit RS blend microparticles (Hasan et al., 2007).

4.2 Aims

- To assess the stability of peptide 1 throughout the GI tract using simulated and porcine gastrointestinal fluids and human faecal fluids
    - To use this knowledge for optimal targeting and rational design of oral peptide 1 delivery vehicles
    - To gain an insight into the oral stability of small peptides
- To produce oral peptide 1 formulations based on the intestinal stability study, previous studies of peptide intestinal permeability, existing delivery strategies for chemotherapeutic peptides and research into peptide oral delivery strategies
    - To characterise their physical characteristics (size, polydispersity, morphology, charge), loading and encapsulation efficiency
    - To assess their *in vitro* peptide 1 release
    - To assess the impact of varying method parameters
    - To select method parameters which produce formulations with the highest probability for successful oral delivery
- To improve the permeability and peptide release profile by additional coatings/enteric encapsulation

4.3 Materials

Peptide 1 was supplied by Thermo Scientific. Pepsin from porcine gastric mucosa, 469 units/mg solid, 924 units/mg protein, pancreatin from porcine pancreas, activity at least 3x USP specifications and trifluoroacetic acid were from Sigma Aldrich. Hydrochloric acid 37%, specific gravity 1.18, and glacial acetic acid (100%) were from BDH. Acetonitrile HPLC grade was from Fisher Scientific. Pig gastric and intestinal fluids were from freshly slaughtered pigs and immediately frozen and store at -80ºC. Human faecal fluids were from healthy individuals not taking antibiotics.

Faecal basal media materials: Bacteriological peptone and yeast extract were from Oxoid. Sodium chloride, L-cysteine hydrochloride, vitamin K, resazurin sodium salt and sodium hydroxide were from Fisher Scientific. Dipotassium hydrogen orthophosphate and magnesium sulphate 7-hydrate were from BDH. Calcium chloride dihydrate was from VWR. $NaHCO_3$ and haemin were from Sigma Aldrich. Bile salts were from Fluka Analytical. Tween 80 was from Fluka Chemika.

Poly (lactic-co-glycolic acid) (PLGA), 5050 DLG 2A was from Lakeshore Biomaterials (USA). Polyvinyl alcohol (PVA), 87-89% hydrolysed, and monobasic potassium phosphate were from Sigma Aldrich. Dichloromethane (DCM) was from VWR. Ethanol, 96% v/v, was from BDH. Chitooligosaccharide (1-4) 2-amino-2-deoxy-β-D-glucose, (chitosan) average molecular weight 5K was from KittoLife.

MDA-MB-231 breast cancer cells were from the American type culture collection (ATCC), Manassas, USA. Crystal violet ACS reagent, anhydrous dye >=90% was from Sigma Aldrich. PrestoBlue™ cell viability reagent, Gibco 0.25% trypsin-EDTA, Gibco Leibovitz's L15 medium, Penicillin-Streptomycin liquid, and fetal bovine serum were from Invitrogen

Eudragit L100 was a gift from Degussa/ Evonik (Darmstadt, Germany), sorbitan sesquioleate (Alacel 83) and phosphate buffered saline tablets (PBS) were from Sigma Aldrich. Liquid paraffin BP was supplied by JM Loveridge Plc. Sodium phosphate, tribasic, anhydrous was from Alfa Aesar. n-hexane was from Fisher Scientific.

4.4 Methods

4.4.1 Peptide 1 intestinal stability

100µl of a 10mg/ml peptide 1 solution was added to 1.9ml of simulated gastric fluid (SGF) with and without pepsin and simulated intestinal fluid (SIF) with and without pancreatin (final peptide 1 concentration of 0.5mg/ml). These were placed in a Gallenkamp shaking incubator at 37±0.5°C and agitated at 100rpm.

Samples (0.15ml) were removed after 0, 15, 30, 60, 90 and 120 minutes and added to either 0.45ml 0.002M NaOH to raise the pH of acidic fluids or 50% acetic acid to lower the pH of SIF to halt the reaction (final peptide 1 concentration 0.125mg/ml). These samples were analysed for peptide 1 content using HPLC. SGF and SIF were prepared as described in chapter 2, section 2.4.2.

100µl of a 10mg/ml peptide 1 solution was added to 1.9ml of gastric (pH 2.31), duodenal (pH 6.55), jejunal (pH 6.79), ileal (pH 6.86) and descending colonic fluids (pH 7.06) from a pig, giving a final peptide 1 concentration of 0.5mg/ml. The pig intestinal fluids were prepared by centrifuging at 10,000rpm for 10 minutes and using the supernatant for testing. These mixtures were placed in a Gallenkamp shaking incubator at the parameters used for the simulated fluids. Samples (0.15ml) were removed after 0, 15, 30, 60, 90 and 120 minutes and added to either 0.45ml of 0.002M NaOH to raise the pH of acidic fluids or 50% acetic acid to lower the pH of small and large intestinal fluids and halt the reaction (final peptide 1 concentration 0.125mg/ml). These samples were filtered using 0.45µm filters (Millex GP, Millipore, Ireland) and analysed for peptide 1 content using HPLC.

0.05ml of a 10mg/ml peptide 1 solution was added to 0.95ml of human faecal slurry and placed in a Gallenkamp shaking incubator at the parameters used in the previous tests (final peptide 1 concentration 0.5mg/ml). Samples (0.15ml) were removed after 0, 15, 30, 60, 90 and 120 minutes and added to 0.45ml 50% acetic acid to halt the reaction (final peptide 1 concentration 0.125mg/ml). These samples were centrifuged at 10,000rpm for 10 minutes and the supernatants analysed for peptide 1 content using HPLC. The human faecal slurry was prepared as described in chapter 2, section 2.4.2.1.

Peptide 1 recovery in the withdrawn samples was calculated using the equation below. The theoretical concentration of the sample is the concentration of peptide 1 assuming 100% recovery.:

$$\text{Peptide 1 recovered (\%)} = \frac{Measured\ peptide\ 1\ concentration\ (\mu g/ml)}{Theoretical\ peptide\ 1\ concentration\ (\mu g/ml)} \text{x100}$$

4.4.1.1 Statistics

The peptide 1 degradation data from gastric and small intestinal fluids were analysed using a simple regression model using least squares. Assuming a zero order degradation rate, the concentration of peptide 1 in the samples was plotted against time. The slope of this line is defined as –k, the rate constant is k. This is used to determine the half life (t ½) of the peptide in the intestinal fluids using this equation where C[0] is the concentration of peptide 1 (µg/ml) at time 0:

t ½= $C[0] \div 2k$

The data from peptide 1 degradation in porcine colonic and human faecal fluids were analysed as a first order reaction. The natural log (ln) of the concentration of peptide 1 was plotted against time. The slope of this line is defined is –k, the rate constant is k.

Half life of the peptide is calculated using this equation:

t½= $\ln(2) \div k$

The slope of the regression line was assessed for significance by one way ANOVA. Comparisons between the rate constants and half lives of peptide 1 in different intestinal fluids were performed by one way ANOVA. Values of $p \leq 0.05$ were considered significant. All statistical analyses were determined using Minitab 15.

4.4.2 HPLC method

Samples were run in a mobile phase of 0.1% (v/v) trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile (5-45%) at 25°C. Samples were separated with a C18 column and detected at 220nm.

4.4.3 Formation of PLGA nanoparticles

The method used to produce PLGA nanoparticles was a double emulsion solvent evaporation process based on those previously used to encapsulate protein and peptide drugs (Garinot et al., 2007, Yang et al., 2012, Pan et al., 2002, Wu et al., 2012b). The process is outlined in figure 4.1.

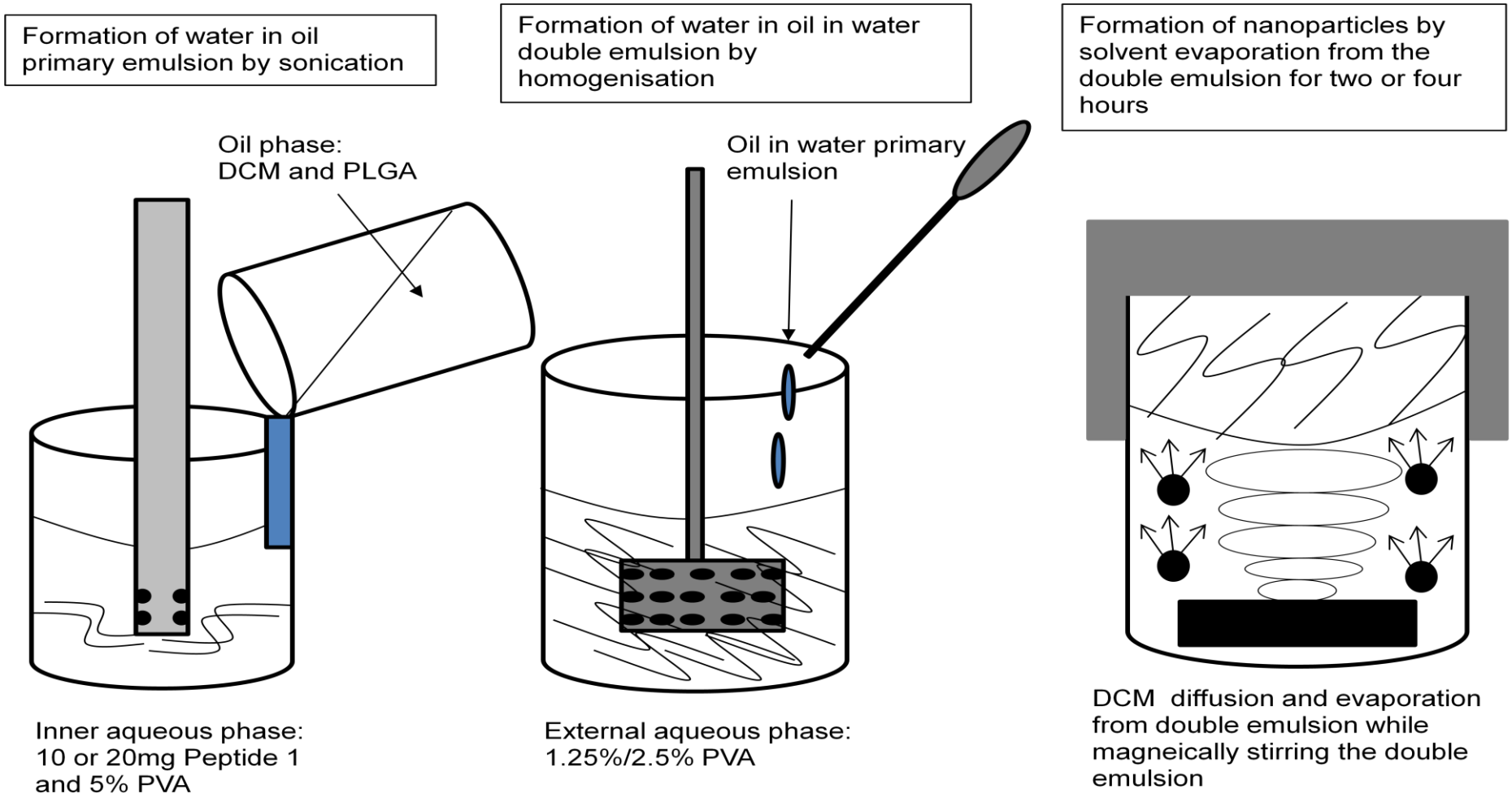


Figure 4.1 *Double emulsion solvent evaporation method used to produce PLGA nanoparticles encapsulating peptide 1*

PLGA (200mg) samples were dissolved in DCM (5ml). An inner aqueous phase (1ml) of 5% PVA, a stabiliser, was added to the PLGA solutions to form blank nanoparticles. For peptide 1 nanoparticles, peptide 1 (10 or 20mg) was dissolved in the inner aqueous phase and left for 20 minutes prior to primary emulsification giving ratios of PLGA to peptide of 10:1 and 20:1. These were emulsified with an Ultra Turrax T25 homogeniser at 24000rpm for one minute and sonicated at 17 microns amplitude with a Sanyo Soniprep 150 probe sonicator for five minutes to form a primary water in oil emulsion. The emulsions were added dropwise to an outer aqueous phase (40ml) and emulsified at 10000rpm with a Silverson L4RT mixer for 5 minutes. The outer aqueous phase varied in PVA concentration; either 1.25% PVA, 2.5% PVA.

All emulsification and sonication steps were carried out with the samples on ice to protect the peptide from thermal denaturation (Zambaux et al., 1998). The double emulsions were magnetically stirred for either 2 or 4 hours during solvent evaporation. The nanoparticles were separated by centrifuging at 21000 rpm, RCF 41415 at 15°C for 20 minutes in a Sigma 3K30 centrifuge. Supernatants were poured off and retained for analysis of peptide 1 content by HPLC. The nanoparticles were resuspended and washed in water and centrifuged again using the same parameters. The supernatants were retained and analysed for peptide 1 content by HPLC. Pellets were finally re-suspended in DI water (5ml).

The supernatants from the centrifugation of the double emulsions and from washing the nanoparticles were analysed for peptide 1 content using HPLC and this was subtracted from the initial amount of peptide 1 added. Encapsulation efficiency (EE) and drug loading were calculated using these equations:

$$\text{EE (\%)} = \frac{Peptide\ initially\ added\ (mg) - Peptide\ 1\ in\ supernatants\ (mg)}{Peptide\ 1\ initially\ added\ (mg)} \text{ x100}$$

$$\text{Drug loading} = \frac{Peptide\ 1\ initally\ added (mg) - Peptide\ 1\ in\ supernatants\ (mg)}{Weight\ of\ freeze\ dried\ nanoparticles\ (mg)} \text{ x1000}$$

(Drug loading =μg peptide 1/mg nanoparticles)

Each nanoparticle sample was dispersed in DI water for size, polydispersity and zeta potential measurements using a Malvern Zetasizer nano Z-S.

The nanoparticle suspensions were freeze dried using a VirTis Advantage freeze dryer using the parameters described in chapter 2, section 2.4.8. The resulting powder was weighed and the yield determined using the equation below.

$$\text{Yield (\%)} = \frac{Weight\ of\ freeze\ dried\ nanoparticles\ (mg)}{Weight\ of\ excipients\ added\ (mg)} \text{x100}$$

The morphology and size of the freeze dried nanoparticles were examined by scanning electron microscopy (SEM) (Philips XL30, Eindhoven, Holland). Nanoparticles were fastened on a SEM stub using carbon adhesive pads and then coated with gold using an Emitech K550 sputter coater. Routine, high vacuum imaging at 10/5kV was used.

4.4.3.1 *In vitro* peptide 1 release

Nanoparticles (10mg) with encapsulated peptide 1 were placed in a glass vial with 1ml PBS, pH 7.4, in triplicate. Samples were placed in a Gallenkamp shaking incubator at 100rpm, 37°C. After 30 minutes, 2, 4, 8 and 24 hours samples were centrifuged at 10,000rpm for 10 minutes and 0.5ml of the supernatant analysed for peptide 1 content by HPLC. 0.5ml of fresh PBS was added to replace the 0.5ml removed, the nanoparticles were resuspended and the samples returned to the shaking incubator. Those nanoparticles prepared after a solvent evaporation period of 2 hours were also sampled after 1 and 2 weeks.

$$\text{Peptide 1 release (\%)} = \frac{Cumulative\ amount\ of\ peptide\ 1\ released\ (\mu g)}{Total\ peptide\ 1\ (\mu g)}$$

4.4.4 Chitosan coated PLGA nanoparticles

Chitosan coated PLGA nanoparticles were prepared as described above with a 1:20 ratio of peptide 1 to PLGA but with a 1% 5K chitosan/1.25% PVA external aqueous phase. Size and morphology of the nanoparticles were assessed as described previously. The yield was calculated in the same way but the weight of excipients also included the chitosan added. The peptide 1 loading and encapsulation efficiency were determined as before. *In vitro* peptide 1 release from the chitosan coated nanoparticles (20mg) was performed in PBS as described for the uncoated particles and monitored over 2 weeks.

4.4.5 Peptide 1 efficacy

MDA-MB-231 (ATCC HTB-26) breast cancer cells were cultured in Leibovitz's L-15 medium with 15% fetal bovine serum and 1% penicillin-streptomycin solution in an incubator without CO_2 at 37°C. 2×10^5 cells per well were plated in 24 well plates in 100µl culture medium, to these either 1µM of peptide 1 alone, peptide 1 encapsulated in PLGA nanoparticles, peptide 1 encapsulated in chitosan coated PLGA nanoparticles, blank or blank chitosan coated PLGA nanoparticles were added in 100µl culture medium. Some of the cells were left without peptide or formulation. After 24 hours incubation the cells were trypsinised and transferred to 12 well plates. After 6 days further incubation the cells were tested for viability. This was to determine the effect of peptide 1 on breast cancer cell proliferation and the effects on peptide 1 efficacy caused by its encapsulation in PLGA nanoparticles.

After removing and refreshing the culture media PrestoBlue™ cell viability reagent was added to the cells and incubated for 2 hours at 37°C. This reagent is a resazurin based solution that is reduced by metabolically active cells changing colour to red to give a measure of cell viability. 100µl samples were plated in a 96 well plate and their absorbance at 550nm measured using a Bio-Tek Powerwave XS plate reader.

The medium with the viability reagent was removed and the cells washed twice with PBS. The cells were fixed with methanol for 5 minutes. This was removed and 0.05% w/v crystal violet solution added to the cells to stain remaining cells. After 30 minutes the stain was removed and the cells washed twice with PBS. After drying, methanol was added to solubilise the dye, transferred to a 96 well plate and absorption measured at 550nm using a plate reader as before.

4.4.6 Enteric microparticles

To overcome any immediate burst release of peptide 1 from the PLGA nanoparticles in the stomach the strategies of enteric coating and microencapsulation of nanoparticles were combined.

Due to the ability of Eudragit L100 microparticles prepared using the Kendall *et al*. method to encapsulate lactase and insulin without damaging them, elicit a pH dependent release and provide protection from pepsin they were selected to encapsulate peptide 1 loaded PLGA nanoparticles. As this method uses ethanol to dissolve Eudragit L100, rather than DCM, it will be suitable to encapsulate intact nanoparticles as PLGA is not soluble in ethanol.

Enteric microparticles encapsulating peptide 1 alone and PLGA nanoparticles with peptide 1 were prepared with the substitution of peptide 1 or peptide 1 loaded PLGA nanoparticles for prednisolone (Kendall et al., 2009). The amounts of excipients used were scaled down for the reduced amounts of peptide 1 and peptide 1 loaded nanoparticles available. Eudragit L100 (300mg) was dissolved in ethanol (3ml). Peptide 1 (20mg) was suspended in the ethanol to prepare microparticles with a drug to polymer weight ratio of 1:15. PLGA nanoparticles (30mg), with a 1:20 peptide 1:PLGA initial ratio prepared with 1.25% PVA outer aqueous phase after a solvent evaporation of 2 hours, were added to the polymeric solution to prepare microparticles with a nanoparticle to microparticle weight ratio of 1:10.

The polymeric suspensions with either peptide 1 or peptide 1 PLGA nanoparticles were emulsified into liquid paraffin (20ml), containing 1% (w/w) of sorbitan sesquioleate (Arlacel 83) as an emulsifying agent, using a Heidolph RZR1 stirrer at 1500rpm. Stirring proceeded for 18 hours at room temperature to allow solvent evaporation and particle solidification. The microparticles formed were recovered by vacuum filtration through a Pyrex sintered glass filter (pore size 4; 5-15µm) and washed three times with *n*-hexane (50ml). The microparticle formulations were prepared in triplicate. Blank microparticles containing no peptide 1 and microparticles with blank nanoparticles (30mg) were also prepared using the same parameters

The yield and size of the microparticles, measured using a Malvern Mastersizer, were determined as described in chapter 2, section 2.4.6.1. The morphology and size of the microparticles were examined by scanning electron microscopy (SEM) (Philips XL30, Eindhoven, Holland), with routine, high vacuum imaging at 5kV as described in chapter 2, section 2.4.6.2.

4.4.6.1 Loading and encapsulation efficiency

Microparticles (5/10mg) with encapsulated peptide 1 were placed in pH 6.8±0.05 phosphate buffer for 2 hours in a Gallenkamp shaking incubator to break down the microparticles and determine the peptide 1 loading per mg of microparticles and the efficiency of its encapsulation in the microparticles. The amount of peptide 1 released was determined by HPLC and the equations below were used to calculate peptide 1 loading and encapsulation efficiency.

$$\text{Peptide 1 loading per mg microparticles (µg/mg)} = \frac{Total\ peptide\ in\ sample\ (\mu g)}{Amount\ of\ microparticles\ (mg)}$$

$$\text{Encapsulation efficiency (\%)} = \frac{Measured\ peptide\ 1\ loading\ (\mu g/mg)}{Theoretical\ peptide\ 1\ loading\ (\mu g/mg)} \text{x100}$$

The theoretical peptide 1 loading is the loading of peptide 1 if all the peptide loaded nanoparticles were encapsulated in the microparticles.

These tests were not performed with the microparticles containing nanoparticles as only the microparticles would be broken down using this method. Instead the peptide 1 loading in the microparticles encapsulating nanoparticles was theoretically determined assuming complete encapsulation of the peptide loaded nanoparticles in the microparticles

Obviously it would be better to experimentally and accurately determine the actual peptide 1 content in the microencapsulated PLGA nanoparticles. However, attempts to do this by extracting the polymer into an organic phase and peptide into an aqueous phase led to the formation of a white precipitate at the interface of these phases which seemed to prevent peptide extraction. The most important aspect of this work was to demonstrate if the enteric microencapsulation was able to prevent peptide 1 release in acid. Once this had been achieved other methods for determining peptide 1 content could be explored.

4.4.6.2 *In vitro* release

Peptide 1 PLGA nanoparticles, peptide 1 Eudragit L100 microparticles and peptide 1 PLGA nanoparticles encapsulated in Eudragit L100 microparticles were placed in 0.75 ml 0.1N HCl, pH 1.2 ± 0.05, for 2 hours to simulate gastric conditions. The pH was then raised to pH 6.8 ± 0.05 by the addition of 0.25ml 0.2M tribasic sodium phosphate to simulate the small intestine. Samples were placed in a Gallenkamp shaking incubator at 100rpm, 37±0.5°C. After 30 minutes, 2, 4, 8 and 24 hours samples were taken from the nanoparticle mixture, centrifuged at 10,000rpm for 10 minutes and 0.5ml of the supernatant analysed for peptide 1 release by HPLC. After 30 minutes, 2 hours and 2 hours and 45 minutes samples were taken from the microparticle mixtures, centrifuged and analysed in the same way. 0.5ml of 0.1N HCl/pH 6.8 phosphate buffer were added to replace that removed, the particles resuspended and the samples returned to the shaking incubator after each sampling time point. Cumulative peptide 1 release was calculated as in section 4.4.4.

4.4.7 Statistics

Differences in particle characteristics caused by altering the method parameters, chitosan coating or enteric microencapsulation were assessed for significance by one-way ANOVA using the Minitab 15 program. Results were considered to be significant for p values ≤0.05.

4.5 Results and Discussion

4.5.1 Peptide 1 intestinal stability

The stability of peptide 1 was assessed in simulated and pig intestinal fluids and in human faecal fluids. This was used to design oral formulations of peptide 1.

4.5.1.1 Gastric fluids

More than 70% of intact peptide 1 was recovered from SGF, with and without pepsin, and porcine gastric fluid after 2 hours incubation, table 4.2. In SGF without pepsin there was more than 90% intact peptide 1 recovered suggesting it has high stability at gastric pH possibly due to its small size and lack of higher structure which can be disrupted at this pH.

Intact peptide 1 gradually reduced during incubation in SGF with pepsin from 96% to 75% in a significant zero order degradation ($p \leq 0.05$). This suggests there is some pepsin digestion of peptide 1. Pepsin cleaves between hydrophobic, preferably aromatic amino acids. Peptide 1 doesn't contain these amino acids adjacently but it appears pepsin was still able to digest some of the bonds slowly. Despite the lack of preferred theoretical pepsin digestion sites in teriparatide it was completely degraded within 5 minutes with pepsin suggesting it has a broad substrate spectrum (Werle et al., 2006).

Table 4.2 *Peptide 1 recovery after incubation in SGF and porcine gastric fluids. Data represents means ± SD.*

Sample (minutes)	Peptide 1 recovered (%) SGF	Peptide 1 recovered (%) SGF + pepsin	Peptide 1 recovered (%) pig gastric fluid
0	98.6 ± 22.4	95.8 ± 8.5	79.3 ± 3.6
15	96.1 ± 20.0	92.8 ± 7.5	82.9 ± 18.1
30	92.6 ± 21.5	86.3 ± 7.5	71.2 ± 9.2
60	92.1 ± 23.0	77.0 ± 12.6	90.9 ± 77.2
90	94.4 ± 30.9	77.4 ± 9.0	82.0 ± 9.9
120	96.8 ± 28.2	74.5 ± 6.1	86.1 ± 7.1

There was an immediate 20% loss of peptide 1 when placed in porcine gastric fluid. Recovery values of the peptide then fluctuated around 80%. Fluctuation may be due to differences in sampling caused by the viscous gastric fluid preventing homogenous peptide 1 distribution. To develop a delivery system which allows as high a concentration of intact peptide 1 to reach the absorbing membranes of the small intestine as possible protection from gastric enzymes would be beneficial.

The degradation rate constant (k) of peptide 1 was significantly greater ($p \leq 0.05$) in SGF with pepsin than in SGF or porcine gastric fluid, table 4.3. Active pepsin concentration may be lower in the porcine gastric fluid than in SGF with pepsin. Possibly it was denatured during storage reducing its activity or may be present in porcine gastric fluids at a lower concentration than in the simulated fluid.

The presence of pepsin in the porcine gastric fluids is dependent on which part of the stomach it is taken from. Possibly the fluids were removed from a part with little pepsin content and fluids from another segment may have been more proteolytic. The negative degradation rate constant and half life of peptide 1 in porcine gastric fluid actually indicate an accumulation of peptide 1 rather than degradation, table 4.3. This may be due to sampling problems with the viscous fluids or an interaction of the peptide with constituents of the gastric fluids preventing its complete extraction.

Table 4.3 *Degradation rate constants (k) and half lives of peptide 1 in SGF and porcine gastric fluids. Data represents means ± SD.*

Sample	**Slope (k)**	**Half life (minutes)**
SGF	0.01 ± 0.34	963.18 ± 1726.45
SGF + pepsin	0.68 ± 0.13	359.36 ± 42.09
Gastric fluid	-0.46 ± 0.21	-519.89 ± 305.24

4.5.1.2 Small intestinal fluids

There was a gradual, significant zero order decline in peptide 1 concentration over 2 hours to 76% in SIF without pancreatin, table 4.4. This indicates the pH or composition of this fluid may cause a gradual loss of peptide 1 structure. With pancreatin there was no intact peptide 1 recovered at any time point, only four degradants; the products of peptide 1 digestion.

Table 4.4 *Peptide 1 recovery in SIF and porcine small intestinal fluid. Data represents means ± SD.*

Sample (min)	Peptide 1 recovered (%) SIF	Peptide 1 recovered (%) duodenal fluid	Peptide 1 recovered (%) jejunal fluid	Peptide 1 recovered (%) ileal fluid
0	92.5 ± 5.7	78.1 ± 1.5	21.8 ± 5.7	45.0 ± 5.3
15	95.1 ± 7.3	71.1 ± 7.5	18.3 ± 9.1	37.0 ± 0.4
30	90.7 ± 3.7	70.2 ± 2.9	36.7 ± 7.5	27.1 ± 3.0
60	80.7 ± 5.5	66.4 ± 2.5	29.3 ± 4.0	19.8 ± 1.9
90	77.9 ± 3.8	63.7 ± 1.1	22.9 ± 1.3	13.6 ± 2.6
120	76.2 ± 5.1	56.6 ± 5.0	17.3 ± 2.0	8.6 ± 2.8

The stability of peptide 1 in the porcine small intestinal fluids tended to decline along the small intestine, table 4.4. There was a gradual decline in peptide 1 recovery to 57% after 2 hours in the duodenal fluids. There was an immediate loss of peptide 1 upon mixing with jejunal and ileal fluids, to 22% and 45% respectively, and then a decline to 17% and 9% respectively. This loss in activity could be due to the increasing pH along the small intestine as peptide 1 was shown to be more unstable at pH 6.8 than at pH 1.2. Increasing peptide 1 degradation in the jejunal and ileal fluids compared to the duodenal fluids could be due to increasing amounts of degradative enzymes. This pattern of degradation is similar to that of GnRH in brushtail possum intestinal extracts where it was degraded more in the jejunal and ileal extracts than duodenal samples (Wen et al., 2002b). Analysis of the proteolytic activity of these samples revealed there was more enzymatic activity in the jejunal and ileal samples than the duodenal samples (Wen et al., 2002c).

Analysis of the peptide 1 sequence indicated multiple bonds were vulnerable to digestion by the small intestinal enzymes trypsin, chymotrypsin and elastase so it is unsurprising it was degraded in these samples, figure 4.2. These results indicate peptide 1 could be more successfully orally delivered if it is targeted for absorption from the proximal small intestine where there is reduced proteolytic activity.

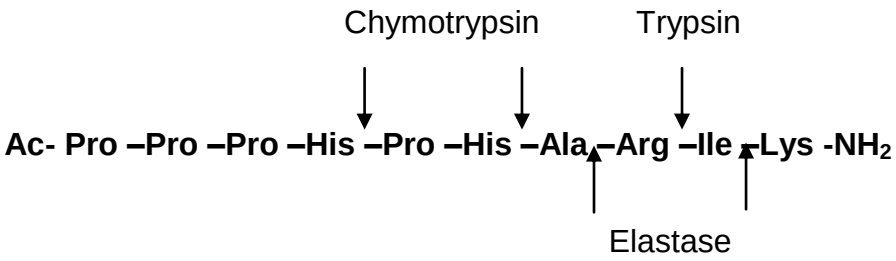


Figure 4.2 *Peptide bonds of peptide 1 susceptible to chymotrypsin, trypsin and elastase cleavage*

Peptide 1 degradation followed a significant zero order degradation in porcine duodenal and ileal fluids. Degradation of peptide 1 in jejunal fluids after 30 minutes incubation until completion of the experiment also followed a significant zero order degradation. Only these significant results were used for comparison of degradation in the intestinal fluids.

There was significantly faster degradation of peptide 1 in all three small intestinal fluids than porcine gastric fluids and the half life of peptide 1 was significantly reduced in the jejunal and ileal fluids, tables 4.3 and 4.5. This suggests peptide 1 is more vulnerable to degradation in the small intestine than in the stomach possibly due to the nature or concentration of the enzymes there. The degradation rate of peptide 1 was faster in the jejunal and ileal fluids than SIF due to the presence of enzymes, table 4.5. The duodenal fluids have a lower peptide 1 directed proteolytic activity than the other small intestinal fluids and also, surprisingly, than SIF without any enzymes. Possibly the constituents of SIF or its slightly higher pH destabilise the peptide. The enzyme activity of the duodenal fluids may also have declined during storage.

Table 4.5 *Degradation rate constants (k) and half lives of peptide 1 in simulated and porcine small intestinal fluids Data represents means ± SD.*

Sample	Slope (k)	Half life (minutes)
SIF	0.63 ± 0.20	386.90 ± 103.36
Duodenal fluid	0.36 ± 0.32	631.35 ± 1277.72
Jejunal fluid	0.95 ± 0.37	105.80 ± 27.88
Ileal fluid	1.32 ± 0.29	86.45 ± 11.89

Peptide 1 degradation in SIF with pancreatin was faster than in the porcine small intestinal fluids. The simulated fluids seem to have a greater proteolytic activity and therefore may not be representative of what would happen *in vivo*. However the degradation results from the porcine intestinal fluids may be an underestimate of the stability of the peptide in these intestinal segments. The fluids were removed from the lumen of the intestinal segments so any intestinal wall associated enzymes would not be present. As the samples are then centrifuged and only the supernatants used for testing any enzymes associated or entangled with the solid matter would not be present to digest peptide 1.

4.5.1.3 Colonic fluids

There was an immediate loss of peptide 1 upon addition to the porcine colonic fluid and human faecal slurry; 44% and 24% respectively, table 4.6. After 15 minutes incubation there was only 16% and 27% intact peptide 1 left in the pig colonic fluid and human faecal slurry respectively. For the remainder of the experiment there was a gradual loss of peptide 1 in the porcine colonic fluids, only 2% remained intact after 2 hours. Loss of peptide 1 followed a significant first order degradation. Unlike the zero order degradation of peptide 1 in the gastric and small intestinal fluids degradation in the colonic fluids is dependent on its concentration and slowed as it was depleted over time.

Table 4.6 *Peptide 1 recovery in porcine colonic and human faecal fluids. Data represents means ± SD.*

Sample (min)	**Peptide 1 recovered (%) pig colonic fluid**	**Peptide 1 recovered (%) human faecal slurry**
0	66.4 ± 5.1	76.2 ± 9.2
15	15.7 ± 7.8	26.8 ± 0.7
30	15.7 ± 4.7	32.3 ± 0.4
60	9.7 ± 0.7	41.2 ± 1.6
90	2.5 ± 2.0	38.2 ± 2.5
120	2.0 ± 0.5	30.9 ± 2.2

The loss of peptide 1 could be due to the higher pH of the colonic fluid and/or the presence of microbial enzymes and fermenting bacteria.

GnRH was less degraded in the colonic fluids of a brushtail possum and these fluids were less proteolytic than the small intestinal fluids (Wen et al., 2002b, Wen et al., 2002c). Possibly peptide 1 is more susceptible to bacterial mediated fermentation in the colon than GnRH despite their similar size. The colonic fluids from a pig may contain more fermenting bacteria or microbial proteases than those from the brushtail possum.

Degradation of peptide 1 in the human faecal slurry did not produce a significant zero or first order reaction rate. Peptide 1 was rapidly degraded during the first 15 minutes but stabilised with 30% remaining, producing a very low reaction rate constant, table 4.7. Possibly the enzymes or bacteria responsible for its degradation were inactivated and so peptide 1 depletion stopped. This may not reflect what would happen *in vivo* where conditions for enzyme and bacterial activity would be optimal. Peptide 1 was also rapidly degraded initially in the porcine colonic fluids but was then continuously degraded until only 2% remained. Peptide 1 had a significantly different ($p \leq 0.05$) calculated half life in these fluids, table 4.7. Possibly the enzymes and bacteria of the pig colonic fluids are more stable or concentrated and therefore active for longer *in vitro* than those in the human faecal fluids. As the colonic fluids are taken directly from the colon whereas the human faecal fluids are prepared from the faeces expelled from the colon they may have differing bacterial and enzyme activities.

Table 4.7 *Degradation rate constants (k) and half lives of peptide 1 in porcine colonic and human faecal fluids calculated by regression of a first order reaction Data represents means ± SD.*

Sample	**Slope (k)**	**Half life (minutes)**
Porcine colonic fluid	0.03 ± 0.00	27.11 ± 3.55
Human faecal slurry	0.00 ± 0.00	224.02 ± 48.50

The degradation rate of peptide 1 was significantly slower in the colonic fluids than in the gastric and small intestinal fluids, this is due to a slowing of the reaction rate as peptide 1 is degraded, tables 4.3, 4.5 and 4.7. The half life of peptide 1 was significantly reduced in the colonic fluids compared to the porcine gastric and small intestinal fluids due to initial rapid degradation of peptide 1.

The different peptide 1 degradation pattern in colonic fluids may be due to both bacterial mediated fermentation and enzymatic degradation occurring rather than just enzymatic digestion. The rapid initial degradation of peptide 1 in the porcine colonic and human faecal fluids may make the large intestine an unsuitable target for peptide 1 delivery.

4.5.1.4 Overall intestinal stability

Figure 4.3 shows the recovery of intact peptide 1after incubation in the various intestinal fluids. Peptide 1 is more stable in the proximal than distal regions of the intestinal tract suggesting the peptide should be targeted for absorption from the upper regions of the small intestine. Of the small/large intestinal fluids peptide 1 degradation rate was slowest and half life longest in the duodenal fluids. The further it travels down the GI tract the more likely it is to be rapidly degraded and have reduced time for intact peptide absorption. Assessment of peptide 1 stability in human intestinal fluids would provide a better prediction of its *in vivo* behaviour. While peptide 1 had a similar stability profile to GnRH in small intestinal fluids it was more unstable in colonic fluids than GnRH. This may be due to differences in the animal from which the fluids were extracted. It may also show that despite similarities in size and number of amino acids stability is also dependent on individual amino acid sequences.

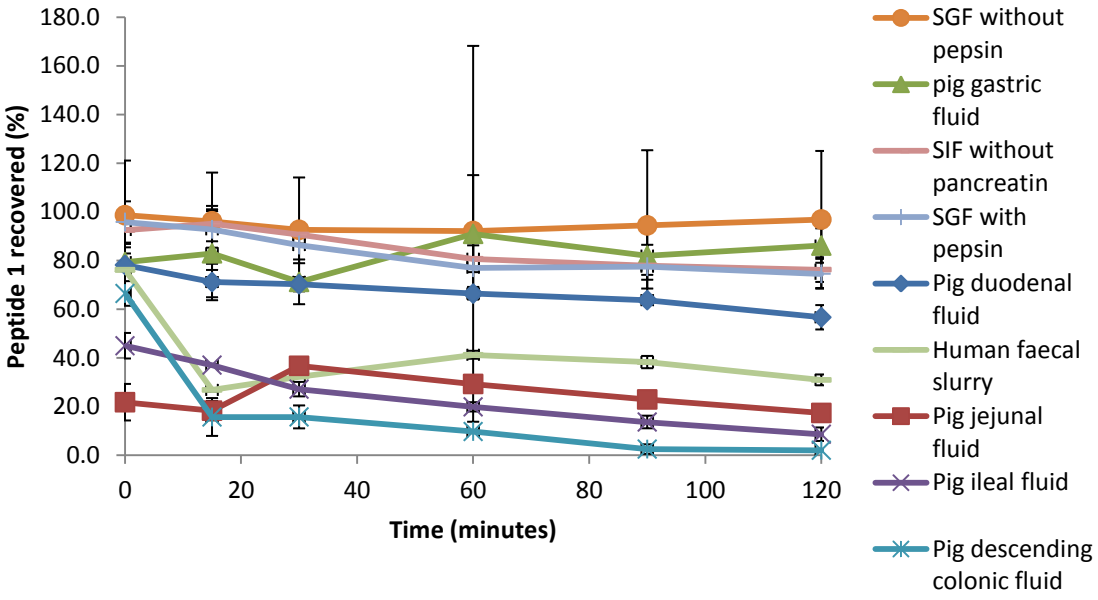


Figure 4.3 *Peptide 1 recovered after incubation in simulated, porcine and human intestinal fluids. Error bars show mean ± SD.*

4.5.2 PLGA nanoparticles

The intestinal stability studies demonstrated a huge obstacle to the oral delivery of peptide 1 is its susceptibility to digestion by intestinal enzymes. An oral delivery system may need to provide protection from pepsin in the stomach to ensure as high a concentration of intact peptide as possible is present when it reaches the small intestine. Protection from small intestinal enzymes would also be beneficial to allow peptide 1 absorption for as long as possible. Peptide intestinal permeability studies have demonstrated their low absorption (Adjei et al., 1993, Zheng et al., 1999b).

Peptide 1 was encapsulated in PLGA nanoparticles for its oral delivery for their ability to protect and enhance the permeability of an encapsulated peptide (Yoo and Park, 2004, Panyam et al., 2003, Cohen-Sela et al., 2009, Westedt et al., 2007). PLGA particles have demonstrated their suitability and compatibility for delivering other chemotherapeutic peptides as sustained release, injectable depot formulations (Crotts and Park, 1998, Dai et al., 2005). They have also been used to encapsulate a variety of protein and peptide drugs increasing their oral bioavailability (Yoo and Park, 2004, Uchida et al., 1994, Challacombe et al., 1992, Gupta et al., 2007, Italia et al., 2007, Sun et al., 2011b, Cui et al., 2006b, Carino et al., 2000, Sun et al., 2011a, Teply et al., 2008, Pan et al., 2002, Li et al., 2004, Wu et al., 2012b)

4.5.2.1 Method development

PLGA nanoparticles encapsulating peptide 1 were produced using a double emulsion solvent evaporation method which has frequently been used to encapsulate therapeutic proteins and peptides (Okada, 1997, Garinot et al., 2007, Yang et al., 2012, Pan et al., 2002, Wu et al., 2012b, Gupta et al., 2007, Teply et al., 2008, Cheng et al., 2006, Li et al., 2004). Varying parameters of this method affected the characteristics of the PLGA particles produced and these in turn had a profound impact on their *in vivo* success.

The size of PLGA particles was critical to their permeability (Gaumet et al., 2009, Desai et al., 1996, Qaddoumi et al., 2004). *In vitro* studies revealed that particles >300nm remained outside cells so to maximise uptake ideally particles should be smaller than 300nm (Gaumet et al., 2009). A high peptide 1 encapsulation efficiency and low initial burst release were also aimed for.

DCM was selected to dissolve PLGA in the oil phase of the double emulsion as it produced smaller particles with a higher encapsulation efficiency than ethyl acetate (Cui et al., 2006b). When forming the emulsions an increased homogenisation speed was shown to produce smaller particles (Luan et al., 2006, Guo and Gemeinhart, 2008). Therefore the highest possible speeds of the homogenisers and greatest amplitude of the sonicator were used. PVA was included in the inner aqueous phase of the double emulsion to stabilise small aqueous phase droplets containing peptide 1, which would be more easily encapsulated within the oily polymer droplets. This increased encapsulation efficiency and reduced initial burst release of BSA (Yang et al., 2001).

The duration of solvent evaporation from the double emulsion was varied as it was shown to affect the encapsulation efficiency and drug release from PLGA particles (Luan et al., 2006). Increasing the solvent evaporation time from 30 minutes to 24 hours increased the initial drug release from the PLGA particles.

The effect of varying the concentration of PVA in the outer aqueous phase of the double emulsion has been researched. Generally an increased concentration produced smaller PLGA particles (Nam et al., 2000, Sun et al., 2011b, Zambaux et al., 1998, Lamprecht et al., 1999, Yang et al., 2001), however there are some conflicting results concluding the opposite (Shi et al., 2009). An increased PVA concentration has also been shown to decrease the encapsulation efficiency so the benefits and disadvantages of an increased concentration need to be balanced (Nam et al., 2000). Two different PVA concentrations were trialled to discover the optimal conditions for producing small (<300nm) nanoparticles with a high encapsulation efficiency.

The amount of peptide 1 added to the inner aqueous phase was also varied. An increased peptide concentration in the inner aqueous phase was previously shown to increase encapsulation efficiency but also increased initial burst release (Luan et al., 2006, Sun et al., 2011b).

4.5.2.2 Solvent evaporation duration

Neither the size nor the polydispersity of the particles was significantly affected by varying the solvent evaporation duration from 2 to 4 hours, table 4.8. The particles produced using both evaporation durations were approximately 300nm. Previous research has found that particle absorption is size dependent and those larger than 300nm were not internalised by cells (Gaumet et al., 2009, Desai et al., 1996, Qaddoumi et al., 2004). Other method parameters may need to be adjusted to ensure the particles produced are smaller than 300nm.

All the nanoparticles were negatively charged as PLGA is an anionic polymer, table 4.8. The particles with positively charged peptide 1 did have a more positive charge indicating its presence. Zeta potentials became more negative with an increase in stirring time, significantly ($p \leq 0.05$) more for particles loaded with 20mg of peptide 1. This could indicate positively charged peptide held on the surface of the particles was lost due to the increased stirring time or that the peptide is more deeply embedded within the particles formed during the longer evaporation period. This could be the result of the oily, polymeric droplets coalescing during the extended evaporation period enabling the formation of a thicker polymeric layer between the peptide and the surface of the particle. However the particles were not significantly larger which might be expected if this were the case.

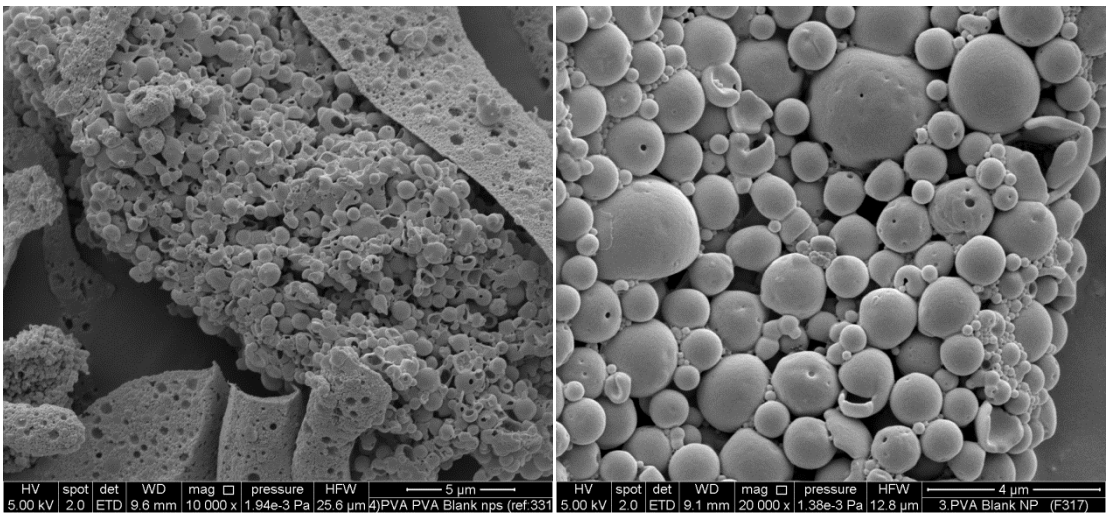
Encapsulation efficiency was decreased by an increased evaporation time, significantly ($p \leq 0.05$) so for those formed with 20mg peptide 1, table 4.8. This may be due to the increased time available for the hydrophilic peptide to migrate into the outer aqueous phase and avoid encapsulation. The prolonged period of stirring may have detached some of the surface adsorbed peptide decreasing the apparent encapsulation efficiency. This hypothesis is also indicated by the more negative charge of the nanoparticles, due to loss of positive, surface adsorbed peptide 1. Despite reduction of encapsulation efficiency caused by the increased solvent evaporation duration the peptide 1 loading of these particles increased. This was because the yield of the particles was reduced so there was more peptide per mg of nanoparticles.

Table 4.8 *Size, polydispersity, yield, zeta potential, encapsulation efficiency and peptide 1 loading of blank and peptide 1 loaded PLGA nanoparticles prepared with a solvent evaporation duration of 2 or 4 hours. Data represents means ± SD.*

Nanoparticles, evaporation duration	**Size (nm)**	**PDI**	**Yield (%)**	**Zeta potential (mV)**	**Peptide 1 loading (ug/mg)**	**Encapsulation Efficiency (%)**
Blank 2 hrs	271.34 ± 40.54	0.38 ± 0.08	54.0 ± 4.1	-24.87 ± 4.67		
Blank 4 hrs	289.66 ± 24.89	0.36 ± 0.04	69.3 ± 3.8	-26.48 ± 1.95		
Peptide 1 10mg 2hrs	293.02 ± 46.46	0.40 ± 0.11	48.5 ± 2.8	-19.93 ± 3.71	60.13 ± 4.38	62.1 ± 6.7
Peptide 1 10mg 4 hrs	313.10 ± 51.22	0.48 ± 0.09	43.8 ± 1.2	-22.09 ± 1.78	53.92 ± 8.19	54.6 ± 7.6
Peptide 1 20mg 2 hrs	304.79 ± 58.65	0.43 ± 0.08	47.8 ± 9.2	-17.53 ± 1.57	100.18 ± 13.42	57.5 ± 5.0
Peptide 1 20mg 4hrs	265.98 ± 50.03	0.36 ± 0.09	34.9± 9.4	-22.86 ± 2.25	131.93 ± 27.16	48.6 ± 3.9

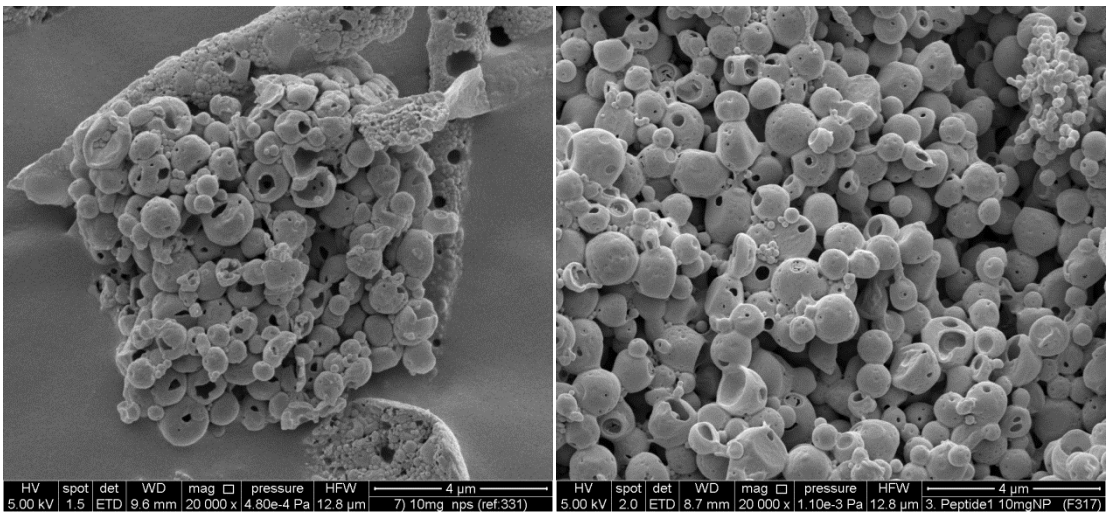
Encapsulation efficiency and peptide 1 loading were calculated by subtracting the amount of peptide in supernatants from centrifugation of the emulsions from the total peptide 1 added initially. This won't account for any peptide losses or degradation during formation caused by its exposure to solvent and shear stresses so may give an over estimated loading and encapsulation efficiency. It would be better to break down the particles and determine how much intact peptide 1 is associated with them. However, attempts to do this by extracting PLGA into DCM and peptide 1 into an aqueous phase resulted in the formation of a white precipitate between the phases from which the peptide could not be extracted. Degradation of peptide 1 may also have occurred at the solvent/aqueous interface. Problems with extracting peptides from PLGA particles using this method have been encountered before (Blanco and Alonso, 1997).

The nanoparticles formed after 4 hours solvent evaporation appear to be larger and more spherical than those formed after 2 hours, figures 4.4-4.6. The prolonged evaporation period may have allowed coalescence and larger particle formation. The longer stirring duration may have caused more spherical particles to form. However size analysis revealed there were no significant differences in the size of the particles. This may be due to aggregation of the particles formed after 2 hours solvent evaporation.



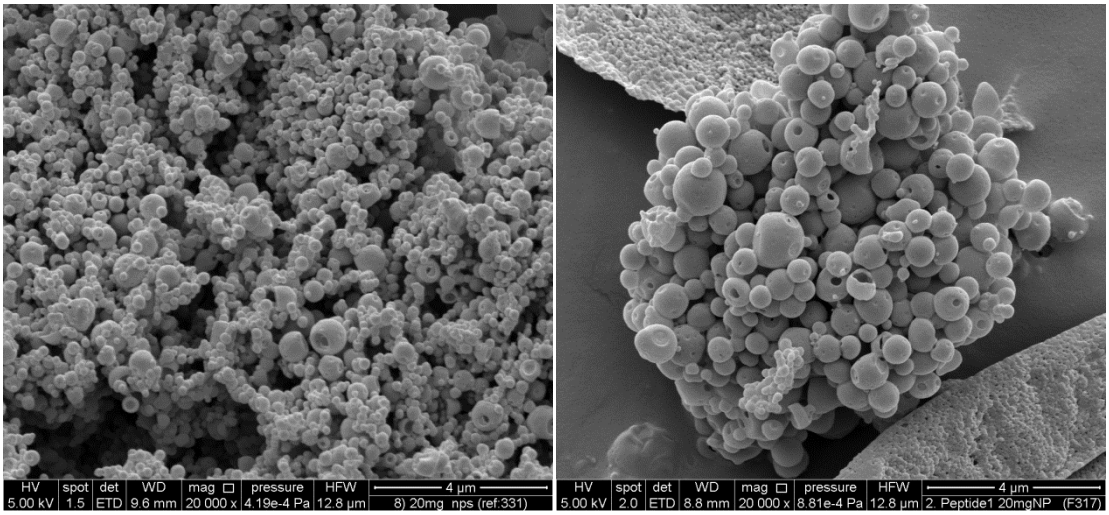
4.4 (a) ***4.4 (b)***

Figure 4.4 *SEM images of blank PLGA nanoparticles prepared after (a) 2 hours solvent evaporation, (b) 4 hours solvent evaporation*



4.5 (a) ***4.5 (b)***

Figure 4.5 *SEM images of 10mg peptide 1 loaded PLGA nanoparticles prepared after: (a) 2 hours solvent evaporation, (b) 4 hours solvent evaporation*



4.6 (a) ***4.6 (b)***

Figure 4.6 *SEM images of 20mg peptide 1 loaded PLGA nanoparticles after: (a) 2 hours solvent evaporation (b) 4 hours solvent evaporation*

Some of the images show the presence of visible pores in the nanoparticle's surface figures 4.4 (b), 4.5 (a) & (b) and 4.6 (b). There appear to be more in nanoparticles initially loaded with 10mg of peptide 1. Peptide 1 in the inner aqueous phase may have increased the osmotic pressure between the aqueous phases causing the solvent to burst from the particles rupturing them. This has previously occurred when the drug concentration of the inner aqueous phase was increased (Lamprecht et al., 1999).

Pores appeared in the particles formed after both evaporation durations but there seem to be more in those prepared by the longer evaporation period possibly as there is greater opportunity for pore formation.

4.5.2.3 *In vitro* release

There was a slight decrease in the proportion of peptide released from the particles formed by a longer solvent evaporation period, but not significantly so, figure 4.7. This may have been due to the loss of surface adsorbed peptide by the increased stirring duration which would have reduced, comparatively, initial release.

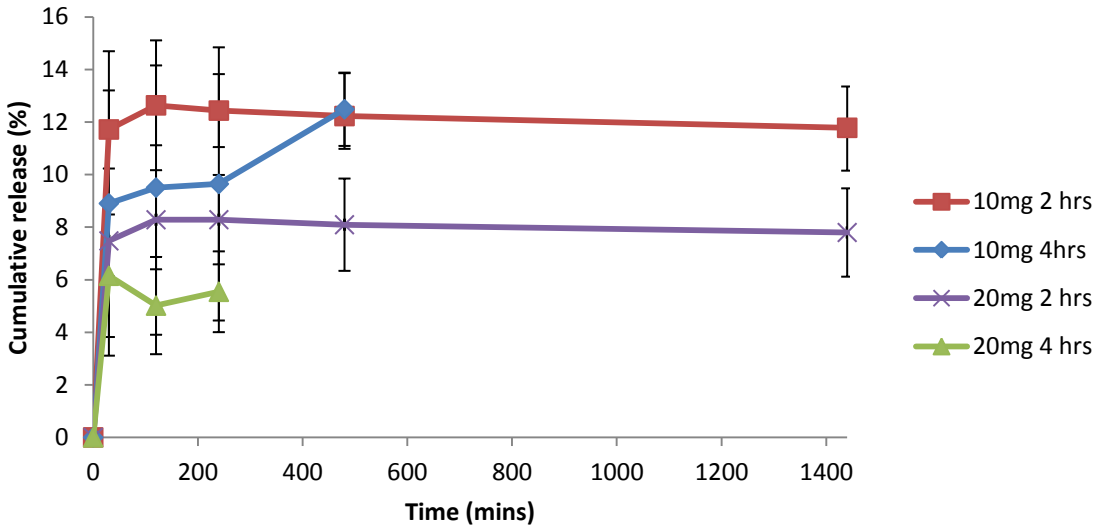


Figure 4.7 *Cumulative release of peptide 1 from PLGA nanoparticles prepared after 2 or 4 hours solvent evaporation. Error bars show mean ± SD.*

Previous investigations found an increased evaporation time of 24 hours produced particles with a higher initial release compared to those prepared with a 30 minute solvent evaporation time (Luan et al., 2006). During the longer evaporation period the peptide may have partitioned from the interior of the particles to the surface providing a greater burst release. The 4 hour evaporation period may not have been long enough for this to occur to an extent to significantly increase initial release.

There was an immediate release of peptide 1 from the nanoparticles upon addition to PBS. This may be the detachment of surface adsorbed peptide which may be present due to an electrostatic interaction between the positively charged peptide and negatively charged particle.

It may also be the result of transit of hydrophilic peptide 1 from the inner aqueous phase to the external aqueous phase of the double emulsion, localising it to the particle surface.

Peptide 1 release from the nanoparticles prepared after 2 hours solvent evaporation was also measured after one and two weeks. There was no further peptide 1 release from these particles. The peptide may still be within the particles and may be released after this period as PLGA particles can provide a sustained GnRH agonist release over 1 and 3 months, table 4.1. The peptide released may have been degraded before it could be measured. It would be useful to test if peptide 1 is stable in PBS over prolonged periods or is degraded following its release. This apparent large incomplete release may also be due to an overestimate of peptide 1 loading calculated by the subtraction method. A better method for determining encapsulation efficiency and peptide loading is needed to determine exactly how much peptide has been encapsulated.

Incomplete peptide release from PLGA particles has previously been reported (Wu et al., 2012b, Blanco and Alonso, 1998). The positively charged encapsulated protein in these cases was thought to interact with the degrading microspheres of negatively charged PLGA preventing its release. This may be occurring here as peptide 1 is positively charged. It would be useful to place peptide 1 and PLGA in PBS to see if there is an interaction between them which could restrict peptide 1 extraction. To prevent this interaction and enable complete release additional excipients could be included in the formulation.

For future work the shorter evaporation time, 2 hours, was selected due to the higher encapsulation efficiency. It would be useful to further reduce the solvent evaporation period to see if encapsulation efficiency could be increased. However, there is a risk that solvent evaporation may not be complete after shorter evaporation periods resulting in incomplete polymer precipitation and nanoparticle formation.

4.5.2.4 External aqueous phase PVA concentration

Reduction of PVA concentration from 2.5% to 1.25% significantly ($p \leq 0.05$) reduced particle size and polydispersity of blank and peptide 1 loaded nanoparticles, table 4.9.

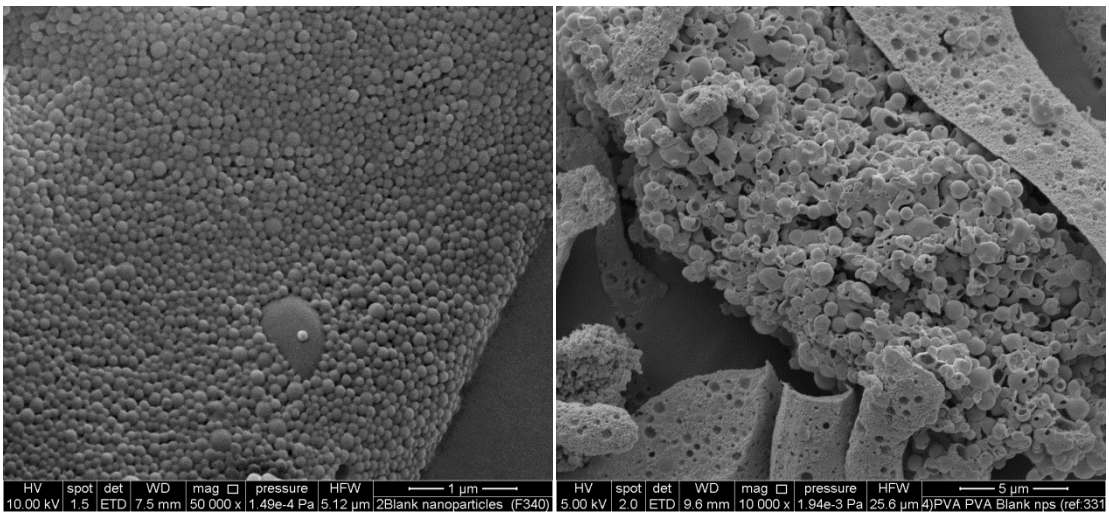
Table 4.9 *Size, polydispersity, yield, zeta potential, encapsulation efficiency and peptide 1 loading of blank and peptide 1 loaded PLGA nanoparticles prepared in a 1.25% or 2.5% PVA outer aqueous phase. Data represents means ± SD.*

Nanoparticles, outer aqueous phase PVA concentration	**Size (nm)**	**PDI**	**Yield (%)**	**Zeta potential (mV)**	**Peptide 1 loading (ug/mg)**	**Encapsulation Efficiency (%)**
Blank 1.25% PVA	174.19 ± 7.69	0.18 ± 0.04	41.3 ± 7.0	-26.71 ± 3.35		
Blank 2.5% PVA	271.34 ± 40.54	0.38 ± 0.08	54.0 ± 4.1	-24.87 ± 4.67		
Peptide 1 10mg 1.25% PVA	175.28 ± 11.95	0.18 ± 0.07	45.4 ± 5.8	-21.29 ± 4.36	60.76 ± 3.24	62.6 ± 1.6
Peptide 1 10mg 2.5% PVA	293.02 ± 46.46	0.40 ± 0.11	48.5 ± 2.8	-19.93 ± 3.71	60.13 ± 4.38	62.1 ± 6.7
Peptide 1 20mg 1.25% PVA	170.56 ± 10.09	0.15 ± 0.07	42.5 ± 9.8	-16.19 ± 2.81	96.14 ± 15.47	54.8 ± 0.9
Peptide 1 20mg 2.5% PVA	304.79 ± 58.65	0.43 ± 0.08	47.8 ± 9.2	-17.53 ± 1.57	100.18 ± 13.42	57.5 ± 5.0

In previous studies an increased PVA concentration generally decreased particle size, possibly due to increased stabilisation of the emulsion droplets, which is the opposite of what has been found here (Nam et al., 2000, Sun et al., 2011b, Zambaux et al., 1998, Lamprecht et al., 1999, Yang et al., 2001). However, another study also found a lower PVA concentration decreased PLGA particle size (Shi et al., 2009). It was concluded here a reduced PVA concentration reduced the viscosity of the outer aqueous phase allowing faster solvent evaporation and more rapid particle formation. This may prevent the formation of larger particles or coalescence of polymer droplets.

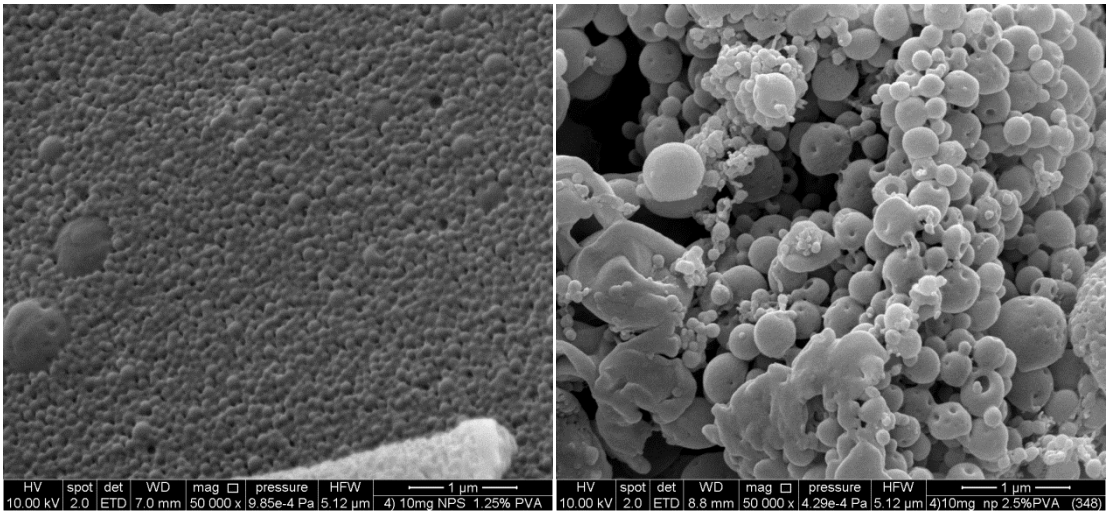
Charge, yield, encapsulation efficiency and loading were not significantly affected by the change in outer aqueous phase PVA concentration, table 4.9. Another study found that a reduction of PLGA particle size and interfacial tension caused by an increased PVA concentration reduced the encapsulation efficiency (Nam et al., 2000). Fortunately here neither an increased PVA concentration nor decreased particle size significantly reduced peptide 1 loading or encapsulation efficiency.

A reduction in outer aqueous phase PVA concentration clearly produced smaller and more uniform blank and peptide loaded nanoparticles, figures 4.8-10. There were some visible pores in the nanoparticles formed in an outer aqueous phase of 2.5% PVA (figures 4.9 (b), 4.10 (b)) but these were not visible in particles produced with 1.25% PVA. The increased viscosity of the 2.5% PVA outer aqueous phase may have slowed solvent evaporation and allowed pores to form. The presence of pores may allow immediate peptide release upon oral administration which could be degraded before it can be absorbed.



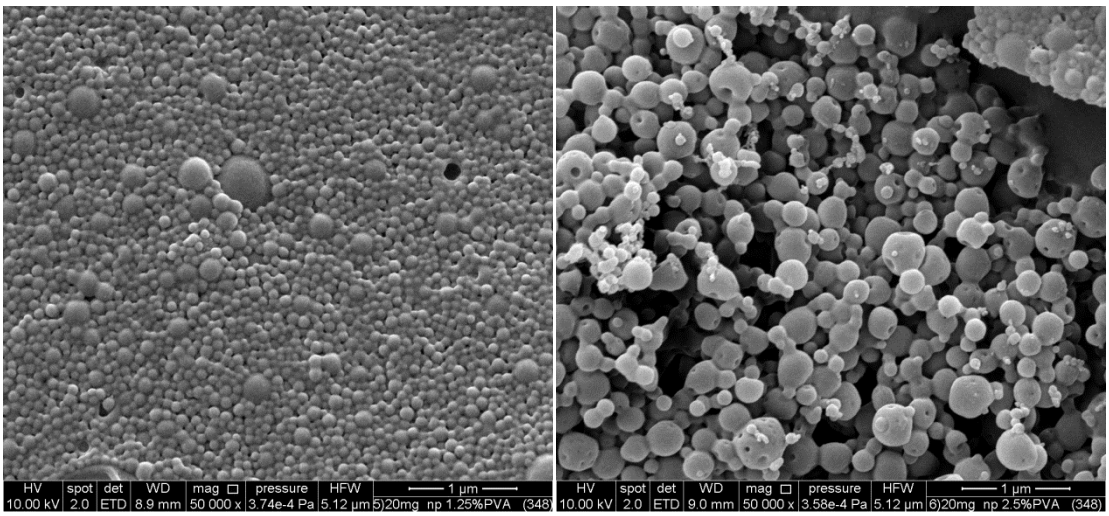
4.8 (a) ***4.8 (b)***

Figure 4.8 *SEM images of blank PLGA nanoparticles prepared in (a) 1.25% PVA and (b) 2.5% PVA*



4.9 (a) ***4.9 (b)***

Figure 4.9 *SEM images of 10mg peptide 1 loaded PLGA nanoparticles prepared in (a) 1.25% PVA and (b) 2.5% PVA*



4.10 (a) **4.10 *(b)***

Figure 4.10 *SEM images of 20mg peptide 1 loaded PLGA nanoparticles prepared in (a)1.25% PVA and (b) 2.5% PVA*

4.5.2.5 *In vitro* release

There was no significant difference in release of peptide 1 from particles formed with different outer aqueous phase PVA concentrations, figure 4.11.

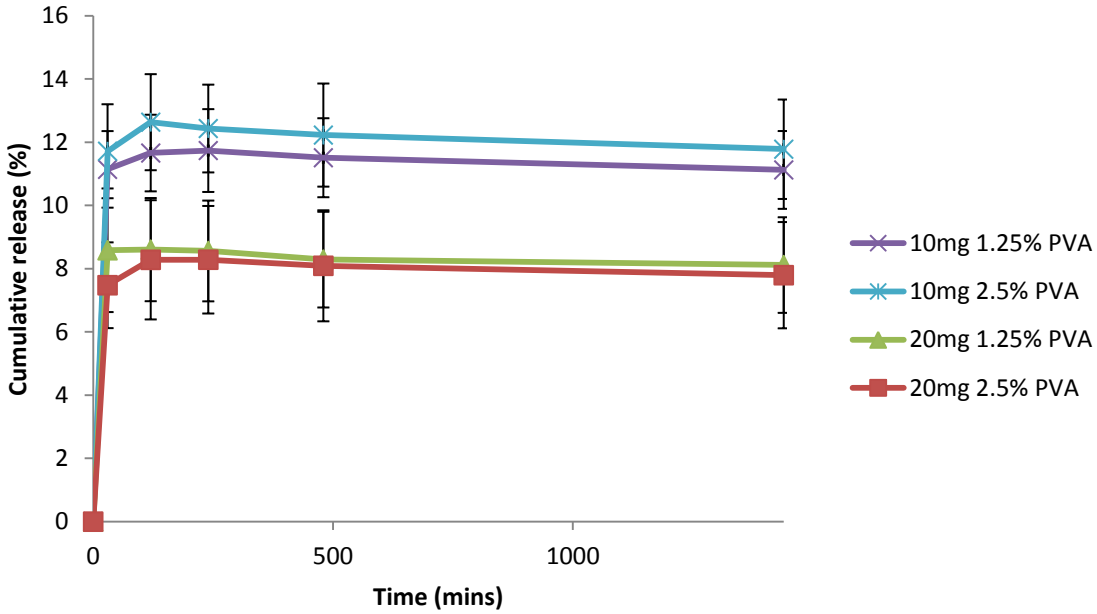


Figure 4.11 *Cumulative peptide 1 release in pH 7.4 phosphate buffer from PLGA nanoparticles prepared in an outer aqueous phase of 1.25% or 2.5% PVA. Error bars show mean ± SD.*

There was an immediate release of approximately 10% of the loaded peptide from the nanoparticles as was reported in section 4.5.4.1.

This again may be due to the detachment of peptide 1 weakly associated with the particle surface and could be vulnerable to enzymatic digestion when orally delivered. There was no further peptide 1 release after one or two weeks. The reasons for this incomplete peptide release were described in section 4.5.2.3.

The lower PVA concentration, 1.25%, was selected for future use as it produced significantly smaller nanoparticles which would have an increased probability for absorption into the bloodstream. It would be useful to further decrease PVA concentration to investigate if particle size could be further reduced and minimise any residual PVA in the nanoparticles.

4.5.2.6 Inner aqueous phase peptide 1 concentration

Peptide 1 concentrations in the inner aqueous phase of the nanoparticles produced in the studies above were 10mg/ml and 20mg/ml. The size of the nanoparticles was not significantly affected by changes in the peptide concentration, tables 4.8 and 4.9.

The charge of peptide 1 loaded nanoparticles was significantly ($p \leq 0.05$) more positive than for the blank nanoparticles, tables 4.8 and 4.9. This indicates the presence of positively charged peptide 1. An increase in peptide 1 concentration from 10 to 20mg/ml, in particles produced with an outer aqueous phase of 1.25% PVA, also produced significantly more positive particles.

An increased peptide 1 concentration produced nanoparticles with a significantly ($p \leq 0.05$) greater peptide 1 loading, tables 4.8 and 4.9. However the encapsulation efficiency was not significantly increased, as it had been in previous studies (Luan et al., 2006, Sun et al., 2011b). In fact encapsulation efficiency of peptide 1 was significantly ($p \leq 0.05$) reduced by increasing the peptide concentration of particles produced with a 1.25% PVA outer aqueous phase. Possibly there is a finite amount of peptide that can be encapsulated with a certain amount of polymer and any more added will not be encapsulated, reducing the encapsulation efficiency.

The presence of peptide 1 appeared to increase the pores visible on the surface of the nanoparticles and has been observed previously (Lamprecht et al., 1999), figures 4.5, 4.6, 4.9 and 4.10.

This may be caused by an increased osmotic pressure between the inner and external aqueous phases causing the particles to rupture and pores to form. The presence of pores might be expected to increase immediate peptide release, however this was not seen, figures 4.7 and 4.11. In fact an increased peptide 1 concentration significantly reduced the percentage of peptide 1 released from particles prepared in an outer aqueous phase of 2.5% after 2 hours solvent evaporation. The amount of peptide 1 released from both sets of particles was similar but due to the increased loading of the particles prepared with 20mg of peptide 1 the proportion of its load released was reduced. The amount of peptide 1 released may represent the amount of peptide 1 associated with the surface of the nanoparticles which was the same for both as they are similar sizes.

In further tests it would be useful to assess if PLGA encapsulation can increase peptide 1 stability with gastrointestinal enzymes, *in vitro* permeability and *in vivo* peptide 1 oral bioavailability.

4.5.3 Chitosan coated PLGA nanoparticles

To enhance the permeation of peptide 1 loaded PLGA nanoparticles they were coated with chitosan. Coating the PLGA nanoparticles with chitosan significantly ($p \leq 0.05$) increased their size and polydispersity, table 4.10. The increased size indicates that chitosan has coated the particles. It may also be due to increased viscosity of the external aqueous phase decreasing shear stress on the PLGA organic phase, therefore producing larger emulsion droplets (Guo and Gemeinhart, 2008). The greater polydispersity of the particles suggest the particles may be coated to varying extents with chitosan. The mucoadhesivity of the chitosan may also have caused the formation of some larger aggregates of particles. The particles loaded with peptide 1 had a mean size greater than 300nm which may prevent their cellular uptake and therefore reduce their absorption into the systemic circulation (Gaumet et al., 2009, Desai et al., 1996, Qaddoumi et al., 2004). This may be negated by the ability of chitosan to adhere to the intestinal membranes and open paracellular channels between cells.

Table 4.10 *Size, polydispersity, yield and charge of blank and peptide 1 loaded PLGA nanoparticles with a chitosan coating. Data represents means ± SD.*

Chitosan coated nanoparticles	**Size (nm)**	**PDI**	**Yield (%)**	**Zeta potential (mV)**
Blank	291.91 ± 49.76	0.49 ± 0.11	17.6 ± 1.6	26.56 ± 9.05
Peptide 1	350.13 ± 44.70	0.58 ± 0.13	21.6 ± 1.8	31.42 ± 1.12

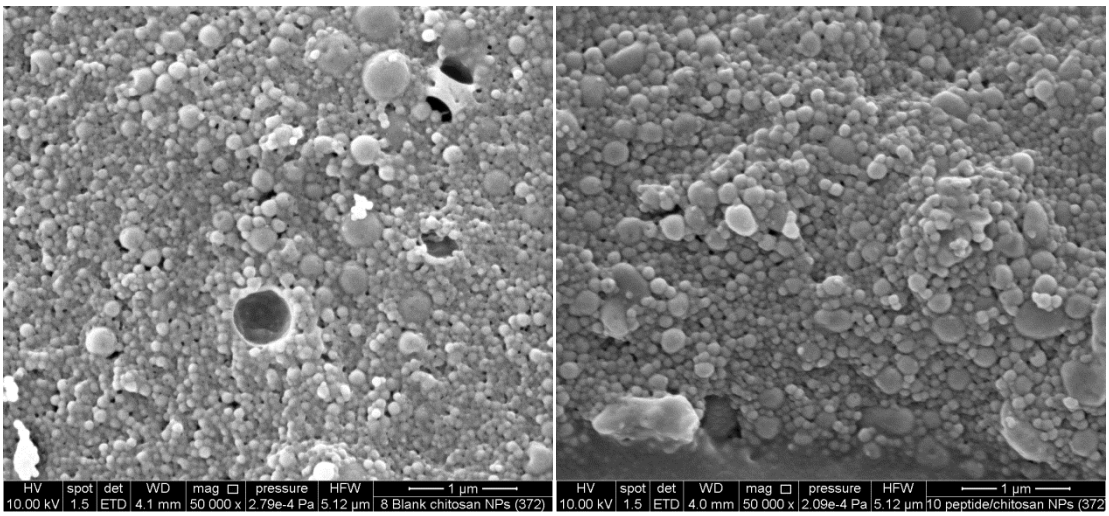
The positive zeta potential is another indication of successful chitosan coating, table 4.10. This should promote interaction with the negatively charged cell membranes of the intestinal tract and may increase oral bioavailability of the peptide. The increased positive charge of the peptide 1 loaded particles compared to the blank particles indicates the presence of the positively charged peptide. The yield of the coated particles is significantly ($p \leq 0.05$) lower than for the uncoated particles but this may be misleading as the yield calculation includes all the chitosan added. Not all the chitosan would have coated the nanoparticles and would have been lost accounting for the lower yields.

The chitosan coated nanoparticles in figure 4.12 are slightly larger and less spherical than the uncoated nanoparticles, figure 4.8 (a). This may indicate chitosan coating of the nanoparticles.

Chitosan coated nanoparticles had a significantly ($p<0.05$) lower peptide 1 loading and encapsulation efficiency than the uncoated nanoparticles, table 4.11. This may be due to electrostatic repulsion between the positively charged peptide and chitosan.

Table 4.11 *Peptide 1 loading and encapsulation efficiency in PLGA nanoparticles with a chitosan coating. Data represents means ± SD.*

Chitosan coated nanoparticles	**Peptide 1 loading (ug/mg)**	**Encapsulation Efficiency (%)**
Peptide 1	35.09 ± 1.28	46.3 ± 4.9



4.12 (a) ***4.12 (b)***

Figure 4.12 *SEM images of chitosan coated PLGA nanoparticles (a) blank (b) 10mg peptide 1 loaded*

4.5.3.1 *In vitro* release

Chitosan coated particles released a very small proportion (≤1%) of their encapsulated peptide, significantly ($p \leq 0.05$) less than the uncoated particles in PBS, table 4.12. This could be due to a slower erosion rate of the chitosan coated particles and longer diffusion pathway of the peptide out of the nanoparticles. Lower peptide release may also be due to less peptide on the surface of the nanoparticles due to electrostatic repulsion between chitosan and peptide 1.

Table 4.12 *Peptide 1 release from chitosan coated PLGA nanoparticles in PBS. Data represents means ± SD.*

Chitosan coated nanoparticles	30 mins release (%)	120 mins release (%)	240 mins release (%)	480 mins release (%)
Peptide 1	1.0 ± 0.7	0.6 ± 0.3	0.8 ± 0.5	0.5 ± 0.1

There was no further measurable peptide release after 8 hours. Samples were taken after 1 and 2 weeks but there was no peptide detected. This incomplete release may be due to the reasons previously described in section 4.5.2.3.

It would be useful to investigate if the chitosan coating can increase the *in vitro* absorption and *in vivo* oral bioavailability of peptide 1 compared to uncoated PLGA nanoparticles and the peptide alone.

4.5.4 Peptide 1 efficacy

The efficacy of the chemotherapeutic peptide, peptide 1, was assessed with breast cancer cells. Peptide 1 loaded PLGA nanoparticles and chitosan coated nanoparticles were added to the cells to assess if their production had impaired the therapeutic action of peptide 1. The viability of the cells was measured to assess if the peptide was able to successfully limit their growth compared to controls with no formulation added. Blank nanoparticles were also added to cells to determine their toxicity alone.

Two different tests were carried out to assess cell viability. The viability reagent PrestoBlue™ is reduced by metabolically active cells to a red colour. However, this did not occur in any of the samples and the absorbance values for the cells with or without formulation were not significantly different, table 4.13. This indicates none of the cells were active. Crystal violet was used to stain viable cells which were fixed by methanol. Again there were no significant differences in the absorbance values for the cells incubated alone or with a formulation. The absorbance values were also very low suggesting there were very few viable cells left.

Table 4.13 *Absorbance values from PrestoBlue™ and crystal violet cell viability assays performed on MDA-MB-231, breast cancer cells, alone and with peptide 1 formulations. Data represents means ± SD.*

Formulation added to cells	PrestoBlue-Absorbance	Crystal violet-Absorbance
No formulation	0.44 ± 0.02	0.07 ± 0.00
Blank PLGA nanoparticles	0.45 ± 0.02	0.09 ± 0.01
Blank chitosan coated nanoparticles	0.45 ± 0.02	0.08 ± 0.01
Peptide 1	0.46 ± 0.01	0.09 ± 0.02
PLGA nanoparticles with peptide 1	0.46 ± 0.02	0.09 ± 0.02
Chitosan coated peptide 1 nanoparticles	0.44 ± 0.02	0.08 ± 0.01

It appears the cells may have died during testing and therefore the effect of peptide 1 either alone or released from the nanoparticles on the proliferation of the breast cancer cells could not be assessed. Possibly the cells should have been tested for viability within a few days rather than a week. Further work to determine the efficacy of peptide 1 released from the PLGA nanoparticles should be conducted with prostate and breast cancer cell lines to determine if their formulation hindered its efficacy.

4.5.5 Enteric microencapsulation

The *in vitro* release studies showed encapsulated peptide 1 was immediately burst released from the nanoparticles. To minimise degradation of prematurely released peptide 1 enteric coating and microencapsulation of nanoparticles were combined.

In chapters 2 and 3 the proteins lactase and insulin were successfully encapsulated in enteric Eudragit L100 microparticles using a method developed for the encapsulation of low molecular weight drugs (Kendall et al., 2009). While these particles were not impermeable to acid they provided protection from pepsin and a pH dependent release. Peptide 1 is not hydrolysed at gastric pH, possibly due to its small size and simple structure. It is slowly degraded by pepsin so encapsulation in these microparticles should ensure a greater amount reaches the small intestine intact. However, here peptide 1 would be vulnerable to enzymatic digestion. Enteric microencapsulation of peptide 1 loaded PLGA nanoparticles would ensure the peptide is protected from small intestinal enzymes when released there and would benefit from the permeation enhancing effects of encapsulation in PLGA particles and chitosan.

The novel use of the Kendall *et al* method to encapsulate peptide 1 loaded PLGA nanoparticles in enteric Eudragit L100 microparticles should provide pepsin protection to any peptide exposed on the nanoparticle surface or any peptide that would have been burst released in the stomach. The acid stability of peptide 1 also means any acid permeating the microparticles would not degrade its primary structure. The rapid pH dependent release upon entry in the small intestine will also provide a high local concentration of the nanoparticles which could promote uptake by an increased concentration gradient.

This method is compatible with PLGA nanoparticle encapsulation as Eudragit L100 is dissolved in ethanol, which PLGA is not soluble in, so they will remain intact in the polymeric suspension.

4.5.5.1 Microparticle size, span, yield and morphology

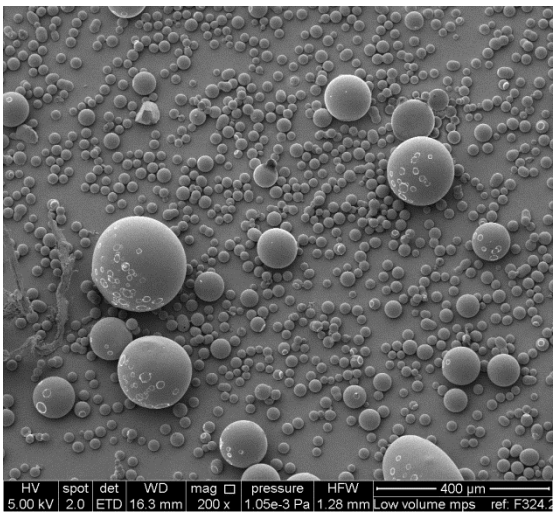
The microparticles formed were micron sized and all had a dv, 0.5 less than 100μm, except those produced with peptide 1 alone, table 4.14. The smaller particles produced with the peptide 1 nanoparticles may be the result of residual PVA acting as an emulsifier. The span of the microparticles is quite large and may indicate the presence of some aggregates (>1). None of the particle yields are greater than 80%, this may be a result of reducing the amounts of excipients relative to that used by Kendall *et al*. in their optimised method.

Table 4.14 *Size, span and yield of blank, peptide 1, peptide 1 PLGA nanoparticles and blank nanoparticles loaded in Eudragit L100 microparticles. Data represents means ± SD.*

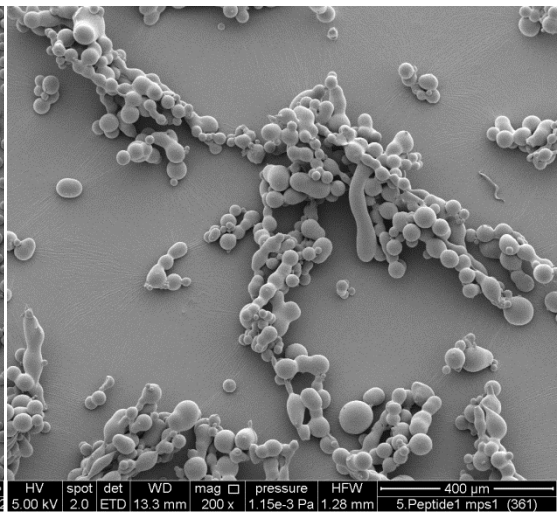
Microparticles	Size (μm)	Span	Yield (%)
Peptide 1	352.11±82.27	1.43±0.35	42.64±4.66
Blank	54.41 ± 2.13	3.84 ± 0.11	73.10
Peptide 1 nanoparticles	41.05 ± 6.67	5.38±2.55	65.3 ±13.1
Blank nanoparticles	86.17 ± 19.42	3.54±1.99	70.2

Blank Eudragit L100 microparticles were spherical but some were very large, figure 4.13 (a). Eudragit L100 microparticles with encapsulated peptide 1 were aggregated together to form chains of smaller, individual microparticles, figure 4.13 (b). Due to the larger size of these aggregates they may not empty as rapidly from the stomach as smaller particles. Possibly additional surfactant is required to ensure this aggregation doesn't occur.

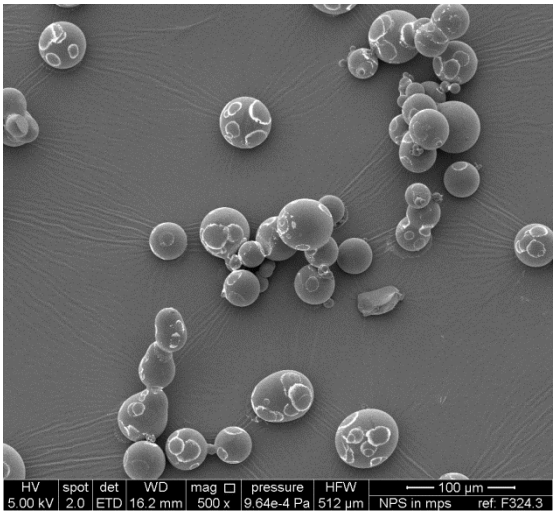
The nanoparticle loaded microparticles were fairly spherical, uniform and less than 100μm, figures 4.13 (c) and (d). They should empty rapidly and uniformly from the stomach immediately releasing the nanoparticles in the small intestine, creating a high local concentration. There were no visible nanoparticles indicating that they have been encapsulated in the microparticles.



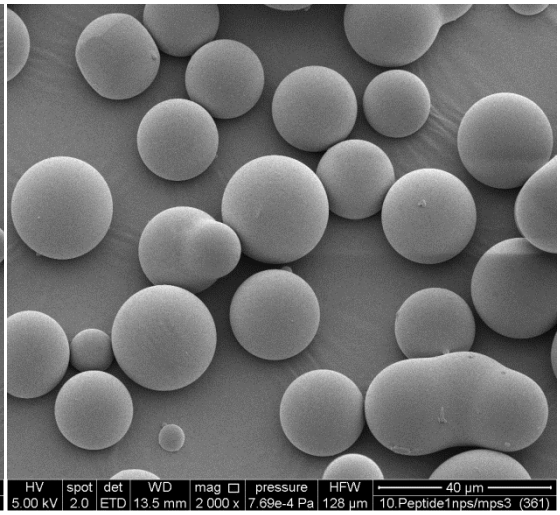
4.13 (a)



4.13 (b)



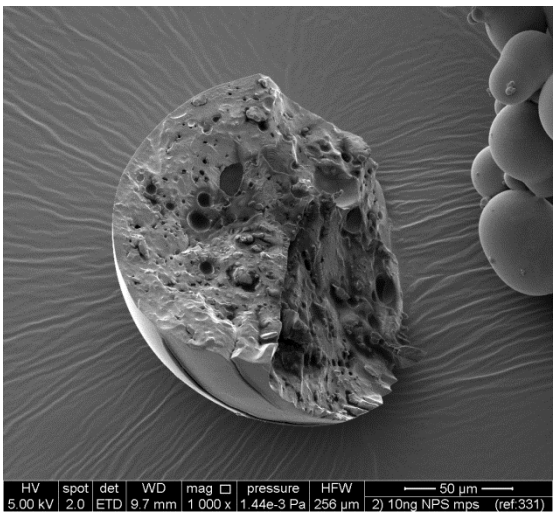
4.13 (c)



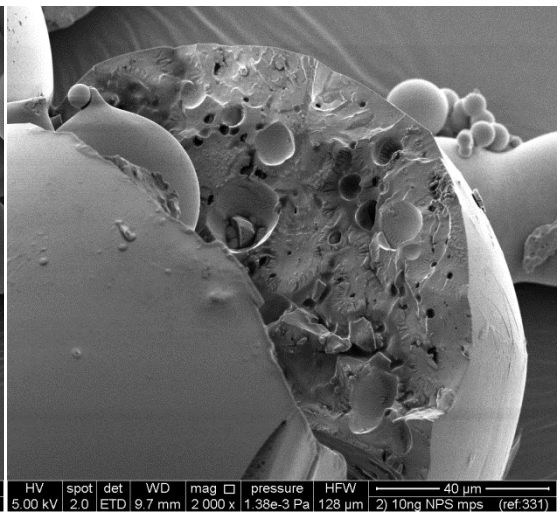
4.13 (d)

Figure 4.13 *SEM images of Eudragit L100 microparticles (a) blank, with encapsulated (b) peptide 1 (c) blank PLGA nanoparticles (d) peptide 1 PLGA nanoparticles*

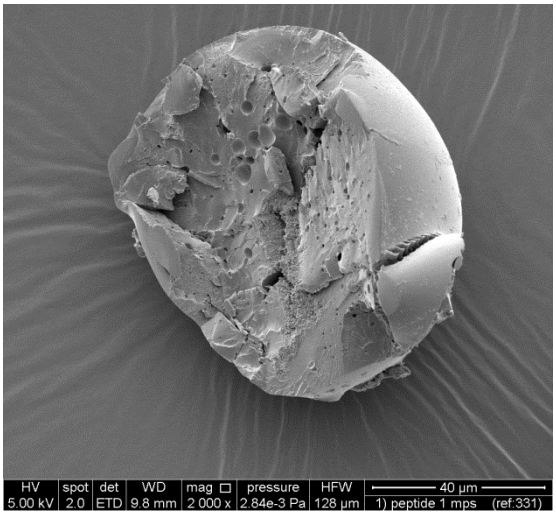
Split microparticles with encapsulated peptide 1 or peptide 1 nanoparticles revealed a porous interior, figure 4.14 (a), (b) and (c). No nanoparticles could be discerned in the microparticles, figure 4.14 (a) and (b).



4.14 (a)



4.14 (b)



4.14 (c)

Figure 4.14 *SEM images of split Eudragit L100 microparticles (a) & (b) with encapsulated peptide 1 PLGA nanoparticles, (c) with encapsulated peptide 1*

The microparticles with encapsulated nanoparticles do not appear to be different to the microparticles without nanoparticles. Possibly it would be difficult to identify any nanoparticles within the polymer matrix of the microparticles and at this magnification.

4.5.5.2 Peptide 1 loading and encapsulation efficiency

Encapsulation efficiency of peptide 1 alone was less than 50%, table 4.15. This may be due to; peptide 1 partitioning to the liquid paraffin phase, reducing the amounts of excipients to that of the Kendall et al. method and the possible

incomplete formation of discrete particles. Peptide 1 loading of the microparticles containing peptide 1 nanoparticles was not experimentally determined but calculated assuming 100% encapsulation of the nanoparticles. Encapsulation efficiency of peptide 1 was much less than for lactase and insulin in chapters 2 and 3. Possibly its smaller size enables it to escape encapsulation.

Table 4.15 *Peptide 1 loading and encapsulation efficiency in Eudragit L100 microparticles. Data represents means ± SD.*

Microparticles	**Peptide 1 loading (µg/mg)**	**Encapsulation efficiency (%)**
Peptide 1	25.70±2.01	41.12±3.21
Peptide 1 nanoparticles	5.31 ± 0.19	-

4.5.5.3 *In vitro* release

There was an immediate burst release of peptide 1 from the PLGA nanoparticles in 0.1N HCl, figure 4.15. There was a significantly ($p<0.05$) higher proportion of peptide 1 immediately released than in PBS, pH 7.4. Faster erosion of PLGA particles in acid has been observed previously and attributed to acid hydrolysis of the PLGA polymer (des Rieux et al., 2007). This may be detrimental to the bioavailability of orally administered peptide 1 as it would first encounter the acidic pH of the stomach and may leave it vulnerable to pepsin digestion.

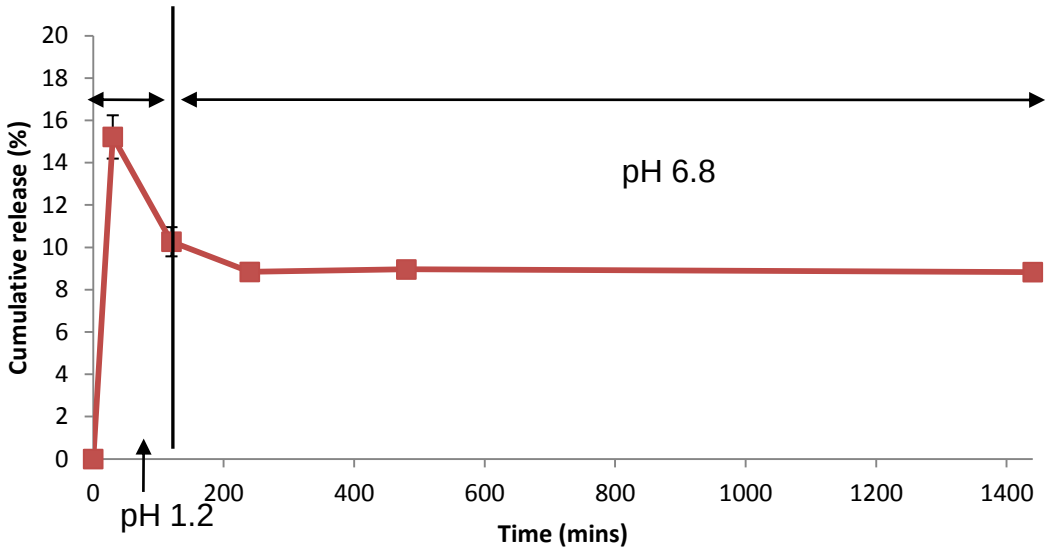


Figure 4.15 *Peptide 1 release from PLGA nanoparticles initially loaded with 10mg peptide 1 in 0.1N HCl for 2 hours followed by pH rise to pH 6.8. Error bars show mean ± SD.*

Enteric microparticles encapsulating peptide 1 alone released <10% of peptide 1 in acid, figure 4.16. At pH 6.8 there was almost complete release, 97%. This demonstrates the ability of these microparticles to prevent release of peptide 1 in the stomach avoiding pepsin digestion. However, when released in the small intestine it would be vulnerable to enzymatic digestion and the formulation lacks permeation enhancers.

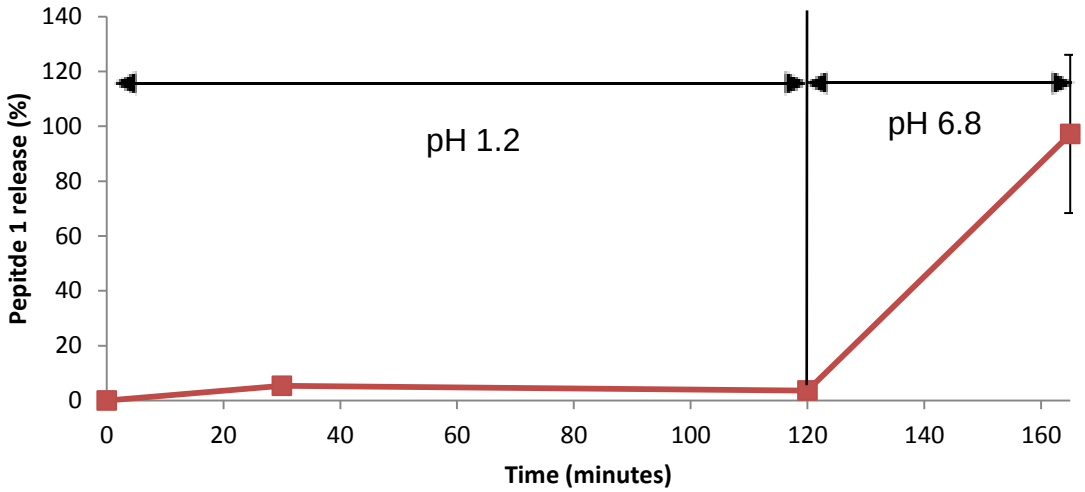


Figure 4.16 *Peptide 1 release from Eudragit L100 microparticles in 0.1N HCl for 2 hours and 45 minutes in pH 6.8 phosphate buffer. Error bars show mean ± SD.*

Enteric microparticles with encapsulated peptide 1 nanoparticles released <2% of peptide 1 in acid, figure 4.17. At pH 6.8 there was a 7% release of peptide 1. The enteric microparticles restricted peptide 1 burst release in acid so should protect it from pepsin digestion when orally administered. It would be useful to test the particles with pepsin and determine if they can increase *in vivo* oral bioavailability compared to peptide 1 alone or when encapsulated in PLGA nanoparticles only. The peptide release upon pH rise mirrors its burst release in PBS. Peptide 1 burst released in the duodenum would be more stable than if released further along the small intestine due to its lower proteolytic activity. Enteric microparticles are preferable to larger enteric dosage forms for their rapid release of encapsulated entities in the small intestine. Release from larger enteric dosage forms may be delayed until further along the small intestine due to their smaller surface area. This would release nanoparticles into a more proteolytic environment and increase the likelihood of peptide degradation.

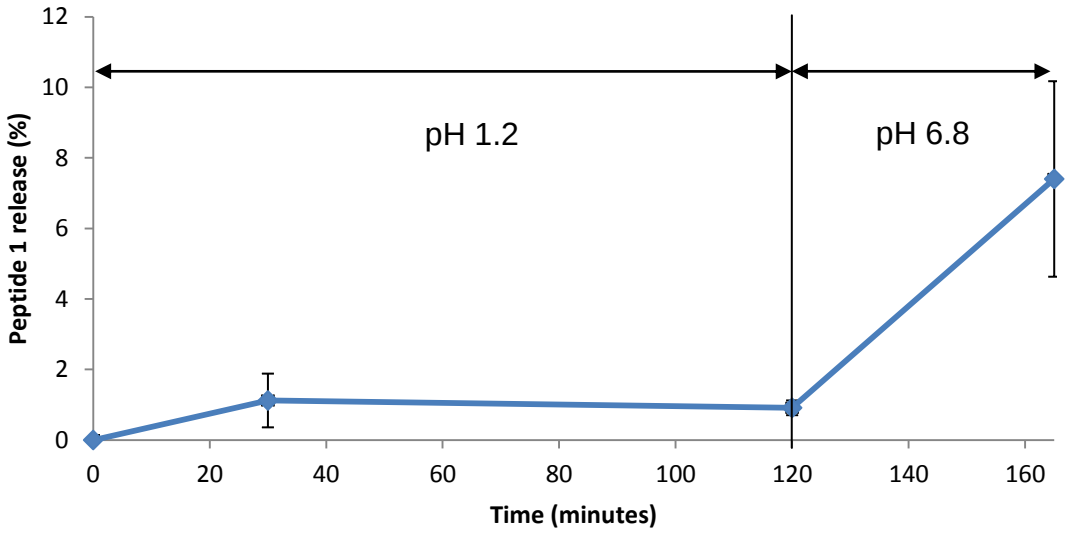


Figure 4.17 *Peptide 1 release from PLGA nanoparticles encapsulated in Eudragit L100 microparticles in 0.1N HCl for 2 hours and 45 minutes in pH 6.8 phosphate buffer. Error bars show mean ± SD.*

The release pattern of peptide 1, if not the percentages released, from Eudragit L100 microparticles and from PLGA nanoparticles encapsulated within microparticles were very similar. This could mean the peptide and nanoparticles have been encapsulated as separate entities and not together.

It could be argued that as the profiles are so similar there is little benefit in first encapsulating the peptide in nanoparticles. However, some of the peptide should remain within the nanoparticles and be protected in the small intestine and absorbed associated with the PLGA particles unlike peptide 1 alone.

Peptide 1 release from PLGA nanoparticles encapsulated in enteric microparticles in acid was significantly ($p \leq 0.05$) reduced compared to its release from unencapsulated nanoparticles, figure 4.18. Upon pH rise peptide 1 was burst released from the nanoparticles released from the microparticles. By delaying the peptide burst release until the upper small intestine pepsin digestion in the stomach will be avoided. The duodenum has been shown to be more favourable for peptide stability so release here is better than in the distal small intestine. Peptide 1 release from the nanoparticles was again very low over 24 hours, this incomplete release may be due to reasons outlined in section 4.5.4.1.

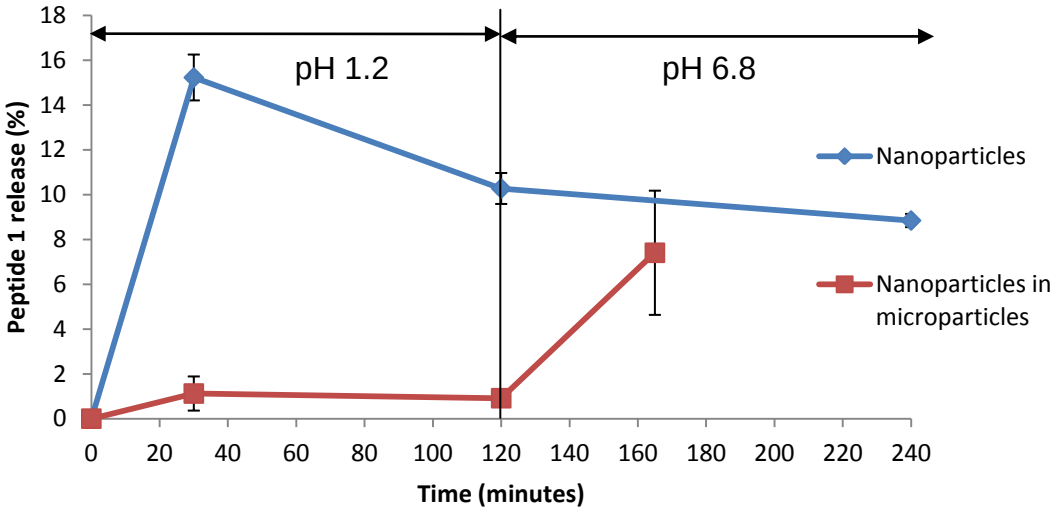


Figure 4.18 *Peptide 1 release from PLGA nanoparticles and from PLGA nanoparticles encapsulated within Eudragit L100 microparticles after 2 hours in 0.1N HCl and 45 minutes in pH 6.8 phosphate buffer. Error bars show mean ± SD.*

4.6 Conclusion

The feasibility of delivering peptide 1 orally was investigated by incubation with simulated and porcine intestinal fluids and human faecal fluids. These studies revealed that the primary structure of peptide 1 was stable at gastric pH but was susceptible to digestion by GI enzymes. This instability was not uniform along the GI tract. Peptide 1 was most stable in gastric and duodenal fluids and was increasingly digested along the small intestine and in the porcine colonic and human faecal fluids.

To provide protection from intestinal degradation and increase its absorption into the systemic circulation peptide 1 was encapsulated in PLGA nanoparticles. The parameters of the double emulsion solvent evaporation method used to form the particles were varied to produce nanoparticles with optimal characteristics for oral delivery.

The longer solvent evaporation duration of 4 hours significantly reduced the encapsulation efficiency of peptide 1 in the nanoparticles, initially loaded with 20mg peptide 1, from 57.5 to 48.6%. The increased evaporation time may have allowed hydrophilic peptide 1 to partition into the external aqueous phase evading encapsulation. The extended stirring time may have caused loss of surface associated peptide so for these reasons the shorter evaporation time of 2 hours was selected for further PLGA nanoparticle production.

The lower PVA concentration, 1.25%, in the outer aqueous phase significantly reduced nanoparticle size to less than 200nm and, as a small particle size is critical for absorption, this concentration was selected for use. Formation of smaller particles might be due to faster solvent evaporation and particle formation due to the decreased viscosity of an external aqueous phase. Successful peptide loading was indicated by the charge of the nanoparticles becoming increasingly positive when positively charge peptide 1 was included in the formulations.

Chitosan coating of the PLGA nanoparticles was conducted to enhance intestinal permeation. Chitosan coating significantly increased nanoparticle size, to more than 300nm, which may hamper its permeation. However, its other permeation enhancing effects may counteract this.

Chitosan coating significantly reduced peptide 1 loading, from 61 to 35µg/mg, and encapsulation efficiency, from 63% to 46%, compared to the uncoated particles. This may be due to electrostatic repulsion between positively charged chitosan and peptide 1. Peptide 1 release *in vitro* was significantly reduced from 11% in 30 minutes from the uncoated particles to only 1% from the chitosan coated nanoparticles. This may be due to an increased diffusion pathway.

In vitro the PLGA nanoparticles immediately released approximately 10% of the peptide 1 load in 30 minutes, followed by very little further release up to 2 weeks later. This pattern of *in vitro* release does not bode well for oral peptide 1 bioavailability. Any peptide 1 immediately released upon oral administration may be subject to pepsin digestion in the stomach and further enzymatic digestion in the small intestine. The peptide not released but remaining within the nanoparticles may, based on these results, not ever be released and so would be unable to halt tumour growth. The lack of any further detectable peptide release could be a result of the peptide degrading when it is released. Incomplete release may also be caused by the positively charged peptide interacting with negatively charged PLGA.

The novel use of the Kendall *et al* method to encapsulate peptide 1 loaded PLGA nanoparticles in enteric Eudragit L100 microparticles significantly reduced peptide 1 burst release in acid compared to peptide 1 nanoparticles, from 15% to 1%, which should prevent its digestion in the stomach. The rapid pH dependent release in the small intestine should provide a high concentration of the nanoparticles which could promote uptake by an increased concentration gradient. There was still a burst release of peptide 1 from the released nanoparticles which *in vivo* may be vulnerable to digestion in the small intestine but at least this would occur in the duodenum where peptide 1 was shown to be more stable than the other small intestinal segments.

Chapter 5

Final conclusions and future work

5.1 Conclusion

Very few protein/peptide drugs are currently orally available due to acidic and enzymatic instability in the GI tract and their large size and hydrophilicity limiting their absorption from the intestinal tract into the bloodstream. Oral delivery strategies are being actively pursued by academic and industrial research groups to increase oral bioavailabilities from less than 1% to 30-50% (Shaji and Patole, 2008).

One of the aims of this project was to compare the intestinal stability of protein and peptide drugs of varying size using simulated and porcine gastrointestinal fluids and a human faecal slurry. Those studied in this project were a large protein, lactase, 464 kDa, a small protein, insulin, 5.8 kDa, and a small peptide, peptide 1, 1.2 kDa. Lactase was immediately denatured at fasting stomach pH whereas the primary structures of insulin and peptide 1 were not unstable. This may be due to disruption of the weak interactions holding together the more complex structure of lactase by ionisation changes caused by the low pH. The 2 polypeptide chains of insulin are primarily held together by stronger disulphide bonds which may not be affected at low pH. The simpler structure of peptide 1 may make it more stable at low pH.

Lactase was stable with intestinal enzymes possibly due to inaccessibility of its peptide bonds to the digestive enzymes. The smaller protein insulin was immediately digested by gastric, small intestinal and large intestinal enzymes. This may be due to greater accessibility of its peptide bonds to digestive enzymes and possible loss of the disulphide bonds holding its polypeptide chains together by the presence of glutathione. Peptide 1 was degraded by enzymes in all GI fluids but more gradually than insulin over a two hour period. This may be due to there being less bonds susceptible to digestion as it simply has less amino acids.

To overcome acid instability of lactase and pepsin digestion of insulin they were encapsulated in enteric Eudragit L100 microparticles. Oral delivery of both these drugs must be carefully coordinated with food consumption as they must be available to digest it or control the effects of its digestion. Delayed drug release at small intestinal pH and delayed efficacy caused by food has previously been observed with enteric tablets or capsules.

By reducing dosage form size this delayed release can be avoided as the greater surface area can provide a faster and more uniform drug release in the small intestine and their ability to mix with the contents of the stomach and rapidly empty avoids delays caused by food. This should also help to minimise variability in absorption and bioavailability and allow effective and rapid control of symptoms.

The preparation of enteric micro and nanoparticulates can pose a threat to the integrity of protein and peptide drugs. An oil in oil emulsion solvent evaporation method previously used for encapsulation of small molecular weight drugs in enteric Eudragit microparticles was used due to its use of the less toxic ethanol to dissolve the polymer and its lack of any high shear homogenisation steps. It also required no control of temperature making it a far simpler method and possibly more attractive for future commercial use. Lactase and insulin were both successfully encapsulated in Eudragit L100 microparticles by this method with a high yield and encapsulation efficiency, >70%, and uniformly sized particles less than 100µm were produced which should allow rapid and uniform gastric emptying and drug release.

Eudragit L100 microparticles were able to control the release of lactase and insulin in a pH dependent manner, once lactase particle size had been reduced by spray drying allowing a deeper encapsulation. Below pH 6 there was <30% release of encapsulated drug, this release was probably protein drug held at the surface of the particles. The particles were able to protect insulin from pepsin digestion, 80% remaining intact after 2 hours with the enzyme in SGF and 49% after 2 hours in porcine gastric fluid, however encapsulated lactase was completely inactivated after incubation of the microparticles in 0.1N HCl.

As it was established that most of the encapsulated lactase remained within the particles it was hypothesised acid may be able to enter the particles. An investigation into the porosity and morphology of the particles revealed a porous interior and by using an SEM with a new back scatter detector, crystal-like structures were observed on the surface of the particles. It was concluded these are most likely to be residual surfactant and they disappeared when incubated in acid. This may provide a means for acid to enter the particles as they appear not to be solely composed of the acid resistant polymer.

To overcome acid permeability antacids were co-encapsulated to protect lactase. Co-encapsulated magnesium hydroxide provided the greatest protection of lactase, 9.2% remaining intact after 2 hours in acid, superior to any of the oral lactase supplements tested during this study.

Insulin release at pH 6.8 was rapid and complete within 45 minutes. This should enable fast insulin absorption *in vivo* and rapid control of post prandial glycemia. Release of insulin from Eudragit L100 particles was faster than from particles of the same polymer prepared with an outer aqueous phase and DCM as the solvent to dissolve the polymer. Possibly the faster evaporation of DCM than ethanol did not allow as many or as large pore formation so their breakdown was slower. It would be useful to explore further the effects of solvents and outer aqueous phase on the morphology of the particles.

As insulin is also very unstable with small intestinal enzymes a protease inhibitor, citric acid, was included in the microparticles. Its release in the small intestine should provide intestinal protection. Citric acid reduced insulin degradation with pancreatin but caused large aggregates of microparticles to form. The inclusion of magnesium hydroxide with lactase in the microparticles also caused aggregation of the particles. This may have been due to a plasticising effect on the Eudragit L100 polymer. More work is needed to find an optimal ratio to form microparticles.

The recently discovered chemotherapeutic peptide 1 was the most stable of the proteins/peptide tested, possibly due to its smaller size. However, it is still large compared to commonly orally delivered low molecular weight drugs so will still encounter permeability limitations and is gradually degraded by enzymes throughout the GI tract. PLGA particles are currently used for the injectable, prolonged release of chemotherapeutic peptides of a similar size and structure to peptide 1. Due to this compatibility and ability of PLGA nanoparticles to provide enzyme protection, without causing digestive disruption, and increased permeability, without disrupting intestinal cell membranes, they were selected for formulation of peptide 1.

All the PLGA nanoparticles produced a rapid peptide 1 burst release in PBS followed by very little further release over 24 hours and up to 2 weeks in some cases, overall less than 20% was released.

Incomplete release may be caused by destruction of the peptide when released or possibly interaction of cationic peptide 1 with anionic, degrading PLGA . To limit burst release of peptide 1 in the stomach, where it would be gradually degraded by pepsin, the peptide 1 loaded PLGA nanoparticles were encapsulated in Eudragit L100 microparticles using the method previously used for lactase and insulin. The microparticles produced were <100μm and reduced peptide 1 release in acid from ~16% to <2%. Peptide 1 burst release would be delayed until the small intestine where it would have an increased chance of being absorbed than from the stomach. The rapid and uniform release of the nanoparticles may also create a high local concentration promoting their absorption into the bloodstream.

The commercial oral protein/peptide drug delivery system which has progressed furthest so far is Tarsa Therapeutics' salmon calcitonin formulation which has successfully completed phase III clinical trials. This is an enteric coated tablet with an enzyme inhibitor, citric acid, and permeation enhancer, acylcarnitine. Enteric coated tablets/capsules have previously suffered from delayed release at small intestinal pH and may be more affected by the presence of food in the stomach than smaller dosages. This may create variability in drug absorption, for calcitonin this may not be too detrimental as it has a large therapeutic window. However for those drugs with a narrow therapeutic window or whose availability must be carefully coordinated with food this may not be acceptable.

This work has demonstrated that the method of Kendall *et al* can successfully encapsulate protein and peptide drugs of widely varying size without impairing the activity of these notoriously fragile macromolecules in Eudragit L100 microparticles. They provided pH dependent release, pepsin protection, rapid release at small intestinal pH, may be less delayed by the presence of food than larger enteric tablets/capsules and may reduce variability in drug absorption seen with larger dosages. They were also able to encapsulate nanoparticles with encapsulated peptide and co-encapsulate citric acid and antacids. This may allow formulation of a modular delivery system incorporating enteric protection with permeation enhancement and enzyme inhibitors.

5.2 Future work

It would be useful to test the stability of the protein/peptide drugs in human intestinal fluids to gain a better insight into their *in vivo* behaviour. Testing more protein/peptide drugs of varying sizes and structure complexities would provide more information about where they are stable and unstable in the GI tract and may enable more tailored oral formulation development based on size.

Further studies to increase the protection of acid labile protein/peptide drugs in Eudragit L100 microparticles by co-encapsulation of antacids could be conducted. It would also be useful to optimise the ratio of co-encapsulated enzyme inhibitors to Eudragit L100 and investigate their ability to increase protection *in vitro* in enzyme solutions and with animal intestinal fluids.

It would be beneficial to explore the permeability of protein/peptide drugs with Caco-2 cells and attempt incorporation of permeability enhancers within the microparticles to explore their potential to increase absorption. Increased permeation caused by encapsulation in PLGA nanoparticles should also be studied and the effects of chitosan coating of these particles should be investigated.

It would be useful to conduct *in vivo*, oral bioavailability studies in animals to investigate if enteric microencapsulation can increase the bioavailability of protein/peptide drugs encapsulated alone or loaded in polymeric nanoparticles. The bioavailability, inter/intra subject variability in absorption and delay between administration and onset of action should be compared to enteric tablets/capsules to investigate the effects of dosage form size reduction. The effects of food on the action of enteric capsules/tablets and microparticles should also be compared.

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