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Ollscoil na hÉireann, Corcaigh
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**Oral insulin dosage forms: Assessment of solid-state
stability, compaction behaviour and permeation
enhancers' impact on dissolution performance**

Thesis presented by

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for the degree of

Doctor of Philosophy

University College Cork

School of Pharmacy

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Bateman

2025

"The struggle itself toward the heights is enough to fill a man's heart. One must imagine Sisyphus happy."

Albert Camus

The Myth of Sisyphus and Other Essays

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property.

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Author Contribution

All work was performed independently by the author, with the following exceptions:

Chapter 3

Dr. Shaza Darwish from the Pharmaceutical Manufacturing Technology Centre, Bernal Institute, University of Limerick performed DVS experiments. Theodoros Giakoumis from the School of Pharmacy, University College Cork performed SEM imaging.

Chapter 5

Vaishnavi Honavar from the Bernal Institute, University of Limerick performed particle imaging.

Signed: *Adrian Fagan*

Date: 18/08/25

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Publications and Presentations

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- **Fagan, A.**, Bateman, L.M., O'Shea, J.P., Crean, A.M., 2024. Elucidating the Degradation Pathways of Human Insulin in the Solid State. *J. Anal. Test.* 8 (3), 288–299. <https://doi.org/10.1007/s41664-024-00302-5>.
- **Fagan, A.**, Bateman, L.M., O'Shea, J.P., Crean, A.M., 2025. Kinetics of human insulin degradation in the solid-state: An investigation of the effects of temperature and humidity. *J. Pharm. Sci.* 114 (2), 1368–1375. <https://doi.org/10.1016/j.xphs.2024.11.032>.
- **Fagan, A.**, Bateman, L.M., Crean, A.M., O'Shea, J.P., Taylor, L.S., 2025. Impact of Permeation Enhancers on the Release of Insulin from Tablets in Biorelevant Media. *Mol. Pharm.* 22 (7), 3999–4008. <https://doi.org/10.1021/acs.molpharmaceut.5c00249>.
- **Fagan, A.**, Bateman, L.M., O'Mahony, M., Crean, A.M., O'Shea, J.P., 2025. Direct compression peptide tablets: insights into the importance of permeation enhancer processibility and peptide stability. *I. J. Pharm.* 685, 126257. <https://doi.org/10.1016/j.ijpharm.2025.126257>.

Oral presentations

- **Andrew Fagan**, Lorraine Bateman, Joseph O'Shea, Abina Crean, Lynne Taylor
Peptide release from minitables in biorelevant media: Impact of permeation enhancers
5th European Conference on Pharmaceutics, Porto, Portugal, 2025

Poster Presentations

- **Andrew Fagan**, Lorraine Bateman, Joseph O'Shea, Abina Crean

Elucidating the mechanisms of insulin deamidation and covalent aggregation
in the solid state

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Abstract

Since the isolation of insulin in the 1920's, there has been significant interest in developing oral peptide dosage forms given their numerous advantages, including non-invasive administration and improved patient compliance. Despite recent progress, as evidenced by the approval of products such as Rybelsus[®] and Mycapssa[®], the poor stability of peptides and their limited permeability continue to restrict oral bioavailability and hinder widespread adoption of this approach. Among the approaches developed to address the oral bioavailability challenge, the use of permeation enhancers (PEs) has proven the most successful. However, from a formulation development perspective, the co-formulation of peptides and PEs presents a number of technical challenges that remain poorly investigated. Furthermore, the stability of peptides to storage and processing conditions is a primary concern during oral dosage form development and manufacture. Accordingly, this thesis aimed to assess the importance of stability and permeation enhancers in the development of peptide tablets, using insulin as a model peptide and sodium decanoate (C10) and salcaprozate sodium (SNAC) as model PEs.

In the first part of this thesis, we sought to investigate the impact that storage and processing conditions have on the solid-state stability of insulin. Insulin was observed to degrade similarly in the solid-state as in solution, where the primary chemical degradation pathway was observed to be deamidation, while aggregation proceeded via a disulfide exchange mechanism. Interestingly, there was no evidence of unfolding occurring in the solid-state. The rates at which the different physical and chemical degradation pathways occurred appeared to follow Arrhenius-like kinetics. Overall, temperature had the largest effect on degradation, with reaction rates increasing as storage temperature increased. Humidity, on the other hand, was found to have a

temperature dependent effect, where moisture had minimal impact on degradation rates at 25°C and 40°C, but had an important effect at 60°C. This temperature-dependent effect of humidity was determined to be due to a change in moisture sorption characteristics at 60°C relative to 25°C and 40°C, which increased the overall reactivity of the material. In addition to temperature and humidity, the effect of high compaction pressures on the stability of insulin was also assessed. Compaction pressure was found to have no significant impact on the conformational stability of insulin in the solid-state or on reconstitution, nor was there any evidence of aggregation occurring during compaction.

In the next part of the thesis, the role of PEs in the development of insulin compacts was examined. We firstly assessed the suitability of C10 and SNAC for processing via direct compression. Overall, SNAC displayed superior tableability, compactibility and compressibility profiles, due to its greater tendency to deform plastically and its greater capacity for strong interparticulate bonding. SNAC did, however, display poor flow character. C10, on the other hand, possessed passable flow character but demonstrated an inability to form strong compacts at suitable porosities, as exhibited by its poor tableability, compactibility and compressibility profiles. The compaction properties of both C10 and SNAC were found to be substantially improved on addition of MCC and PVP as commonly used direct compression excipients, and formulations consisting of 72% C10 or SNAC, 20% MCC/ 5% PVP and 3% insulin enabled acceptable direct compression compacts to be produced. Next, we sought to explore the influence of having such high C10 and SNAC contents present in the formulations on the release characteristics of insulin from the compacts produced. Overall, C10 and SNAC were found to have a profound impact on insulin release behaviour, demonstrating an ability to elevate the pH at the surface of the minitables, thereby

increasing insulin solubility at the dissolution interface in comparison to the bulk media and increasing its total release from the compacts.

In conclusion, in this thesis we have demonstrated the important role that insulin stability and PEs have on the successful development of an oral insulin product. From a stability perspective, our results indicate that the insulin raw material used would be suitable for handling for short periods of time in the controlled environmental conditions typically used during tableting. Furthermore, we have highlighted the impact that PEs have on the performance of insulin compacts, and it is clear that careful formulation design will be key for optimising the manufacturability and release kinetics of an oral peptide dosage form containing PEs.

Abbreviations

%P	% Degradation product
% RH	% Relative humidity
ρ_b	Bulk density
ρ_{tap}	Tapped density
ρ_t	True density
AA	Acetic acid
ACN	Acetonitrile
Asn	Asparagine
Asp	Aspartic acid
ATR	Attenuated total reflectance
BSA	Bovine serum albumin
C10	Sodium decanoate
CAGE	Choline geranate
CD	Circular dichroism
CE	Circle equivalent
CI	Carr's Compressibility Index
CIDs	Covalent insulin dimers
Cys	Cysteine
DoE	Design of experiments
DSC	Differential scanning calorimetry
DTT	Dithiothreitol
DVS	Dynamic vapour sorption
ER	Axial elastic relaxation index
FA	Formic acid
FaSSIF	Fasted state simulated intestinal fluid
FaSSGF	Fasted state simulated gastric fluid
FeSSIF	Fed state simulated intestinal fluid
FTIR	Fourier transform infrared
GIT	Gastrointestinal tract
Gln	Glutamine
GLP-1	Glucagon-like peptide 1

Glu	Glutamic acid
Gly	Glycine
GRAS	Generally regarded as safe
HMWP	Higher molecular weight product
HMWS	Higher molecular weight species
HR	Hausner Ratio
HCl	Hydrochloric acid
iso-Asp	iso-Aspartic acid
IVIG	Immunoglobulin G
KH ₂ PO ₄	Potassium dihydrogen phosphate
L-arg	L-arginine
LC–MS	Liquid chromatography – mass spectrometry
LiCl	Lithium chloride
Lys	Lysine
MS	Mass Spectrometry
MCC	Microcrystalline cellulose
MCFA	Medium chain fatty acid
MgCl ₂	Magnesium chloride
MP	Mobile phase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NaH ₂ PO ₄	Sodium phosphate monobasic
Na ₂ HPO ₄	Sodium phosphate dibasic
NaTC	Sodium taurocholate
PE	Permeation enhancer
Phe	Phenylalanine
pI	Isoelectric point
PDB	Protein data bank
PVP	Polyvinylpyrrolidone
P _y	Yield pressure
RP-HPLC	Reverse-phase HPLC
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography

SEM	Scanning electron microscopy
SIF-sp	Simulated intestinal fluid without pancreatin
SNAC	Salcaprozate sodium
TJ	Tight junction
vPSD	Volume-weighted cumulative particle size distribution
XRD	X-ray diffraction

Chapter 1: General Introduction

1.1. Therapeutic peptides

Biopharmaceutical drugs, or biologics, are pharmaceutical products derived from biological sources. Biologics have garnered significant attention for their potential to aid in the treatment of a variety of indications that have been unresponsive to small molecule therapies, including metabolic and oncological disorders (Wang *et al.*, 2022). Indeed, the biopharmaceutical industry has experienced exponential growth since the 1980s, as highlighted by 2023 drug sales, where 8 of the top 10 selling drugs were biologics, up from just 1 in 2003 (Verdin, 2024; Herper, 2004). Of particular interest in recent years is the use of peptides for the development of next generation pharmaceuticals. Compared to larger biologics, peptide therapeutics offer lower production costs and lower immunogenicity. Additionally, the unique structures of peptides endows them with a number of desirable properties, including excellent efficacy, high target selectivity and reduced systemic toxicity, making peptides highly attractive alternatives to traditional small molecule therapies (Fosgerau and Hoffmann, 2015; Kumar *et al.*, 2024).

1.1.1. Routes of administration

At present, the majority of peptide therapeutics available on the market require parenteral administration, via either intravenous, subcutaneous or intramuscular injection. This is highlighted by a recent review from Ganesh *et al.*, in which it was shown that 65% of all products available on the market and 75% of products in the development pipeline were injectable formulations (Ganesh *et al.*, 2021). While injections provide the advantage of rapidly delivering drugs to the systemic circulation by bypassing the gastrointestinal tract (GIT) and first-pass metabolism, alternative

routes of administration may be desirable due to suboptimal patient compliance with dosage regimens. This is due to pain and local irritation at the injection site, fear associated with needles and the potential requirement for multiple daily injections (Zorec *et al.*, 2013). Pulmonary and nasal administration routes have been investigated in the form of dry powder inhalables and powder or solution based-nasal sprays, however, these approaches have yet to achieve widespread clinical and commercial success (Karimi *et al.*, 2022; Luo *et al.*, 2024). For the majority of drugs, the oral route remains a preferred method of delivery owing to high patient compliance and low production costs and as such, considerable attention has been given by both academia and industry to the development of oral dosage forms of peptides in recent years (Murdan *et al.*, 2023; G. Chen *et al.*, 2022). This is highlighted by the successful approval of several oral peptide products including Rybelsus[®], a tablet form of semaglutide, and Mycapssa[®], a capsule form of octreotide.

1.1.2. Insulin as a model therapeutic peptide

The isolation of insulin in the early 20th century was a ground-breaking achievement in the history of drug discovery, catalysing the advent of the biopharmaceutical industry. While human glucagon-like peptide 1 (GLP-1) analogues, such as semaglutide and liraglutide, have emerged as successful candidates for the management of obesity and diabetes, insulin and its analogues remain vitally important therapeutics, dominating the global peptide drug market (Yu *et al.*, 2018; Muttenthaler *et al.*, 2021). With the growing interest in developing alternative dosage forms for peptides, insulin serves as an excellent model peptide to investigate the feasibility of these approaches due to its availability and the breadth of research conducted on the molecule over the past century. Additionally, for a peptide, insulin is structurally unique, having the size of a large peptide but also several higher order

structural features characteristic of a protein, allowing it to potentially act as a bridge in the translation of these approaches to larger protein therapeutics in the future.

1.1.3. Insulin tablets

The non-invasive nature and cost-effective production of tablets makes them a more desirable alternative to injectables. Moreover, the attraction of insulin tablets extends beyond convenience for patient and manufacturer alike as delivery of insulin via the parenteral route is known to be associated with resistance, immunogenicity, and weight gain. Oral absorption, on the other hand, could offer a reduction in these unwanted side effects (Fonte *et al.*, 2013; Wong, Martinez and Dass, 2016). Although therapeutic insulin has been available for over 100 years, a safe and efficacious insulin tablet has yet to be produced. While multiple attempts have been made to successfully develop an insulin tablet, as highlighted by the cases of Eligen[®] (Emisphere Technologies) and IN-105 (Nobex and Biocon), these products have failed to proceed beyond Phase III clinical trials, and further progress has been hindered by the poor physical and chemical stability of the insulin molecule and its poor permeability across the intestinal epithelium (Spoorthi Shetty *et al.*, 2023). As such, oral insulin in general remains elusive.

1.2. Insulin stability

The stability of the insulin molecule plays a critical role not only in its biological activity and potency, but may also contribute to potentially deleterious immunogenic side effects (Eon-Duval, Broly and Gleixner, 2012). As such, an understanding of all the factors influencing the stability of insulin is vitally important to ensure therapeutic efficacy and safety *in vivo*. The insulin molecule can degrade through a number of

well-known physical and/ or chemical processes that are herein described (Manning *et al.*, 2010).

1.2.1. Structure of the insulin molecule

Insulin is a dipeptide, consisting of an A chain with 21 amino acids and a B-chain with 30 amino acids. The two chains are held together by the presence of 2 inter-chain disulfide bridges at A7-B7 and A20–B19, with a further intra-chain disulfide bridge present on the A-chain from amino acids A6-A11. The insulin secondary structure is primarily helical in nature, as shown in Figure 1.1. The A chain forms two nearly anti-parallel α -helices (A1-A9 and A12-A19), while the B chain forms a single α -helix (B9-B19), followed by a β -turn and a β -strand (B21-B30), although this region may be variable depending on molecular conformation (Bryant *et al.*, 1993). The insulin molecule further folds into an overall 3-dimensional (3D) structure, characteristic of a traditional compact globular protein, in which both hydrophilic and hydrophobic residues populate the surface, and a number of hydrophobic residues reside in the hydrophobic core. It is this 3D structure that is recognised by insulin receptors in the body and confers it with its therapeutic efficacy (Lawrence, 2021).

Depending on the solution conditions, insulin can adopt multiple self-association states. At neutral pH and high concentrations, the insulin molecule is stabilised by the formation of dimers and hexamers. Hexamer formation can also be initiated at lower concentrations by the introduction of Zn^{2+} ions at pH 4 – 8, whereby three dimers assemble into the hexameric structure stabilised by 2 Zn^{2+} ions (Brange and Langkjoer, 1993). However, at low and high pH, insulin has a net positive and net negative charge respectively, and under these conditions electrostatic repulsion acts as a barrier to molecular self-association, resulting in the monomer predominating (Kadima *et al.*, 1993; Attri, Fernández and Minton, 2010).

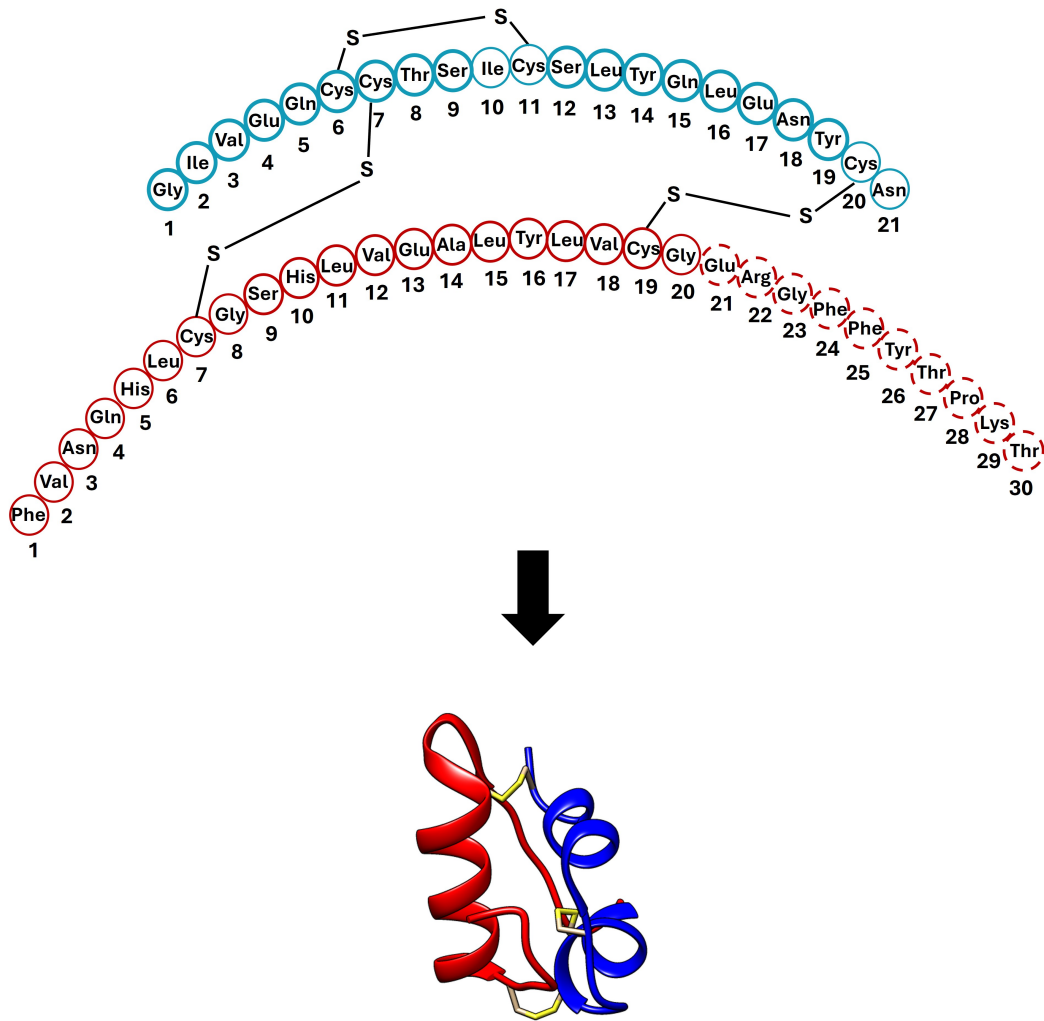


Figure 1.1. Folding of insulin primary structure (top) into 3D monomeric form (bottom). The A chain is shown in blue, and the B chain is shown in red. Secondary structural features are indicated, where alpha helices are shown in bold, and the β -strand is shown as dashed lines. 3D representation was generated on UCSF Chimera, using protein data bank (PDB) entry 3I40.

1.2.2. Physical degradation

Physical degradation refers to conformation changes related to the secondary, tertiary, and quaternary structure. This is manifested primarily by unfolding, aggregation and fibrillation.

Unfolding

Exposure to certain environmental factors such as heat and shaking can disrupt the hydrogen bonding, hydrophobic, electrostatic and van der Waals interactions between peptide chains that hold the insulin 3D structure together, causing a loss of overall shape/ fold (Huus *et al.*, 2005). This is known as unfolding or denaturation. As discussed in Section 1.2.1, the 3D conformation of the insulin molecule determines its interaction with the insulin receptor, therefore, loss of its folded structure renders insulin therapeutically inactive.

Aggregation and Fibrillation

Partial unfolding of the insulin monomer can result in exposure of hydrophobic residues to the surface. To minimise surface energy, the hydrophobic residues tend to interact non-covalently with hydrophobic residues on the opposite surface of adjacent insulin monomers, causing the partially unfolded insulin monomers to aggregate and form oligomers. Persistent aggregation results in the formation of highly ordered fibre-like structures consisting of tightly interacting intermolecular β -sheets, which tend to precipitate in solution (Brange *et al.*, 1997; Nielsen *et al.*, 2001). These are called amyloid fibrils and have been linked to a number degenerative diseases including Alzheimer's and Parkinson's disease (Ow and Dunstan, 2014).

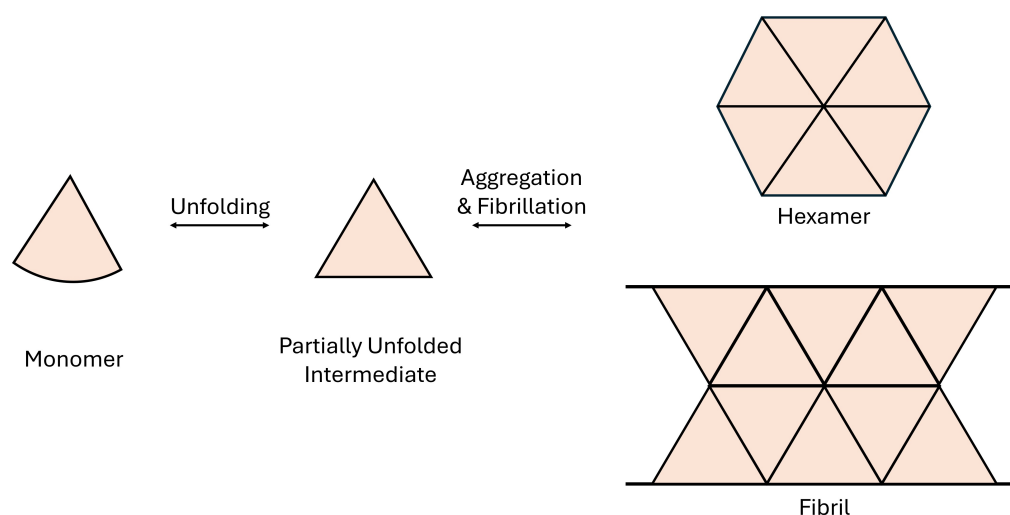


Figure 1.2. Unfolding of insulin monomer resulting in the formation of a partially unfolded intermediate and subsequent aggregation or fibrillation.

1.2.3. Chemical degradation

Chemical degradation refers to irreversible changes in primary structure and can occur through a variety of pathways, including deamidation, transamidation and disulfide interchange (Brange and Langkjoer, 1993; Lai and Topp, 1999).

Deamidation

Deamidation involves the hydrolysis of side chain amide groups of asparagine (Asn) and glutamine (Gln) residues to carboxylic acids. In the process, Asn residues are converted to aspartic (Asp) or isoaspartic acid (isoAsp) and Gln residues are converted to α -glutamic (α -Glu) and γ -glutamic acid (γ -Glu). Insulin contains six possible deamidation sites, Gln A5, Gln A15, Asn A18, Asn A21, Asn B3 and Gln B4, however, in general Asn residues are much more susceptible to deamidation than Gln residues (Andrasi *et al.*, 2020a). The most prominent sites of deamidation are the Asn A21 and Asn B3 residues, depending on the environmental conditions. A proposed mechanism of deamidation for Asn residues via a succinimide intermediate is shown in Figure 1.3A.

Transamidation

Further to deamidation, the Asn A21 or Asn A18 residues are also susceptible to nucleophilic attack by N-terminal phenylalanine (Phe) B1 and glycine (Gly) A1 amino groups or by lysine (Lys) B29 amino groups on adjacent insulin molecules resulting in the irreversible formation of covalent insulin dimers (CIDs) (Flores-Fernández, Solá and Griebenow, 2009). Although higher order oligomers such as trimers and tetramers have been observed, in general the transamidation reaction concludes at the formation of CIDs (Figure 1.3A). Polymerisation reactions are often the result of disulfide exchange (Brange, Havelund and Hougaard, 1992).

Disulfide Exchange

An alternative mechanism by which insulin forms covalent aggregates is through disulfide exchange. Insulin contains six cysteine (Cys) residues, all of which are involved in either inter- or intra-chain disulfide bonds. The surface exposed A7-B7 disulfide bridge may be broken in the presence of reducing agents, or may undergo a β -elimination reaction, resulting in the formation of free thiol groups. The free thiols formed can further interact with free thiol groups on adjacent insulin molecules to form new intermolecular disulfide bridges (Brange, Havelund and Hougaard, 1992; Costantino, Langer and Klibanov, 1994a; Zhang, Chou and Moo-Young, 2011). This is shown in Figure 1.3B. Interchain disulfide bonds are integral to the conformational stability of insulin, therefore, breaking the A7-B7 disulfide bond causes an unfolding of the molecule and increases the susceptibility of other disulfide bonds to undergo further disulfide exchange reactions, potentially leading to polymerisation (Costantino, Langer and Klibanov, 1994a).

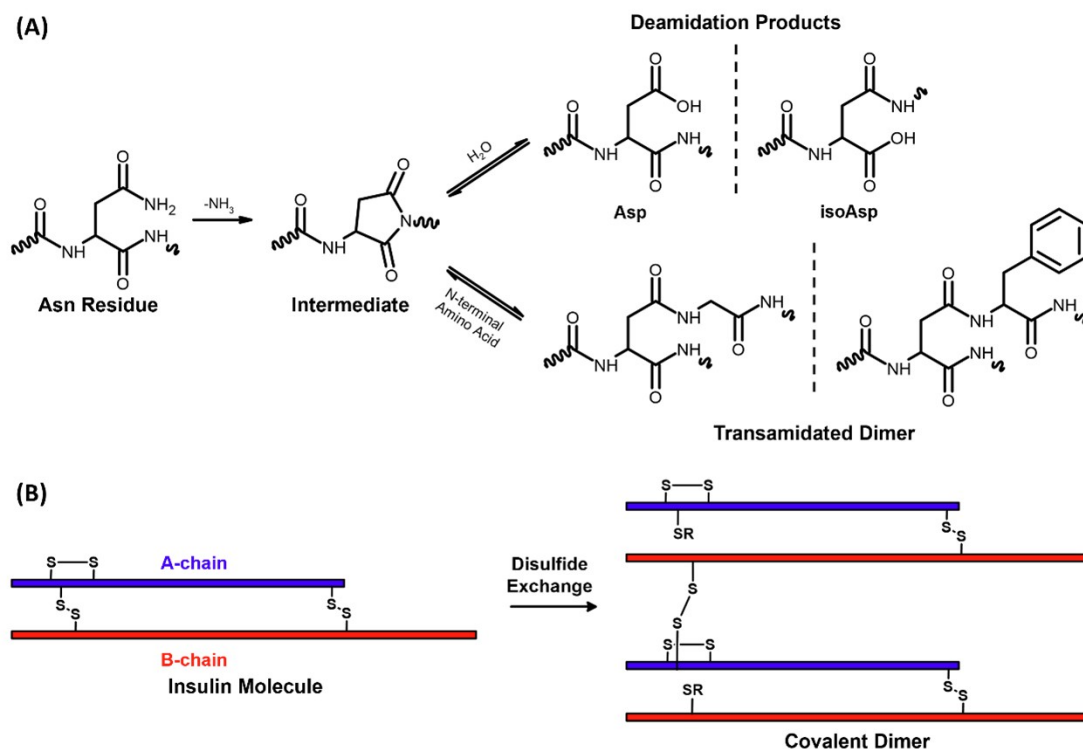


Figure 1.3. Schematic representation of (A) chemical degradation occurring via a cyclic intermediate pathway resulting in the formation of deamidation products and/or transamidated dimers, and (B) disulfide exchange pathway for formation of HMWPs.

1.2.4. Factors influencing physical and chemical stability

Both the physical and chemical stability of insulin are intrinsically dependent on the properties of the molecule and indeed are connected to one another, i.e., molecular conformation affects susceptibility to chemical attack, while changes in primary structure likewise affect secondary, tertiary, and quaternary structures (Zapadka *et al.*, 2017). Beyond the properties of the molecule, various environmental factors are known to impact the stability of insulin such as heat, shaking and pH.

1.2.5. Solid-state stability

To overcome the significant stability concerns described above, attempts have been made to formulate peptides in the solid-state using drying techniques such as freeze drying, spray drying and spray freeze drying (Emami *et al.*, 2018). It is considered that by removing water from the system through drying there is a reduction in overall molecular mobility and flexibility, and the potential for peptide molecules to undergo unfolding and aggregation is thereby minimised. However, stability remains an important consideration in the solid-state, and while the stability of insulin in solution has been well-characterised, much less research has been performed on the solid-state to date (Brange, 1992; Brange and Langkjaer, 1992; Brange *et al.*, 1992; Brange, Havelund and Hougaard, 1992; Brange and Langkjoer, 1993; Strickley and Anderson, 1996).

Degradation pathways in the solid-state

In comparison to the solution state, the restricted mobility of insulin molecules in the solid-state greatly influences the type of degradation that can occur. For example, chemical degradation reactions such as deamidation, which require only short-range conformational flexibility, predominate over physical degradation processes such as aggregation, which require much greater molecular mobility. Aggregation is observed in the solid state, although, it is largely the result of covalent cross-linking due to disulfide exchange and requires elevated residual moisture content in order to increase conformational freedom (Costantino, Langer and Klibanov, 1994a; Flores-Fernández, Solá and Griebenow, 2009).

Factors affecting solid-state stability

There are a number of environmental factors that influence the solid-state stability of insulin, of which the most notable are temperature, humidity and light (Yoshioka and Aso, 2005; Strickley and Anderson, 1996, 1997; Terryn *et al.*, 2006a). These are summarised in Table 1.1.

Table 1.1. Potential factors affecting solid-state stability of insulin.

Stressor	Effect on solid state	Degradation Pathway
High Relative Humidity	Sorbed water acts as plasticiser and increases molecular mobility	Deamidation (Strickley and Anderson, 1996; Pikal and Rigsbee, 1997; Lai and Topp, 1999)
	Acts as reactant in deamidation reaction	Covalent di- and polymerisation (Costantino, Langer and Klivanov, 1994a; Strickley and Anderson, 1996)
High Temperature	Increased molecular vibrations, decrease in activation energy for reaction	Deamidation (Pikal and Rigsbee, 1997; Sadrzadeh <i>et al.</i> , 2010)
		Covalent aggregation (Pikal and Rigsbee, 1997; Sadrzadeh <i>et al.</i> , 2010)
Light	Reduction of disulfide bonds Formation of radicals	Disulfide bond cleavage and oxidative damage (Terryn <i>et al.</i> , 2006a)

It is well-known that exposure of solid peptide materials to elevated temperatures can increase susceptibility to chemical degradation due to a lowering of the activation energy required for reaction and/ or increased molecular mobility in the system (Lai and Topp, 1999). Given that peptides commonly exist in the amorphous state, the physical properties of the material are impacted by the glass transition temperature (T_g) and, as such, increasing environmental temperatures above the T_g likely has a

significant impact on reactivity. For insulin, the rates of deamidation and covalent aggregation have both been observed to increase as temperature is increased, appearing to follow Arrhenius- like kinetics over a temperature range above and below the T_g (Sadrzadeh *et al.*, 2010; Costantino, Langer and Klibanov, 1994a; Pikal and Rigsbee, 1997).

High moisture contents in solid peptide materials have also been found to cause increased reactivity, with water playing a complex and multifaceted role in degradation processes. Firstly, water can have a plasticising effect, increasing the mobility of molecules in the solid state. Water can also act as a reactant in degradation pathways, such as deamidation, and at high concentrations can further serve as a reaction medium/ solvent. Insulin has been observed to undergo significant amounts of deamidation and covalent aggregation in the solid-state, with the extent of degradation being dependent on moisture content (Costantino, Langer and Klibanov, 1994a). At low moisture contents, deamidation is the predominant degradation pathway, while at high moisture contents, covalent dimer formation is favoured relative to deamidation, suggesting that the role of water shifts from a reactant to a plasticiser as moisture content is increased (Strickley and Anderson, 1996, 1997).

Finally, exposure of insulin to radiation with energies similar to those used for radio-sterilisation has been found to induce low levels of disulfide cleavage and oxidative damage (Terry *et al.*, 2006a). However, for the most part, degradation in the solid-state is primarily impacted by storage temperature and relative humidity.

1.2.6. Analytical techniques for stability characterisation

As described above, the pathways of insulin degradation are multifaceted and to completely characterise the degradation products, a multitude of complementary

analytical techniques are required, as summarised in Table 1.2. To investigate physical degradation pathways, circular dichroism (CD) spectroscopy and differential scanning fluorimetry (DSF) are often used to assess secondary structural changes, while size exclusion chromatography (SEC) and dynamic light scattering (DLS) are used to detect the presence of higher molecular weight products (HMWPs) such as aggregates. The most powerful techniques used to detect primary structural changes are reverse phase high performance liquid chromatography (RP-HPLC) combined with mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy.

The majority of these techniques are solution-based, requiring reconstitution of the sample prior to analysis. There are techniques available to measure solid-state changes, such as Fourier Transform Infrared Spectroscopy (FTIR) which can be used for direct detection of secondary structural changes in solid-state peptides. Moreover, techniques such as differential scanning calorimetry (DSC) and x-ray diffraction (XRD) can be used to gain insight into bulk material properties, such as T_g and crystallinity, however, these are low resolution techniques and atomic level information cannot be obtained without the use of solution-state techniques such as MS and NMR.

Table 1.2. Insulin degradation pathways and common techniques for characterisation.

Degradation Pathway	Residues involved	Characterisation Technique
Deamidation	Predominantly Asn A21 and Asn B3	RP-HPLC (Najjar <i>et al.</i> , 2014) MS (Rojsajakul, 2007; Andrasi <i>et al.</i> , 2020a) NMR (Jin <i>et al.</i> , 2014)
Transamidation	Asn A18, Asn A21, Phe B1, Gly A1 and Lys B29	SEC (Brange, Havelund and Hougaard, 1992) MS (Nettleton <i>et al.</i> , 2000) Light Scattering (Hjorth <i>et al.</i> , 2016)
Disulfide Exchange	Cys A7-Cys B7	SEC (Brange, Havelund and Hougaard, 1992) MS (Nettleton <i>et al.</i> , 2000) Light Scattering (Hjorth <i>et al.</i> , 2016)
Unfolding	-	CD Spectroscopy (Sklepari <i>et al.</i> , 2016) DSF (Housmans <i>et al.</i> , 2023) FTIR (Housmans <i>et al.</i> , 2023) NMR (Jin <i>et al.</i> , 2014)
Non-covalent Aggregation	Surface exposed hydrophobic residues	SEC (Brange, Havelund and Hougaard, 1992) Light Scattering (Hjorth <i>et al.</i> , 2016) CD (Bouchard <i>et al.</i> , 2000) NMR (Jin <i>et al.</i> , 2014)

1.3. Tableting of insulin

While stabilising peptides in the solid-state has proven an effective method of prolonging shelf-life, there remain a number of critical factors that need to be understood when translating peptide powders to efficacious tablets. Firstly, tableting can be a complex process involving multiple unit operations, such as blending,

granulation and compaction, wherein powders are often subject to high temperatures, humidities and pressures (Li *et al.*, 2023). It is important, therefore, to assess how the handling and processing conditions to which the material is exposed during each step affect not only peptide molecular stability, but also the physical properties of the material. For example, it is well-known that the initial moisture content of a pharmaceutical powder can impact its compaction properties, which, in turn, influences the physicomachanical properties of the tablets produced (Nokhodchi and Javadzadeh, 2007). Furthermore, careful consideration must be given to the composition of the tablet formulation, and excipients should be selected to ensure that the final powder blend displays suitable bulk material properties for compaction, while also ensuring that insulin is released in a bioavailable and therapeutically active form.

1.3.1. Tableting process

As mentioned above, tableting can be a complex, multi-step process in which the unit operations required vary depending on the specific properties of the raw materials selected. In general, there are three main processing techniques used during tableting: direct compression, wet granulation and dry granulation, shown schematically in Figure 1.4. Of these, direct compression is the simplest and most effective process, where compression occurs directly after blending of drug and excipient powders. Although simple, direct compression is not suitable for all materials, and additional steps, i.e., granulation, may be required prior to compression (Jivraj, Martini and Thomson, 2000). In granulation, inter-particulate bonds are created between finely powdered particles to create larger secondary particles called granules, which exhibit better content uniformity, flowability and compaction properties in comparison to the constituent powders. In the case of wet granulation, a liquid granulating agent, such as polyvinyl pyrrolidone (PVP), is introduced to encourage interactions between

particles. The resultant wet granules require drying before proceeding to compaction (P. Chen *et al.*, 2022). On the other hand, for moisture sensitive materials, dry granulation techniques such as roller compaction or slugging can be employed to form granules via compaction (Vadaga *et al.*, 2024). Once produced, granules often require milling for particle size reduction and additional post-granulation excipients are added prior to compaction on a tablet press. Further steps such as coating and embossing may also be performed after tablets are produced.

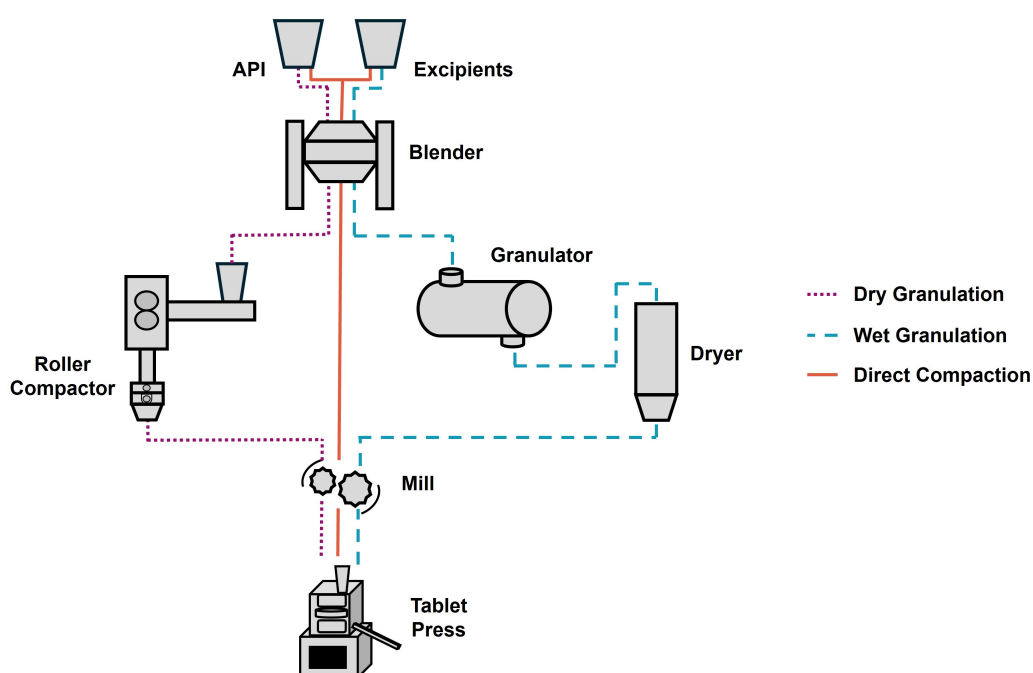


Figure 1.4. Schematic overview of main processing techniques used during tableting.

1.3.2. Impact of processing conditions on stability

Although the intricate processing steps described in Section 1.3.1 are often required to improve the bulk properties of the materials used during tableting, this inevitably leads to the introduction of a number of stressors which may negatively impact the stability of sensitive peptide materials such as insulin.

Granulation

During wet granulation, the use of a liquid granulating agent exposes powder material to high moisture content, which is known not only to influence the physical and chemical stability of insulin but may also encourage unwanted interactions between insulin and other excipients necessarily present in the formulation (Strickley and Anderson, 1996, 1997). Additionally, the drying step required to reduce moisture content to an acceptable level may expose the material to elevated temperatures which can further influence the stability of insulin, as shown in Table 1.1 (Monaco *et al.*, 2021; Sadrzadeh *et al.*, 2010). Although dry granulation eliminates the need for high moisture contents and temperatures, it does require powder compaction.

Compaction

Both dry granulation and the compaction of the final blend in the tablet press require the application of high pressures (Vadaga *et al.*, 2024). Although there are no published studies investigating the impact of compaction pressures on insulin material, there have been a number of investigations into the effect of compaction pressures on larger proteins. In an investigation by Wei *et al.*, it was found that lysozyme, a small globular protein with a molecular weight of 14 kDa, was stable to compression pressures as high as 300 MPa. On the other hand, bovine serum albumin (BSA), a larger protein with a molecular weight of 66 kDa, was found to undergo aggregation at pressures as low as 25 MPa, while no secondary structural changes were observed. This tendency to aggregate was also observed to be dependent on the size of the BSA particles, where more aggregation was noted for powders with smaller particle sizes (Wei *et al.*, 2019). In a separate investigation into the effects of compaction pressure on the stability of intravenous immunoglobulin (IVIG), compaction pressure did not appear to cause significant aggregation of the IVIG, although secondary structural

changes were observed. The effect of compaction pressure on IVIG was similarly found to be dependent on particle size (Lu *et al.*, 2021). It is clear, therefore, that compaction pressures have a protein specific effect on stability, suggesting that an in-depth characterisation of stability during compaction will be vital for the development of an effective insulin tablet.

1.3.3. Direct compression insulin tablets

Due to the significant stress conditions introduced during processes such as granulation, it is apparent that minimising the number of steps involved in tableting by using a direct compression approach would be prudent to reduce potential sources of instability. While granulation offers the inherent advantage of improving powder properties, direct compression performance is directly related to the properties of the materials themselves. As such, the composition of direct compression formulations plays a critical role in ensuring that the powder blend exhibits adequate flow and compaction properties. However, an additional challenge is presented in the case of insulin, where the chosen formulation also plays a key role in successful absorption of insulin after release in the GIT.

1.3.4. Oral bioavailability challenges

As outlined in Section 1.1.3., the development of an effective oral peptide dosage form has, in general, been hindered by two key challenges: poor gastrointestinal stability and poor oral bioavailability. While the intricate structures of peptides, such as insulin, provide them with valuable therapeutic properties, their structural complexity also leaves them highly susceptible to denaturation in the acidic environment of the stomach and hydrolytic degradation due to the presence of gastric and intestinal enzymes. Moreover, the large size and hydrophilic nature of peptides restricts

paracellular transport across gastrointestinal epithelia and thereby limits peptide permeability (Zhang *et al.*, 2024). The combined effect of these two factors has, as a result, limited overall bioavailability of peptide drugs to < 2% (Nicze *et al.*, 2024). These barriers to oral absorption are shown in Figure 1.5a.

Gastrointestinal pH

The physicochemical properties of insulin makes it highly susceptible to pH variations experienced in the GIT, which ranges from acidic in the stomach (pH 1 – 2.5) to neutral/ weakly basic in the intestines (pH 6.5 – 8) (Yamamura *et al.*, 2023). The acidic environment of the stomach is known not only to promote disulfide bond cleavage, leading to denaturation of the insulin molecule, but can similarly facilitate deamidation (Chen *et al.*, 2018). Moreover, insulin solubility is dependent on a number of environmental factors, including pH, meaning that transition from an acidic environment in the stomach to a neutral environment in the intestines may reduce solubility or even induce precipitation (Grossmann and McClements, 2023).

Gastric- and intestinally- secreted enzymes

The presence of proteolytic enzymes, such as pepsin, trypsin and chymotrypsin, along the GIT leaves insulin highly susceptible to rapid degradation (Marschütz and Bernkop-Schnürch, 2000). Additional enzymes present in the intestinal mucus layer and bound to brush border membranes are also known to exhibit specific peptidase activity, thus reducing the chances of insulin reaching the epithelial wall intact (Haddadzadegan, Dorkoosh and Bernkop-Schnürch, 2022).

Physical barriers

A thick layer of mucus surrounding the intestinal epithelium acts as a physical barrier to the absorption of insulin into the systemic circulation. This hydrogel layer,

composed of a 3-dimensional network of negatively charged glycoproteins, serves to trap foreign species, such as pathogens, and effectively hinder them from reaching epithelial cells (Lundquist and Artursson, 2016). The mucus layer, however, is also very effective at binding large molecules, such as insulin, immobilising them in the hydrogel network, leading to rapid clearance from the GIT.

If successful penetration of the mucus layer is achieved, the epithelium itself presents another physical barrier to insulin absorption. Transport across the epithelium can occur via a transcellular or paracellular pathway. Typically, passive diffusion is limited to small, lipophilic molecules, and, therefore, transcellular transport of peptides occurs via carrier- or receptor- mediated mechanisms. As a result, the paracellular route is favoured for transport of peptides. However, paracellular transport is primarily facilitated by tight junctions between epithelial cells, which compose < 1% of the total epithelial surface. In addition, auxiliary agents are often required to enable transient opening of tight junctions to permit peptide transport (Haddadzadegan, Dorkoosh and Bernkop-Schnürch, 2022; Zhang *et al.*, 2024).

1.3.5. Strategies to improve oral bioavailability

Recent years have seen the adoption of several effective strategies to address the physical and chemical barriers to absorption in the GIT. As these approaches have been thoroughly reviewed elsewhere, a brief description of some of the most promising approaches are provided in this section and shown schematically in Figure 1.5b (Dan, Samanta and Almoazen, 2020; Han *et al.*, 2024; Bruno, Miller and Lim, 2013).

Structural modification

Altering the structure of the insulin molecule through modification of accessible amino acid residues or conjugation with lipophilic moieties may be an effective strategy to improve stability to proteolytic degradation while also enhancing permeability (Kamelnia *et al.*, 2024). For example, conjugation of ethoxycarbonyl methionine, α -lipoic acid and dehydrocholic acid groups to amino acids such as lysine and glycine have been observed to protect insulin from hydrolytic cleavage under acidic conditions and from tryptic degradation (Saunders and Offord, 1977; Huang and Huang, 2005, 2006). Use of a biocompatible polymer, such as polyethylene glycol (PEG), has also proved effective in preventing proteolytic degradation in intestinal fluids. Furthermore, PEG-methacrylic acid copolymer systems have shown mucoadhesive properties, allowing them to bind to the intestinal mucosa, which prolongs insulin exposure at the intestinal wall (Coolich *et al.*, 2023). Structural modification, however, can be a challenging and costly strategy, making the use of a dosage form composed of unaltered insulin in combination with functional excipients potentially a more time- and cost-effective approach (Buckley, Hubálek and Rahbek, 2016).

Enzyme inhibitors

A common method to prevent insulin digestion by proteolytic enzymes in the GIT is the addition of enzyme inhibitors to deactivate specific enzymes in the stomach and/or intestines. Aprotinin and trypsin inhibitors, such as soybean and lima bean trypsin inhibitors, are leading polypeptide-based enzyme inhibitors used for their ability to specifically and effectively target trypsin and chymotrypsin (Bruno, Miller and Lim, 2013). Beyond the targeting of specific enzymes, alteration of the local pH at the site of peptide release has been used to inhibit enzyme activity. For example, inclusion of

citric acid with calcitonin in a tablet was found to inhibit intestinal peptidases (Binkley *et al.*, 2012). As intestinal peptidases are most effective at neutral to basic pH values, citric acid acts to lower the environmental pH, thus inhibiting their activities.

Permeation Enhancers

Although inhibition of enzyme activity is important to ensure that insulin released from an oral dosage form reaches the systemic circulation intact, additional components are required to enable transport across the epithelial wall. Permeation enhancers (PEs) are molecules that have been successfully used to transiently alter the permeability of epithelial cells, thereby facilitating improved transcellular and paracellular transport of peptides. There are several classes of PEs which have been used to improve peptide permeability, each of which vary in their structures and mechanisms of action. Chelating agents, such as ethylenediaminetetraacetic acid (EDTA), are often used to increase peptide paracellular transport. By chelating extracellular Ca^{2+} ions, EDTA causes a cell signalling cascade resulting in the disbandment and opening of tight junctions (Bohley and Leroux, 2024). Another class of PEs are bile salts, such as sodium taurocholate and sodium glycholate, which disrupt the integrity of cell membranes by interacting with membrane bound lipids, enhancing peptide transport via both paracellular and transcellular routes (Moghimipour, Ameri and Handali, 2015). Ionic and non-ionic surfactants, including sodium caprate and polysorbate 20, have also been used to alter membrane permeability and improve peptide absorption from the GIT. Surfactants exhibit detergent like action, in which cell membranes are fluidised by insertion of surfactant monomers into the phospholipid bilayers (Maher, Geoghegan and Brayden, 2023). Medium-chain fatty acids (MCFAs) can display similar surfactant-like action to alter membrane permeability (Kim, Park and Na, 2022). Various other types of molecules, including

zonula occludens toxin and peptides, have shown promising permeation enhancing activity (Fasano *et al.*, 1997; Cox *et al.*, 2001; Kim *et al.*, 2023).

There have been a number of clinical trials involving different types of PEs conducted over recent decades. In general, the PEs used have shown good biocompatibility, with several well-established PEs having gained food additive status or being designated Generally Regarded As Safe (GRAS) (Brayden *et al.*, 2020). However, there remain long-term safety concerns associated with continuous perturbation of the gastrointestinal epithelia that need to be investigated.

Novel delivery systems

Beyond the traditional formulation approaches, many attempts have been made to develop novel drug delivery systems (DDSs) to improve the overall oral bioavailability of insulin. One such approach is through encapsulation of insulin within colloidal micro- and nanoparticulate carrier systems composed of either polymeric, lipidic or inorganic materials (Al Tahan, Al-Khattawi and Russell, 2025). Carrier systems offer the advantage of being highly versatile as the constituent materials, e.g. polymers, are often easily functionalised with chemical moieties, enabling the properties of the system to be selectively tuned. This allows for not only controlled and/ or targeted release of insulin but also protects it from degradation in the GIT and increases its absorption into the systemic circulation (Han *et al.*, 2024; Hamman, Enslin and Kotzé, 2005; Zheng *et al.*, 2025). Ionic liquids have also shown promise for oral delivery of insulin (Raptis *et al.*, 2025). Ionic liquids are salts with melting points below 100°C and have physicochemical and biological properties that can be easily modified according to choice of cation/ anion (Liu *et al.*, 2022). In one example, a choline geranate (CAGE) ionic liquid showed a marked enhancement in the oral bioavailability of insulin in rats, while preventing enzymatic degradation in

the GIT. Furthermore, insulin was found to be stable in the formulation after storage at room temperature for 2 months (Banerjee *et al.*, 2018). Several other approaches, such as microneedles for oral insulin delivery, have been investigated, however, many of these delivery systems have yet to show meaningful clinical viability, with mostly non-clinical data being reported, and as a result, clinical translation of these strategies may still be years away.

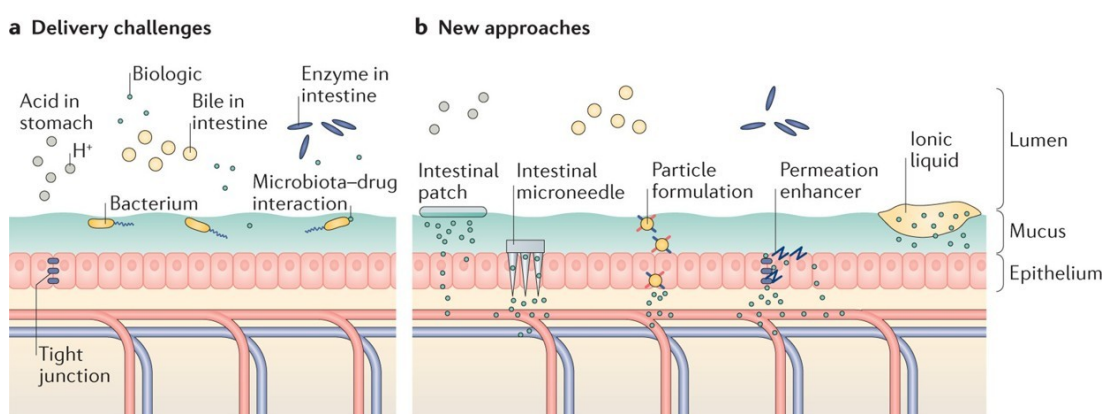


Figure 1.5. (a) Biological barriers to oral absorption of peptides and (b) approaches to overcome these biological barriers. Reproduced from (Anselmo, Gokarn and Mitragotri, 2019).

1.3.6. Permeation enhancers for oral insulin delivery

With the successful approval of products such as Rybelsus[®] and Mycapssa[®], PEs have provided a breakthrough in the oral delivery of peptides, proving the most effective strategy to enhance the absorption of peptides in the GIT to date. As described in Section 1.3.5, there are several types of PEs, which aid in the transport of peptides across the epithelial membrane via a paracellular route, by opening tight junctions, or via a transcellular route, by disrupting cell membrane integrity or increasing the hydrophobicity of peptides. Of these PEs, two of the most widely tested in human

trials are sodium caprate (C10) and salcaprozate sodium (SNAC), the structures of which are given in Figure 1.6 (Twarog *et al.*, 2019).

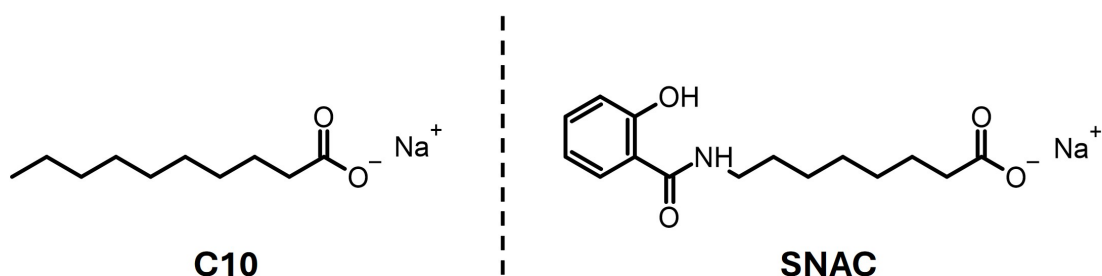


Figure 1.6. Structure of C10 and SNAC.

C10

C10 is the salt form of the MCFA capric acid and has been approved as a food additive in both the EU and US (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS) *et al.*, 2018). As part of the Gastrointestinal Permeation Enhancement Technology (GIPET[®]) developed by Merrion Pharma, C10 incorporated in oral dosage forms was assessed in clinical trials for delivery of a variety of biologics, including insulin (Walsh *et al.*, 2011). Moreover, insulin tablets containing C10 as a permeation enhancer have also been developed by Novo Nordisk (I338) and Biocon (IN-105), however, no oral peptide products containing C10 have proceeded beyond Phase III clinical trials as yet (Halberg *et al.*, 2019; Khedkar *et al.*, 2019; Ballantyne *et al.*, 2023). Promising results have been observed from recent Phase III clinical trials performed by Merck on its novel MK-0616 candidate, in which C10 is coformulated with a PCSK9 inhibitor enlicitide decanoate for the treatment of hypercholesterolemia, and may prove to be the first approved oral peptide product containing C10.

Various investigations have been conducted to elucidate the mechanism by which C10 enhances permeation. Initial investigations proposed a paracellular mechanism via tight junction modulation, however, subsequent investigations have observed

detergent like activity, where C10 enhances permeation through membrane insertion and perturbation (Kim, Park and Na, 2022). The exact mechanism of action remains unclear, and it is possible that a combination of paracellular and transcellular transport is involved.

From what is known about C10 activity, the enhancement effect is hypothesised to be exerted through the monomeric form and is, therefore, dependent on medium composition and its concentration in solution. As the pKa of C10 is approx. 5, at acidic pH values, C10 will exist in the unionised form, which is known to precipitate out as an oily phase due to the formation of large colloidal structures and lamellar bilayers (Berg *et al.*, 2022). On the other hand, at pH values above the pKa, C10 exists predominantly in the ionised form, which can exert a permeation enhancing effect. In the ionised form, self-association into micelles is restricted by electrostatic repulsion between charged head groups and the critical micelle concentration (CMC) is high. However, depending on the ionic strength of the surrounding medium, the CMC may be lowered and the amount of monomeric form available to interact with the epithelium will be reduced (Twarog *et al.*, 2019). Additionally, bile salts present in the intestinal medium may sequester C10 monomers into mixed micelles, further limiting its action.

SNAC

SNAC is the N-acetylated amino acid derivative of salicylic acid that was discovered as part of a large library of Eligen[®] carrier compounds developed by Emisphere Technologies and later acquired by Novo Nordisk. It has been used to successfully develop an oral B12 formulation and more recently a tablet form of semaglutide (Rybelsus[®]) for the management of obesity and diabetes (Aroda, Blonde and Pratley, 2022).

While similar in structure to MCFAs such as C10, the hydrophilic salicylamide region of SNAC prevents efficient membrane insertion and SNAC does not exhibit detergent like activity. Moreover, although extensive research has been conducted, the mechanism of action of SNAC is poorly understood (Bohley and Leroux, 2024). There is little evidence to suggest a paracellular mechanism, and a transcellular mechanism is thus hypothesised. It is proposed that SNAC forms non-covalent complexes with peptides, increasing their hydrophobicity and permeability, without meaningfully altering the membrane structure (Kim, Park and Na, 2022). This mechanism is thought to be peptide dependent and is likely incomplete (Twarog *et al.*, 2019). In the case of Rybelsus[®], SNAC was observed to form a complex with semaglutide, enhancing its permeability. An important aspect of the release and absorption of semaglutide is the ability of SNAC to elevate the local pH environment at the surface of the tablet, preventing enzymatic degradation and promoting monomerisation of semaglutide. Moreover, it is interesting to note that, in the case of semaglutide it is proposed that absorption occurs exclusively in the stomach, not in the intestines as with the majority of drugs (Buckley *et al.*, 2018).

1.3.7. Impact of permeation enhancers on tableting and release properties

Understanding the mechanisms by which PEs influence the absorption of peptides is critical to maximise oral bioavailability. However, it is also fundamentally important to investigate how the physical properties of PE materials impact the compaction behaviour of the formulation, and the release profiles of the tablets produced.

Compaction properties

Both C10 and SNAC are amenable to processing via conventional tableting methods, allowing for efficient and scalable production of peptide tablets. However, high

concentrations of C10 and SNAC are required to have a noticeable impact on peptide permeability and, as a result, a large proportion of the tablet formulation must be composed of the PE (Twarog *et al.*, 2019). For example, 75% of the Rybelsus® formulation is composed of SNAC. In the context of a direct compression process, the bulk properties of the formulation will be greatly influenced by the individual properties of the PE. For plastic materials, such as microcrystalline cellulose (MCC), the primary mechanism of powder consolidation during compaction is through plastic deformation of the particles, resulting in soft, ductile tablets with low compaction pressures required for consolidation. Brittle materials, such as mannitol, on the other hand, undergo powder consolidation via fragmentation of larger particles into smaller fragments, and exhibit poorer compressibility in comparison to plastic materials (Zhang *et al.*, 2017). As such, PEs must exhibit acceptable mechanical properties for direct compression or additional processing steps, such as dry granulation, may be required. Although a significant amount of research has been conducted on PEs, there is limited published literature available investigating the tableting behaviour of PEs to date.

Release behaviour

As described by the biopharmaceutical classification system (BCS), the properties that influence the absorption of a drug are solubility, dissolution and permeability (Samineni, Chimakurthy and Konidala, 2022). Being highly soluble but poorly permeable, hydrophilic peptides, such as insulin, are categorised as BCS Class III drugs. While the use of PEs has enabled improved permeability, if the dissolution rate of a peptide from the dosage form is slow, the amount of drug available for diffusion across the epithelia will be minimal and absorption will be low. There are several factors that influence the dissolution rate of a drug, including drug particle surface

area, solubility in the dissolution media and the concentration of the drug in the surrounding medium. A number of equations have been proposed to model drug dissolution, of which the Nernst-Brunner equation is one of the most widely used. This is given in Equation 1.1 below.

$$\frac{dM}{dt} = \frac{S \cdot D}{\delta} (C_s - C_t) \quad \text{Equation 1.1.}$$

where dM/dt represents the dissolution rate, S is the particle surface area, D is the diffusion coefficient, δ is the diffusion boundary layer thickness, C_s is the drug solubility in the dissolution medium, and C_t is the concentration of the drug in the bulk medium at time t .

It is important, therefore, to fully characterise the impact of permeation enhancers on the dissolution rate of peptides from dosage forms in which they are present.

As described above, the low efficacy of C10 and SNAC requires high doses to be present in each tablet. As such, the release behaviour of peptide tablets will be heavily influenced by the C10 and SNAC present. However, few investigations into the impact of PEs on the release behaviour of peptides from tablets have been published. It is possible to propose, according to the properties of peptides and PEs, that the composition of gastric and intestinal media will likely play an important role in their dissolution characteristics. Moreover, besides SNAC, it is unclear whether PEs such as C10 also possess the ability to modify the local pH environment surrounding tablets, and this will also play an important role in the solubility of the peptide and, therefore, its dissolution rate.

Furthermore, several considerations have highlighted the importance of the release kinetics of tablets for optimising the performance of PEs in the GIT (Maher and Brayden, 2021). Firstly, for PEs to exert an enhancing effect, they must present at the

epithelial wall above a particular threshold concentration. Thereafter, there is a short period of time within which the peptide must also present at the target site to take advantage of the enhancing effect. This timeframe is impacted by rapid absorption and/ or dilution of the PE in the GI media and transit of the dosage form away from the site of action. Furthermore, the duration for which the peptide and the PE are present at the site of action influences the amount of peptide absorbed (Bækdal *et al.*, 2021; Baluom, Friedman and Rubinstein, 1998; Sutton *et al.*, 1993). As a result, synchronised release of peptide and PE from tablets is vital for ensuring co-localisation at the epithelial wall.

1.4. Aims and Objectives

The overarching aims of this thesis were as follows:

- To develop a comprehensive understanding of the stability of human insulin in the solid-state.
- To take a holistic approach to the development of a direct compression insulin formulation with a particular focus on understanding the influence of permeation enhancers on formulation design and *in vitro* dissolution performance.

To achieve these aims, the following objectives were established:

Chapter 2:

- To develop and optimise a suite of characterisation techniques for investigating the physical and chemical degradation pathways of insulin.
- To compare the degradation pathways observed in solution with those in the solid-state.

Chapter 3:

- To investigate the effects of humidity and temperature on insulin degradation kinetics in the solid-state, utilising the techniques developed in Chapter 2.

Chapter 4:

- To rationalise a direct compression formulation for the preparation of insulin minitabets using a mixture design of experiments approach.

Chapter 5:

- To assess the impact of permeation enhancers and medium composition on the *in vitro* dissolution behaviour of the minitabets produced in Chapter 4.

Chapter 2: Elucidating the degradation pathways of human insulin in the solid state

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2.1. Author Contributions

As lead author, I was responsible for developing and validating experimental methodologies, carrying out experimental investigations and performing formal analysis of the data. Furthermore, I was responsible for writing the original draft of the paper. Lorraine Bateman, Joseph O'Shea and Abina Crean were responsible for supervising the progress of experiments, reviewing and editing of the manuscript and funding acquisition.

2.2. Abstract

While there have been significant advances in the development of peptide oral dosage forms in recent years, highlighted by the clinical and commercial success of approved

peptides such as Rybelsus®, there remain several barriers in the way of broad range applicability of this approach to peptide delivery. One such barrier includes the poor physical and chemical stability inherent to their structures, which persists in the solid state although degradation typically occurs at different rates and via different pathways in comparison to the solution state. Using insulin as a model peptide, this work sought to contribute to the development of analytical techniques for investigating common insulin degradation pathways. Chemically denatured, deamidated and aggregated samples were prepared and used to benchmark circular dichroism spectroscopy, reverse phase HPLC and size exclusion chromatography methods for the investigation of unfolding, chemical modifications and covalent aggregation of the insulin molecule respectively. Solid state degraded samples were prepared by heating insulin powder at 60°C and 75% relative humidity for 1, 3, 5 and 7 days, and the degradation profiles of the samples were evaluated and compared with those observed in solution. While no unfolding was observed to occur, significant deamidation and covalent aggregation were detected. Reductive disulfide bond cleavage using dithiothreitol allowed for separation of the insulin A- and B-chains, offering a facile yet novel means of assessing the mechanisms of deamidation and covalent aggregation occurring in the solid state.

2.3. Highlights

- Deamidation and covalent aggregation observed to occur on storage of insulin in the solid-state at elevated temperature and humidity conditions.
- Insulin covalent aggregation occurred via a disulfide exchange pathway.
- Insulin deamidation observed to occur exclusively on the A-chain.

2.4. Introduction

Due to their remarkable efficacy, reduced systemic toxicity and high tolerability proteins and peptides have emerged as highly effective therapies, particularly in the treatment of oncological and metabolic disorders (Leader, Baca and Golan, 2008; Fosgerau and Hoffmann, 2015). While the majority of peptide drugs on the market require delivery via subcutaneous injection, there has been increasing interest in alternative routes of administration, in particular the oral route (Cunningham and Tanner, 2020; Long *et al.*, 2020; Haddadzadegan, Dorkoosh and Bernkop-Schnürch, 2022). This is highlighted by the recent FDA approval of oral formulations of octreotide (Mycapssa[®]), semaglutide (Rybelsus[®]) and desmopressin (Minirin[®]). Another interesting therapeutic peptide that would greatly benefit from an oral approach to delivery is insulin, which has received considerable attention for advancing novel approaches to peptide delivery (Zhao *et al.*, 2022; Wong, Al-Salami and Dass, 2017; Bernstein, 2008; Sonia and Sharma, 2014). While much of the focus during oral peptide development has been on the improvement of intestinal absorption and oral bioavailability, another major hurdle in the way of capitalising on the many advantages of peptide therapeutics that must be addressed is their poor physical and chemical stability.

Poor stability of peptides during processing, storage and on delivery into the body, can not only result in a decrease in overall therapeutic efficacy but is also associated with increased unwanted immunogenic side effects, such as disruption of normal protein-protein interactions and development of amyloid diseases (Eon-Duval, Broly and Gleixner, 2012; Bouchard *et al.*, 2000; Jha, Deepalakshmi and Udgaonkar, 2012). There are two main categories of degradation that peptides may undergo: physical and

chemical (Brange and Langkjoer, 1993; Manning *et al.*, 2010). Physical degradation refers to conformational changes related to secondary, tertiary and quaternary structures and includes unfolding and aggregation. Chemical degradation, on the other hand, refers to primary structural changes such as deamidation (Zhang, Chou and Moo-Young, 2011; Andrasi *et al.*, 2020b). The physical and chemical stabilities of peptides are intrinsically dependent on the properties of the molecule and indeed are connected to one another, i.e., molecular conformation affects susceptibility to chemical attack, while changes in primary structure likewise affect secondary, tertiary, and quaternary structures (Zapadka *et al.*, 2017).

While the stability of insulin has been well characterised in solution, less work has been performed on the molecule in the solid-state and the resultant implications for processing and manufacture of solid oral dosage forms. In solution, various external factors, such as agitation, temperature and freeze-thawing, along with the solution conditions, e.g., pH and ionic strength, significantly impact the stability of insulin. For example, the presence of air-liquid interfaces as a result of shaking is known to cause insulin to undergo aggregation and fibrillation, whilst chemical degradation in the form of deamidation can be initiated in acidic environments (Sluzky, Klibanov and Langer, 1992; Brange and Langkj er, 1993). In the solid-state, on the other hand, the tightly fixed nature of the molecules restricts their mobility and, therefore, the type and extent of the degradation that can occur may be altered relative to the solution state. As such, chemical degradation pathways, which require only short-range conformational flexibility, predominate over physical degradation processes, which require much greater molecular mobility (Lai and Topp, 1999). For example, insulin aggregation is observed to occur in the solid-state, however, it is largely the result of intermolecular covalent interactions between insulin molecules and often requires

elevated residual moisture content to increase conformational flexibility (Brange *et al.*, 1992; Costantino, Langer and Klibanov, 1994a; Flores-Fernández, Solá and Griebenow, 2009; Costantino, Langer and Klibanov, 1994b). Additionally, in contrast to the solution-state, temperature, humidity and light are the predominant factors affecting stability in the solid-state, along with the solution conditions prior to lyophilisation (Costantino, Langer and Klibanov, 1994a; Strickley and Anderson, 1996, 1997; Terryn *et al.*, 2006b; Flores-Fernández, Solá and Griebenow, 2009).

Clearly a comprehensive understanding of the various degradation pathways of a peptide drug substance plays an important role in formulation design and development of solid oral dosage forms. To date, excipient choice has focused primarily on improving the oral bioavailability of peptides through the use of permeation enhancers and enzyme inhibition (Brayden *et al.*, 2020). However, understanding the stability of the peptide drug substance to the drug product manufacturing processes and indeed to the long-term storage conditions is essential to inform rational excipient screening and selection.

A large body of work has been performed in this field on the development and application of a multitude of complementary analytical methods for investigating the physical and chemical stability of human insulin (Bennion and Daggett, 2003). This has highlighted the importance of deamidation and covalent aggregation in both the solution and solid-state. However, while the mechanisms of deamidation have been well characterised, opposing covalent aggregation pathways have been observed in the solid-state, including transamidation and disulfide exchange (Costantino, Langer and Klibanov, 1994a; Strickley and Anderson, 1996). As physicochemical characterisation of peptides is performed in the solution-state, in this paper the authors

first sought to build upon previous work by benchmarking commonly used analytical techniques with positive control samples that have followed known degradation pathways in the solution-state. Circular dichroism (CD) spectroscopy and size exclusion chromatography (SEC) were chosen to investigate unfolding and aggregation respectively, while reverse phase high performance liquid chromatography (RP-HPLC) and mass spectrometry (MS) have been chosen to investigate chemical modifications. These techniques were then translated to the solid state to highlight their efficacy for elucidating the degradation pathways of samples exposed to high temperature and humidity conditions in the solid state, with the techniques offering an alternative approach to solid-state characterisation to enable a platform screening approach. Reductive cleavage of disulfide bonds using dithiothreitol as a reducing agent was used as a simple but highly effective tool to confirm the mechanism of deamidation and covalent aggregation occurring in the solid-state samples.

2.5. Experimental

2.5.1. Materials

Recombinant human insulin and all reagents used during analysis were procured from Merck KGaA, unless otherwise stated.

2.5.2. Degraded Sample Preparation

Standard recombinant human insulin samples were reconstituted in 0.1% formic acid (FA), pH 2.6. Insulin samples were exposed to a range of conditions to induce degradation. Chemical denaturation was performed by incubating insulin samples at 1 mg/ mL in 6M urea, prepared in 0.1% FA, at room temperature for 4 hours (Bennion

and Daggett, 2003; Povarova, Kuznetsova and Turoverov, 2010). To initiate aggregation, 4 mg/ mL samples, prepared in 0.1% FA, were heated at 60°C for 18 hr. Deamidated samples were prepared by storing insulin at 10 mg/ mL in 0.1% FA for 1 month (Rojasajakul, 2007). All samples were diluted to 1 mg/ mL as required prior to analysis. All samples were prepared and measured in triplicate.

2.5.3. Solid-State Sample Preparation

Subsequently, 7 mg insulin samples were stored at 60°C/ 75% relative humidity (RH) for 1, 3, 5 and 7 days to promote solid state degradation. Kilner® jars were used to store samples in a sealed environment, with constant RH conditions maintained using saturated sodium chloride solutions. The samples were placed in bespoke 3D printed Eppendorf holders (3D Jake) inside the Kilner® jars, and the stability chambers were placed in an oven at 60°C. Prior to analysis the solid-state degraded samples were reconstituted in 0.1% FA to a target concentration of 1 mg/ mL and centrifuged for 2 min at 14,000 rpm using a Mikro 120 Centrifuge (Hettich) to separate insoluble particles. All samples were prepared and measured in triplicate.

2.5.4. Disulfide Bond Cleavage

Dithiothreitol (DTT) was used as a reducing agent to separate the insulin A- and B-chains via disulfide cleavage. DTT was prepared in 100 mM phosphate buffer pH 8.0 with 6M urea and added to samples at a final DTT concentration of 50 mM. Urea was used to unfold the protein to enable exposure of the disulfide bonds to the surface of the protein and ensure complete reduction. All insulin standard and degraded samples were prepared in the 50 mM DTT solutions at a target concentration of 1 mg/ mL. Where degradation was performed in the solution state, as in the case of the deamidated samples, the samples were diluted to a final insulin concentration of 1 mg/

mL and final DTT concentration of 50 mM. The samples were then heated on a ThermoMixerTM Comfort (Eppendorf) at 65°C for 30 min at 300 rpm. Samples were centrifuged for 2 min at 14,000 rpm using a Mikro 120 Centrifuge (Hettich) and analysed in triplicate.

2.5.5. Circular Dichroism Spectroscopy

CD spectroscopy was performed using a ChirascanTM CD spectrometer (Applied Photophysics) fitted with an AWC 100 cooler (Julabo) and a TC 125 temperature controller (Quantum Northwest Inc.). The spectral measurement range used was 200-260 nm, with a bandwidth of 1 nm, a step-size of 1 nm and a time-per-point of 0.5 s. Each spectrum was recorded in triplicate, background subtracted and averaged. The spectrum of a blank sample was also recorded and subtracted from the averaged sample spectra. 0.01 cm pathlength Quartz Suprasil demountable cells (Hellma Analytics) were used. Data visualisation was performed using OriginPro 2022b graphing and analysis programme (OriginLab Corporation) and spectral smoothing was performed using the Savitzky-Golay method, with a polynomial order of 2 and 8 points to the window.

2.5.6. Size Exclusion Chromatography

An Agilent 1260 Series HPLC system with an AdvanceBio SEC 130Å, 2.7 µm, 7.8 x 300 mm (Agilent Technologies) column were used for the chromatographic separations. The mobile phase (MP) consisted of 65% L-arginine (1 g/ L)/ 20% acetonitrile (ACN)/ 15% acetic acid. An isocratic elution method was used, with a total run time of 12 min required. A flow rate of 1 mL/ min and injection volume of 5 µL were used. 280 nm was used as a protein specific detection wavelength. OriginPro

2022b (OriginLab Corporation) was used for graphing chromatograms, and GraphPad Prism 8.4.3 was used for data analysis and graphical representation of results.

2.5.7. Liquid Chromatography – Mass Spectrometry

For RP-HPLC, an Agilent 1260 Series HPLC system was used, while LC-MS analysis was carried out using a Waters Acquity I-class UPLC coupled to a Vion IMS Mass Spectrometer (Waters Corporation), m/z range of 0 - 1250 m/z . All analysis was performed in positive ion mode. A Poroshell 120 SB-C18, 2.7 μm , 4.6 x 150 mm (Agilent Technologies) column was used for chromatographic separations. MP A consisted of 95% H_2O , 5% ACN and 0.1% formic acid (FA) and MP B consisted of 5% H_2O , 95% ACN, 0.1% FA. The FA was used as a weak ion-pairing reagent for adequate adsorption of protein to the stationary phase, also adjusting the MP pH to approximately 2.6. A gradient elution method was used, where MP B was linearly increased from an initial content of 20% to 35% over 4 min, returned to 20% MP B in 1 min and held for 3 min. The flow rate was 1 mL/ min and a total injection volume of 5 μL was used. The HPLC column was heated at 60°C. 280 nm was used as a protein specific detection wavelength. Visualisation and data analysis were performed as in section 2.4.6.

2.6. Results and Discussion

In order to track the different degradation pathways that insulin may follow under stressed conditions, the first part of this investigation sought to establish the ability of several techniques to characterise changes to the insulin native structure. Insulin samples were subjected to chemical denaturants, heat and acidic conditions to stimulate degradation and compared subsequently with standard reference samples for identification of changes to the native insulin structure.

Firstly, far-UV CD spectroscopy was assessed for its ability to discriminate between insulin in its native fold and in unfolded states. A representative spectrum of a 1 mg/mL insulin standard sample is shown in black in Figure 2.1.

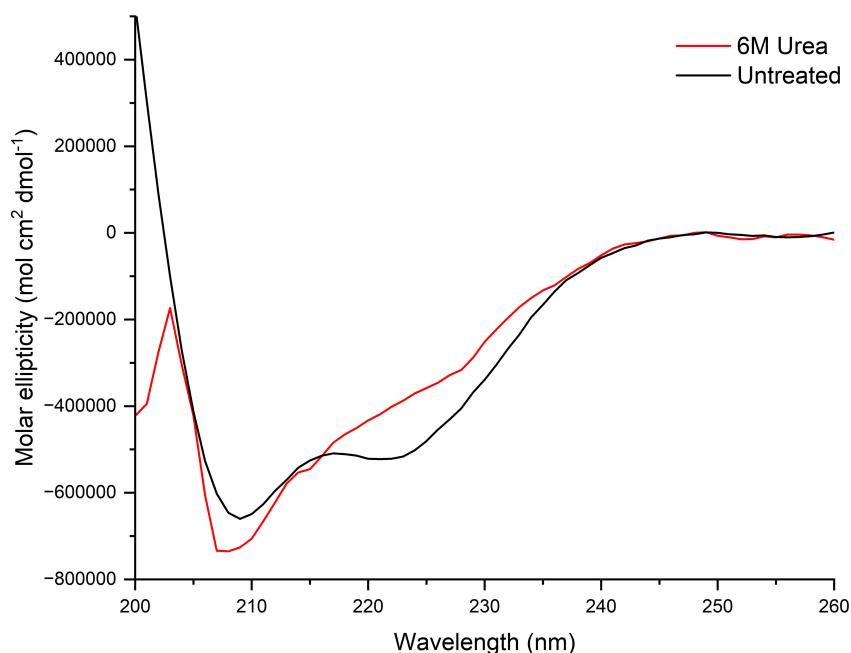


Figure 2.1. Representative far-UV CD spectra of a 1 mg/mL insulin sample in 6M urea (red) and an untreated 1 mg/mL insulin standard sample (black).

The presence of two negative bands at ~ 223 and 209 nm indicated that native insulin under these conditions was predominantly helical in nature, as expected (Bryant *et al.*, 1993). Chemical denaturation was performed by incubation of insulin in 6M urea at room temperature for 4 hours. Urea is known to cause a disruption of native hydrogen bonds along the polypeptide backbone, thus causing peptide and protein unfolding (Bennion and Daggett, 2003). This can be clearly observed in the spectrum of a chemically degraded 1 mg/mL insulin sample shown in red in Figure 2.1. The unfolding was manifested by the loss of the negative band at 223 nm and the complete loss of spectral resolution below 200 nm (not shown). The negative band at 209 nm was present in the chemically degraded insulin spectrum, indicating that the insulin

molecule was resistant to complete unfolding by high urea concentrations. The presence of the 209 nm band suggests some helical content was retained, shown previously to be the result of the formation of a partially unfolded monomeric species, known to precede aggregation and fibrillation (Ahmad *et al.*, 2004).

The next degradation pathway investigated was aggregation and as such, SEC was used to separate native insulin from higher molecular weight species (HMWS) that may form during degradation. The compendial mobile phase, consisting of 65% L-arginine (L-Arg), 20% ACN and 15% acetic acid, was utilised in this investigation. Under these conditions any non-covalent aggregates present in solution will dissociate and insulin exists exclusively as a monomer, while interactions with the stationary phase are minimised (Tantipolphan *et al.*, 2010). Therefore, any additional peaks present in the chromatogram are the result of covalent aggregation or fragmentation that occurred under stressed conditions.

A representative chromatogram of a 1 mg/ mL insulin standard sample is given in black in Figure 2.2. The insulin monomer appeared as a single peak in the spectrum and was monodisperse under these conditions. Degraded samples were also prepared by heating 4 mg/ mL insulin at 60°C overnight. The samples were diluted to 1 mg/ mL and analysed using SEC. A representative chromatogram of a heat stressed sample is given in red in Figure 2.2. As expected, the insulin monomer was again observed to be present, however, an additional peak of lower intensity was found to elute approximately 1 min earlier than the monomer peak, indicating the presence of a HMWS in the sample. It is clear, therefore, that the stressed conditions caused insulin to undergo covalent aggregation, where the HMWS present were likely covalent insulin dimers (CIDs) (Hjorth *et al.*, 2016).

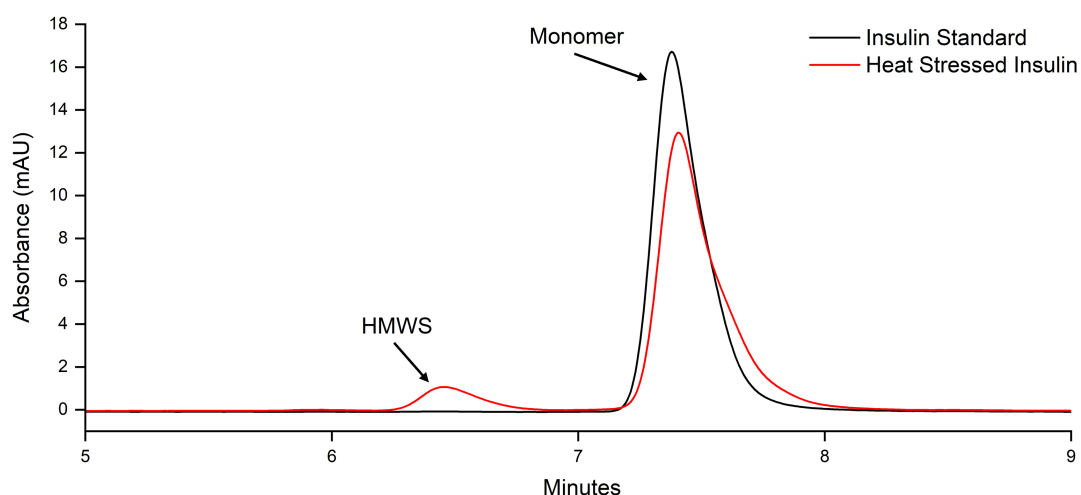


Figure 2.2. Representative SEC chromatogram of 1 mg/ mL insulin standard sample (black) and 4 mg/ mL insulin sample heated at 60°C overnight and diluted to 1 mg/ mL prior to analysis (red).

Finally, LC-MS was investigated for its ability to separate and identify primary structural modifications of the insulin molecule. A particularly important chemical degradation pathway for insulin is deamidation. To promote deamidation, 10 mg/ mL insulin samples were prepared in 0.1% FA and stored at room temperature for 1 month (Rojasajakul, 2007). The deamidated samples were diluted to 1 mg/ mL prior to analysis with LC-MS.

A representative mass spectrum of a 1 mg/ mL standard insulin sample is given in Figure 2.3. The predominant ions present appeared at 968.79 and 1162.34 m/z . Given the low pH of the solution (pH $\sim$ 2.6), it is likely that these peaks represent insulin in the +6 and +5 charge states, respectively. Two unique species were observed to elute in the RP chromatograms of the deamidated samples (see Figure S2.1 in Appendix 1). These were separated on the reverse phase column, and the +5 charge ions of the native insulin and the deamidated species are given in Figure 2.4a and Figure 2.4b, respectively.

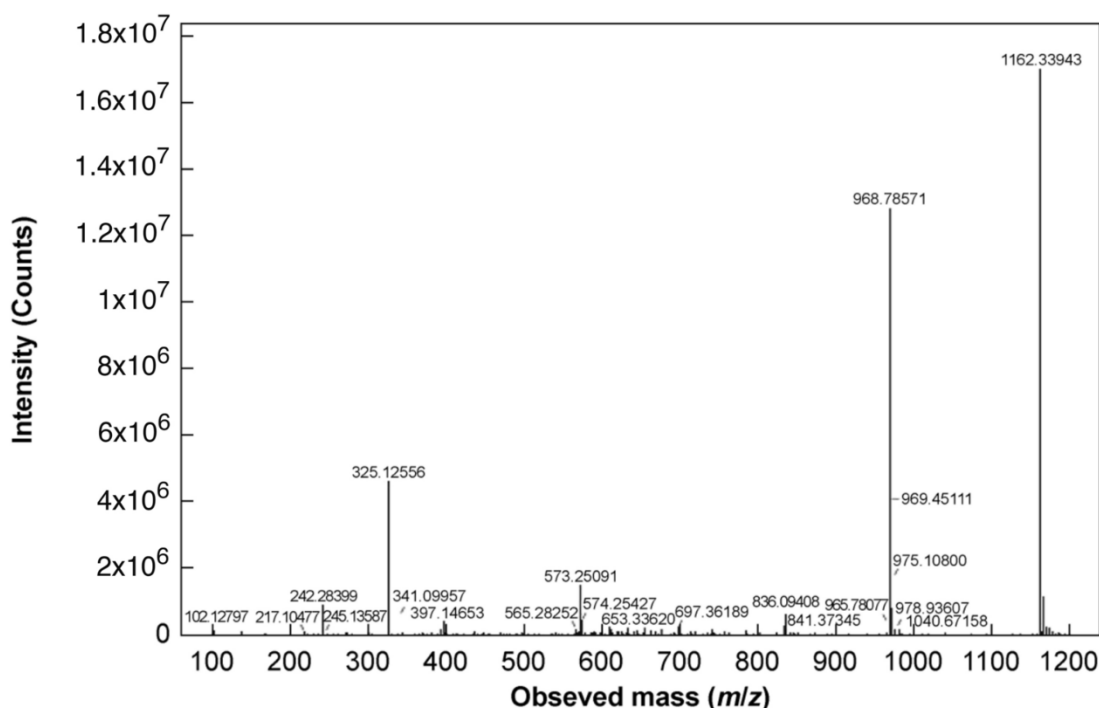


Figure 2.3. Representative mass spectrum of 1 mg/mL insulin standard sample, in the m/z range of 0 – 1250 m/z . MS analysis was conducted in positive ion mode.

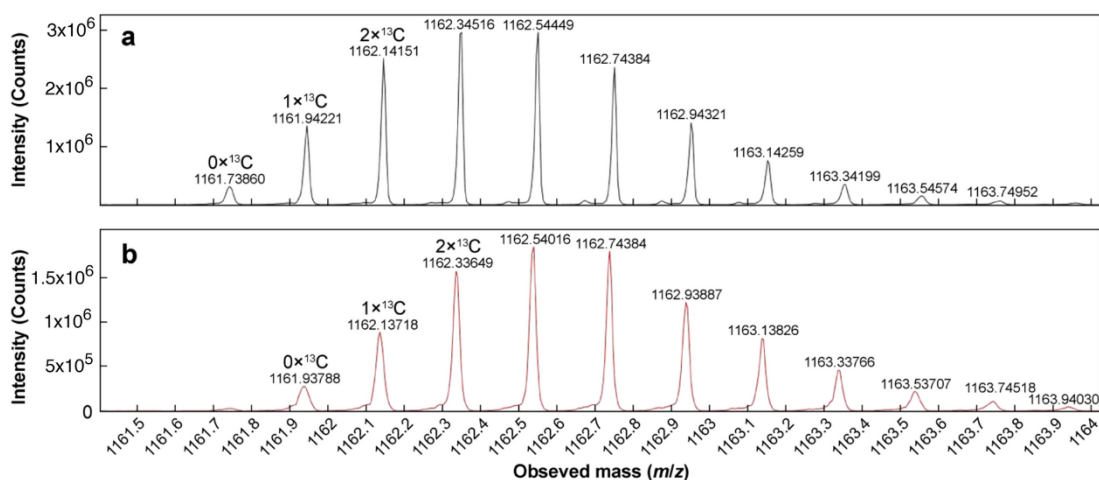


Figure 2.4. Peak clusters for the +5 charge states from representative mass spectra of (a) native insulin and (b) degraded species. The monoisotopic peak and the ^{13}C contributions for the first two isotopic peaks are indicated. MS analysis was conducted in positive ion mode.

From Figure 2.4a, the monoisotopic peak of the +5 charge state peak cluster for the native insulin was found to appear at 1161.74 m/z , while the peak cluster was centred

at 1162.35 m/z , similar to that seen previously with the insulin standard sample in Figure 2.3. However, in the mass spectrum of the degraded species, seen in Figure 2.4b, a clear shift in the positions of all the ions can be seen, with the monoisotopic peak appearing at 1161.94 m/z , while the centre of the peak cluster shifted to 1162.54 m/z . This corresponded to an increase in 0.2 m/z for the +5 charge state, and an overall increase in mass of 1 Da for the degradant species relative to the native insulin. Given that deamidation causes hydrolysis of an amide group to a carboxyl group, an increase in mass by 1 Da was expected and is confirmation that the new species observed is indeed the product of deamidation (Gervais, 2016). Analysis of the +6 charge states also indicated an increase in mass of 1 Da, as shown in Table S2.1 in Appendix 1.

To identify the site of deamidation, insulin was separated into its constituent A- and B-chains using DTT. DTT is a dithiol reducing agent commonly used to reversibly reduce disulfide bonds in proteins (Alliegro, 2000). To ensure complete reduction, DTT was used in large excess and combined with 6M Urea to unfold the insulin molecule and expose all disulfide bonds to the surface. Representative chromatograms of standard and deamidated insulin samples reduced with 50 mM DTT are given in Figure 2.5.

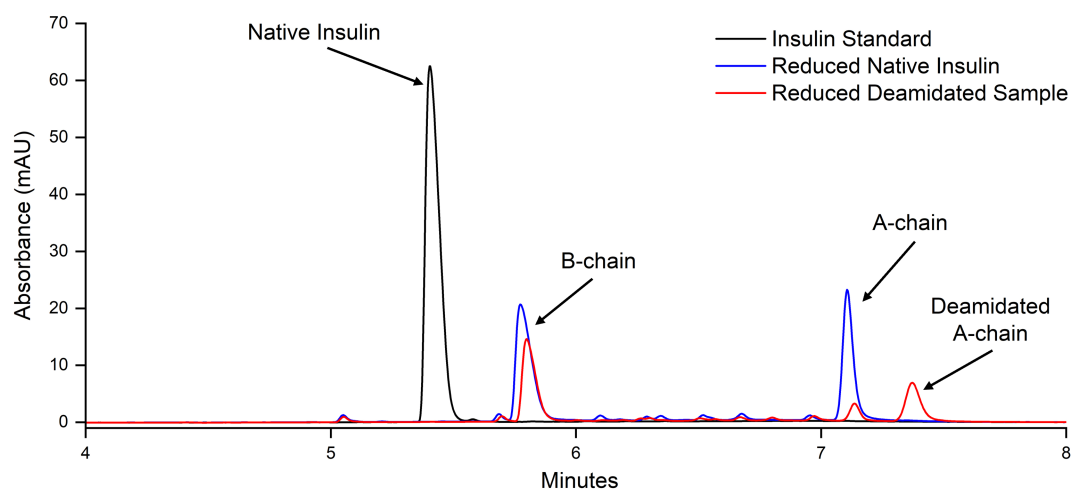


Figure 2.5. Representative RP-HPLC chromatograms of a 1 mg/ mL insulin standard (black) sample and native (blue) and deamidated (red) insulin samples in 50 mM DTT and 6M Urea heated at 65°C and stirred at 300 rpm for 30 min.

A single peak was observed to elute at 5.4 min in the chromatogram of a standard, untreated insulin sample, representing the native insulin molecule, as seen in Figure 2.5. However, complete loss of the peak at 5.4 min occurred on treatment of a standard insulin sample with 50 mM DTT, while 2 new species eluted at 5.8 min and 7.1 min, respectively. The loss of the native insulin peak and appearance of two unique peaks indicated successful cleavage of the disulfide bridges and presence of the separated A- and B-chains in solution. The identities of the peaks were elucidated with MS analysis, where representative mass spectra of the peaks at 5.4 min and 7.1 min are given in Figure 2.6a and Figure 2.6b, respectively. The predominant ions present in the mass spectrum of the peak at 5.4 min appeared at 1144.24 m/z , 858.43 m/z and 686.75 m/z . As the B-chain has a molecular weight of approx. 3400 Da, these ions likely represent the +3, +4 and +5 charge states of the B-chain. As seen from Figure 2.6b, multiple charging was evident from the presence of ions of low intensity at 1203.5 m/z and 808.99 m/z , suggesting the existence of the A-chain in +2 and +3 charge states.

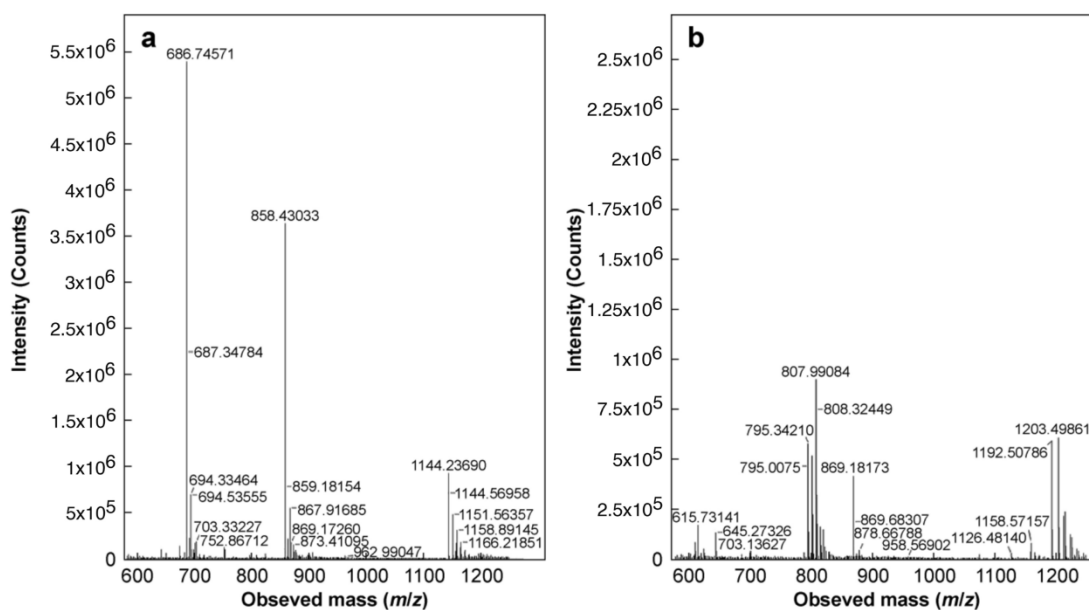


Figure 2.6. Representative mass spectra of (a) the peak at 5.4 min and (b) the peak at 7.1 min in the RP chromatograms over a mass range of 600 – 1250 m/z for a native insulin sample treated with 50 mM DTT and 6M urea heated at 65°C and stirred at 300 rpm for 30 min. MS analysis was conducted in positive ion mode.

The reduced deamidated insulin was also found to undergo complete disulfide cleavage, with the B- and A-chains eluting at 5.8 and 7.1 min, respectively, as was observed in the reduced native sample. However, as shown in red in Figure 2.5, the intensity of the A-chain peak was reduced significantly, and an additional species eluted at 7.4 min. As amide groups are more polar than carboxylic acid groups, the deamidated form of the insulin chains are predicted to have a greater affinity for the reverse phase column than the native forms, and it is likely, therefore, that the peak at 7.4 min represented the deamidated A-chain. This was confirmed from the mass spectrum of the peak at 7.4 min, where a clear shift of 0.33 m/z in the peak cluster of the +3 charge state can be seen relative to that of the peak at 7.1 min. This corresponded to an overall mass increase of 1 Da on the A-chain, which was shown previously to occur during deamidation. No evidence of deamidation was found on

the B-chain, confirming that the deamidation occurred exclusively on the A-chain. Representative mass spectra showing the peak clusters for the +3 charge states of the peaks at 7.1 min and 7.4 min of the deamidated samples are shown in Figure 2.7a and Figure 2.7b.

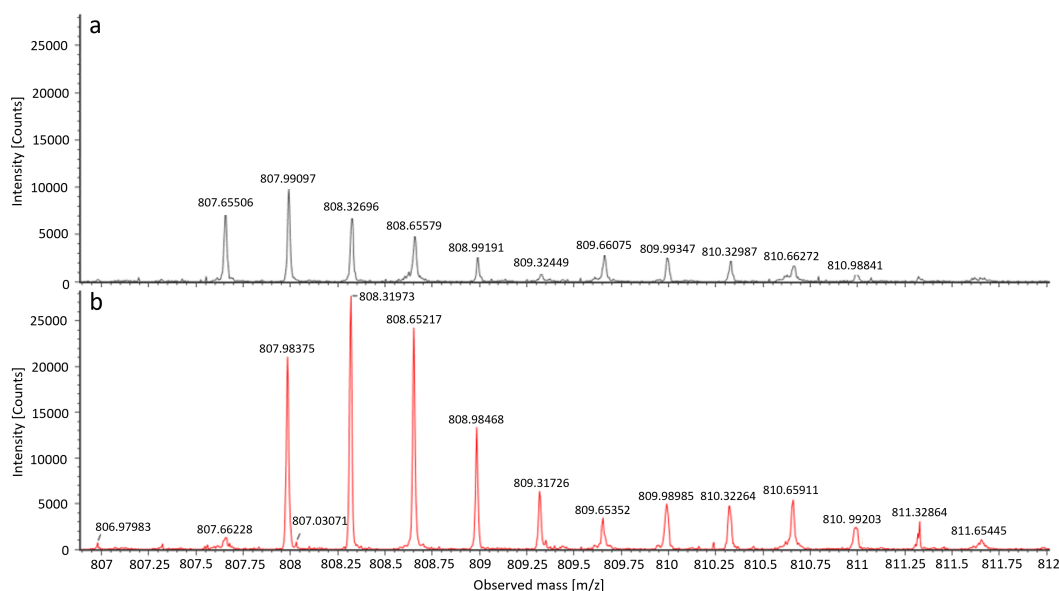


Figure 2.7. Peak clusters for the +3 charge states of the insulin A-chain from representative mass spectra of (a) peak present at 7.1 min and (b) peak present at 7.4 min for a deamidated insulin sample treated with 50 mM DTT and 6M urea heated at 65°C and stirred at 300 rpm for 30 min. MS analysis was conducted in positive ion mode.

Subsequent to establishing the techniques described above and demonstrating their utility in characterising various known degradation products of insulin, the authors sought to determine whether these techniques could be used effectively to investigate degradation in the solid state. To do so, insulin was incubated at 60°C/ 75% RH in the solid state for 1 week. The samples were prepared in 0.1% FA at 1 mg/ mL and analysed after 1, 3, 5 and 7 days using CD, SEC and RP-HPLC.

Visual inspection of the samples, shown in Figure 2.8a, found that the insulin samples underwent discolouration over time, transitioning from a bright white to a dull yellow colour. This change in colour was coupled with an increase in the number of insoluble particles present in the samples over the incubation period. These physical changes to the samples served as direct evidence of degradation occurring.

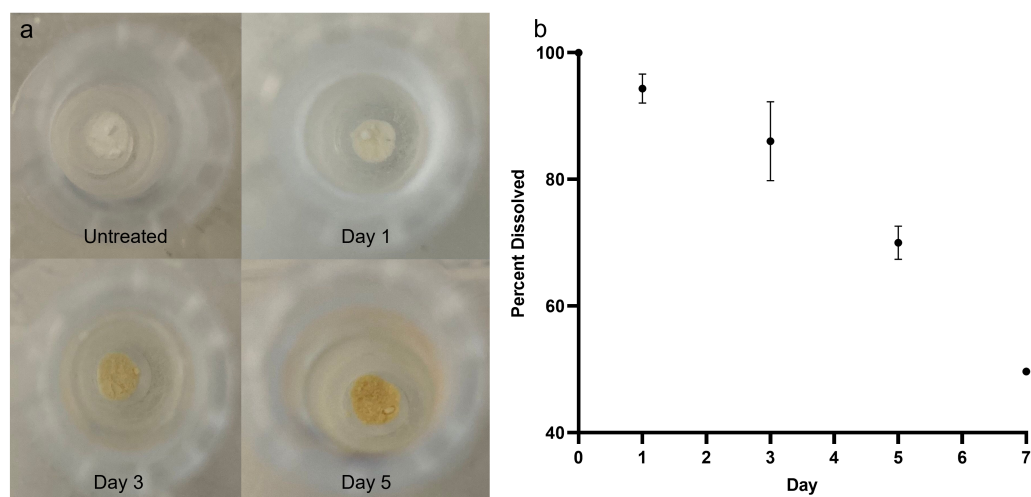


Figure 2.8. (a) Appearance of solid-state insulin samples and (b) percent of insulin dissolved upon exposure to 60°C/ 75% RH for 1, 3, 5 and 7 days, as determined by RP-HPLC (n = 3).

To assess the nature of the degradation, the secondary structure of the samples after reconstitution was first investigated using CD spectroscopy, where representative spectra of the samples at each time point and an untreated sample are given in Figure 2.9. No qualitative differences in spectral features were observed in the spectra of the samples stored at 60°C/ 75% RH and the untreated samples, indicating any degradation that occurred in the solid-state did not majorly affect the ability of the insulin molecule to fold into an ordered structure. To investigate if there were quantitative differences between the spectra, the relative intensities of the bands at 223 nm and 209 nm were determined (Table 2.1.). All of the stressed samples were found

to have a reduction in the 223 nm peak relative to native insulin in acidic conditions. Changes in the 223:209 nm ratio have been found to be related to the association state of insulin in solution, and, therefore, the formation of aggregates as a result of the stressed conditions may have resulted in a change in the hydrophobicity of the environment surrounding the helices of the stressed samples and caused a decrease in the 223 nm bands (Sklepari et al., 2016).

Table 2.1. Relative intensities of the 223 nm and 209 nm bands in the far-UV CD spectra of insulin samples exposed to 60°C/ 75% RH for 1, 3, 5 and 7 days and untreated samples, n = 3.

Sample	223:209 nm
Day 1	0.79:1
Day 3	0.81:1
Day 5	0.79:1
Day 7	0.79:1
Untreated	0.84:1

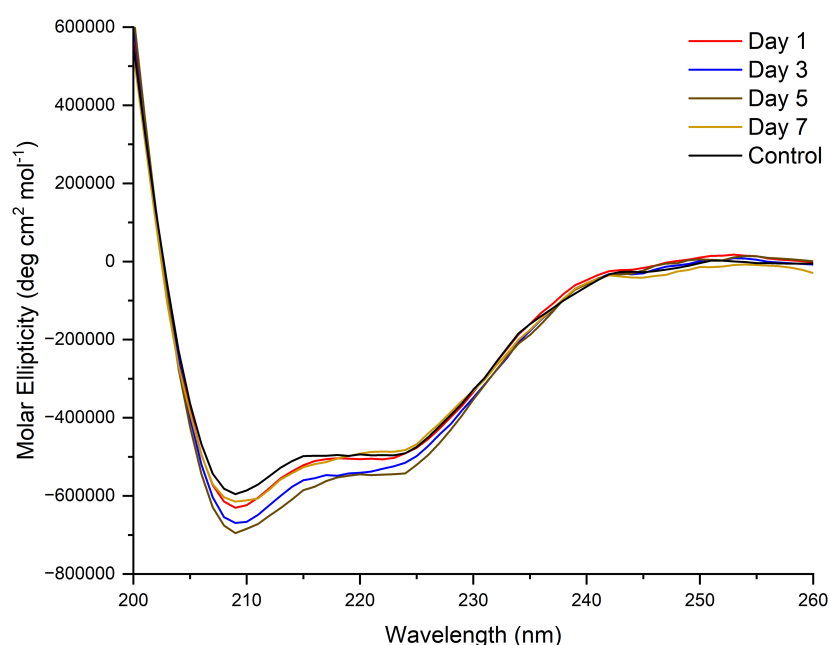


Figure 2.9. Representative far-UV CD spectra of 1 mg/ mL insulin samples heated in the solid state at 60°C/ 75% relative humidity (RH) for 1 day (red), 3 days (blue), 5 days (brown) and 7 days (yellow) and a 1 mg/ mL untreated sample (black).

To assess the aggregation profile of the samples, SEC was performed. The results of the SEC analysis are given in Figure 2.10. Initially, the samples were observed to primarily form dimers, with the proportion of dimers present in the samples after Day 1 found to be 6.5%. The proportion of dimers grew rapidly between Day 1 and Day 3, composing 17% of the samples by Day 3. This initial rapid growth, however, was observed to slow and by Day 7 dimers composed 21.1% of the samples. Larger oligomers were also found to be present by Day 3, composing 4.6% of the samples, while this increased to 11.1% by Day 7 (see Figure S2.2 in Appendix 1). These results, coupled with the increase in insoluble particles over time, indicated that exposure to the high temperature and humidity conditions caused the samples to undergo significant aggregation over the duration of the study.

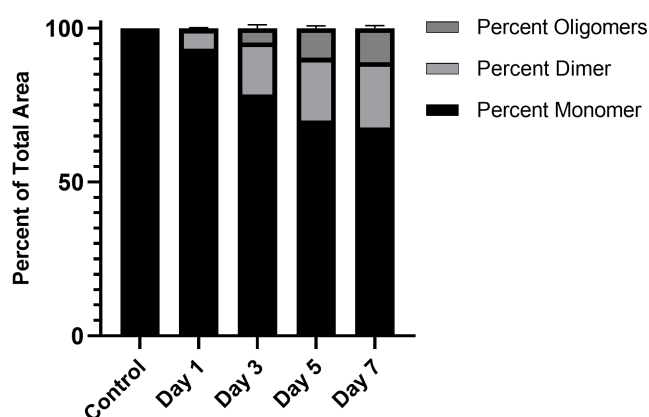


Figure 2.10. Peak areas of species present in SEC chromatogram of 1 mg/mL samples heated in the solid-state at 60°C at 75% RH for 1, 3, 5 and 7 days, along with an untreated sample as a percentage of the total peak areas, $n = 3$.

The mechanism by which insulin undergoes covalent aggregation in the solid state is complex. It has been previously reported by Strickley and Anderson that exposure to high moisture contents can lead to the formation of CIDs via a transamidation reaction (Strickley and Anderson, 1996, 1997). In the proposed mechanism, the C-terminal

carboxylic acid of the Asn A21 residue undergoes intramolecular nucleophilic attack on the amide side chain to form a cyclic anhydride intermediate, which can further react with water to form an Asp A21 deamidation product or with N-terminal Gly A1 or Phe B1 residues on adjacent insulin molecules to form Asp A21-Gly A1 and Asp A21-Phe B1 covalent dimers (Strickley and Anderson, 1996). In contrast, Costantino *et al.* have reported that insulin forms covalent aggregates via disulfide exchange, whereby the surface exposed A7-B7 disulfide bridge undergoes a β -elimination reaction to produce free thiols, which promote the formation of intermolecular disulfide bridges with adjacent insulin molecules (Costantino, Langer and Klibanov, 1994a). However, the solubility of the samples in different reducing and non-reducing solutions was the basis for the observations made, while no discriminatory analytical techniques were used to confirm the nature of the intermolecular interactions occurring. Given that the transamidation reaction stops with the formation of CIDs, it is likely that disulfide exchange is responsible for the formation of the oligomeric species observed in this investigation, while both mechanisms may still be occurring simultaneously to form CIDs (Brange and Langkjoer, 1993).

To investigate the mechanism of covalent aggregation, disulfide bond reduction with DTT was performed. As shown previously, reduction of the disulfide bonds in insulin with a large excess of DTT causes complete separation of the A- and B-chains. If the aggregation occurred via disulfide exchange, the aggregates should be broken down into the constituent A- and B-chains of the insulin molecules involved and will be separated by SEC. If, however, species larger than A- and B-chains are observed to elute, this is indicative of a nonreducible aggregation pathway occurring, i.e., transamidation. The SEC chromatograms of a native insulin sample and the solid-state degraded samples reduced with 50 mM DTT are given in Figure 2.11.

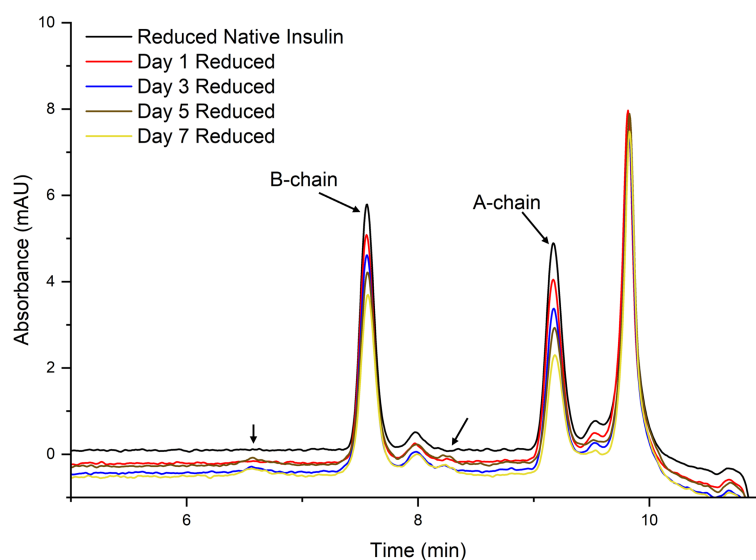


Figure 2.11. Representative SEC chromatograms of a native insulin sample (black) and insulin samples heated in the solid state at 60°C at 75% RH for 1 (red), 3 (blue), 5 (brown) and 7 (yellow) days, treated with 50 mM DTT and 6M urea and heated at 65°C and stirred at 300 rpm for 30 min.

From Figure 2.11, minimal differences were observable between native insulin and the solid-state degraded insulin on reduction with DTT. Given their presence in all samples, the peaks at 7.6 min, and 9.2 min were determined to be the reduced B- and A-chains respectively due to their retention times, while the minor peaks at 7.9 min and 9.5 min were also attributed to the B- and A-chains due to their absence from all negative control samples, although the identity of these species are unknown. The peak at 9.8 min was observed to be present in all insulin and negative control samples and was attributed to the phosphate buffer salts present in the reaction buffer. There were, however, two additional peaks at very low intensity present at 6.6 min and 8.2 min in the solid-state degraded samples that were absent from the reduced native insulin samples. These peaks were absent from the reduced Day 1 samples, however, were present in the reduced Day 3, Day 5, and Day 7 samples, indicating the presence of new species at very low quantities in the degraded samples on prolonged exposure to

the high temperature and humidity conditions used. These peaks may represent covalently linked A- and B-chains that were unaffected by the reducing conditions and suggest that there may have been a low level of transamidation occurring in the solid-state degraded samples, however, there is minimal evidence for this, and the predominant mechanism of covalent aggregation was via disulfide exchange.

Finally, RP-HPLC was performed to determine if chemical degradation was also initiated by the high temperature and humidity conditions. The results of the RP-HPLC analysis are given in Figure 2.12, where it can be seen that the insulin samples underwent significant chemical degradation. Similar to the SEC results, the degradation was observed to proceed quickly at first and slowed between Day 5 and Day 7. As described previously, one of the most prominent chemical degradation pathways for the insulin molecule is deamidation, and this was indeed observed to occur in the solid state, making up 10.1% of the samples by Day 7.

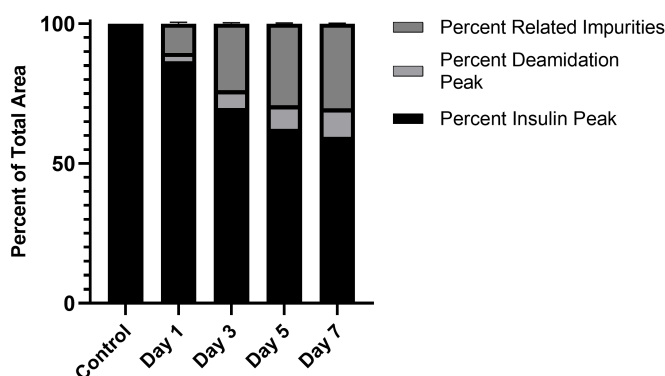


Figure 2.12. Peak areas of species present in RP-HPLC chromatogram of 1 mg/ mL samples heated in the solid state at 60°C at 75% RH for 1, 3, 5 and 7 days, along with an untreated sample as a percentage of the total peak areas, $n = 3$.

Deamidation in the solid state is known to occur predominantly via the Asn A21 residue, as detailed above, although it has been seen to occur at the Asn B3 residue at

75% RH (Pikal and Rigsbee, 1997). Deamidation was observed to be occurring on the A-chain for the solid-state degraded samples, as confirmed by reductive cleavage of the insulin A- and B-chains, described previously. Representative chromatograms of a native insulin sample and the solid-state degraded samples reduced with 50 mM DTT are given in Figure S2.3 in Appendix 1.

Multiple additional degradation peaks were observed to elute after the native insulin peak in the RP chromatograms of the solid-state degraded samples, labelled Percent Related Impurities in Figure 2.12 (see representative chromatogram of Day 7 sample in Figure S2.4 in Appendix 1). However, LC-MS analysis did not indicate the presence of any new species between 0-1250 m/z in this region. This suggests that the additional peaks may be due to the presence of the covalent dimers and oligomers observed in the size exclusion chromatograms. Indeed, the proportion of dimers and oligomers present in SEC analysis of the samples accounts well for the proportion of the additional peaks observed in the RP-HPLC analysis. Given the large size of these species, they may have been outside of the instrument m/z range and thus were not detected.

As can be seen from the above discussion, the pathways by which insulin undergoes degradation in the solid-state are similar to those observed in the solution-state. Although no unfolding was observed, as expected given the reduced conformational flexibility available in the solid state, the solid-state degraded samples were observed to undergo a significant amount of deamidation and covalent aggregation. Therefore, analytical techniques used for characterisation of peptides and proteins in solution can be translated to characterising degradation in the solid state, offering much higher

resolution and smaller sample sizes than techniques such as microscopy and thermal analysis, which are commonly used for characterising the solid-state.

2.7. Conclusion

The physical and chemical stability of insulin plays an important role not only in its therapeutic activity but also its immunogenicity. As such, this investigation sought to characterise several common degradation pathways that the insulin molecule undergoes. A short stability study was performed in which solid-state insulin samples were exposed to high temperatures and humidities for 1 week. The results of the stability study indicated as expected, that high temperature and humidity conditions have a significant effect on the stability of the insulin molecule in the solid-state. While CD spectroscopy showed that the native fold of the insulin molecule was unaffected, given the reduced conformational flexibility afforded by the solid state, the SEC and RP-HPLC analyses identified a large amount of covalent aggregation and deamidation, respectively. In particular, DTT enabled confirmation that disulfide exchange was the predominant covalent aggregation mechanism occurring in the solid-state under high temperature and humidity. This offers an advantage over the previous indirect approach of assessing sample solubility in different reducing and non-reducing solutions, allowing for direct detection of changes occurring on insulin's constituent chains. These results underlined the efficacy of the techniques studied for characterising the solid-state degradation pathways of insulin, offering an informative platform approach to investigating solid state degradation of peptides during oral drug product development.

Chapter 3: Kinetics of human insulin degradation in the solid-state:

An investigation of the effects of temperature and humidity

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3.1. Author Contributions

As lead author, I was responsible for developing and validating experimental methodologies, carrying out experimental investigations and performing formal analysis of the data. Furthermore, I was responsible for writing the original draft of the paper. Lorraine Bateman, Joseph O'Shea, and Abina Crean were responsible for supervising the progress of experiments, reviewing and editing the manuscript, and funding acquisition.

3.2. Abstract

With the growing interest in the development of oral peptide dosage forms, a comprehensive understanding of the factors affecting the stability of solid peptide drugs is critical. This study used human insulin as a model peptide to examine the individual and interactive effects of temperature and humidity on its solid-state stability. Insulin samples were stored at various combinations of temperature (25°C, 40°C, and 60°C) and humidity (11%, 33% and 75%) over 6 months and their stability was assessed. The primary degradation pathways observed were deamidation and covalent aggregation. The rates at which each degradation product formed were determined empirically and modelled using the humidity-corrected Arrhenius equation. Temperature was found to have a major impact on both deamidation and covalent aggregation rates, with the reaction rates increasing as the storage temperature was increased. However, the effect of humidity was found to be temperature dependent, where moisture induced degradation was minimal at 25°C and 40°C, but was an important factor at 60°C. This was further investigated using DVS, where a clear difference in the moisture sorption characteristics of the material was observed at 60°C relative to 25°C and 40°C. The findings of this investigation suggested that the effect of moisture on insulin deamidation and covalent aggregation rates was not a function of water content but rather the nature of the interaction between water and the material, and the physical state of the material may play a crucial role in this interaction.

3.3. Highlights

- Individual and combinatorial effect of temperature and humidity on solid-state stability of insulin over time assessed using humidity-corrected Arrhenius model.

- Significant amount of deamidation and covalent aggregation observed to occur.
- Rates of deamidation and covalent aggregation increased as storage temperature increased; effect of humidity found to be temperature dependent.
- Temperature dependent effect of humidity observed to be the result of a change in moisture sensitivity and overall moisture sorption characteristics of insulin samples stored at 60°C relative to 25°C and 40°C.

3.4. Introduction

For decades biopharmaceuticals, such as peptide, antibody and nucleic acid-based therapeutics, have received considerable attention for their potential to address unmet therapeutic needs (Kesik-Brodacka, 2018). Biopharmaceuticals offer distinct advantages, including high potency and high target selectivity, reducing the dose required to be administered and preventing unwanted side effects. These desirable therapeutic properties are attributed to their unique, complex structures, which allow for interactions to occur at only select sites of action and, therefore, enable target specific activity (Crommelin *et al.*, 2003). Their distinct structures, however, contain an inherent fragility, making them highly susceptible to degradation and loss of activity (Akbarian and Chen, 2022; Ingle and Fang, 2023). One of the most successful and indeed therapeutically important biopharmaceuticals is insulin, which is a peptide hormone used in the treatment and management of diabetes mellitus (Sims *et al.*, 2021). At present insulin is administered primarily via subcutaneous injection, however, a non-invasive route of delivery remains desirable (Zhang *et al.*, 2024).

As with other biopharmaceuticals, insulin is susceptible to physical and chemical degradation during production, dosage form manufacturing and storage. Attempts to overcome this limitation by formulating insulin in the solid-state have been reported using technologies such as spray drying, freeze drying and spray-freeze drying with varying results (Emami *et al.*, 2018). In the solid-state, by removing most of the water from the system through these drying processes, insulin experiences a reduction in both local and global molecular mobility, thereby reducing the potential for insulin molecules to lose their structural integrity and undergo deleterious interactions with other insulin molecules. However, insulin has been observed to follow similar degradation pathways in the solid-state as in the solution state, although differences in

molecular mobility and chemical environment result in different degradation kinetics. While considerable attention has been given to the development of an oral dosage form of peptides, including insulin, the major focus of research in this area has been on improving the poor oral bioavailability of peptides through the use of permeation enhancing excipients, such as salcaprozate sodium, and novel formulation strategies, such as ionic liquids (G. Chen *et al.*, 2022). However, there remains a gap in the understanding of how factors, such as storage and processing conditions, impact the stability of the solid form of peptide drug substances. Therefore, stability remains an important consideration in solid dosage form development.

Independent investigations have highlighted the effects of humidity, temperature and light on the degradation of insulin in the solid-state, however, no systematic investigation of the individual and combined effects of temperature and relative humidity on the extent and kinetics of insulin degradation has been performed to date (Sadrzadeh *et al.*, 2010; Strickley and Anderson, 1996, 1997; Flores-Fernández, Solá and Griebenow, 2009; Terryn *et al.*, 2006a). This investigation seeks to address this gap in knowledge, where solid-state insulin samples were exposed to a variety of temperature and humidity conditions. A previous investigation in our group identified deamidation, occurring via the insulin A-chain, and covalent aggregation, as a result of a disulfide exchange mechanism, to be the predominant degradation pathways occurring in the solid-state at high temperature and humidity conditions (Fagan *et al.*, 2024). These degradation pathways were further tracked in this investigation, where the individual and combinatorial effects of temperature and humidity on the rates of each degradation pathway were determined using the humidity-corrected Arrhenius equation. The rate constants associated with each degradation product were derived

empirically. Further, dynamic vapour sorption was utilized to characterize the moisture sorption behaviour of insulin and the effects of temperature on that behaviour.

3.5. Experimental Section

3.5.1. Materials

A single batch of recombinant human insulin powder (Lot No. 21J130), procured from Merck KGaA was used in this investigation. The material was observed to lack regular features with a large distribution of particle sizes above and below 50 μm , as confirmed using scanning electron microscopy (SEM), shown in Figure 3.5 below. All solvents and chemicals used for chromatography, including H_2O , acetonitrile (ACN), formic acid (FA), acetic acid (AA) and l-arginine (L-arg), were either HPLC or ACS reagent grade and were procured from Merck KGaA. The lithium chloride (LiCl), magnesium chloride (MgCl_2) and sodium chloride (NaCl) salts used for humidity control were all ACS reagent grade and were supplied by Merck KGaA.

3.5.2. Stability Study Samples

The stability study was performed by storing insulin samples at combinations of temperature (25°C, 40°C and 60°C) and humidity (11%, 33% and 75% RH) for 1, 3, 5 and 7 days, 2 weeks, 1 month, 3 months and 6 months. Kilner[®] jars were used to store samples in a sealed environment, with constant % relative humidity (% RH) conditions maintained using saturated salt solutions. LiCl, MgCl_2 and NaCl were used to maintain 11%, 33% and 75% RH, respectively (Greenspan, 1977). The samples were placed in bespoke 3D printed Eppendorf holders (3D Jake) inside the Kilner[®] jars, and the stability chambers were placed in ovens with controlled temperatures of 25°C, 40°C and 60°C. A schematic representation of the experimental set up can be seen in Figure S3.1 in Appendix 2. Prior to analysis, all samples were reconstituted to

a target concentration of 1 mg/ mL in 0.1% FA, pH 2.6, and centrifuged at 14000 rpm using a Mikro 120 Centrifuge (Hettich) for 2 mins. Samples were prepared and analysed in triplicate.

3.5.3. Chromatography

Chromatography was used to determine the respective amounts of degradation products formed over time, where deamidation products were characterised with reverse phase HPLC (RP-HPLC), and aggregates were characterised with size exclusion chromatography (SEC). An Agilent 1260 Series HPLC system was used for all size exclusion and reverse phase HPLC analyses. A gradient elution method was utilized for RP-HPLC analyses. Mobile phase A (MPA) consisted of 95% H₂O, 5% ACN and 0.1% FA, while mobile phase B (MPB) consisted of 5% H₂O, 95% ACN and 0.1% FA. MPB was linearly increased from 20% to 35% over a 4 min period, returned to 20% MPB in 1 min and held for 3 min. A flow rate of 1 mL/min and total injection volume of 5 µL were used. The separations were performed using a Poroshell 120 SB-C18, 2.7 µm, 4.6 x 150 mm (Agilent Technologies) column, which was heated at 60°C. 280 nm was used as a protein specific detection wavelength.

SEC was performed using the compendial mobile phase consisting of L-arg (1 g/ L), ACN and AA (65:20:15 v/v), with a flow rate of 1 mL/ min and injection volume of 5 µL. An isocratic elution method was used, requiring a total run time of 12 min, and 280 nm was used as the detection wavelength. Separation was carried out using an AdvanceBio SEC 130Å 2.7 µm, 7.8 x 300 mm (Agilent Technologies) column (Fagan *et al.*, 2024).

GraphPad Prism 8.4.3 was used for data analysis and graphical representation of results.

3.5.4. Water Sorption Analysis

The DVS Resolution (Surface Measurement Systems Ltd.) instrument was used to perform dynamic water vapour sorption (DVS) analysis. Humidity ramping experiments were performed on insulin powder samples at 25°C, 40°C and 60°C, respectively. The samples were first allowed to dry at 0% humidity for 6 hours. The samples were then exposed to linearly increasing humidity values up to 90% RH. A ramp rate of 10% RH per hour was used. The net change in mass versus % RH was plotted to determine the change in water sorption by insulin as a function of humidity. Graphing and analysis were performed using OriginPro 2022b (OriginLab Corporation).

3.5.5. Scanning Electron Microscopy (SEM)

The morphology of the insulin powder material used in this investigation were examined using a Jeol JSM-5510 scanning electron microscope (Jeol Ltd.). Micrographs were collected at x50 and x400 magnifications.

3.5.6. Degradation Kinetics

To model the effects of temperature and humidity on the rate of formation of degradation products, the linearised humidity-corrected Arrhenius equation was used (Naveršnik and Jurečič, 2016). This is shown in Equation 3.1, where k is the rate constant of the degradation pathway, A is the pre-exponential factor, E_a is the activation energy of the reaction, R is the gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is Temperature and B is the moisture sensitivity constant (between 0 – 0.1).

$$\ln k = \ln A - \left(\frac{E_a}{R}\right) \frac{1}{T} + B(RH) \quad \text{Equation 3.1.}$$

Prior to assessing the effects of temperature and humidity, the rate constant of each degradant was determined. The degradation pathways did not follow common reaction kinetics, such as first or second order. Instead, the relationship between the % of degradation product formed (%P) and time could be described using the power law shown in Equation 3.2.

$$\%P = kt^n \quad \text{Equation 3.2.}$$

$$\log(\%P) = \log k + n \log t \quad \text{Equation 3.3.}$$

Using the linear form in Equation 3.3, simple linear regression allowed for determination of the power of time, n, from the slope and the reaction rate constant, k, from the intercept.

3.6. Results and Discussion

3.6.1. Degradation Pathways

Solid-state insulin samples were stored at various combinations of temperature and humidity and sampled at specific time points over 6 months. Both deamidation and covalent aggregation were observed to occur, as determined using RP-HPLC and SEC, respectively. The extent of formation of these products in addition to the loss in total native insulin content over time are shown in Figure 3.1.

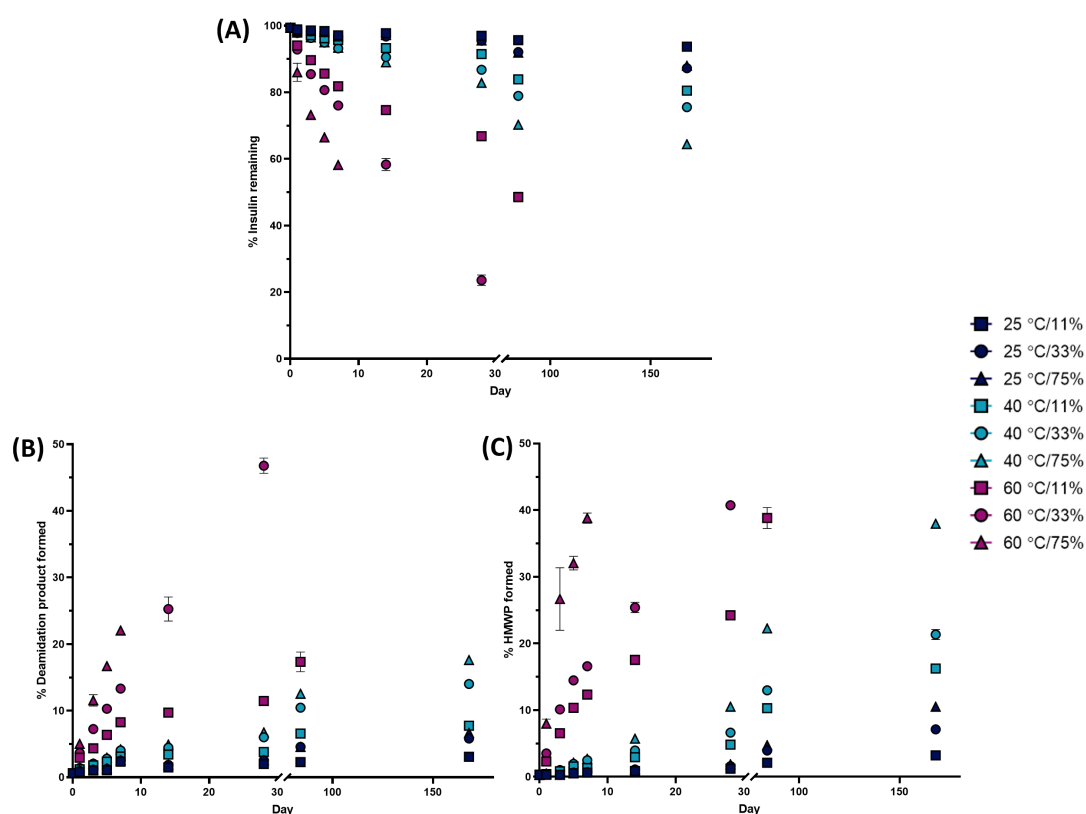


Figure 3.1. RP-HPLC results showing (A) % native insulin remaining, (B) % deamidation product formed, and SEC resulting showing (C) % higher molecular weight products (HMWPs) formed over time on exposure to different temperature and humidity conditions. All results were calculated as a percentage of total peak areas present in the RP and SEC chromatograms. Environmental conditions are given in the legend in the form temperature (°C)/ RH (%).

Insoluble particles were found to form in the samples over time, causing a decrease and in some cases a complete loss of the insulin solubility of these samples. Once a complete loss in solubility occurred for a particular set of samples, one more timepoint was collected and the stability study was halted for those conditions as no more HPLC analyses could be performed. This was found to occur predominantly in the samples stored at 60°C, where the time it took for a sample to become insoluble decreased with increasing RH, as can be seen in Figure S3.2 in Appendix 2. Formation of insoluble

particles in the solid-state have been reported previously to be the result of aggregation (Costantino, Langer and Klibanov, 1994b). To confirm this hypothesis, SEM analyses were performed to identify changes in particle morphology occurring due to extended storage at high temperature and humidity conditions. SEM micrographs of an untreated insulin sample, and an insulin sample stored at 60°C/ 75% RH for 2 weeks are given in Figure 3.2. The untreated, neat insulin material appeared to lack regular features and displayed a large particle size distribution, as seen in Figure 3.2A and Figure 3.2B. The sample stored at the elevated temperature and humidity conditions also displayed a large particle size distribution, however, it is evident from Figure 3.2C and Figure 3.2D that the insulin particles underwent significant aggregation and agglomeration during storage at 60°C/ 75% RH. It is likely that this change in particle morphology resulted in the decrease in sample solubility observed in this investigation.

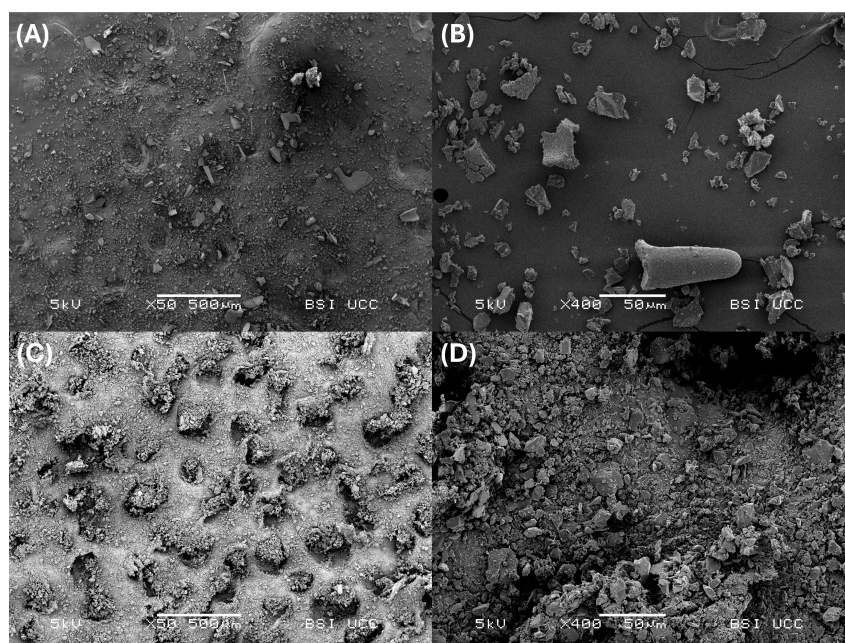


Figure 3.2. SEM micrographs of untreated insulin powder are given in (A) at X50 magnification and (B) at X400 magnification, while micrographs of insulin stored at 60°C/ 75% RH for 2 weeks are given in (C) at X50 and (D) at X400 magnification.

The degradation products exhibited non-linear degradation profiles in this investigation, as observed from Figure 3.1. Deamidation appeared to occur slowly initially in the samples stored at 25°C and 40°C, remaining below 5% of the total sample composition after 1 week of storage. However, the amount of deamidation product present increased after 2 weeks, where the rate of formation appeared to increase with increasing humidity at both 25°C and 40°C. On the other hand, deamidation occurred rapidly at 60°C, and the extent of degradation increased markedly from 11% to 75% RH. Similar degradation behaviour was observed for the formation of HMWPs. It is notable that in addition to the total amount of HMWPs increasing as the temperature and the RH conditions were elevated, there was also a change in the composition of the HMWPs, as can be seen from the results summarised in Table 3.1. At 25°C, the HMWPs were composed primarily of dimers, however, as the humidity was increased, the proportion of oligomeric species present increased over time. This trend was also evident at 40°C and 60°C, with the effect being more pronounced at higher temperature. This may be accounted for by an increase in molecular flexibility and mobility of reactive species as temperatures and humidities were increased, allowing for polymerisation reactions to occur more readily. A representative chromatogram of a sample stored at 60°C and 11% RH for 28 days is given in Figure S3.3 in Appendix 2.

Table 3.1. Average proportion of total HMWP content accounted for by dimers at different temperature and relative humidity conditions over the duration of the stability study. The presence of dimers and larger oligomers were determined by SEC, $n = 3$.

	Proportion of HMWPs present as dimers (%)								
Time	25/11	25/33	25/75	40/11	40/33	40/75	60/11	60/33	60/75
(Day)	(°C/%)	(°C/%)	(°C/%)	(°C/%)	(°C/%)	(°C/%)	(°C/%)	(°C/%)	(°C/%)
1	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	92.3
3	100.0	100.0	100.0	100.0	100.0	100.0	94.5	90.4	67.0
5	100.0	100.0	100.0	100.0	100.0	100.0	89.2	84.7	61.2
7	100.0	100.0	100.0	100.0	100.0	100.0	87.3	80.6	53.1
14	100.0	100.0	100.0	100.0	100.0	94.1	82.1	73.9	N/A
28	100.0	100.0	100.0	94.5	96.0	88.1	70.8	48.8	N/A
84	100.0	95.8	95.0	88.5	90.9	74.1	46.3	N/A	N/A
168	100.0	93.1	88.8	83.4	75.1	49.2	N/A	N/A	N/A

It is interesting to note that Strickley and Anderson previously reported that degradation of lyophilised insulin was governed by the formation of a cyclic anhydride intermediate, with two potential reaction pathways available: interaction with H₂O to form the A21 deamidated product, or interaction with an N-terminal amino acid on a neighbouring insulin molecule to form a transamidated dimer (Strickley and Anderson, 1996, 1997). However, a previous investigation in our group identified disulfide exchange as the predominant mechanism of covalent aggregation at 60°C/75% RH, indicating that deamidation was the favoured reaction pathway occurring via the cyclic intermediate (Fagan *et al.*, 2024). Alternatively, deamidation may have occurred via direct hydrolysis, without the formation of the intermediate species. This is further supported by the results of this stability study, whereby the formation of the

deamidation product would be expected to level off over time if it was in direct competition with HMWP formation. However, the formation of both the deamidation product and the HMWPs can be seen to increase over time under all conditions investigated, suggesting the degradation mechanisms did not occur via a common intermediate.

3.6.2. Degradation Kinetics

From the above discussion, it appeared that both the temperature and humidity conditions at which the samples were stored had an impact on the degradation pathways investigated. To assess the relative contributions of temperature and humidity to the rates of degradation, the humidity-corrected Arrhenius equation, shown in Equation 3.1, was used. To do so, first the reaction rate constants for each degradation pathway were determined. The overall reduction in native insulin content and indeed the formation of the individual degradation products did not appear to follow common zero, first or second order reaction kinetics. Pikal and Rigsbee, and Sadrzadeh *et al.* have previously proposed a square root of time kinetic model to account for the degradation kinetics of insulin in the solid-state at different temperatures, providing a mechanistic framework to explain this behaviour (Pikal and Rigsbee, 1997; Sadrzadeh *et al.*, 2010). However, the square root time kinetic model was suboptimal to account for the degradation behaviour observed in this investigation. As such, an empirical power law model was used to describe the change in % degradation products formed (%P) with time, as described in Equation 3.3. The rate constants derived from this analysis are found in Table 3.2.

Table 3.2. Rate constants determined for the formation of each degradation product in insulin samples stored under different temperature and humidity conditions.

Conditions		Deamidation Product		HMWP	
Temperature (°C)	RH (%)	k (%/day)	Model R ²	k (%/day)	Model R ²
25	11	0.720	0.943	0.239	0.994
25	33	0.738	0.943	0.225	0.969
25	75	0.751	0.923	0.203	0.918
40	11	1.139	0.944	0.403	0.987
40	33	1.277	0.992	0.511	0.984
40	75	1.234	0.989	0.482	0.996
60	11	2.965	0.982	2.948	0.948
60	33	3.187	0.978	2.369	0.945
60	75	5.047	1.000	8.734	0.965

3.6.3. Effect of Temperature on Rate Constants

The effect of temperature on the rate constants for each degradation product at the three humidity conditions studied can be seen graphically in Figure 3.3.

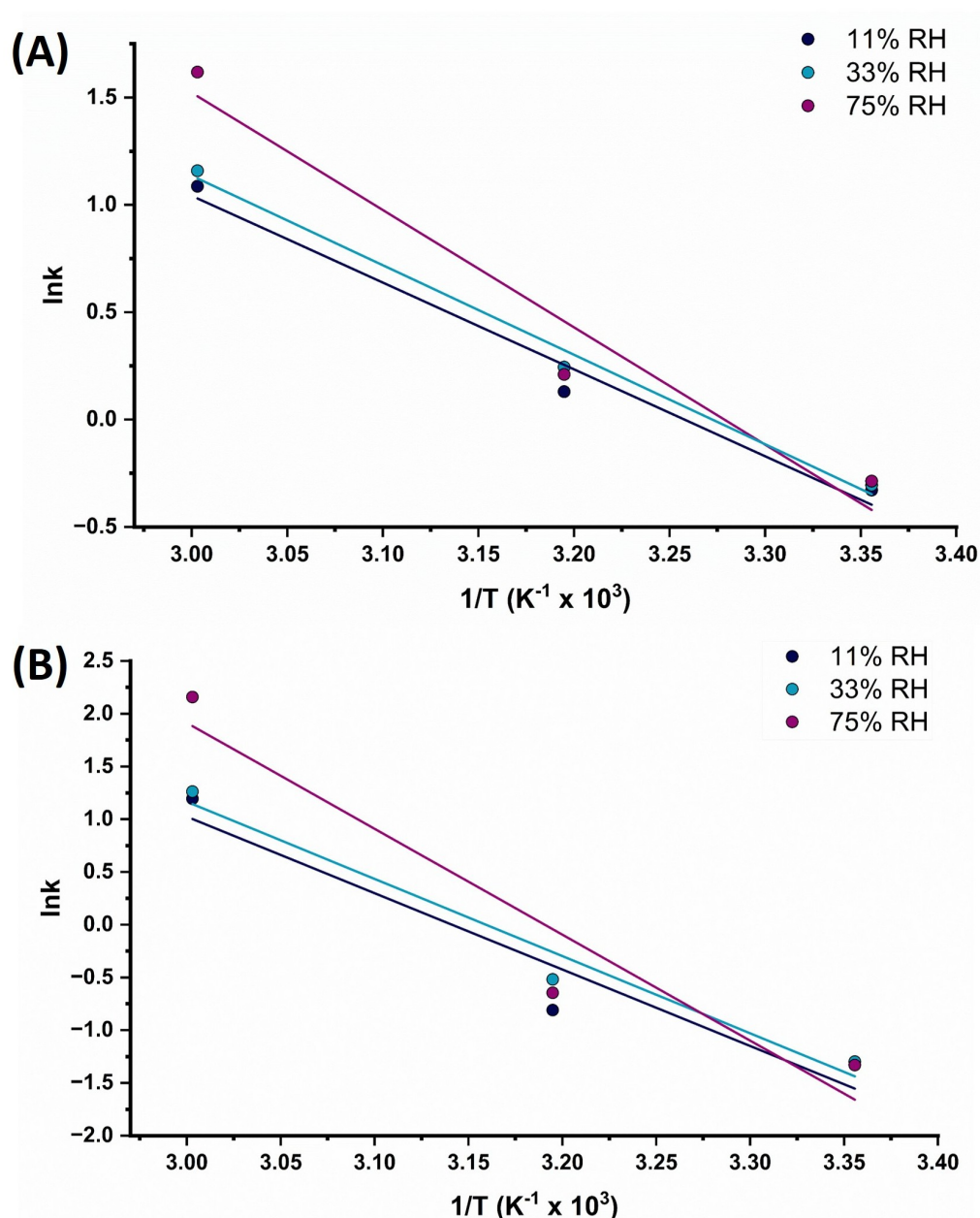


Figure 3.3. Change in rate constant as a function of temperature at three different humidity conditions for (A) deamidation product and (B) HMWPs.

As expected, increasing the temperature caused an increase in reaction rate constants for both degradation products identified. This is in agreement with a previous investigation performed by Sadrzadeh *et al.*, in which amorphous spray-dried insulin samples were exposed to a wide range of temperatures from 25 – 120°C at constant humidity (Sadrzadeh *et al.*, 2010). The rates of deamidation and covalent aggregation

were assessed using a square root of time model and were found to follow Arrhenius-like kinetics with degradation rates increasing as storage temperature increased.

As seen in Figure 3.3, at 25°C and 40°C, the reaction rate constants did not differ significantly at 11%, 33% and 75% RH, however, at 60°C, the reaction rate constants were significantly increased at 75% RH, relative to 11% and 33% RH ($p < 0.0001$). This effect appeared to be more pronounced for HMWP formation than deamidation as is evident from Table 3.2, where the rate constants for the deamidation pathway are higher than the aggregation pathway under all conditions studied except at 60°C/ 75% RH. At 60°C/ 75% RH the predominant degradation mechanism switched from deamidation to covalent aggregation, indicating that the covalent aggregation pathway occurring was more susceptible to the effects of moisture at this temperature than was the deamidation mechanism. These results suggest that temperature was a significant factor impacting both the deamidation rate and covalent aggregation rate in the solid-state, however, the effect of humidity appeared to be temperature dependent.

3.6.4. Effect of Humidity on Rate Constants

To quantify the effect of humidity on degradant formation, the moisture sensitivity constant, B , in the Arrhenius Equation was determined using the rate constants identified for each degradation product. The moisture sensitivity constant is a value between 0-0.1 that estimates the susceptibility of a material to degradation driven by moisture, where the smaller the value, the less sensitive the material is to the effects of moisture. As can be seen from Figure 3.4, for both degradation products observed, the humidity sensitivity constants were similar and near zero at 25°C and 40°C, indicating that humidity had minimal effect on the degradation rate constants at these temperatures. However, at 60°C, a notable increase in the magnitude of the humidity

sensitivity constant was observed relative to those at 25°C and 40°C. Both deamidation and HMWP formation appeared to be sensitive to moisture at 60°C, however, the effect was more pronounced for the covalent aggregation pathway, suggesting that moisture had a greater role in HMWP formation than in deamidation. It was hypothesised, therefore, that a physical change in the material during storage at 60°C, such as that observed in Figure 3.2, likely impacted the bulk material's sensitivity to moisture and consequently increased its susceptibility to degradation.

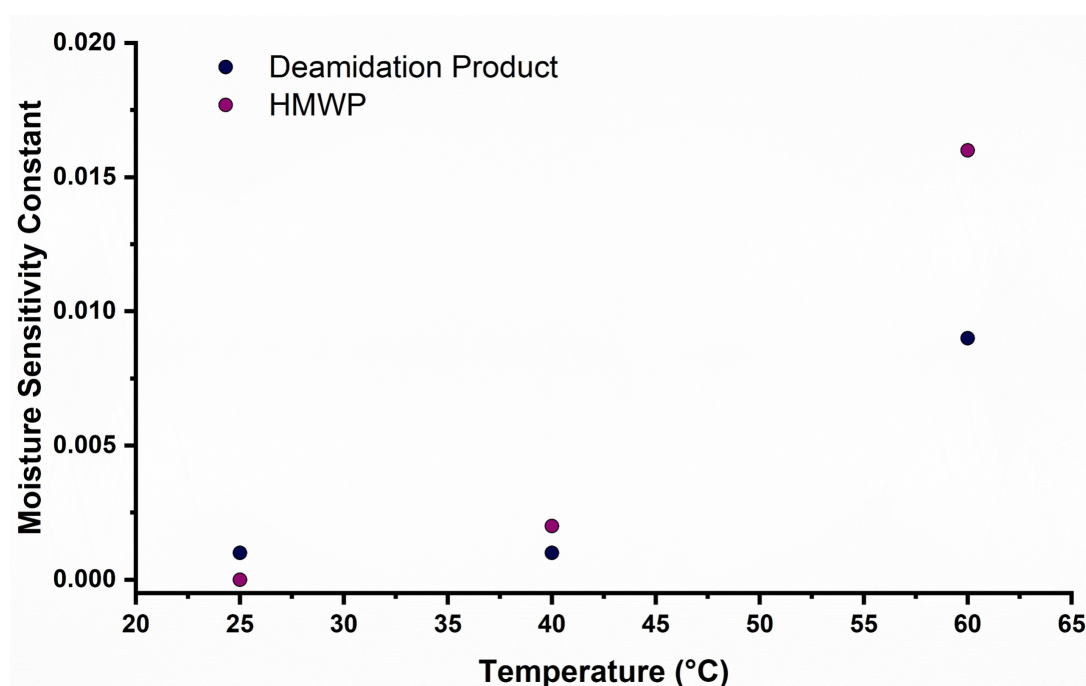


Figure 3.4. Effect of temperature on the moisture sensitivity constants associated with the deamidation products and HMWPs.

It is well known that the interaction of water with solid materials can have a major influence on both the physico-chemical and bulk properties of the material (Chakravarty and Nagapudi, 2021). The role of water, however, is complex and it can act in a number of ways when interacting with a solid material, including as a plasticiser, increasing the local and global mobility of molecules within the material, as a reactant in degradation processes and as a solvent/ reaction medium (Ohtake, Feng

and Shalaev, 2018). In the case of peptide and protein powders, the interaction with water is expected to be particularly complex, given their naturally heterogeneous physical and chemical structure (Shamblin, Hancock and Zografi, 1998). Further, it has been reported that water activity, which is a measure of the amount of water that is freely available to react, increases as the equilibrium temperature of the environment is increased, while the overall moisture content of a material decreases and, therefore, temperature is a confounding factor when studying water-solid interactions (Allada *et al.*, 2017; Labuza and Altunakar, 2020). As such, to assess the moisture sorption characteristics of insulin in the solid-state at 25°C, 40°C and 60°C, humidity ramping experiments were performed using DVS, and the results are given in Figure 3.5A.

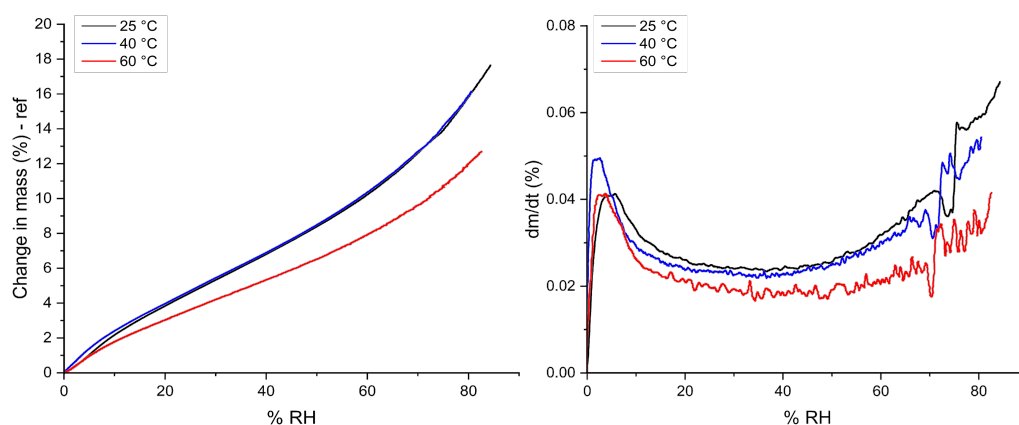


Figure 3.5. (A) Percent change in mass of insulin powder with increasing humidity at 25°C (black), 40°C (blue) and 60°C (red). Humidity was ramped from 0-90 % RH at a rate of 10% RH per hour. (B) Change in mass of insulin samples over time as humidity was increased from 0-90% at 25°C (black), 40°C (blue) and red 60°C (red).

As can be seen, the mass of the insulin samples, which is indicative of the moisture content, increased at increasing humidity independent of the chamber temperature. Additionally, the change in mass over time displayed a sigmoidal relationship at each temperature, with the rate of change in mass appearing to decrease at ~ 5% RH and

increase above $\sim 50\%$ RH for all samples. This can be seen clearly in Figure 3.5B, which shows the rate of change in mass of the insulin samples as a function of %RH. This increase in rate of moisture uptake at $\sim 50\%$ RH is indicative of a change in the nature of the moisture sorption, potentially a change from surface adsorption to bulk absorption. A possible explanation for this is that the water acts as a plasticiser, causing an increase in global molecular mobility, increasing the void volume and therefore increasing the surface area for absorption. However, given that the reaction rates of both degradation products did not appear to be impacted by humidity at 25°C and 40°C (Figure 3.4), the increase in rate of moisture sorption at elevated humidity did not appear to play a role in the degradation processes investigated, suggesting similar water activities at each temperature.

While overall the insulin samples showed a similar trend in sorption behaviour, there was a clear difference in the moisture sorption characteristics of the insulin sample heated at 60°C relative to those heated at 25°C and 40°C . At 60°C the rate of moisture uptake appeared to be slower and, indeed, the total amount of moisture absorbed by the insulin sample was reduced in comparison to the samples at 25°C and 40°C , respectively. The observed reduction in amount of moisture retained at elevated temperature is in agreement with the literature previously described above, and a possible explanation for this may be the agglomeration of insulin particles at 60°C , observed in Figure 3.2, causing a reduction in overall surface area for moisture sorption. In addition to the overall change in moisture sorption characteristics of the material at 60°C , relative to 25°C and 40°C , the increase in the moisture sensitivity observed in Figure 3.4 suggests that there was a change in the association state of water which increased its reactivity at this temperature. These findings highlight that the role of moisture in the degradation of insulin is not necessarily a function of the

total amount of moisture present, but rather the nature of the interaction between water and the material.

There have been numerous investigations studying the effects of humidity on the solid-state stability of insulin, however, the temperature dependent effect of humidity has not previously been reported. In their investigations, Costantino *et al.* and Flores-Fernandez *et al.* described the negative effect of moisture on lyophilized insulin samples, causing aggregation and secondary structural changes to occur (Costantino, Langer and Klibanov, 1994a; Flores-Fernández, Solá and Griebenow, 2009). However, while the samples were exposed to a variety of humidity conditions, ranging from 11% to 96% RH, the sample chambers were also stored at an elevated temperature of 50°C. It was assumed that the changes observed occurred due to the increased moisture content as the % RH was increased, however, physical changes in the samples which influenced their moisture sorption behaviour as a result of storage at 50°C cannot be ruled out. In another set of studies, Strickley and Anderson examined the effect of humidity and sample pH prior to lyophilization on the mechanism of degradation in the solid-state, where degradation rates were found to increase as % RH was increased from 11 – 96% at 35°C (Strickley and Anderson, 1996, 1997). In our investigation, however, the effect of humidity on degradation rates was found to be temperature dependent, where humidity was not found to be an important factor affecting degradation rates at 25°C and 40°C while at 60°C it had a pronounced effect. This is in agreement with the results reported by Pikal and Rigsbee, where it was observed that the rate constants for deamidation and covalent dimerisation of amorphous insulin samples stored at 25°C and 40°C were not affected by moisture, although 60°C was not investigated in this study (Pikal and Rigsbee, 1997).

It is important to note that the insulin samples prepared in these previous investigations underwent lyophilisation at different pH and solution conditions prior to storage at various temperature and humidity conditions. In this study, however, no lyophilisation was performed, instead insulin powder was tested as provided by the supplier. Considering the contrasting results described above, it is possible that the moisture sensitivity of insulin is affected not only by temperature, but also by lyophilisation, perhaps due to differences in the physical state of the material prepared by freeze drying and that without freeze drying that impacts the interaction between water and the material. As seen in this investigation, DVS is an invaluable tool for assessing the moisture sorption behaviour of materials, however, thermal analyses may also offer a key insight into bulk behavioural changes, such as phase transitions, that can occur as a function of temperature and humidity. Differential scanning calorimetry (DSC) is a technique often utilised to monitor physical changes in amorphous materials, where the temperature at which the material transitions from a glassy state to a rubbery state, called the glass transition temperature (T_g), is an important property that may be affected by temperature and humidity conditions. However, the use of T_g as a means of assessing the physical properties of large peptides and proteins remains problematic as the glass transition of these materials is typically spread over a very broad temperature range and the associated change in heat capacity is small relative other amorphous small molecule and polymeric materials (Pikal, Rigsbee and Roy, 2007). As such, few studies have been performed to assess the solid-state properties of insulin using DSC to date. Further investigations in this area may provide a means of overcoming this limitation and would prove invaluable for understanding the impact that storage conditions and indeed processing conditions have on the physical

properties of peptide and protein materials, offering a way to minimise potential stability concerns during storage and manufacturing.

The above findings suggest that, in addition to the storage conditions, the solid form and lyophilization conditions have a key role to play in the susceptibility of insulin to degradation in the solid-state. It is clear, therefore, that a holistic approach should be taken to understand the concomitant factors affecting the degradation of insulin and other peptides in the solid-state, and this will be vital for informing oral dosage form development, including formulation design, storage conditions and packaging requirements.

3.7. Conclusion

Given the renewed interest in oral peptide delivery in recent years, a holistic understanding of all factors affecting stability is vital for informing rational dosage form development. In this investigation, the authors sought to investigate the effects of temperature and humidity on the degradation kinetics of human insulin in the solid-state. Insulin samples were stored at 9 different combinations of temperature and humidity for 6 months, and the degradation products were investigated using RP-HPLC and SEC. Deamidation and covalent aggregation were observed to be the primary mechanisms of degradation under the conditions investigated. The effects of temperature and humidity on the degradation rates of both products were determined using the humidity-corrected Arrhenius equation, deriving the degradation rate constants empirically. The degradation rates were observed to increase as the storage temperature increased, as expected, however, the degradation rates were found to have a temperature dependent sensitivity to moisture. To characterise this further, DVS was used to assess the moisture sorption characteristics of human insulin at each temperature investigated. The total moisture content and rates of moisture uptake were

observed to decrease at 60°C, relative to those observed at 25°C and 40°C, respectively. This difference in moisture sorption behaviour offered an insight into the importance of temperature on the moisture sensitivity of the insulin molecule, which is a key consideration when developing an oral insulin dosage form, however, further investigations are required to fully understand these behavioural changes. A cognisance of the impact of storage and processing conditions on the degradation of insulin will be key for the development of a successful oral dosage form, and the results from this investigation offer an insight into the individual and potential combinatorial effects of temperature and humidity on insulin stability which may help to inform handling conditions and potential excipient requirements during formulation development.

Chapter 4: Direct compression peptide tablets: insights into the importance of permeation enhancer processibility and peptide stability

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4.1. Author Contributions

As lead author, I was responsible for developing and validating experimental methodologies, carrying out experimental investigations and performing formal analysis of the data. Furthermore, I was responsible for writing the original draft of the paper. Lorraine Bateman, Marcus O'Mahony, Abina Crean and Joseph O'Shea, were responsible for supervising the progress of experiments, equipment access, reviewing and editing the manuscript, and funding acquisition.

4.2. Abstract

Much of the remarkable advancements in oral peptide delivery have been achieved through the use of chemical permeation enhancers, such as sodium caprate (C10) and salcaprozate sodium (SNAC). However, co-formulation of peptides with permeation enhancers in an oral product introduces several processing and formulation challenges which require further investigation. This investigation sought to rationalise the development of direct compression insulin compacts, using C10 and SNAC as model permeation enhancers, respectively. The physical and mechanical properties of C10 and SNAC were first assessed to evaluate their suitability for processing via direct compression. Overall, C10 displayed passable flow character, however, poor tabletability, compactibility and compressibility profiles were obtained. SNAC, on the other hand, exhibited superior compaction properties, though its flow character was poor. Improvement in the compaction properties of both materials were observed on addition of commonly used direct compression excipients microcrystalline cellulose (MCC) and polyvinylpyrrolidone (PVP) K30, and formulations consisting of 72% C10/ SNAC, 20% MCC, 5% PVP and 3% insulin were selected for production of direct compression compacts at compaction pressures of 100 and 200 MPa. The compacts produced exhibited complete release within 30 min, and this release behaviour was not significantly affected by the compaction pressure used. Furthermore, the stability of insulin after compaction at 200 MPa, and on storage of the compacts at 40°C/ 75% RH for 1 month was assessed. Insulin displayed excellent physical stability to mechanical stress, where no evidence of unfolding or aggregation was identified. Moreover, on storage of the formulations at accelerated stability conditions for 1 month, a significant reduction in overall deamidation and aggregation tendency was observed on blending of insulin within the direct compression

formulations in comparison to raw insulin material stored under the same conditions, independent of the permeation enhancer used. These results offer a key insight into the influence that formulation components have on the manufacturability of direct compression peptide formulations and the stability of the peptide during compaction and storage.

4.3. Highlights

- The suitability of C10 and SNAC materials for direct compression was determined.
- C10 material exhibited passable flow and poor compaction properties, while SNAC exhibited acceptable compaction properties but poor flow.
- Addition of MCC and PVP improved compaction properties of both C10 and SNAC.
- Insulin was found to be stable to high compaction pressures.
- Physical and chemical stability of insulin improved in direct compression formulations during storage compared to raw material.

4.4. Introduction

Recent decades have seen considerable investment in the discovery and development of therapeutic peptides for the treatment of a variety of chronic indications (Fosgerau and Hoffmann, 2015). Therapeutic peptides often demonstrate improved selectivity, efficacy and toxicity profiles in comparison to small molecule therapies, while in some cases also offering greater stability and cheaper manufacturing costs than larger protein-based therapeutics (Xiao *et al.*, 2025). Despite longstanding bioavailability challenges, substantial advances have been made in the development of oral peptide formulations as an alternative to injectable products which currently dominate the market (Ganesh *et al.*, 2021). Among the leading strategies to enhance the oral bioavailability of peptides is the incorporation of permeation enhancers (PEs) into dosage forms to facilitate improved peptide permeation across the gastric and intestinal epithelia. This approach culminated in the breakthrough approval of Rybelsus[®], a tablet form of semaglutide, in 2019 (Maher *et al.*, 2019; Aroda, Blonde and Pratley, 2022). Due to their success, considerable attention has been paid to understanding the mechanisms of action and *in vivo* efficacies of different PE candidates (Maher and Brayden, 2021; Kim, Park and Na, 2022). However, the unique formulation and processing requirements associated with the co-formulation of peptides with PEs present several technical challenges that remain insufficiently investigated in the literature.

Given their numerous advantages, such as low manufacturing costs and good stability, tablets are the most prevalent oral dosage form (Sohail Arshad *et al.*, 2021). The tablet manufacturing process can be complex, involving several inter-connected unit operations, in which the raw material attributes and processing conditions play a critical role in determining the quality of the final drug product (Shi *et al.*, 2021). Of

the main processing techniques used to produce tablets, direct compression is the simplest and most cost-effective, where compression occurs directly after blending of the drug and excipient powders. However, without the use of additional processing steps, such as granulation, the manufacturability of a direct compression formulation is fundamentally dependent on the physical and mechanical properties of the constituent raw materials (Chen, Aburub and Sun, 2019). It has been noted that for some of the most widely tested PEs in the clinic, such as sodium caprate (C10) and salcaprozate sodium (SNAC), a large concentration is required to have a noticeable impact on peptide permeability and, therefore, to produce a direct compression formulation would require a significant portion to be composed of the PE (Twarog *et al.*, 2019; Niu *et al.*, 2025). As a result, the bulk properties of such direct compression formulations, e.g. flowability, compressibility and compactibility, will be largely dominated by the properties of the individual PE. Although investigations have described bile salt and surfactant-based PEs as possessing poor cohesive properties, leading to poor compressibility, there have been no published studies investigating the compaction behaviour and overall suitability of widely used PEs for direct compression formulations to date (Fan *et al.*, 2019; Pedersen *et al.*, 2023).

Beyond understanding the processibility of PE raw materials, the sensitivity of peptides to degradation during processing is a primary concern in the development of efficacious peptide tablets. The compaction of peptides is a relatively understudied area, with little known about the effects of the thermal, shear and mechanical stresses associated with tableting on the physical and chemical stability of peptides. While there is limited data published on the impact of compaction on the stability of peptides, proteins have been shown to exhibit structure-specific lability to compaction pressure. In an investigation by Wei *et al.*, bovine serum albumin (BSA) was found to undergo

significant pressure-induced aggregation, with the extent of aggregation being particle size-dependent, while lysozyme, on the other hand, was resistant to degradation at pressures as high as 300 MPa (Wei *et al.*, 2019). Compaction pressure has similarly been observed to impact the secondary structures and bioactivities of trypsin and immunoglobulin G (IVIG) (Klukkert *et al.*, 2015; Lu *et al.*, 2021). Moreover, other biologics, such as siRNA, have been shown to exhibit a loss in bioactivity after tableting (Busignies *et al.*, 2020). A further factor to be considered is the susceptibility of peptides to degradation during storage after tableting. Our group has previously investigated the impacts of temperature and humidity on the rates of human insulin degradation in the solid-state, however, the stability of peptides in tablets with high PE contents during storage has not been investigated (Fagan, Bateman, O'Shea, *et al.*, 2025). As peptide stability is intrinsically related to its biological activity, a comprehensive understanding of all external factors affecting primary structure, conformational state and aggregation tendency is imperative to successfully develop and control the manufacturing of a direct compression formulation (Bock, Gavenonis and Kritzer, 2013).

In this study, the authors sought to rationalise the development of a direct compression peptide formulation, where insulin was used as a model peptide and C10 and SNAC were chosen as model PEs for the investigation. Furthermore, due to availability and cost constraints of insulin, minitables were chosen as the dosage form of preference. Firstly, the flow behaviour and compaction properties of the C10 and SNAC raw materials were characterised to assess their suitability for tableting via direct compression. Additional commonly used tableting excipients, microcrystalline cellulose (MCC) and polyvinylpyrrolidone (PVP) K30, were then co-formulated with insulin and the PEs at different proportions, and a mixture design of experiments

approach was utilised to determine an optimal formulation for the production of insulin compacts. The chosen formulation was further compacted at two different compaction pressures, 100 and 200 MPa, and the impact of compaction pressure on the dissolution behaviour of insulin from the compacts produced was assessed in simulated intestinal fluid without pancreatin (SIF-sp). Finally, insulin stability during compaction at high pressure and on storage at accelerated conditions of 40°C/ 75% RH for 1 month was assessed.

4.5. Experimental Section

4.5.1. Materials

Insulin (recombinant human), obtained from Merck KGaA (Germany), was used as a model peptide in this investigation. Sodium decanoate (C10) and salcaprozate sodium (SNAC) were acquired from Merck KGaA (Germany) and BOC Sciences (New York, USA) respectively. Due to the morphology of the C10 material procured being large, flaky and brittle agglomerates, manual grinding was performed using a pestle and mortar to create uniform particles for processing. A representative image of the C10 raw material before and after grinding is shown in Figure S4.1 in Appendix 3. SNAC material was processed and characterised as received. The solid state of the insulin, C10 and SNAC was characterised by powder X-ray diffraction (PXRD), with diffractograms and experimental details provided in Figure S4.4 in Appendix 3. Microcrystalline cellulose (MCC), Avicel PH102 grade, and polyvinylpyrrolidone (PVP K30), Kollidon 30 LP grade, used for tableting were donated by International Flavors & Fragrances (IFF) Cork, previously FMC Biopolymer (Ireland) and BASF (Germany), respectively. Potassium phosphate monobasic (KH_2PO_4) and sodium hydroxide (NaOH) used for preparation of SIF-sp buffer were reagent grade and were obtained from Merck KGaA (Germany). Water (H_2O), acetonitrile (ACN), formic acid

(FA), l-arginine (L-arg), anhydrous sodium phosphate monobasic (NaH_2PO_4) and anhydrous sodium phosphate dibasic (Na_2HPO_4) used for chromatography were all reagent or HPLC grade and were obtained from Merck KGaA (Germany).

4.5.2. Powder Characterisation

Particle size analysis

Particle size measurements were performed by microscopic image analysis using a Morphologi G3 (Malvern, UK). The circle equivalent (CE) diameters representing 10% (d10), 50% (d50) and 90% (d90) of the volume-weighted cumulative particle size distribution (vPSD) were determined. Span was used as an indicator of particles size distribution and particle shape was assessed using the aspect ratio.

Density

The bulk (ρ_b) and tapped (ρ_{tap}) density of each material was determined using the method described in Tsolaki et al. (Tsolaki, Healy and Ferguson, 2024). True density (ρ_t) measurements were performed using an AccuPycII 1340 (Micromeritics Instrument Corporation, USA) helium pycnometer. The mean sample volume of 10 measurements was used to calculate true density.

Powder flowability

Powder flowability was assessed using Carr's Compressibility Index (CI) and Hausner Ratio (HR), shown in Equations 4.1 and 4.2, respectively.

$$CI = 100 \left(\frac{\rho_{tap} - \rho_b}{\rho_{tap}} \right) \quad (\text{Equation 4.1})$$

$$HR = \left(\frac{\rho_{tap}}{\rho_b} \right) \quad (\text{Equation 4.2})$$

4.5.3. Tableting

Compaction studies were performed using a STYL'One Evo (Medelpharm, France) compaction simulator fitted with 4 mm flat-faced Euro D tooling. Die filling height was kept constant during tableting and die filling was performed manually by accurately weighing and transferring the required amount of powder to the die prior to compaction to produce compacts with a target weight of 25 mg. The top and bottom die were lightly brushed with magnesium stearate (MgSt) for lubrication before filling. Default compression cycle 1 of the STYL'One Evo (Medelpharm, France) at 25% machine speed was used for the compaction studies, with an average dwell time of 7 ms. Compaction pressures of 50, 100, 150, 200 and 250 MPa were applied. All compaction experiments were performed in triplicate.

Compact Characterisation

Tablet thickness, diameter and breaking force were measured using a PharmaTest PTB511 Hardness Tester (Pharma Test, Germany).

Tablet tensile strength was calculated using Equation 4.3.

$$\sigma = \frac{2F}{\pi DT} \quad (\text{Equation 4.3})$$

Where σ is the tensile strength, F is the breaking force, D is tablet diameter and T is tablet thickness.

Tablet porosity (ε) was calculated using Equation 4.4.

$$\varepsilon = 1 - \frac{\rho_a}{\rho_t} \quad (\text{Equation 4.4})$$

Where ρ_a is the apparent density of the tablet and ρ_t is the true density of the powder.

The elastic behaviours of C10 and SNAC were investigated by calculating the axial elastic relaxation index (ER) for each material. This was calculated using Equation 4.5 (Armstrong and Haines-Nutt, 1972).

$$ER (\%) = 100 \left(\frac{h_1 - h_0}{h_0} \right) \quad (\text{Equation 4.5})$$

where h_1 is the tablet thickness 24 hr after compaction and h_0 is the table thickness under maximum pressure inside the die.

To assess the compaction behaviour of the C10 and SNAC raw materials, each powder was compacted at a range of compaction pressures. The tensile strength and porosity of each compact was determined, and the compaction triangle was then used to assess the tableability, compactibility and compressibility of C10 and SNAC (Joiris *et al.*, 1998).

Tableability describes the relationship between the tensile strength of a compact and the applied compaction pressure. This was modelled using the linear relationship proposed by Newton *et al.*, shown in Equation 4.6 (Newton *et al.*, 1971).

$$\sigma = C_p P + b \quad (\text{Equation 4.6})$$

where C_p is the tableability parameter, P is the compaction pressure and b is a constant.

Compactibility describes the relationship between the tensile strength of a compact and its porosity. The Ryshkewitch-Duckworth equation was used in this study to model compactibility (Duckworth, 1953; Ryshkewitch, 1953). This is defined as:

$$\sigma = \sigma_0 e^{-k\varepsilon} \quad (\text{Equation 4.7})$$

where σ_0 is the tensile strength of a tablet at zero porosity and k is a constant representing the bonding capacity of a material.

Compressibility describes the relationship between the porosity of a compact and the applied compaction pressure. In this study, the Heckel model was used to assess compressibility (Ilić *et al.*, 2013). This is expressed as shown in Equation 4.8.

$$\ln\left(\frac{1}{1-D}\right) = \ln \varepsilon = K.P + A \quad (\text{Equation 4.8})$$

where D is the relative density of the tablets, K (Heckel coefficient) and A are the regression coefficients determined from the linear portion of the curve.

The reciprocal of K, 1/K, gives an estimation of the yield pressure (P_y) of a material, which is a measure of its compressibility.

4.5.4. Formulation Design Optimisation

To determine an optimal formulation for the production of direct compression insulin compacts, a mixture design of experiments (DoE) was employed. In addition to PEs and insulin, MCC and PVP K30 were selected as commonly used tableting excipients to assess their impact on tableting performance. Given that PEs are required to be present at high concentrations to exert an oral bioavailability enhancement, the PEs were chosen to constitute the bulk of the formulation, with contents ranging from 72 – 97% w/w. The MCC and PVP K30 contents were varied between 0 – 20% w/w and 0 – 5% w/w, respectively, while insulin was constrained at 3% w/w for all formulations. Accordingly, a constrained extreme vertices design was created using Minitab® 20.2 (Minitab LLC, United Kingdom), where the proportions of PE, MCC and PVP were the components of the design, compaction pressure served as a process variable, and tensile strength was the response variable. The design was augmented with a centre point and axial points, and 3 replicates for each design point were used. The simplex design plot is shown in Figure 4.1. From this design 9 different

formulations were prescribed, as shown in Table 4.1, where identical formulations were evaluated for both C10 and SNAC.

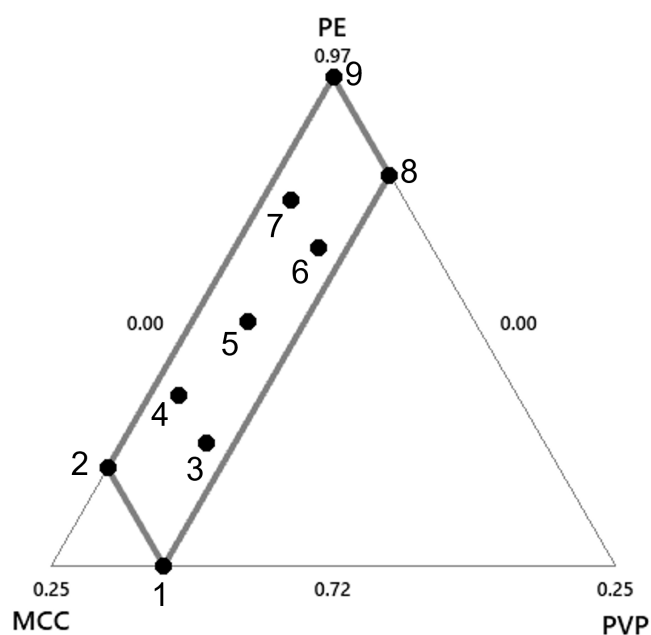


Figure 4.1. Simplex design plot for constrained mixture DoE with three levels. Each numbered point represents a blend with varying concentrations of PE, MCC and PVP K30. Insulin concentration was set constant at 3% w/w.

Table 4.1. Overview of component proportions for mixture DoE and compaction pressure levels investigated.

Formulation	PE	MCC	PVP	INS	Compaction		Replicates
					Pressure (MPa)		
					Low	High	
1	0.72	0.2	0.05	0.03	100	200	3
2	0.77	0.2	0	0.03	100	200	3
3	0.7825	0.15	0.0375	0.03	100	200	3
4	0.8075	0.15	0.0125	0.03	100	200	3
5	0.845	0.1	0.025	0.03	100	200	3
6	0.8825	0.05	0.0375	0.03	100	200	3
7	0.9075	0.05	0.0125	0.03	100	200	3
8	0.92	0	0.05	0.03	100	200	3
9	0.97	0	0	0.03	100	200	3

Approximately 200 mg of each formulation were prepared by accurately weighing the formulation components using an analytical balance and blending using a pestle and mortar. Compacts were produced at a target weight of 25 mg at two different compaction pressures, 100 and 200 MPa. The tensile strengths of the tablets were determined using a PharmaTest PTB511 Hardness Tester (Pharma Test, Germany). Regression analysis with a stepwise selection of terms ($\alpha = 0.05$) was performed using Minitab[®] 20.2 (Minitab LLC, United Kingdom) to determine the impacts of formulation components and compaction pressure on tablet tensile strengths. Further, multiple mixture contour plots were utilised for selection of the optimal formulation

for both C10 and SNAC containing compacts. The selected formulation subsequently underwent further stability and dissolution testing.

4.5.5. Dissolution Testing

Dissolution was performed using a Distek 2100B system (Distek, New Jersey, USA) fitted with 100 mL vessels and paddles (Dissolution Accessories, the Netherlands). Compacts were placed in 40 mL of SIF-sp in the dissolution vessels and stirred at a paddle speed of 150 rpm. 350 μ L samples were taken manually at 1, 5, 10, 15, 30, 45, 60 and 120 min timepoints, with an equivalent volume of buffer being replaced in each vessel after sampling to maintain a constant volume throughout. Samples were centrifuged at 18626 RCF for 2 min using a Mikro 120 Centrifuge (Hettich Lab, Germany) to remove particulates, and the supernatant was collected for analysis. All experiments were performed in triplicate.

4.5.6. Stability Testing

To determine if insulin was susceptible to pressure-induced degradation, raw insulin powder was compacted at 200 MPa, and changes to secondary structure and aggregation state were investigated. Additionally, to assess the impact of the formulation chosen in Section 4.5.4. on the stability of insulin over time, compacts were stored at accelerated conditions of 40°C/ 75% RH for 1 month.

4.5.7. Characterisation of Degradants

Prior to analysis, all degraded samples were reconstituted in 0.1% FA (pH 2.6) to a target concentration of 1 mg/ mL. Samples were centrifuged at 18626 RCF for 2 min using a Mikro 120 Centrifuge (Hettich Lab, Germany) to remove particulates, and the supernatant was collected for analysis.

Quantification of insulin and assessment of aggregation tendency during dissolution and stability testing was performed using size exclusion chromatography (SEC), and degree of deamidation during stability testing was assessed using reverse-phase HPLC (RP-HPLC), the methods for which have both been described previously (Fagan *et al.*, 2024; Fagan, Bateman, Crean, *et al.*, 2025).

Fourier transform infrared (FTIR) spectroscopy was used to investigate changes in the secondary structure of insulin in the solid-state after compaction. Attenuated total reflectance (ATR) IR analysis was performed using a Spectrum Two™ FT-IR Spectrometer (PerkinElmer, USA). Analysis was performed over a spectral range of 4000 – 400 cm⁻¹, with a resolution of 2 cm⁻¹ and intervals of 0.5 cm⁻¹. 32 scans were averaged to give a spectrum for each sample, and analysis was performed in triplicate.

Circular dichroism (CD) spectroscopy was also used to analyse the secondary structure of insulin in solution, and this method has been described previously (Fagan *et al.*, 2024).

4.6. Results

4.6.1. Physical and Mechanical Properties of C10 and SNAC

In the first part of this investigation, the physical and mechanical properties of C10 and SNAC were characterised to assess their suitability for processing via direct compression.

Particle size analysis and flow behaviour

Automated image analysis was used in this study for particle size and morphology analysis of all the powders used during compaction, and the results are given in Table 4.2. As can be seen, C10 particles had a larger average size, with a d₅₀ of approx. 35

μm, compared to SNAC particles, which had a d50 of approx. 20 μm (vPSDs for C10 and SNAC can be found in Figure S4.2b and Figure S4.3b, respectively, in Appendix 3). The C10 particles were characterised by a slightly narrower size distribution, with a span of 1.21, while SNAC particles had a span of 1.53 and thus a broader size distribution. Furthermore, both the C10 and SNAC particles were observed to be irregularly shaped, as confirmed by the low aspect ratios seen in Table 4.2 and the images provided in Figure S4.2a and S4.3a, respectively in Appendix 3.

HR and CI are two commonly used compendial metrics for predicting a material's flow. As seen in Table 4.2, C10 particles exhibited passable flow character, with a HR of 1.31 and CI of 24%, while SNAC exhibited poor flow, with a HR of 1.36 and CI of 26% (The United States Pharmacopeial Convention, 2024).

Table 4.2. Particle size characterisation, density values, Hausner Ratio and Carr's Compressibility Index for tableting materials. Density values are given as averages ($n = 3$ for ρ_b and ρ_{tap} , $n = 10$ for ρ_t).

Material	Particle Size (μm)			Span	Aspect Ratio	ρ_b (g/cm ³)	ρ_{tap} (g/cm ³)	ρ_t (g/cm ³)	HR	CI (%)	Flow Character
	d10	d50	d90								
C10	14.29	35.08	56.89	1.21	0.70	0.24	0.31	1.14	1.31	24	Passable
SNAC	6.68	20.18	37.58	1.53	0.66	0.26	0.36	1.3	1.36	26	Poor
Insulin	8.22	16.41	28.51	1.24	0.71	0.25	0.39	1.28	1.54	35	Very Poor
MCC	56.89	139.64	242.66	1.33	0.58	0.31	0.39	1.53	1.23	19	Fair
PVP K30	65.31	130.32	197.24	1.01	0.75	0.25	0.36	1.21	1.43	30	Poor

Compaction triangle

To assess the suitability of C10 and SNAC for compaction, their tabletability, compactibility and compressibility profiles were evaluated. To do this, C10 and SNAC powders were individually compacted at compaction pressures of 50, 100, 150, 200 and 250 MPa, and the tensile strengths and porosities of the compacts produced were recorded. The resultant compaction triangle profiles for C10 and SNAC are shown in Figure 4.2.

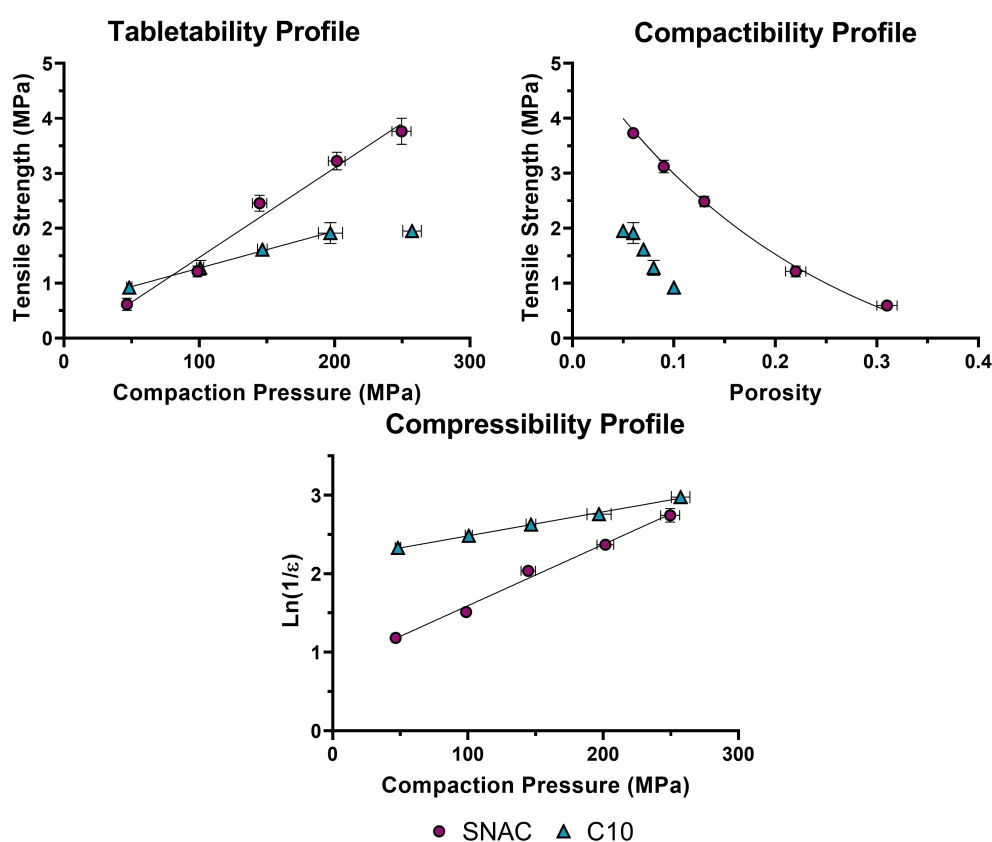


Figure 4.2. Tabletability, compactibility and compressibility profiles for C10 (blue) and SNAC (purple) materials. Error bars represent standard deviation, $n = 3$.

As can be seen from Figure 4.2, SNAC powder exhibited comparatively good tabletability, with acceptable tablets (>1.7 MPa) formed at compaction pressures above 100 MPa, reaching tensile strengths as high as 3.8 MPa. C10, on the other hand, displayed poor tabletability. The low tensile strength values obtained even at the

highest compaction pressure suggested that there was poor cohesion between C10 particles, preventing the formation of strong compacts. To further quantitatively compare the tableability of SNAC and C10, linear regression was performed, and the tableability parameter, C_p , was derived from the slope of the graphs. However, the tensile strengths of the C10 compacts appeared to plateau at pressures above 200 MPa, and, therefore, only the linear region was used in the regression model. As can be seen in Table 4.3, SNAC exhibited a significantly higher C_p value than C10, further confirming the better tableability of the SNAC material.

From the compactibility profiles shown in Figure 4.2, SNAC appeared to fit the Ryshkewitch-Duckworth model well and exhibited good compactibility. This was evidenced by the formation of tablets with acceptable tensile strengths within the desirable porosity range (0.15 ± 0.05) (Leane *et al.*, 2015). C10, on the other hand, did not fit the Ryshkewitch-Duckworth model well. Instead, there appeared to be an inversely proportional relationship between tensile strength and porosity until the plateau in tensile strength described previously. Interestingly, compacts with very low porosity were formed at low compaction pressure, and consequently the porosity range over which the measurements were taken was very narrow. This suggested that the C10 particles formed a highly dense packing arrangement even at low compaction pressures. However, given the low tensile strength values achieved at such low porosities, it is apparent that there was not significant bonding between C10 particles, at least during the period over which the compaction pressure was applied in this investigation (7 ms average dwell time).

The compressibility of C10 and SNAC materials were assessed using the Heckel model. As can be seen from Table 4.3, the compaction data for both C10 and SNAC were well described by the Heckel model. The slope of the SNAC data appeared to be

steeper, as seen in Figure 4.2, therefore resulting in a lower P_y and overall better compressibility compared to C10. In general, higher compressibility and low P_y in the Heckel model suggests that the particles undergo more pronounced plastic deformation (Ilić *et al.*, 2013). As such, the SNAC material used in this investigation can be described as more plastic than the C10 material.

Table 4.3. Fitted tableting parameters obtained from the Newton equation for tabletability, Ryshkewitch-Duckworth equation for compactibility and Heckel equation for compressibility.

Material	Tabletability		Compactibility		Compressibility		
	C_p (MPa)	R^2	σ_0 (MPa)	- k	R^2	P_y (MPa)	R^2
C10	0.0067*	0.9986*	-	-	-	327.01	0.9978
SNAC	0.0163	0.9783	6.44	4.31	0.9965	128.27	0.9912

*tensile strength values at 250 MPa removed due to deviation from linear model.

Elastic Recovery

The axial elastic relaxation indices (%ER) of the C10 and SNAC compacts at each compaction pressure are given in Table 4.4. The C10 compacts showed a higher elastic recovery after tableting than SNAC, where no apparent pressure dependent relationship was observed. These results may be due to the higher plasticity of SNAC, as plastic deformation of the particles can allow for the stress due to compaction to be more readily distributed throughout the compact. C10 particles, on the other hand, were less plastic, and the applied pressure may have caused greater stress on the particles leading to greater expansion after compaction.

Table 4.4. Elastic relaxation index for C10 and SNAC studied at compaction pressures ranging from 50 to 250 MPa. Measurements are given $\pm$ standard deviation, $n = 3$.

Material	% ER					Average
	50 MPa	100 MPa	150 MPa	200 MPa	250 MPa	
C10	5.94 $\pm$ 0.48	7.75 $\pm$ 1.44	6.51 $\pm$ 1.16	6.93 $\pm$ 0.57	8.01 $\pm$ 0.50	7.03 $\pm$ 1.20
SNAC	3.30 $\pm$ 0.37	5.51 $\pm$ 0.21	3.71 $\pm$ 0.89	5.22 $\pm$ 0.19	7.09 $\pm$ 0.28	4.97 $\pm$ 1.44

4.6.2. Formulation Selection

To evaluate the impact of commonly used tableting excipients on the tensile strength of C10 and SNAC compacts, MCC PH102 and PVP K30 were incorporated into the compacts as model direct compression excipients (see Table 4.2 for particle size analysis). The impacts of each formulation component on the tensile strength of the compacts for the C10 and SNAC formulations, as expressed by the coefficients of the regression model, are given in Table S4.1 in the Appendix 3. For the C10 formulations, MCC was found to have the largest impact on tensile strength, while C10 and PVP K30 had smaller effects. This effect of MCC on tensile strength was found to be pressure dependent, where a positive interaction between MCC and compaction pressure was noted. A negative interaction term between MCC and C10 was also observed.

To visualise the contribution of each mixture component to the tensile strength as a function of compaction pressure, the Cox response and mixture contour plots are given in Figure 4.3a-d. The data indicated that an increase in the proportion of MCC present in the formulations resulted in compacts with higher tensile strengths. Similarly, increasing PVP K30 content caused an increase in tensile strength, although to a lesser

extent. The direct opposite effect was observed for C10, where tensile strength was observed to decrease as C10 content increased. Although all formulations compacted at 200 MPa produced tablets with tensile strengths > 1.7 MPa, this was not observed at 100 MPa. The highest tensile strengths achieved at a compaction pressure of 100 MPa was 1.6 ± 0.08 MPa for the formulation consisting of 72% C10, 20% MCC, 5% PVP K30 and 3% insulin. While this is below 1.7 MPa, which is generally accepted as the minimum desirable tensile strength for a tablet, it does represent an approx. 20% increase in tensile strength relative to pure C10 powder compacted at the same pressure, highlighting the positive effect the additional excipients had on the tableability of C10 (Leane *et al.*, 2015). As this formulation resulted in the compacts with the highest tensile strengths at both pressure levels, this was selected for further dissolution and stability testing.

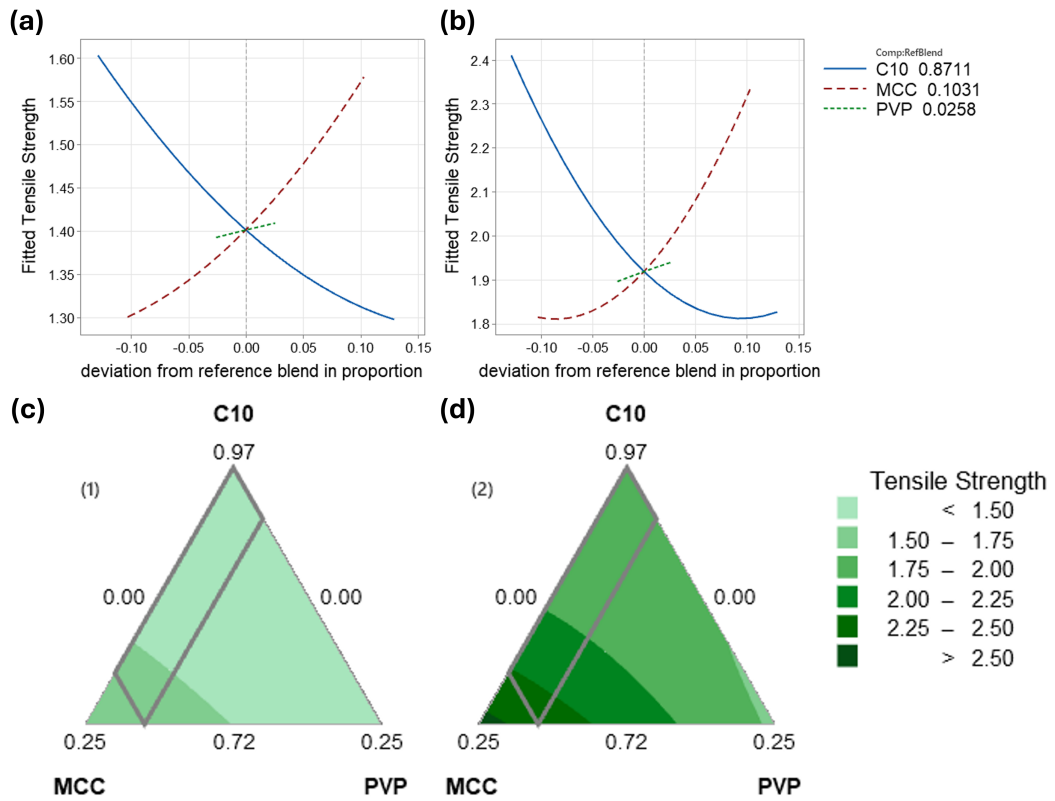


Figure 4.3. Cox response plots showing contribution of C10, MCC and PVP K30 to tensile strength at (a) 100 MPa and (b) 200 MPa. Also shown are mixture contour plots for the formulations at (c) 100 MPa and (d) 200 MPa.

For the SNAC formulations, MCC had the highest regression coefficient of the mixture components, followed by PVP and SNAC. Similarly to the C10 formulations, the effect of MCC was pressure dependent and there was a negative interaction between MCC and SNAC. Other significant terms included in the model were the positive interaction between SNAC and compaction pressure, and the positive ternary interaction between SNAC, PVP K30 and compaction pressure.

From the Cox response plots shown in Figure 4a and 4b, at both compaction pressures increasing the MCC content of the formulations caused an increase in compact tensile strength. This contrasts with the negative effect that SNAC had on tensile strength at both compaction pressures. Further, unlike the C10 formulations, PVP K30 exerted a

strong positive influence on the tensile strengths of the compacts at both compaction pressures. Tablets of tensile strengths > 1.7 MPa could be achieved for formulations with $> 15\%$ MCC at a compaction pressure of 100 MPa, while all formulations formed satisfactory compacts at 200 MPa. Similarly to the C10 tablets, formulations with 20% MCC exhibited the highest tensile strengths, and, therefore, the formulation composed of 72% SNAC, 20% MCC, 5% PVP and 3% insulin was also chosen in this case for further investigations.

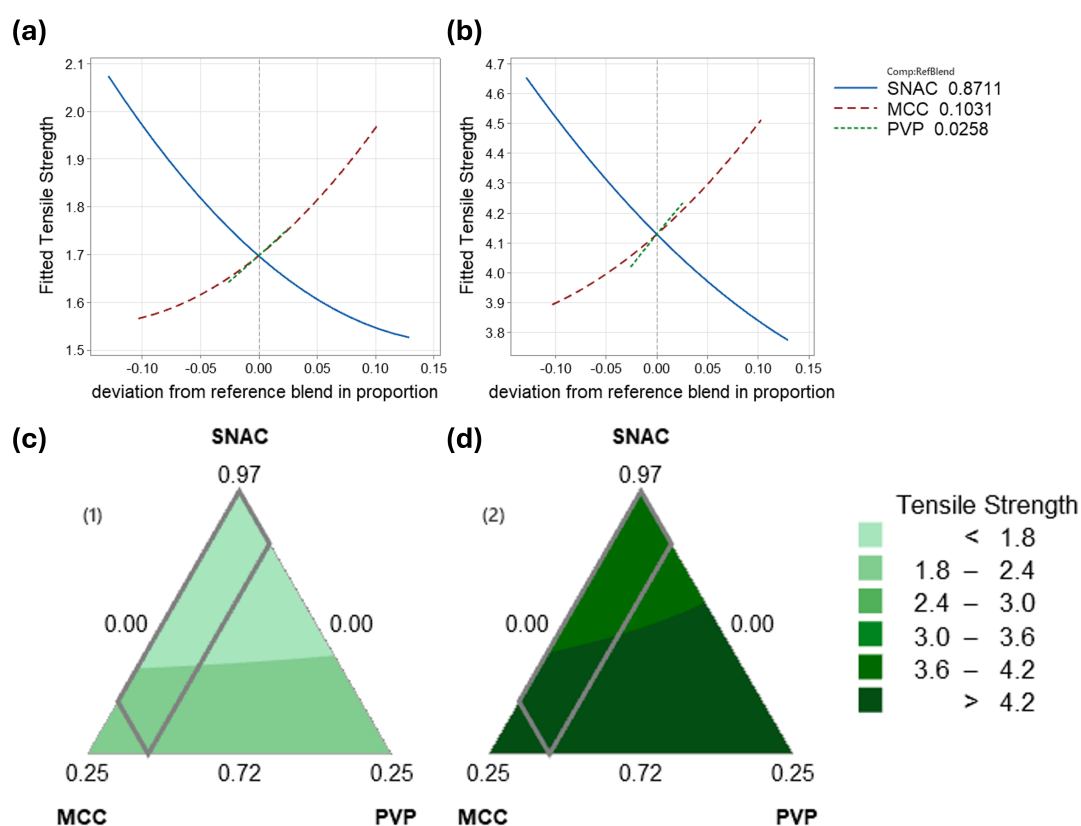


Figure 4.4. Cox response plots showing contribution of SNAC, MCC and PVP K30 to tensile strength at (a) 100 MPa and (b) 200 MPa. Also shown are mixture contour plots for the formulations at (c) 100 MPa and (d) 200 MPa.

4.6.3. Dissolution Testing

The chosen formulations were compacted into approx. 25 mg compacts at 100 and 200 MPa, and their weights, tensile strengths and porosities are reported in Table 4.5. The

dissolution profiles of each of the formulations in SIF-sp are given in Figure 4.5a and 4.5b.

Table 4.5. Characterisation of compacts produced at 100 and 200 MPa compaction pressures for formulations composed of 72% C10/20% MCC/5% PVP/3% insulin and 72% SNAC/20% MCC/5% PVP/3% insulin. Measurements are given $\pm$ standard deviation, $n = 3$.

Formulation	Compaction Pressure (MPa)	Weight (mg)	Tensile Strength (MPa)	Porosity
C10	100	24.1 $\pm$ 0.2	1.5 $\pm$ 0.2	0.08 $\pm$ 0.00
	200	24.3 $\pm$ 0.3	2.5 $\pm$ 0.2	0.05 $\pm$ 0.01
SNAC	100	24.7 $\pm$ 0.1	2.2 $\pm$ 0.2	0.20 $\pm$ 0.01
	200	23.0 $\pm$ 0.1	4.4 $\pm$ 0.2	0.13 $\pm$ 0.01

For the C10-containing compacts, similar dissolution profiles were observed at both compaction pressures, where $> 90\%$ insulin release occurred after approx. 30 min. Similar release profiles were observed for the SNAC-containing compacts. Although it may be expected that drug release would be prolonged by the near doubling of tensile strengths of the compacts observed in Table 4.5, these findings suggest that higher compaction pressures did not significantly alter the release of insulin from formulations containing C10 and SNAC.

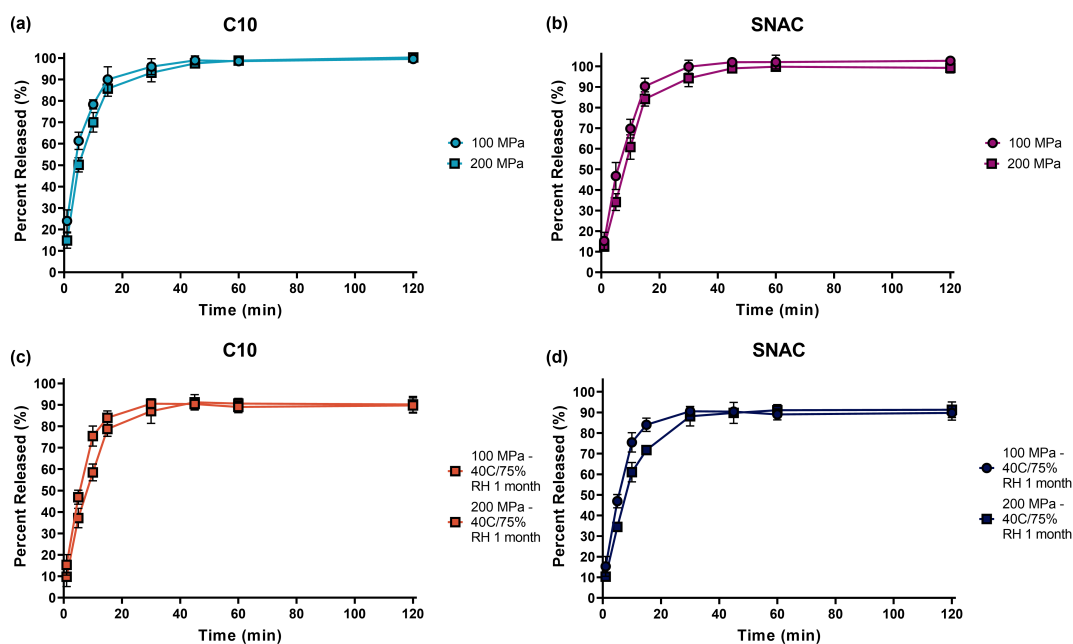


Figure 4.5. Release profiles of insulin from compacts composed of (a) 72% C10/20% MCC/5% PVP/3% insulin and (b) 72% SNAC/20% MCC/5% PVP/3% insulin, compacted at 100 and 200 MPa. Release profiles for (c) C10- and (d) SNAC-compacts stored at 40°C/ 75% RH for 1 month are also given. Error bars represent standard deviation, $n = 3$.

4.6.4. Stability Testing

To determine the impact of compaction pressure on the physical stability of insulin, 25 mg insulin powder was compacted at the higher compaction level of 200 MPa. Secondary structural changes were assessed in the solid-state using ATR-IR, and in solution upon reconstitution to 1 mg/ mL in 0.1% FA solution (pH 2.6) using CD spectroscopy. SEC was used to identify the presence of higher molecular weight species in solution. The results are given in Figure 4.6.

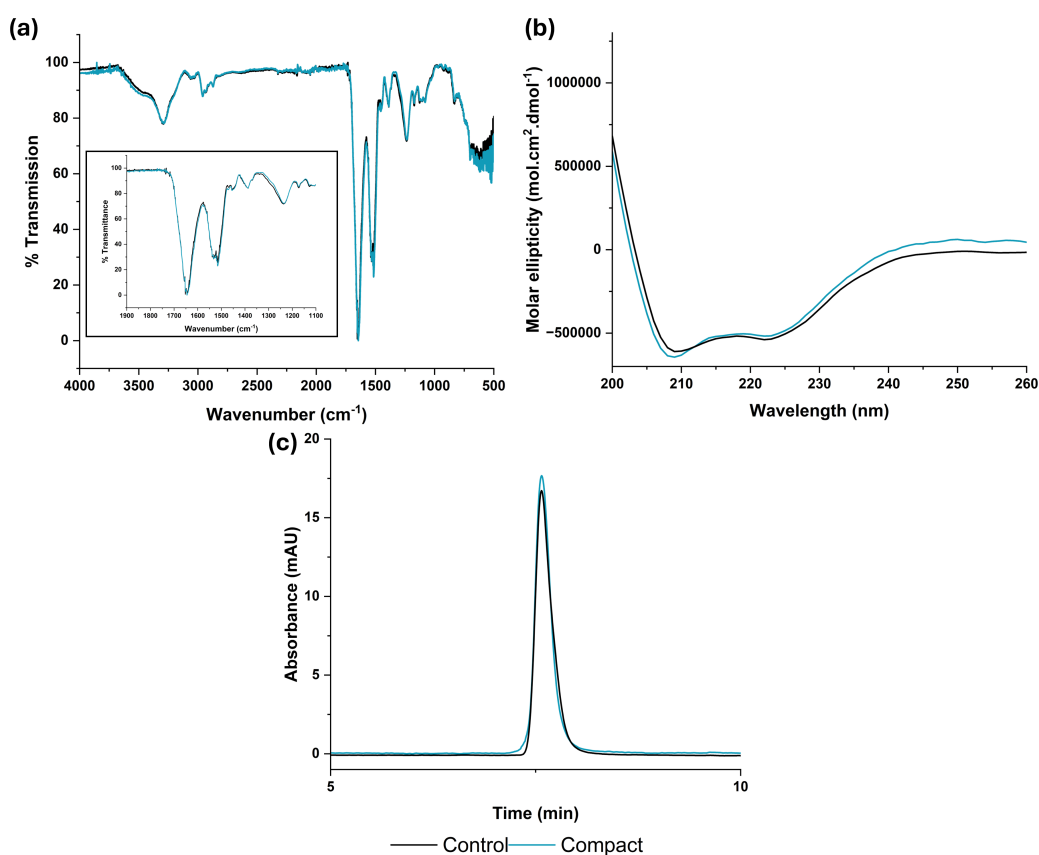


Figure 4.6. Representative (a) ATR-IR spectra (b) CD spectra and (c) SEC chromatograms of insulin compacted at 200 MPa. For CD and SEC analysis, insulin samples were reconstituted in 0.1% FA at a target concentration of 1 mg/mL prior to analysis.

As can be seen from the inset in Figure 4.6a, there were no detectable changes in the amide I, II or III bands in the IR spectra of insulin after compaction compared to a reference sample. Similarly, from the CD spectra in Figure 4.6b, there was no indication of a reduction in helical content, or an increase in disordered structures for the samples compacted at 200 MPa. As a result, there was no evidence to suggest any secondary structural changes occurred in the solid-state or indeed on reconstitution after compaction. Furthermore, the insulin molecules were not observed to undergo aggregation during compaction, as confirmed by SEC. As can be seen from Figure 4.6c, a single monomer peak was present at approx. 7.6 min in the chromatograms of

both the compacted insulin sample and the control sample, indicating that insulin was monodisperse in solution and there were no aggregates present.

To further assess the impacts of storage conditions on the stability of insulin after compaction, the C10- and SNAC-containing compacts were exposed to 40°C/ 75% RH for 1 month. Additionally, to determine if compaction pressure played a role in the degradation profiles observed, the uncompacted formulations were also stored at the same conditions. The extent of insulin chemical and physical degradation was then monitored using RP-HPLC and SEC, respectively, and the results are given in Figure 4.7. There was no evidence of insulin undergoing any chemical or physical degradation in the C10- and SNAC-containing compacts prior to storage, suggesting that neither compaction pressure, nor the excipients triggered insulin degradation. For the C10-containing compacts stored at 40°C/ 75% RH for 1 month, comparable levels of insulin degradation, approx. 2% deamidation and 5.5% aggregation, were observed at each compaction pressure, suggesting that increasing compaction pressure did not alter the susceptibility of insulin to degradation. In contrast, the insulin present in the uncompacted formulation underwent a slightly higher degree of degradation, approx. 2.5% deamidation and 7% aggregation. Similar behaviour was observed for the SNAC-containing formulation, where approx. 2% deamidation and 6% aggregation was detected for the compacts at both compaction pressures, while this increased to 3% deamidation and 9% aggregation for the uncompacted samples. In comparison to unformulated insulin raw material stored at the same conditions, there was an approx. 68% reduction in deamidation products and 45% reduction in the amount of aggregates detected in the C10- and SNAC-containing compacts. Although a greater amount of degradation was detected in the uncompacted C10 and SNAC formulations, this was also significantly reduced in comparison to the insulin raw material.

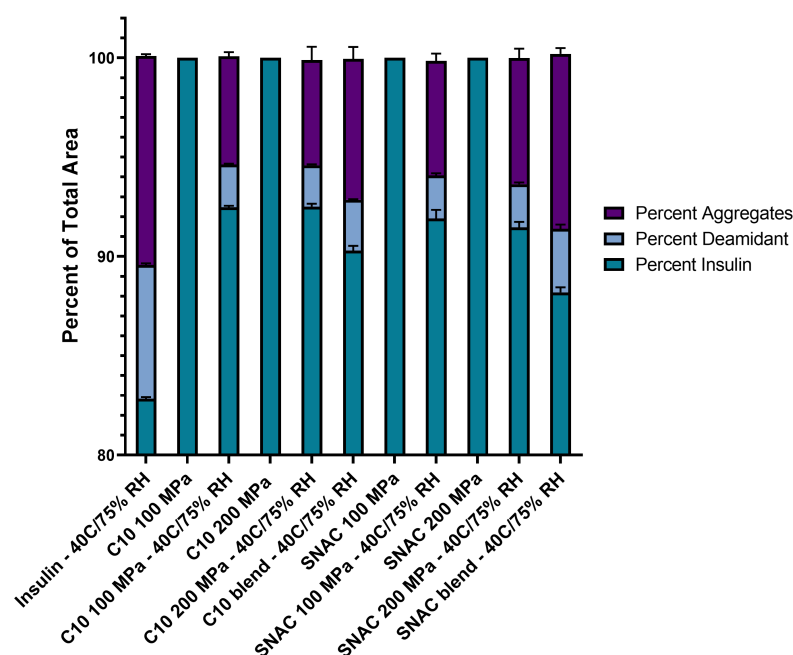


Figure 4.7. Degraded species as a percentage of the total peak areas in RP and SEC chromatograms of compacts and uncompact formulations consisting of 72% C10/20% MCC/5% PVP/3% insulin and 72% SNAC/20% MCC/5% PVP/3% insulin stored at 40°C/ 75% RH for 1 month. Percent insulin and percent deamidant were calculated from the peak areas of the RP chromatograms, while percent aggregates were calculated from the peak areas of the SEC chromatograms. Error bars represent standard deviation, $n = 3$.

Finally, to determine if storage of the compacts under the accelerated conditions had an impact on their release performance, further dissolution testing was performed, as shown in Figure 4.5c and 4.5d. Insulin release did not appear to be inhibited, where maximum release was again observed to occur after 30 min. A reduction in the total insulin release was observed, however, likely due to the formation of the degradation products described above.

4.7. Discussion

Chemical PEs play a critical role in the successful delivery of peptides via the oral route. Of these, C10 and SNAC have proved two of the most successful in the clinic, with the use of SNAC in the Rybelsus[®] formulation enabling improved oral bioavailability of semaglutide (Lewis *et al.*, 2022). It is widely known that to have a notable enhancing effect, C10 and SNAC must be present at the gastric and intestinal epithelia in high millimolar concentrations, therefore, requiring large quantities to be present in oral delivery systems (Maher, Mrsny and Brayden, 2016). As the performance of a direct compression formulation is intrinsically related to the properties of the individual components, high quantities of these PEs may pose a challenge to process development. However, while there has been considerable attention given to understanding the bioavailability of peptides released from oral dosage forms containing C10 and SNAC, there have been no previous publications reporting the compaction behaviour of the PEs (Twarog *et al.*, 2019). As a result, this investigation sought, in part, to contribute to the understanding of C10 and SNAC as direct compression excipients for oral peptide formulations.

Overall, C10 and SNAC displayed distinct physical and mechanical properties. C10 had a larger average particle size and narrower size distribution than SNAC. The C10 particles also exhibited fair flow character under compendial tests. However, these characteristics did not translate to good compaction properties. Instead, C10 powder exhibited poor tabletability and compressibility, and its compactibility profile deviated from the traditional Ryshkewitch-Duckworth model, as seen in Figure 4.2. Given the low tablet porosities observed, it is clear that C10 particles formed a densely packed arrangement under the applied pressures, although, this did not result in strong compacts, likely due to limited interparticulate bonding. This is in agreement with the

results of a previous investigation into the compression properties of sodium dodecyl sulphate (SDS), a molecule with a similar surfactant-like structure to C10. On compaction at a range of pressures from 0-300 MPa, SDS was found to exhibit poor cohesive properties resulting in compacts with low tensile strengths (Chantraine *et al.*, 2007b, 2007a). Conversely, SNAC showed good tabletability, compactibility and compressibility profiles, forming strong compacts with desirable porosities. The SNAC material exhibited a greater plastic deformation tendency, as indicated by the lower P_y value compared to C10. Further evidence of this was provided by the lower average elastic recovery of the SNAC compacts relative to the C10 compacts. The lower tensile strengths of the compacts formed by C10 compared to SNAC suggests a difference in the number and/ or strength of interparticulate interactions occurring during compaction. This may be attributable in part to the differences in chemical structures of C10 and SNAC, shown in Figure 4.8. While C10 has a fatty acid structure, consisting of a saturated 10-carbon chain, SNAC contains additional aromatic, hydroxyl and amide functional groups which may undergo a greater degree of intermolecular hydrogen bonding and van der Waals interactions during compaction (Patel, Kaushal and Bansal, 2006). Moreover, the smaller size of the SNAC particles and their greater tendency to deform plastically likely also contributed to the larger tensile strengths observed. However, this further gave rise to the poor flow character of SNAC.

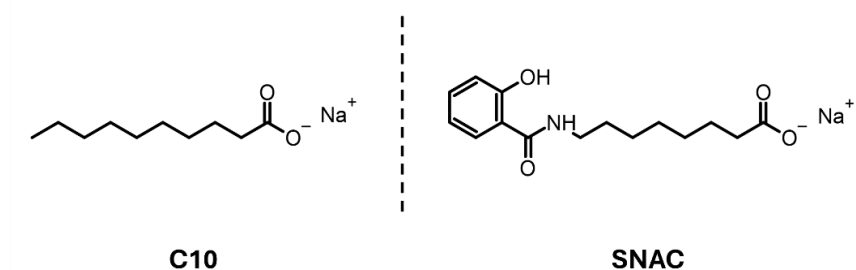


Figure 4.8. Structures of C10 and SNAC.

The poor compaction properties of C10 were found to be improved on addition of the commonly used direct compression excipients MCC and PVP. MCC is known to possess good flow and compaction behaviour, and PVP was chosen as a binder to improve interparticulate interactions (Lahdenpää, Niskanen and Yliruusi, 1997; de Backere *et al.*, 2021). Increasing the proportion of MCC and, to a lesser extent, PVP caused a significant improvement in the tensile strength of C10-containing compacts, where an average increase in tensile strength of 25% was achieved at compaction pressures of 100 and 200 MPa for formulations containing 20% MCC and 5% PVP K30 in comparison to compacts consisting of C10 alone. This enhancement in the tabletability of C10 was likely due to greater interparticulate bonding and improved plastic deformation due to the presence of the PVP and MCC particles. An even greater improvement in tabletability was observed for SNAC, where an 80% increase in tensile strength was observed for formulations containing 20% MCC and 5% PVP K30 compacted at 100 MPa in comparison to the pure SNAC compacts, and a 40% increase was observed for those compacted at 200 MPa. Given the marked improvements in tabletability, formulations consisting of 72% C10/20% MCC/5% PVP/3% insulin and 72% SNAC/20% MCC/5% PVP/3% insulin were further compacted at 100 and 200 MPa and their release profiles were assessed. In contrast to PEs such as SDS and sodium laurate, which have been reported to adversely impact the disintegration properties of tablets and slow dissolution, a previous investigation in our group found that C10 and SNAC had a significant positive impact on the release of insulin from tablets (Maher, Mrsny and Brayden, 2016; Chantraine *et al.*, 2007b; Fagan, Bateman, Crean, *et al.*, 2025). This was determined to be due to high local concentrations of C10 and SNAC causing an elevation in pH at the surface of the compacts, which resulted in increased insulin solubility and, therefore, increased release. Similar behaviour was

observed in this study, where > 90% release of insulin was observed from all compacts by 30 min. Minimal differences were observed in the release profiles of the compacts compacted at 100 MPa compared to 200 MPa, indicating that increasing tablet tensile strength did not inhibit insulin release. It is probable that the surfactant-like properties of C10 and SNAC, in combination with their high solubilities at pH > 5, enabled unaltered release of insulin from the more densely packed compacts.

These results offer a valuable insight into processability of C10 and SNAC for direct compression formulations. Of the two, SNAC appears to have a higher potential, as highlighted by its ability to produce tablets with adequate tensile strengths and desirable porosities over a range of compaction pressures. However, its poor flow character will likely hinder any progression to production scale. While it is possible that careful selection of suitable direct compression excipients may alleviate this, as evidenced by the positive effects of MCC and PVP in this study, the use of additional particle engineering approaches, such as granulation, may prove a more effective strategy going forward. Moreover, such approaches may aid in overcoming the poor compaction properties of C10 and decrease the associated risk of defects, such as capping and lamination, occurring during tableting.

In addition to the processability of PEs, the inherent instability of peptides represents another key challenge in the design and development of effective oral peptide dosage forms. The complex structures of peptides endow them with several unique therapeutic properties, however, this also leaves them highly sensitive to external stressors, such as heat, humidity and mechanical stress. A number of investigations have highlighted the effects of different storage conditions on the solid-state stability of peptides to date, however, little work has been performed to assess the effects of mechanical stress on peptide stability in the solid-state (Lai and Topp, 1999). From compaction studies on

proteins, the impacts of mechanical stress on protein stability appear to be structure- and size-dependent, and this is likely applicable to peptides also. In this way, insulin is an interesting case study for investigating this behaviour as it has the higher order structural features associated with proteins, while often being categorised as a peptide (Brange and Langkjoer, 1993). As seen in Figure 4.6, insulin did not undergo any noticeable changes in conformation or aggregation state in the solid- and solution-states upon compaction at 200 MPa in this study, suggesting that the insulin molecule was stable to the high compaction pressures used during tableting. Furthermore, blending of insulin with the PEs, MCC and PVP had an apparent positive effect on its physical and chemical stability during storage at 40°C/ 75% RH for 1 month. As seen in Figure 4.7, there was a reduction in the amount of deamidation and aggregation that occurred in the insulin molecules present in the blended formulations in comparison to pure insulin powder. The reduction in aggregation tendency may be due to dilution of the insulin molecules in the formulation, which restricted their capacity to interact with one another and self-associate, while the reduction in deamidation may be due to the hygroscopic nature of MCC and PVP (Yassin *et al.*, 2015). It is well known that water plays a critical role in the deamidation of asparagine residues of solid-state insulin samples, either through reaction with a cyclic intermediate or via direct hydrolysis (Strickley and Anderson, 1996, 1997). Therefore, absorption of moisture by MCC and/ or PVP may have limited the amount of water that was freely available to react, thus limiting the deamidation pathway. Another possible explanation for the observed reduction in deamidation may lie in the ability of C10 and SNAC to influence solution pH. It has been noted previously that for amorphous lyophilised insulin samples, the pH at which the samples are prepared prior to drying plays a major role in the degradation pathway observed on storage at different moisture contents. For

example, Strickley and Anderson found that increasing the ‘effective’ pH of lyophilised samples from 3 to 5 resulted in a decrease in overall degradation rates, particularly the deamidation rate (Strickley and Anderson, 1997). A similar mechanism may be occurring in this case, where the ability of C10 and SNAC to elevate the local ‘effective’ pH of the blends to > pH 7 on sorption of large amounts of moisture could reduce the favourability of the deamidation pathway in comparison to the covalent aggregation pathway. Moreover, insulin exhibited greater stability in the compacts during storage than in the uncompacted formulations, suggesting that the compaction of the molecules into a densely packed arrangement had a stabilising effect. This is likely the result of a decrease in the amount of moisture that could penetrate the compacts due to their low porosity, coupled with reduced molecular mobility. It was also evident that the type of PE used did not influence the stability of insulin.

4.8. Conclusion

This investigation sought to assess the suitability of commonly used permeation enhancers, C10 and SNAC, for direct compression peptide formulations. Of the two, SNAC displayed superior tabletability, compactibility, and compressibility profiles. This was hypothesised to be due to its greater plasticity and capacity to form stronger interparticulate interactions. However, its poor flow character poses a challenge for its use at production scale. C10, in contrast, displayed fair flow character but poor tabletability, compactibility and compressibility, as indicated by the formation of low tensile strength tablets with very low porosities. The compaction properties of both PEs were found to be substantially improved on addition of MCC and PVP, where formulations consisting of 72% PE, 20% MCC, 5% PVP and 3% insulin enabled production of acceptable direct compression compacts. These compacts exhibited

immediate release behaviour, which did not appear to be affected by the compaction pressure at which they were produced. Furthermore, the stability of insulin to high compaction pressures and accelerated storage conditions was assessed. As such, the physical stability of insulin after compaction at 200 MPa was analysed. No evidence of unfolding or aggregation was observed, indicating that the insulin molecule was stable to mechanical stress experienced during tableting. Moreover, blending of insulin in the direct compression formulation appeared to have a positive effect on stability during storage at accelerated conditions. A reduction in overall deamidation and aggregation tendency was observed in comparison to raw insulin powder stored under the same conditions, where higher stability was observed for the tablets compacted at 100 and 200 MPa than for the uncompacted formulations. These results highlight the key role that formulation components play not only in the processibility of direct compression peptide formulations, but also the overall stability of the peptide during compaction and storage.

Chapter 5: Impact of permeation enhancers on the release of insulin from tablets in biorelevant media

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5.1. Author Contributions

As lead author, I was responsible for developing and validating experimental methodologies, carrying out experimental investigations and performing formal analysis of the data. Furthermore, I was responsible for writing the original draft of the paper. Lorraine Bateman, Abina Crean, Joseph O'Shea and Lynne Taylor were responsible for supervising the progress of experiments, reviewing and editing the manuscript, and funding acquisition.

5.2. Abstract

The use of chemical permeation enhancers (PEs) to improve the permeation of peptides across gastric and intestinal epithelia has proven an effective strategy in the development of oral dosage forms of peptides. However, there remains a poor understanding of how the presence of PEs impacts the dissolution characteristics of oral formulations containing peptides, nor is it known how the complex composition of biological media can influence their behavior *in vivo*. This investigation sought to examine the effect of two widely studied PEs, sodium caprate (C10) and salcaprozate sodium (SNAC), on the release behavior of a model peptide, insulin, from minitables in a variety of biorelevant media. First, the equilibrium solubilities of insulin, C10, and SNAC were determined in simulated gastric and intestinal media. Insulin, C10, and SNAC all displayed pH-dependent solubility across a physiologically relevant range of pH conditions. Moreover, at high concentrations, C10 was found to overwhelm the buffer capacity of the simulated media, increasing the pH of fasted state simulated intestinal fluid (FaSSIF) from 6.5 to 9.0, fed state simulated intestinal fluid (FeSSIF) from pH 5.0 to 8.8 and fasted state simulated gastric fluid (FaSSGF) from pH 1.6 to 9.2. Similarly, SNAC caused an increase in the pH of FaSSIF from 6.5 to 7.9, FeSSIF from pH 5.0 to 7.7, and FaSSGF from pH 1.6 to 7.6. Relative to in simulated intestinal media, the solubility of insulin was found to increase significantly in media at pH representative of saturated C10 and SNAC solutions, increasing from 0.1 mg/mL in blank FaSSIF to 14.0 mg/mL in phosphate buffer at pH 7.6 and to 23.7 mg/mL in phosphate buffer at pH 9.2, suggesting that the presence of C10 and SNAC at high concentrations could have a considerable favorable impact on insulin solubility. Furthermore, the release profiles of insulin from minitables containing C10 and SNAC were investigated in each of the biorelevant media and compared with the

release profiles of insulin from blank minitabets in the absence of PEs. Insulin release from the blank minitabets was found to be media dependent, following an apparent solubility trend. Complete release of insulin was observed in simulated gastric media; however, only between 67 and 82% release was observed in the simulated intestinal media. On the other hand, on the addition of C10 and SNAC to the formulation, greater than 90% release was observed across all media investigated. This difference in release behavior was determined to be caused by an increase in pH at the surface of the minitabets due to the presence of high local concentrations of C10 and SNAC, respectively, as confirmed by a change in color of a universal indicator solution. These findings offer a key insight into the influence that C10 and SNAC have on the dissolution characteristics of insulin from an oral dosage form in a variety of simulated gastric and intestinal media.

5.3. Highlights

- The impact of C10 and SNAC on the release behaviour of insulin from minitabets in biorelevant media was studied.
- At high concentrations, C10 and SNAC were found to elevate the pH of simulated gastric and intestinal media to between pH 7.6 – 7.9 and pH 8.8 – 9.2, respectively.
- Media dependent insulin release was observed from minitabets in the absence of C10 and SNAC, while in the presence of C10 and SNAC complete release was observed.
- Difference in release behaviour was determined to be due to a local increase in pH at the surface of minitabets in the presence of C10 and SNAC.

5.4. Introduction

Since their advent in the 1920's, therapeutic peptides have received considerable attention as potential alternatives to traditional small molecule therapies in the treatment of oncological, metabolic and genetic diseases, among others (Wang *et al.*, 2022). The structures of peptides are unique and often complex, conferring them with a number of desirable properties, such as high target selectivity, high potency and low inherent toxicity. The preference for oral delivery of pharmaceuticals due to patient acceptance, convenience and cost-effectiveness, has driven extensive innovation in the design of oral delivery systems for peptide therapeutics. However, the structural complexity of peptides means that they are highly susceptible to degradation by digestive enzymes in the gastrointestinal tract (GIT), while their large size and hydrophilicity restricts permeation across the intestinal epithelium, thereby limiting oral bioavailability, typically to below 2% (Nicze *et al.*, 2024). As a result, the majority of peptides are delivered parenterally via injectable dosage forms, and the widespread adoption of oral dosage forms for the delivery of hydrophilic peptides remains elusive.

A number of strategies have been adopted to improve the oral bioavailability of peptides, including the use of nanoparticles, hydrogels or ionic liquids (O'Neill *et al.*, 2015; Banerjee *et al.*, 2018; Fan *et al.*, 2018). However, the clinical and commercial viability of these approaches are yet to be proven. One approach that has gained significant interest for its efficacy and simplicity is the addition of permeation enhancers (PEs) to formulations to improve peptide permeation across the gastric and intestinal epithelia, leading to the recent approval of oral dosage forms of semaglutide and octreotide (Maher and Brayden, 2021). There are several types of PEs, such as bile salts, medium chain fatty acids and chelating agents, which can act on epithelia via a paracellular pathway by opening tight junctions or via a transcellular pathway by

increasing the hydrophobicity of peptides or by disrupting the integrity of cell membranes. Two of the most widely studied PEs clinically include sodium caprate (C10) and salcaprozate sodium (SNAC), where SNAC has been used successfully in Rybelsus® tablets for the oral delivery of semaglutide. While a considerable amount of research has been conducted on both of these PEs to date, focus has been primarily placed on understanding their efficacies and mechanisms of action (Kim, Park and Na, 2022). However, there remains a lack of understanding of how the addition of these PEs to oral dosage forms, such as tablets, impacts the release characteristics of peptides from the dosage form, nor is it understood how the complex composition of the gastrointestinal milieu affects this behaviour.

C10 and SNAC possess a number of physicochemical properties that are likely to impact the release properties of oral formulations in which they are present. Firstly, at pH values at least 2 pH units above their respective pK_as (approximately 5 for both), C10 and SNAC exhibit high aqueous solubility due to extensive ionization, while at pH values below the pK_a, C10 and SNAC exist mainly in the poorly soluble, free acid form (Twarog *et al.*, 2019; Fattah *et al.*, 2020). As a result, it is expected that the dissolution behaviours of C10 and SNAC will vary significantly in gastric and intestinal media. Additionally, it has been reported that at high concentrations, such as those observed at the surface of a tablet, SNAC causes an increase in pH of the surrounding environment (Buckley *et al.*, 2018). However, it is unknown how this shift in pH is impacted by the composition of the surrounding media, nor how the elevated pH at the surface of the tablet impacts the release behaviour of the peptide present in the dosage form. The solubilities of peptides vary depending on a number of environmental conditions, including pH, buffer type and ionic strength (Grossmann and McClements, 2023). In particular, peptide solubility is impacted by its isoelectric

point (pI), the pH at which the peptide possesses a net neutral charge. At the pI, solubility is minimised, while at 1-2 pH units above and below the pI, peptide molecules possess net negative and net positive charges, respectively, causing solubility to increase (Tokmakov, Kurotani and Sato, 2021). Therefore, the solubility and consequently the release profile of a peptide from an oral dosage form may be highly dependent not only on the pH of the media present in the GIT, but also on pH changes which occur due to the presence of a PE such as SNAC or C10.

The purpose of this study was to investigate the impact of two different PEs, C10 and SNAC, on the release properties of minitabets in a variety of biorelevant media, including simulated gastric and intestinal fluids, using human insulin as a model peptide. Firstly, the equilibrium solubilities of insulin, C10 and SNAC were determined in different biorelevant media, and the effects of high concentrations of C10 and SNAC on the pH of the media were evaluated. Insulin minitabets containing a high proportion of either C10 or SNAC were prepared by direct compression. The dissolution performance of the minitabets was assessed in each of the biorelevant media and compared to minitabets prepared in the absence of C10 and SNAC. To ascertain the impact of C10 and SNAC on the pH at the surface of the minitabets, a drop of universal indicator solution was applied to the C10, SNAC and blank minitabets, and a colour change was used to indicate differences in surface pH.

5.5. Experimental Section

5.5.1. Materials

The recombinant human insulin used in this investigation was procured from Merck KGaA (Germany). Two different permeation enhancers were investigated, C10 and SNAC, acquired from Merck KGaA (Germany) and BOC Sciences (New York, USA)

respectively. Microcrystalline cellulose (MCC), Avicel PH102 grade, and polyvinylpyrrolidone (PVP K30), Kollidon 30 LP grade, used for tableting were purchased from FMC Biopolymer (Ireland). Fasted state simulated intestinal fluid (FaSSIF), fed state simulated intestinal fluid (FeSSIF) and fasted state simulated gastric fluid (FaSSGF) were prepared using 3F powder procured from Biorelevant (United Kingdom). The compositions of the biorelevant media are given in Table 5.1.

Table 5.1. Compositions of FaSSIF, FeSSIF and FaSSGF.

Components	FaSSIF	FeSSIF	FaSSGF
Sodium Taurocholate (NaTC)	3 mM	15 mM	0.08 mM
Lecithin	0.75 mM	3.75 mM	0.02 mM
Sodium Dihydrogen Phosphate (NaH ₂ PO ₄)	29 mM	-	-
Sodium Chloride (NaCl)	106 mM	203 mM	34.2 mM
Hydrochloric acid (HCl)	-	-	As necessary for pH adjustment
Acetic Acid (CH ₃ COOH)	-	144 mM	-
Sodium Hydroxide (NaOH)	10.5 mM	50.5 mM	-
pH	6.5	5.0	1.6
Buffer capacity (Osm/ L)	270	670	120

5.5.2. Solubility Study

The saturation solubilities of insulin, SNAC and C10 were evaluated in FaSSIF, FeSSIF, FaSSGF, and their blank equivalents, that is media in the absence of sodium

taurocholate and lecithin. Excess amounts of insulin, C10 and SNAC were added individually to 1 mL of media in 2 mL Eppendorf tubes. Eppendorf tubes were shaken at 350 rpm for 24 hours on a Thermomixer Comfort (Eppendorf, Germany) at 37°C. Prior to analysis, samples were centrifuged using a Mikro 120 Centrifuge (Hettich Lab, Germany) at 18626 RCF for 2 min. All samples were prepared in triplicate.

5.5.3. Minitablet Preparation

Two different minitabket formulations were prepared in this investigation, composed of 72% PE (C10 or SNAC), 20% MCC, 5% PVP K30 and 3% insulin, all by weight. These formulations were designed to be similar to the commercial Rybelsus® formulation in terms of PE content and excipient selection. Rybelsus® consists of 75% w/w SNAC and variable amounts of semaglutide depending on the desired dose. Additionally, Rybelsus® tablets contain MCC and PVP, although the specific proportions of these excipients are not disclosed (U.S. Food and Drug Administration, 2019). A blank formulation was also prepared with 92% MCC, 5% PVP K30 and 3% insulin. The formulation components were blended in a pestle and mortar. Direct compression was performed manually using a Fette E1 tablet press with 4 mm tooling (I Holland – United Kingdom), with tablet weights of 22 ± 3 mg and tablet hardness values > 1 MPa achieved for all formulations.

5.5.4. Dissolution Testing

Dissolution was performed using a Distek 2100B system (Distek, New Jersey, USA) fitted with 100 mL vessels and paddles (Dissolution Accessories, the Netherlands). A total dissolution volume of 40 mL and paddle speed of 75 rpm were used. 350 μ L samples were taken over time intervals of 1, 5, 10, 15, 30, 45, 60 and 120 mins, with an equivalent volume of medium being replaced after sampling. Release rates of

insulin from all minitables were assessed in FaSSIF, FeSSIF, FaSSGF, and their blank equivalents, while the blank minitables were also tested in 0.1M phosphate buffer, pH 7.6, isotonic with FaSSIF. Prior to analysis, samples were centrifuged using a Mikro 120 Centrifuge (Hettich Lab, Germany) at 18626 RCF for 2 min. All samples were prepared in triplicate.

5.5.5. Quantitation

Quantitation was performed using an AdvanceBio SEC 130Å, 2.7 µm, 7.8 x 300 mm (Agilent Technologies, California, USA) column. An isocratic elution method was used, where the mobile phase consisted of l-arginine (1 g/L), acetonitrile (ACN) and 0.15M phosphate buffer (65%/20%/15%, all given as % v/v), adjusted to pH 7.5 with 1M hydrochloric acid (HCl). An injection volume of 10 µL and detection wavelengths of 210 nm and 280 nm were used. The total runtime was 15 min. All samples were analysed in triplicate. OriginPro 2022b and GraphPad Prism 8.4.3 were used for data analysis and graphical representation of results.

5.5.6. pH Measurements

The pH of saturated solutions of insulin, C10 and SNAC in all media investigated was measured using a pH probe. Subsequently, the surface pH of the minitables was assessed by adding a drop of universal indicator solution (Merck, Germany) to each of the blank, C10 and SNAC minitables and images were captured using a Canon EOS 1500D digital camera (Canon, Japan).

5.6. Results

5.6.1. Solubility Study

As the pH and composition of gastric and intestinal media vary widely, it is important to understand the solubility differences of insulin, C10 and SNAC in biorelevant media

to determine how their behaviour may be affected in different regions of the GIT. As such, in this investigation the saturation solubility of each solid was determined in FaSSIF, FeSSIF, FaSSGF and their blank equivalents, i.e. in the absence of sodium taurocholate and lecithin. The solubility values are given in Figure 5.1.

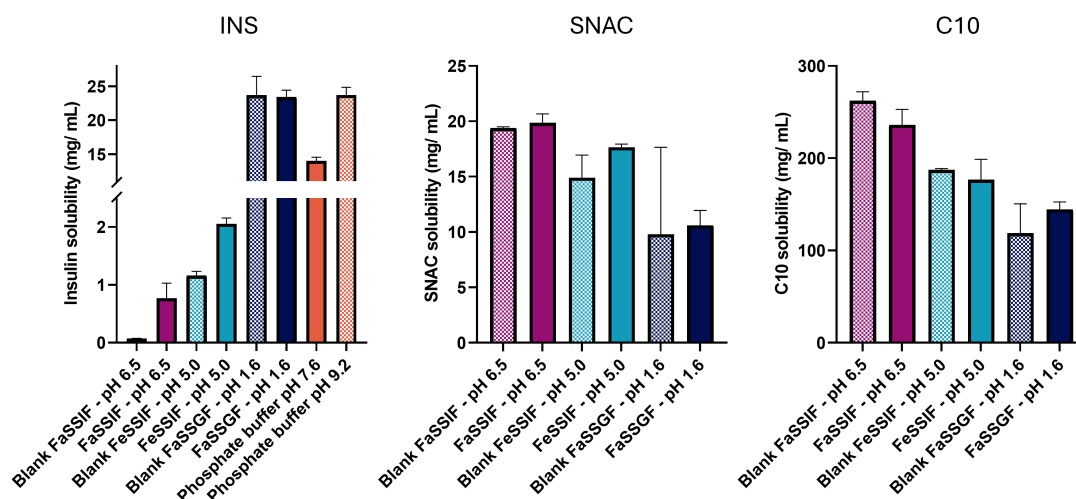


Figure 5.1. Saturation solubility of insulin, C10 and SNAC in FaSSIF, FeSSIF, FaSSGF and their blank equivalents after shaking for 24 hr at 350 rpm and 37°C. Insulin saturation solubility was also investigated in 0.1M phosphate buffer, isotonic with FaSSIF, at pH 7.6 and pH 9.2. Error bars represent standard deviation, $n = 3$.

Insulin exhibited the highest solubility in gastric media, independent of the presence of sodium taurocholate or lecithin. The lowest solubility was observed in blank FaSSIF at 0.1 mg/ mL, while this was observed to increase by a factor of 8 to 0.8 mg/ mL in FaSSIF, suggesting that the solubility of insulin was enhanced by the presence of sodium taurocholate and lecithin in this medium. Similarly, the solubility of insulin approximately doubled from 1.2 mg/ mL in blank FeSSIF to 2.1 mg/ mL in FeSSIF, indicating that the presence of sodium taurocholate and lecithin also had an impact on insulin solubility in this medium. It is interesting to note that according to proximity to the pI of insulin, which is at approx. pH 5.3, the solubility of insulin was expected to be highest in gastric media, which was indeed observed (Thomas and Thevis, 2019).

However, it would also be expected that insulin solubility would be lower in FeSSIF (pH 5.0) than in FaSSIF (pH 6.5), but the converse was observed. Given that FaSSIF is composed of a NaH_2PO_4 – NaOH buffer system, while FeSSIF is buffered by CH_3COOH – NaOH , it is likely that the unexpected behaviour observed was due to a specific ion effect as NaH_2PO_4 has been observed to have a stronger salting out effect on proteins than CH_3COOH (Mazzini and Craig, 2017). Additionally, solubility may have been impacted by the NaCl concentration, which is twice as high in FeSSIF as compared to FaSSIF.

The solubilities of C10 and SNAC were also observed to be pH dependent, although the presence of sodium taurocholate and lecithin did not appear to impact the observed solubility. This suggests that pH dominates PE solubility, rather than the mixed micelles present in the simulated media. Solubilities of both were highest in FaSSIF, which has a pH above the respective pK_as of C10 and SNAC, approximately 5 for each, while at very low pH in gastric media, the solubilities were lowest and more variable, particularly in the case of SNAC. Interestingly, in the presence of excess PE material there was a significant increase in the pH of all media investigated, with C10 increasing the pH to between 8.8 – 9.2, while SNAC caused an increase in pH to between 7.6 – 7.9. Insulin, on the other hand, did not appear to impact media pH. This can be seen clearly in Figure 5.2, which shows the pH of solutions after the addition of excess insulin, C10 and SNAC material. Similar pH values were obtained in both the biorelevant and blank media and as such only the pH values of the biorelevant media are shown in Figure 5.2. These data confirm the previous reports that SNAC elevated the pH of simulated gastric media from acidic to neutral, while also confirming that C10 exhibits similar behaviour, although C10 caused a larger increase in pH than did SNAC (Buckley *et al.*, 2018). Additionally, these results suggested that

the ability of C10 and SNAC to alter pH was independent of the composition and initial pH of the media investigated.

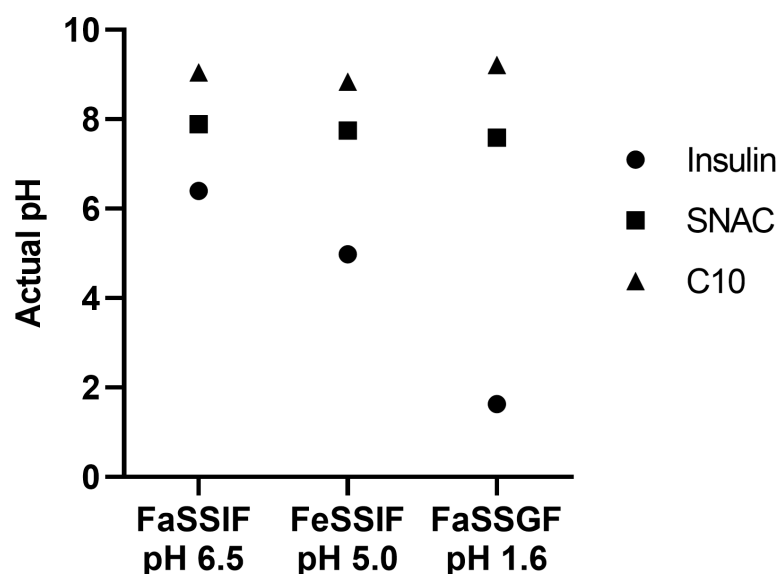


Figure 5.2. pH of saturated solutions of insulin, C10 and SNAC in FaSSIF, FeSSIF and FaSSGF after shaking for 24 hr at 350 rpm and 37°C. Error bars represent standard deviation, $n = 3$.

To determine if the shift in pH caused by C10 and SNAC influenced the solubility of insulin, excess insulin material was added to 0.1M phosphate buffer, isotonic with FaSSIF, at pH 7.6 and pH 9.2 to mimic the pH of solutions containing excess SNAC and C10 material, respectively. While outside its buffering range, 0.1M phosphate buffer was confirmed to be stable at pH 9.2 for at least 48 hr. The saturation solubilities at each pH were recorded and graphed in Figure 5.3. As expected, it appeared that at increasing distance from the pI, represented by a dashed line at pH 5.3 in Figure 5.3, the solubility of insulin was increased. In comparison to the lowest solubility observed in blank FaSSIF, a 140-fold increase in solubility was observed at pH 7.6, while a 240-fold increase was observed at pH 9.2. Solubility at pH 9.2 was approx. twice that at pH 7.6 and was comparable to that observed in simulated gastric media.

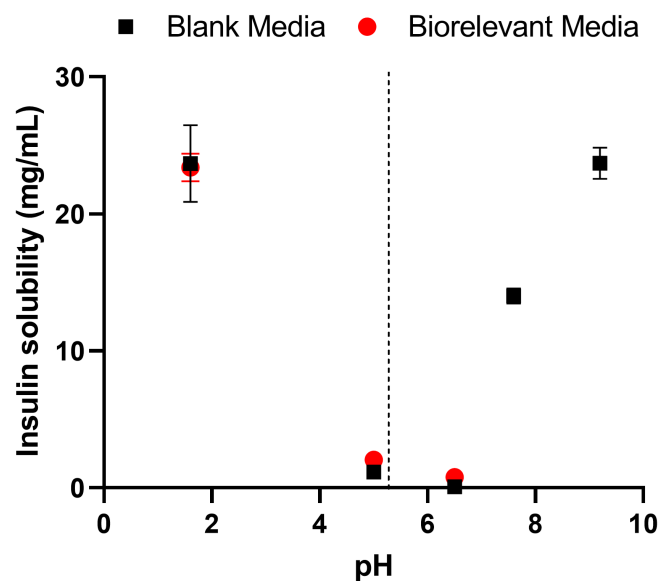


Figure 5.3. Solubility of insulin as a function of pH. The pI of insulin is represented by a dashed line at pH 5.3. Error bars represent standard deviation, $n = 3$.

Overall, pH was observed to have a profound impact on the solubility of insulin across all the media investigated, and while the presence of sodium taurocholate and lecithin appeared to have an effect at pH 5.0 and pH 6.5, the proximity of the pH to the pI was the dominant factor affecting solubility. These results suggested that alterations in pH due to the presence of high concentrations of C10 and SNAC may greatly affect the solubility, and, therefore, release of insulin in gastric and intestinal media. As such, the release rates of insulin from minitables containing C10 and SNAC in biorelevant media were investigated.

5.6.2. Dissolution Testing

To determine the impact of C10 and SNAC on the release rates of insulin, minitables were prepared and dissolution testing was carried out in a variety of blank and biorelevant media described previously. Minitables were produced via direct compression using a single punch tablet press. The formulations were composed of 72% SNAC or C10, 20% MCC, 5% PVP K30 and 3% insulin. Blank minitables were

also prepared in the absence of PEs and were composed of 92% MCC, 5% PVP K30 and 3% insulin.

Blank Minitablets

Dissolution testing was first performed on blank insulin minitables to determine if media composition had an impact on the release profiles of insulin in the absence of PEs, and results are shown in Figure 5.4.

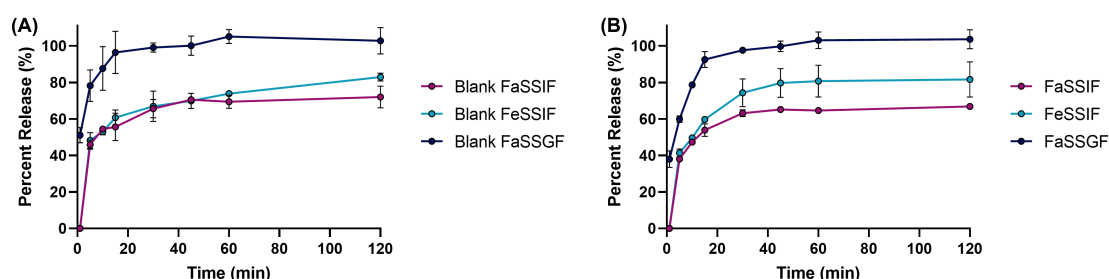


Figure 5.4. Release profiles of insulin from minitables composed of 92% MCC, 5% PVP K30 and 3% insulin in (A) blank FaSSIF (purple), blank FeSSIF (light blue) and blank FaSSGF (dark blue), and (B) FaSSIF (purple), FeSSIF (light blue) and FaSSGF (dark blue). Error bars represent standard deviation, $n = 3$.

Insulin appeared to exhibit solubility dependent release behaviour in both the blank and biorelevant media. Complete release was observed in FaSSGF and blank FaSSGF after 30 min, and while maximum release was also observed after approx. 30 min in FaSSIF, FeSSIF and their respective blank equivalents, complete release was not achieved in these media. Given that the maximum possible concentrations reached during dissolution were far lower than the saturation solubility of insulin in each of the media, the reason for the incomplete release in the simulated intestinal media was unclear. During dissolution, undissolved material was observed to settle at the bottom of the vessels, and it was suspected that incomplete disintegration of the minitables may have restricted insulin release. This phenomenon, known as coning, is a well-

known artifact of dissolution involving insoluble excipients such as MCC (Terashima and Ozeki, 2024). An attempt to increase the amount of insulin released in blank FaSSIF was performed by increasing the paddle rotation speed until total release was observed. This is shown in Figure 5.5.

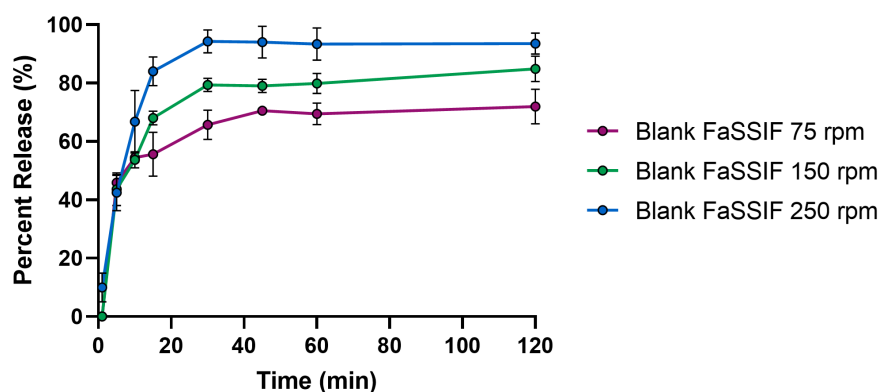


Figure 5.5. Release profiles of insulin from blank minitabets in blank FaSSIF at paddle rotation speed of 75 rpm (purple), 150 rpm (green) and 250 rpm (blue). Error bars represent standard deviation, $n = 3$.

As expected, increasing the paddle speed caused an increase in the total amount of insulin released from the blank minitabets, although the time taken to achieve maximum release remained at approx. 30 min for all paddle speeds. While increasing the paddle speed from 75 rpm to 150 rpm caused a notable increase in total insulin released from 72% to 85%, a very high paddle speed of 250 rpm was required to achieve greater than 90% release. These results suggested that at low rotation speeds, instead of being dispersed in the medium, insoluble MCC powder instead settled at the bottom of the dissolution vessels and may have trapped insulin above its saturation solubility, thereby preventing its dissolution into the bulk medium. To test this hypothesis further, the release profiles of insulin from minitabets in 0.1M phosphate buffer, pH 7.6 were assessed, and are shown in Figure 5.6.

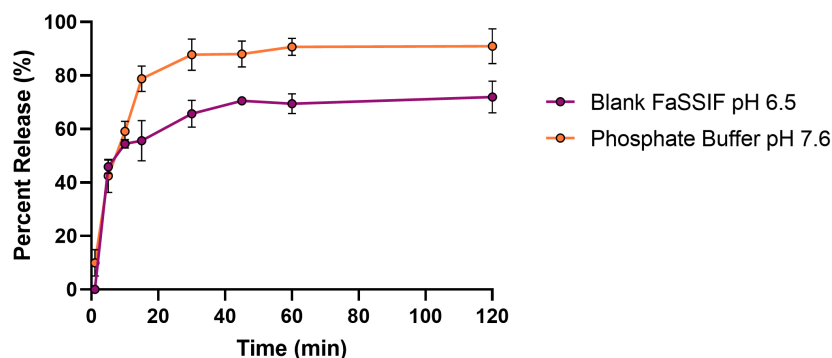


Figure 5.6. Insulin release from blank minitables in blank FaSSIF (purple) and 0.1M phosphate buffer, pH 7.6 (orange). Error bars represent standard deviation, $n = 3$.

Increasing the pH of the dissolution medium to 7.6 was found to increase the release rate and the total amount of insulin released in comparison to blank FaSSIF, with greater than 90% release being achieved. Given that the solubility of insulin was found to be approx. 20-fold higher in phosphate buffer, pH 7.6, than in blank FaSSIF, as shown in Figure 5.3, it is clear that solubility played an important role in the release behaviour of the blank minitables. This is further supported by the complete release of insulin observed in the simulated gastric media in Figure 5.4A and Figure 5.4B and offers a plausible explanation for the limited release found in simulated intestinal media.

Minitables Containing Permeation Enhancers

To determine the impact of PEs on the release of insulin from the minitables, dissolution testing was performed on the minitables containing C10 and SNAC and the insulin release profiles from each are given in Figure 5.7.

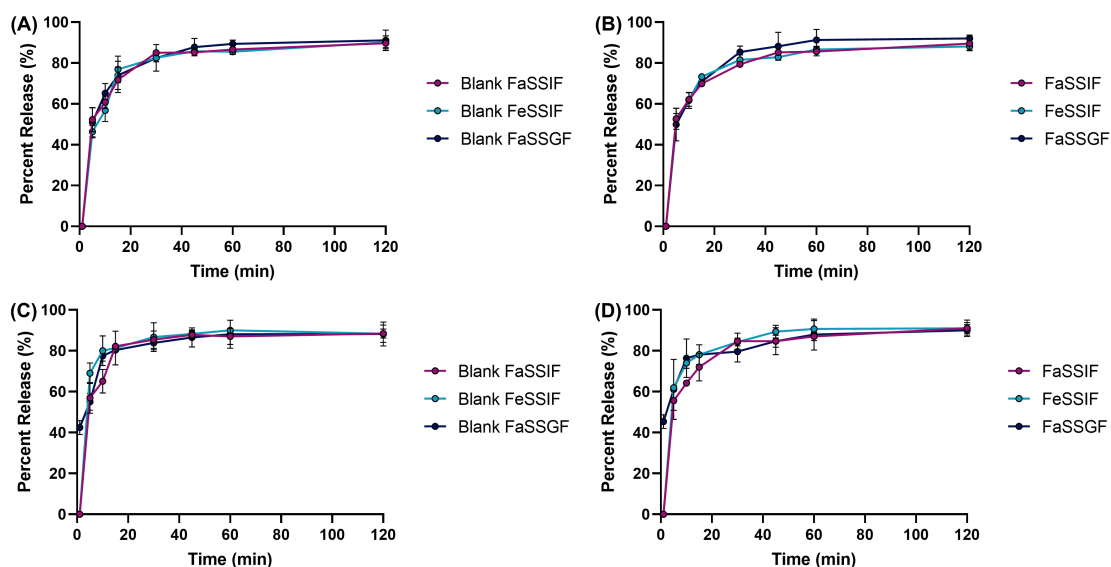


Figure 5.7. Release profiles of insulin from minitables composed of 72% C10, 20% MCC, 5% PVP K30 and 3% insulin in (A) blank FaSSIF (purple), blank FeSSIF (light blue) and blank FaSSGF (dark blue), and (B) FaSSIF (purple), FeSSIF (light blue) and FaSSGF (dark blue). The release profiles of insulin from minitables composed of 72% SNAC, 20% MCC, 5% PVP K30 and 3% insulin are also given in (C) blank FaSSIF (purple), blank FeSSIF (light blue) and blank FaSSGF (dark blue), and (D) FaSSIF (purple), FeSSIF (light blue) and FaSSGF (dark blue). Error bars represent standard deviation, $n = 3$.

As seen in Figure 5.7A and Figure 5.7B, the dissolution media did not appear to affect the release of insulin from the C10-containing minitables, regardless of pH or composition, where near-complete release was observed across all of the media investigated. Similarly, the release of insulin from the SNAC-containing minitables was observed to be independent of the dissolution media, as shown in Figure 5.7C and Figure 5.7D, and maximum release from both the C10- and SNAC-containing minitables was observed to occur after approx. 30 min in all media. This is in direct contrast with the blank minitables, which displayed an apparent solubility-dependent

insulin release. Therefore, it is clear that the presence of C10 and SNAC had a significant impact on the release of insulin from the minitables investigated.

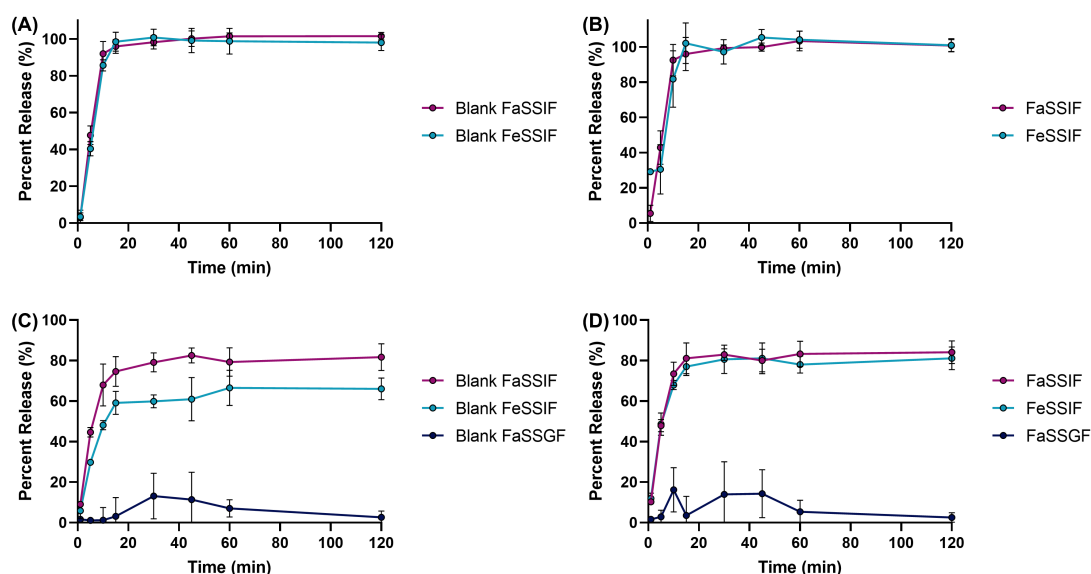


Figure 5.8. C10 release profiles from minitables composed of 72% C10, 20% MCC, 5% PVP K30 and 3% insulin in (A) blank FaSSIF (purple) and blank FeSSIF (light blue) and (B) FaSSIF (purple) and FeSSIF (light blue). The release profiles of SNAC from minitables composed of 72% SNAC, 20% MCC, 5% PVP K30 and 3% insulin are also given in (C) blank FaSSIF (purple), blank FeSSIF (light blue) and blank FaSSGF (dark blue), and (D) FaSSIF (purple), FeSSIF (light blue) and FaSSGF (dark blue). Error bars represent standard deviation, $n = 3$.

The release of C10 and SNAC from the minitables were also monitored, and the release profiles are given in Figure 5.8. C10 was observed to exhibit rapid and complete release in the simulated intestinal media after 15 min, however, no C10 was detectable from the dissolution samples in blank FaSSGF and FaSSGF. On visual inspection, the vessels appeared turbid during dissolution in the simulated gastric media, while in the simulated intestinal media they appeared clear. It was suspected that at low pH C10 was converted to the unionised, free acid form which is known to

form an oily precipitate (Namani and Walde, 2005). This precipitate may have been removed during centrifugation and was not detectable. SNAC was observed to display similar release behaviour, with maximum release achieved after approx. 15 min and minimal release observed in the simulated gastric media. However, unlike C10, complete release of SNAC was not achieved in any of the media investigated. As complete release of insulin was observed from all of the samples, it is unlikely that poor disintegration prevented the release of SNAC in this case. Instead, it is possible that SNAC also formed a second phase in the dissolution vessels which was removed during centrifugation and not detected. To test this, after the final time point was sampled, at the end of dissolution 1M NaOH was added to each of the vessels containing FaSSIF, FeSSIF and FaSSGF to adjust the pH to above 10, and the amount of C10 and SNAC recovered is given in Figure 5.9. Complete recovery of C10 and SNAC was achieved in all of the biorelevant media on increasing the pH. In particular, the appearance of the vessels containing the C10 and SNAC minitablets in the simulated gastric media changed from turbid to clear, suggesting that the second phase formed was disrupted at the elevated pH, enabling complete recovery to be achieved. Similarly, the total recovery of SNAC observed on elevating the pH of FaSSIF and FeSSIF is suspected to be due to the disruption of colloidal self-assemblies or precipitated material.

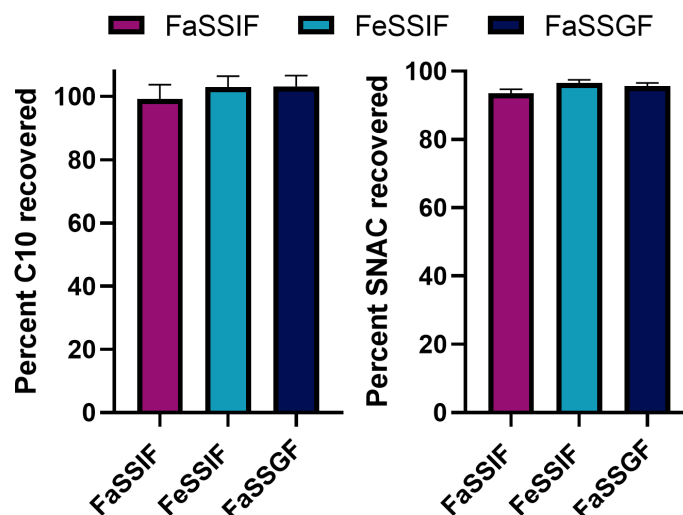


Figure 5.9. Percent C10 and SNAC recovered after dissolution on adjustment of pH of FaSSIF (purple), FeSSIF (light blue) and FaSSGF (dark blue) to basic. Error bars represent standard deviation, $n = 3$.

5.6.3. Surface pH Measurements

The differences in insulin release behaviour observed in the presence of the PEs in comparison to the blank minitables were hypothesised to be due to high concentrations of C10 or SNAC present at the surfaces of the minitables at the onset of dissolution. This would result in the actual pH experienced by insulin in these minitables being higher than the pH of the surrounding dissolution media, potentially reaching as high as pH 9.2 in the presence of C10 and pH 7.9 in the presence of SNAC. Although it was previously confirmed in Figure 5.2 that C10 and SNAC solutions altered the pH of biorelevant media during solubility measurements, there was no direct evidence for this occurring during dissolution as measurement of the bulk media before and after dissolution showed no detectable change in pH. As such, differences in pH at the surfaces of the blank, C10- and SNAC-containing minitables were investigated. To do so, a single drop of universal indicator solution was applied to each

of the minitables, and the colour change was captured using a digital camera, with results shown in Figure 5.10.

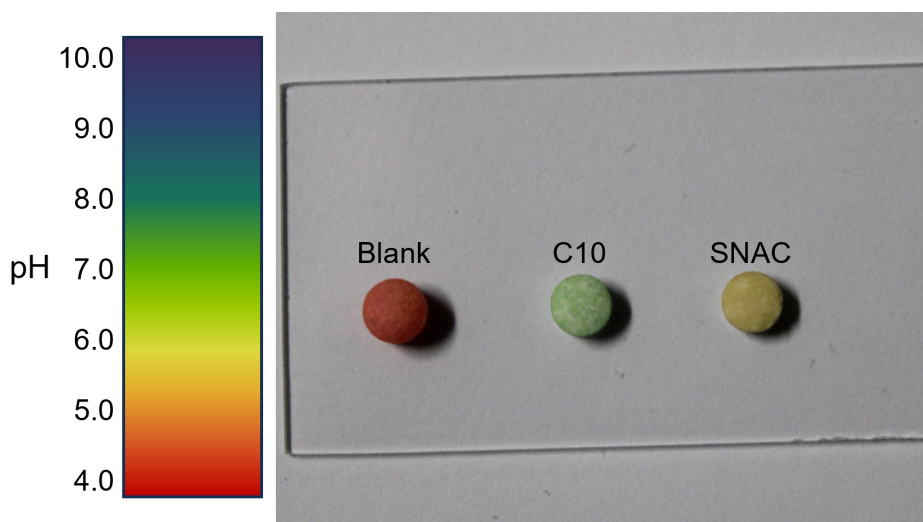


Figure 5.10. Representative image of blank, C10 and SNAC minitables after addition of a single drop of universal indicator solution. Images were captured using a Canon EOS 1500D digital camera. A pH-colour scale is also given.

On application of the universal indicator solution, which is acidic and appears red in colour, to the blank minitables, no colour change was observed. On the other hand, the C10 minitables appeared a dark green after addition of the indicator solution, while the SNAC minitables appeared pale green. This suggested that the pH values at the surfaces of the C10 and SNAC minitables were close to pH 8 and pH 7, respectively, much higher than that of the blank minitables. It is clear, therefore, that the presence of C10 and SNAC considerably altered the surface pH of the minitables, and this likely accounts for the notable differences in release characteristics of the blank minitables compared to the C10- and SNAC-containing minitables.

5.7. Discussion

PEs have garnered considerable attention from both industry and academia for their potential to improve the oral bioavailability of peptide therapeutics, with the approval

of Rybelsus® in 2019 being a breakthrough success for this approach. Although their applicability is limited to peptides with a favourable therapeutic index, long half-life and high potency, the use of PEs in oral dosage forms has proven a simple, yet effective strategy to improve the absorption of peptides from the GIT and has advanced the field of oral peptide delivery further than any other approach to date (Fattah *et al.*, 2020). Popular PEs, such as C10 and SNAC, have been studied extensively, and considerable effort has been placed on elucidating their mechanisms of action, efficacies and safety profiles *in vivo* (McCartney, Gleeson and Brayden, 2016; Maher *et al.*, 2009; Bohley and Leroux, 2024; Fein *et al.*, 2023). However, little focus has been placed on understanding the formulation aspect of this approach, and there remains a dearth of information available in the literature on the release kinetics of PEs and peptides from oral dosage forms and how this behaviour is further influenced by the various components of gastric and intestinal media.

A number of important considerations have been identified as vital to obtain optimal absorption during co-delivery of peptides and PEs to the GIT. Firstly, it is important that there is temporal synchronicity in the release of the peptide and PE from the dosage form. Several studies have highlighted that the efficacy of a PE *in vivo* is dependent on the duration for which the PE and peptide are co-localised at the gastric and intestinal epithelia above a particular concentration (Sutton *et al.*, 1993; Baluom, Friedman and Rubinstein, 1998; Bækdal *et al.*, 2021). As such, for PEs to exert an enhancing effect, a threshold concentration must be achieved at the site of action. Thereafter, there is a short timeframe within which the peptide must also present at the site of action prior to absorption and/ or dilution of the PE in the GI media or transit of the dosage form away from the site of action. Another factor to be considered is the composition of biological media (Larsen *et al.*, 2024). As described above, the efficacy

of a PE is highly dependent on its concentration. Therefore, sequestration of PEs into mixed micellar or vesicular structures with bile salts or phospholipids present in the intestinal fluids may unfavourably alter their activities. Indeed, in an investigation by Roos *et al.* it was observed that the enhancement action of a surfactant-based PE, sodium dodecyl sulphate (SDS), was reduced in FeSSIF relative to FaSSIF, where it was hypothesised that the greater abundance of micellar structures present in FeSSIF may have reduced the amount of SDS available to interact with the epithelium in the rat single-pass intestinal perfusion model used (Roos *et al.*, 2019). On the contrary, in an investigation by Berg *et al.*, the permeation enhancing effect of C10 was found to be unaffected by the presence of bile salts and phospholipids present in simulated intestinal media (Berg *et al.*, 2022). Media composition can also have a profound impact on the properties of peptides. For example, peptides can exist in multiple charge states, both positive and negative, depending on the pH of the environment, and as previously noted in the Introduction, the solubility of a peptide is inherently connected to the number of charges it possesses. However, the charge of a peptide may also affect its absorption across the epithelial wall. As tight junctions (TJs) exhibit charge-dependent permeability, peptide charge may influence paracellular transport, where positively charged molecules tend to pass through TJs more easily than do negatively charged molecules (Almansour *et al.*, 2018; Schoultz and Keita, 2020). Further, diffusion of peptides through the mucus layer has been found to be charge dependent (Li *et al.*, 2013). In addition, it has been reported that two water soluble peptides, octreotide and desmopressin (to a lesser extent) interacted with micelles in biorelevant media, leading to reduced flux across a membrane (Denning, Douglas and Hageman, 2021). Based on the increase in insulin solubility in both FaSSIF and FeSSIF, insulin also appears to interact with micellar species. The nature of these

interactions is unclear at this time. It is clear, therefore, that the release kinetics of the dosage form and the media into which the PEs and peptides are released are critical to maximise peptide bioavailability.

The current investigation sought to bridge this gap in knowledge by investigating the *in vitro* release characteristics of minitabets containing two different PEs, C10 and SNAC, in a number of biorelevant media, using insulin as a model peptide. C10 and SNAC were observed to exert a profound influence over the release of insulin from the minitabets. In the absence of C10 or SNAC, the release of insulin from blank minitabets was found to be media dependent. Complete insulin release was achieved in FaSSGF and blank FaSSGF, however, limited release of insulin was observed to occur in FaSSIF, FeSSIF and their blank equivalents. As shown in Figure 5.3, the solubility of insulin was highly dependent on medium pH, where highest solubility was observed at pH 1.6 and pH 9.2, while close to the pI at pH 5.3 solubility was lowest. These results suggested that insulin release from the blank minitabets in the simulated intestinal media was solubility limited, and this was further confirmed by the increase in insulin release rate in phosphate buffer at pH 7.6 seen in Figure 5.6. In contrast, complete release of insulin was observed in all media from minitabets containing C10 and SNAC.

There is compelling evidence to suggest that the difference in release behaviour noted in the presence of the PEs was the result of a local increase in pH at the surface of the minitabets due to high concentrations of C10 and SNAC at the solid-solution interface. Firstly, in Figure 5.2 it was observed that the pH of all simulated gastric and intestinal media investigated were elevated in the presence of excess C10 and SNAC material, where C10 caused an increase in pH to between pH 8.8 – 9.2 and SNAC caused an increase to between pH 7.6 – 7.9. SNAC has been previously reported to

alter the pH of simulated gastric media, however, similar investigations have not been performed in simulated intestinal media, nor have the effects of C10 on the pH of biorelevant media been studied elsewhere (Buckley *et al.*, 2018). Although there was no detectable change in the pH of the bulk media after dissolution of the minitables, the use of a universal indicator solution provided visual confirmation that the surface of the C10 minitables had an approximate pH of 8 and the surface of the SNAC minitables had an approximate pH of 7. No colour change was observed to occur at the surface of the blank minitables, as seen in Figure 5.10. In comparison to the blank minitables, the pH at the surface of the minitables containing C10 and SNAC was higher than that of the surrounding media, and this elevated pH likely caused a considerable increase in the saturation solubility of insulin above that observed in the simulated intestinal media, allowing for complete release to be achieved. If correct, this hypothesis proposes that for tablets containing large proportions of C10 or SNAC, the pH experienced by insulin during the initial stage of dissolution would be independent of the dissolution medium and instead its solubility and, therefore, release would be a function of the elevated pH caused by high local concentrations of C10 and SNAC. While the current study has focused on dissolution of insulin, the above findings may also provide crucial insights into the release of other therapeutic peptides, such as glucagon-like peptide-1 (GLP-1) analogues, from oral dosage forms in which PEs are present. Depending on their composition and size, GLP-1 analogues, such as semaglutide and exenatide, typically possess a pI in the range of 4.0 – 7.0, and, therefore, exhibit low aqueous solubility at the pH of intestinal fluid (Zhang *et al.*, 2018; Palani *et al.*, 2022; Sandmeier *et al.*, 2024). This suggests that, similarly to insulin, an increase in pH driven by the presence of C10 or SNAC would have an impact on their solubilities and, therefore, release behaviour. However, given that PEs

have previously been observed to form peptide-specific non-covalent and hydrophobic ion-pairing interactions which can reduce the aqueous solubility of peptides, it is not clear without further studies if the behaviour observed in this investigation will translate to other clinically relevant peptides (Bonengel *et al.*, 2018; Buckley *et al.*, 2018).

The dissolution of C10 and SNAC from the minitables was observed to be rapid in simulated intestinal media, with maximum release observed after 15 min. Minimal release of C10 and SNAC was observed in simulated gastric media, and this was suspected to be due to the formation of a second phase which was removed before analysis (Namani and Walde, 2005). Similarly, the incomplete release of SNAC in simulated intestinal media was proposed to be due to the formation of large colloidal self-assemblies/precipitates in solution which were likely removed during centrifugation and not detected. On addition of 1M NaOH and elevation of the media pH to above 10, complete recovery of C10 and SNAC was achieved, suggesting disruption of the vesicular structures/precipitates which enabled detection. It is proposed, therefore, that rapid and complete release of the PEs did indeed occur in each of the biorelevant media. On the other hand, although it was apparent that the PEs were not freely available in their monomeric/micellar form, which may restrict their efficacies, the release of insulin from the minitables was not affected by the dissolution media, indicating that insulin release was purely a function of the elevated pH at the minitab surface during the initial stage of dissolution. Overall insulin release was, however, found to be slower than that of C10 and SNAC, with maximum concentration being reached after 30 min in all media, rather than in 10 or 20 min as observed for C10 and SNAC respectively. From a delivery perspective, the staggered release of insulin and PEs from the minitables in this investigation would likely result

in incongruous presentation at the epithelial walls, leading to suboptimal enhancement of insulin permeability.

It is clear from the above discussion that further work is required to investigate the release mechanisms of peptides from oral dosage forms containing C10 and SNAC, and an understanding of the impact of biologically relevant fluids on release may prove essential for successful dosage form development. A particular focus of research in this area should be placed on improving the co-release of peptides and PEs, for example in this case by slowing the dissolution rate of C10 or SNAC or increasing the dissolution rate of insulin, as this may prove beneficial for achieving optimal oral peptide bioavailability. Additionally, an understanding of how the association state of PEs in different biorelevant media impacts their efficacies will be vital for optimising the design of oral dosage forms, while also informing the need for site specific delivery.

5.8. Conclusion

The use of PEs has become the predominant method of improving the oral bioavailability of peptides. However, while the therapeutic efficacies and safeties of PEs have been well characterised, very little attention has been given to understanding how the addition of PEs impacts the release behaviour of peptides from oral dosage forms or how the composition and pH of the surrounding media further influences this. As such, this investigation of the impact of two widely employed PEs, C10 and SNAC, on the release of insulin from minitables in a variety of biorelevant media revealed potentially noteworthy observations. Firstly, high concentrations of C10 were found to cause an increase in pH to between 8.8 – 9.2, while SNAC was found to elevate the pH to between 7.6 – 7.9, independent of the composition or initial pH of the media. Furthermore, the solubility of insulin was found to increase significantly at pH 7.6 and

pH 9.2, representative of saturated solutions of SNAC and C10 respectively, relative to in simulated intestinal fluid, suggesting that high local concentrations of C10 and SNAC could have a notable impact on insulin solubility. Secondly, while minitables without a PE exhibited medium-dependent insulin release, release of insulin from the C10- and SNAC-containing minitables was found to be independent of the dissolution media. This difference in release behaviour was determined to be the result of an increase in pH at the surface of the minitables containing C10 and SNAC relative to the blank minitables, caused by high concentrations of C10 and SNAC, respectively, at the solid-solution interface. Interestingly, while C10 and SNAC did have an impact on the total amount of insulin released from the minitables, insulin release was found to be slower than that of C10 and SNAC, suggesting a staggered release of insulin and the PEs from the minitables. Overall, it is clear that the presence of C10 and SNAC had a profound influence on the release characteristics of insulin from direct compression minitables, although further work is required to understand and optimise the co-release of peptides and PEs from oral dosage forms in order to achieve substantial improvements in bioavailability.

Chapter 6: General Discussion

6.1. Overview and Summary

Therapeutic peptides have valuable potential in the treatment of a broad range of unmet clinical indications, including autoimmune, metabolic and oncological disorders (Wang *et al.*, 2022). The biopharmaceutical market in general has experienced rapid growth in recent decades, and peptide therapeutics have been positioned to occupy a niche between small molecules and larger biologics (Xiao *et al.*, 2025). As the peptide therapeutic market has grown, there has been increasing interest in alternative administration routes to offer ease of administration, greater patient compliance and greater market coverage (Anselmo, Gokarn and Mitragotri, 2019). As the most common route of drug delivery, the oral route holds great potential for peptide delivery. Accordingly, substantial focus has been given to overcoming the inherent barriers to oral delivery, primarily the poor gastrointestinal stability and permeability of peptides, through the use of novel and innovative formulation approaches. The biggest development in this area came with the successful use of chemical PEs to facilitate improved permeation of peptide therapeutics across the gastric and intestinal epithelia, leading to the approval of products such as Rybelsus[®] and Mycappsa[®]. There are several other oral peptide products currently in the development pipeline which seek to take advantage of PEs, such as Merck's MK-0616 (enlicotide decanoate), which is in Phase III clinical trials (Ballantyne *et al.*, 2023).

Since the potential of PEs for oral peptide delivery was realised, they have become the leading strategy for improving oral peptide bioavailability. To date, considerable focus has been placed on developing a large library of compounds that improve peptide permeability, which include bile salts, surfactants and even peptides, and on

understanding their efficacies *in vivo* (Kim, Park and Na, 2022). However, beyond understanding the *in vivo* performance of PEs, there are several other key factors to be considered in the successful development of an oral peptide product. Firstly, as highlighted in Chapter 1, the requirement for large quantities of PEs to be present in oral formulations means that they must possess adequate manufacturability properties to enable commercial scale production (Leane *et al.*, 2015). For example, in the case of direct compression tableting, PEs must have suitable flow and mechanical properties to enable the production of tablets with acceptable and reproducible weights, content uniformities and tensile strengths. In addition, PEs may also heavily influence the release behaviour of dosage forms in which they are present, thereby impacting the extent and duration of enhanced peptide permeation. Besides the influence of the PEs on the performance of oral dosage forms, another key consideration is the stability of the peptide itself. As well as being stable to the harsh environments of the GIT, peptide structure must not be adversely affected by the storage, handling and manufacturing conditions involved in the production of the dosage form. Given that the therapeutic efficacy of a peptide is intrinsically linked to its stability, ensuring the peptide is maintained in a stable, active form during processing is essential to achieving its therapeutic end point.

In light of these considerations, this thesis aimed to advance the current knowledge of the role that peptide stability and PEs play in the development of oral peptide dosage forms, using insulin as a model peptide and C10 and SNAC as model PEs. Specifically, an in-depth characterisation of the solid-state stability of insulin was performed in Chapter 2, and the role that temperature and humidity play in the rates at which different degradation pathways occur were evaluated in Chapter 3. In Chapter 4, the suitability of C10 and SNAC for processing via direct compression was investigated

by evaluating their mechanical behaviours during compaction, enabling rational design of direct compression formulations with high PE content. The influence of the high contents of PEs on the release of insulin from minitablets was then studied in Chapter 5. Furthermore, the overall effects of blending insulin in direct compression formulations and compacting at high pressures on the stability of the insulin molecule were assessed in Chapter 4. A detailed description of the investigations performed to assess these aims has been provided in the preceding chapters, and a broader discussion of the key findings in the context of the wider literature is provided herein.

6.2. Insulin Stability during Storage and Compaction

The poor long-term stability of biopharmaceuticals in solution presents a major challenge not only for formulation design but also introduces significant economic burdens due to requirement for cold-chain storage and handling. This led to the development of solid-state formulation strategies, such as freeze drying and spray drying, which enable prolonged storage of peptide therapeutics prior to reconstitution and use (Emami *et al.*, 2018; Sharma *et al.*, 2021). Moreover, renewed interest in the development of oral dosage forms of peptides has presented a new necessity for stabilising peptides in the solid-state. Although peptides are generally more stable in the dry, solid-state than in aqueous solutions, degradation may still occur, and stability remains a primary concern for the handling and processing of peptide raw materials for oral dosage form development (Chang and Pikal, 2009).

Insulin is known to degrade via similar pathways in the solid-state as those observed in solution. Deamidation and covalent aggregation have been identified as the primary degradation pathways in the solid-state, the mechanisms of which are described schematically in Figure 1.3. Briefly, deamidation occurs via a two-step process, where firstly a cyclic intermediate is formed at the C-terminal Asn A21 residue, followed by

hydrolysis to form the deamidated product. Alternatively, the intermediate may interact with N-terminal amino acid residues on adjacent insulin chains to form covalent insulin dimers through a transamidation pathway (Strickley and Anderson, 1996, 1997). Covalent aggregates may also be formed through a disulfide exchange reaction in which β -elimination of cysteine residues causes the cleavage of intramolecular disulfide bonds and formation of free thiols. The free thiols formed further catalyse the scrambling of disulfide bonds in adjacent insulin molecules, giving rise to intermolecular disulfide bonds (Costantino, Langer and Klibanov, 1994a). Our investigations have further confirmed that insulin degradation in the solid-state is similar to that observed in solution, as detailed in Chapter 2. Insulin samples were found to undergo significant deamidation and covalent aggregation at elevated temperature and humidity conditions, where LC-MS confirmed deamidation occurred exclusively on the insulin A-chain, while reductive cleavage experiments enabled verification of disulfide exchange as the predominant covalent aggregation mechanism. Interestingly, from analysis of the CD spectra, there was no evidence of any alterations to the backbone secondary structural features, indicating that insulin exhibits excellent conformational stability even during extensive chemical degradation in the solid-state. Secondary structural changes have previously been observed to occur in lyophilised insulin samples stored at elevated humidity conditions, however, there have been no other investigations into the conformational stability of insulin in the solid-state (Flores-Fernández, Solá and Griebenow, 2009). Although these degradation mechanisms have been well characterised previously, the predominance of each pathway and the rates at which they occur are known to be heavily influenced by environmental factors, including temperature and humidity (Lai and Topp, 1999; Costantino, Langer and Klibanov, 1994b).

Exposing insulin material to elevated temperatures significantly impacts its molecular stability by accelerating intrinsic degradation rates and increasing overall molecular mobility, with both deamidation and covalent aggregation pathways following apparent Arrhenius kinetics (Pikal and Rigsbee, 1997; Sadrzadeh *et al.*, 2010). The role of moisture in insulin stability, on the other hand, is more complex and multi-modal given the heterogeneous chemical structure of insulin. Moisture has been found to increase the rates of both deamidation and covalent aggregation of lyophilised insulin samples, acting as a reactant in the deamidation pathway at low humidity but shifting to a plasticising role at high humidity, thereby favouring the covalent aggregation pathways (Strickley and Anderson, 1996, 1997; Costantino, Langer and Klibanov, 1994a). This is not always the case, though, as moisture has also been reported as having no significant impact on deamidation and covalent aggregation rates of lyophilised insulin (Pikal and Rigsbee, 1997). However, there had not previously been a comprehensive investigation of the combined effects of temperature and humidity on insulin degradation kinetics. Accordingly, in Chapter 3, we sought to bridge this knowledge gap by probing the individual and combined effects of temperature and humidity on the rates of deamidation and covalent aggregation in solid-state insulin samples. In our investigation, temperature had a profound impact on the degradation kinetics of amorphous insulin, where increasing temperatures from 25°C to 60°C caused an increase in both the deamidation and covalent aggregation rates. The temperature-dependent changes in the rates of the different pathways were well described by the Arrhenius kinetic model. It was also evident that the degradation products were not in direct competition for a common intermediate, as evidenced by the consistent increase in both deamidation and covalent aggregation products over time at all conditions studied and the degradation profiles elucidated in Chapter 2.

Humidity, on the other hand, was found to have a temperature dependent effect on stability, which has not been previously reported. At 25°C and 40°C, deamidation and covalent aggregation rates did not appear to be affected by humidity, while at 60°C reaction rates were observed to accelerate at increasing humidity. This effect was more pronounced for the covalent aggregation pathway, highlighting the role of water in facilitating intermolecular bond formation. A plausible explanation for this temperature-dependency was offered by DVS analysis, in which there was a clear change in the moisture sorption behaviour of the insulin material at 60°C that gave rise to the apparent increase in sensitivity to degradation due to moisture at this temperature. It is possible that at 60°C, insulin underwent a molecular relaxation process which changed the phase of the amorphous material and increased overall reactivity and, particularly, sensitivity to moisture (Shamblin, Hancock and Zografi, 1998). However, as detailed in Chapter 3, the complex and heterogeneous nature of the insulin structure prevented confirmatory analysis of this phenomenon using thermal analysis, such as DSC. Regardless, it is evident from the above discussion that both temperature and humidity play important but distinct roles in insulin solid-state stability and will be highly influential factors in informing the storage and handling conditions used during oral product development.

Beyond external stressors, insulin stability is also heavily influenced by its physical state and preparatory conditions. In their investigations, Pikal and Rigsbee sought to compare the differences in stability between ‘crystalline’ insulin, that is insulin precipitated near its pI in the presence of zinc, and amorphous lyophilised insulin (Pikal and Rigsbee, 1997). The amorphous insulin was observed to exhibit substantially higher stability than the crystalline form. Most notably, the degradation rates of the crystalline form were highly sensitive to moisture content, while those of

the amorphous form were independent of moisture content. Furthermore, the degradation profiles and rates of lyophilised insulin samples have been found to be dependent on solution pH prior to drying. Stickley and Anderson observed that increasing pH from 3 to 5 caused a reduction in the overall degradation rates of lyophilised samples during storage (Stickley and Anderson, 1996). This pH dependence was accounted for by a proposed reduction in ionisation of the free carboxyl group of the A21 residue as pH was increased, restricting the formation of the cyclic intermediate. In a follow-up study, the authors identified a change in the degradation profiles of the samples according to pH prior to lyophilisation, where at low pH deamidation was favoured, while covalent aggregation was favoured as pH was increased (Stickley and Anderson, 1997). Moreover, the degradation rates of the samples lyophilised at pH 3 – 5 were found to increase as moisture content was increased, whereas the samples lyophilised at pH 7.1 in the aforementioned study by Pikal and Rigsbee were not sensitive to moisture content. In comparison, the insulin raw material in our investigations was used as obtained from the supplier in the amorphous form, and did not undergo any processing prior to performing the experiments. This material clearly displayed distinct degradation profiles and sensitivity to moisture than those of the lyophilised samples described above, thus further highlighting the role that the physical state of the material plays in insulin degradation.

Another consideration in the development of an oral dosage form is the stability of insulin to the high compaction pressures used during tableting. Proteins have been found to exhibit structure-specific sensitivity to compaction pressures, however, no investigations have been conducted to investigate the stability of therapeutic peptides during compaction. As such, in Chapter 4 we sought to characterise the conformational

stability of insulin after compaction at 200 MPa. The insulin molecules proved to be remarkably robust, with no evidence of secondary structural changes occurring in either the solid-state or upon reconstitution, nor was there evidence of aggregation occurring. As a result, insulin was deemed suitable for further tableting experiments, and efforts were made to prepare a suitable direct compression formulation with a high proportion of permeation enhancers. Interestingly, when incorporated in the formulations, consisting of 72% PE (C10 or SNAC), 20% MCC, 5% PVP K30 and 3% insulin, insulin displayed improved stability on storage at 40°C/ 75% RH for 1 month in comparison to the raw material tested in Chapter 3.

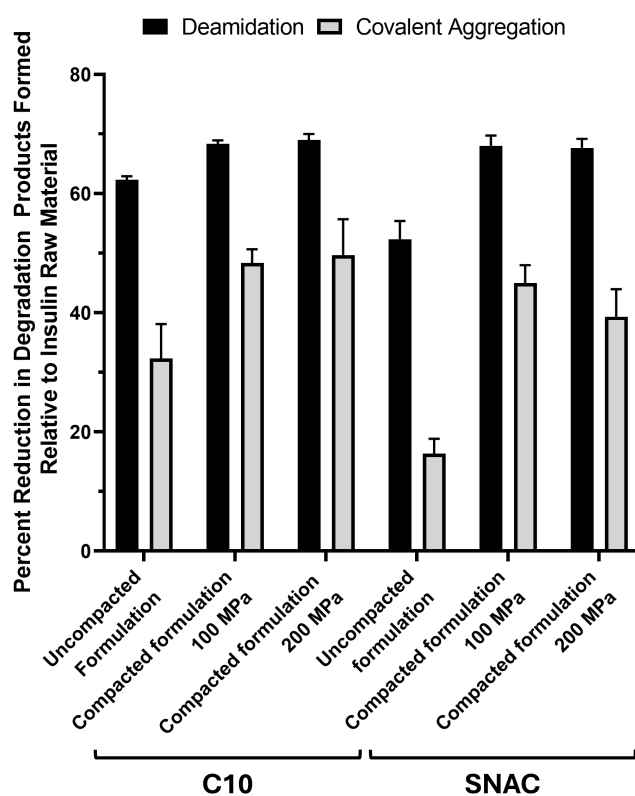


Figure 6.1. Relative percent decrease in total amount of deamidation and covalent aggregation in direct compression insulin formulations with C10 and SNAC, stored at 40°C/ 75% RH for 1 month, compared to raw uncompacted insulin powder.

As can be seen in Figure 6.1, there was a reduction in the extent of both deamidation and covalent aggregation in the formulated samples versus the raw material. Furthermore, a greater stabilising effect was observed on compaction of the formulations into minitables at both 100 and 200 MPa. A number of factors may have contributed to the reduced degradation observed after storage of the formulated insulin samples and, indeed, the compacts. Firstly, as MCC and PVP are known to be hygroscopic, the presence of these excipients in the formulation may have limited the amount of moisture freely available to react, thereby reducing the extent of degradation (Bühler, 2005; Yassin *et al.*, 2015). Moreover, as reported in Chapter 5, C10 and SNAC are slightly basic materials, which elevate the pH of solutions when present in high concentrations. On adsorption of large amounts of moisture, it is possible that the presence of large PE contents gave the formulations an ‘apparent’ pH that was basic and reduced overall degradation rates in a manner similar to that observed in the lyophilised samples described previously. It is also likely that dilution of the insulin molecules within the formulations limited the potential for intermolecular interactions to occur and reduced the amount of covalent aggregation. Finally, the compaction of the formulations into minitables may have improved insulin stability through a reduction in overall molecular mobility, while also limiting the amount of moisture that could penetrate the compacts through densification.

In the context of an insulin tablet, and indeed the broader context of therapeutic peptide tablets, these results contribute several key insights into the importance of stability in the success of such products. Our investigations have shown that the unformulated amorphous insulin raw material investigated would likely display adequate stability over short periods of time in the typical controlled working conditions used during tableting, that is 20-25°C and <60% RH (Haycocks, Goldschmidt and Thomsen,

2021). Less than 2% total degradation was observed at 25°C and humidities up to 75% over 7 days, suggesting that handling of the material for short periods at these conditions would not have deleterious effects on stability. What's more, an improvement in stability to storage conditions could be seen on blending of insulin within direct compression formulations with permeation enhancers and to a greater extent on compaction into tablets.

6.3. Permeation Enhancers in Insulin Tablet Development

PEs have been the focus of a considerable body of research over the preceding decades aiming to enhance the oral bioavailability of peptides. Several of the most promising PEs have been studied extensively in both laboratory and clinical settings, and some have been granted either food additive, GRAS or pharmaceutical excipient status (Maher *et al.*, 2019). Of these, the most popular PEs are C10 and SNAC. However, from a formulation perspective, there remains a critical gap in understanding of several of the key aspects involved in dosage form development and drug delivery for oral formulations containing such PEs. In the case of tableting, the properties of PEs are of fundamental importance for the compaction behaviour of the formulation, and the disintegration and dissolution behaviours of the tablets produced. Accordingly, a primary aim of this thesis was to extend the current body of knowledge on PEs to include these crucial formulation considerations.

The focus of our investigations in Chapter 4 was to characterise the suitability of C10 and SNAC as excipients for a simple direct compression process. To do so, the mechanical properties of the PEs were assessed using a compaction triangle approach, where the materials were compacted at various compaction pressures, and the tensile strengths and porosities of the compacts produced were used to construct the tableability, compactibility and compressibility profiles of C10 and SNAC. Overall,

the C10 material possessed passable flow character, although its mechanical properties were poor. C10 produced compacts with very low porosity, even at the lowest compaction pressures used, suggesting that the C10 particles underwent rearrangement and/ or fragmentation to form a densely packed powder bed under the applied pressures. These low porosities, however, did not translate to high tensile strength tablets, and C10 exhibited poor tabletability and compressibility, while its compactibility profile deviated from the traditional compaction model used. The poor compressibility of C10 material suggested that it did not undergo significant plastic deformation during tableting and instead exhibited characteristics of a more brittle or elastic material. Furthermore, the low tensile strengths observed at very low porosities suggested that there was limited bonding between C10 particles during compaction. This is in congruence with the observation that SDS forms weak compacts at compaction pressures up to 300 MPa due to the poor cohesion between SDS particles, suggesting that the anionic surfactant structures shared by C10 and SDS restricts interparticulate bonding during tableting and prevents formation of compacts with desirable tensile strengths (Chantraine *et al.*, 2007a).

SNAC, on the other hand, had poor flow character but acceptable mechanical properties. SNAC displayed good tabletability, compactibility and compressibility, allowing for tablets with desirable tensile strengths and porosities to be produced. In contrast to C10, SNAC was found to undergo greater plastic deformation and lower elastic recovery after compaction, giving rise to the desirable compaction properties observed. A possible explanation for the stark difference in compaction behaviour of C10 and SNAC may lie in their chemical structures. As shown in Figure 1.6, C10 has a simple MCFA structure, while SNAC is functionalised with a salicylamide group on the end of an 8-carbon MCFA chain. As a result, SNAC has a greater capacity for

intermolecular interactions, which may lead to more substantial cohesive forces between SNAC particles during compaction and, thus, stronger compacts. It is clear, therefore, that SNAC has superior compaction properties to C10, making it a more suitable excipient for direct compression, however, its poor flow character may prove difficult for large scale tablet production without the use of further processing steps or addition of an excipient to improve flow.

A further observation from Chapter 4 was that the blending of C10 and SNAC with commonly used direct compression excipients provided a substantial improvement to their overall compaction performance. MCC and PVP were chosen as a commonly used diluent and binder, respectively, and were blended with C10 and MCC in different proportions to determine the optimal blend for tensile strength enhancement. The addition of MCC, and to a lesser extent PVP, was found to markedly increase the tableability of C10 and SNAC, where 20% MCC and 5% PVP were chosen as the most suitable proportion of each component to produce formulations for further testing. These formulations, consisting of 72% PE (C10 or SNAC), 20% MCC, 5% PVP K30 and 3% insulin, were further compacted into minitables at 100 MPa and 200 MPa, and the percent improvement in tensile strength relative to the raw C10 and SNAC powders are given in Figure 6.2. The formulations chosen were similar in composition to the commercial Rybelsus[®] formulation and provides a realistic representation of a PE content required for peptide bioavailability enhancement *in vivo*.

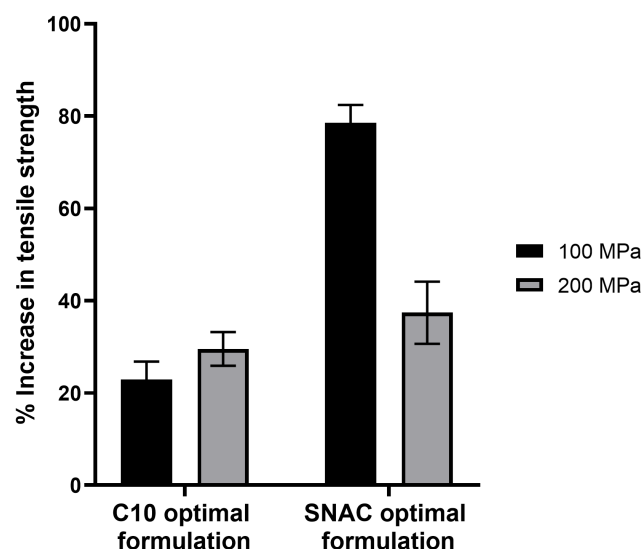


Figure 6.2. Percent increase in tensile strength observed on compaction of formulations consisting of 72% C10/20% MCC/5% PVP/3% insulin and 72% SNAC/20% MCC/5% PVP/3% insulin at 100 MPa and 200 MPa in comparison to the raw C10 and SNAC materials compacted at the same pressures.

It has been reported elsewhere that high SNAC concentrations can cause an increase in the pH of gastric media (Buckley *et al.*, 2018). Furthermore, our investigations have highlighted that this behaviour is consistent across both gastric and intestinal media, regardless of initial pH or ionic strength, and is similarly observed for C10. As peptide solubility is pH dependent, changes in pH due to the presence of high local concentrations of C10 and SNAC may significantly alter the solubility of peptides and thus their dissolution behaviour in the GIT (Grossmann and McClements, 2023). As a result, it was hypothesised that, in addition to impacting the overall compaction performance, the presence of high PE contents in the formulations selected in Chapter 4 would also have a meaningful influence on the release of insulin from the compacts produced. Indeed, several studies have highlighted the critical roles that release kinetics and dissolution media have in maximising peptide bioavailability from oral

dosage forms (Baluom, Friedman and Rubinstein, 1998; Bækdal *et al.*, 2021; Roos *et al.*, 2019). Therefore, in Chapter 5 we sought to investigate the impacts that C10 and SNAC have on the release characteristics of direct compression compacts in biorelevant media. Dissolution testing was performed in FaSSIF, FeSSIF, FaSSGF and their blank equivalents, and the insulin release profiles of the C10- and SNAC-containing compacts were compared with those of blank minitablets prepared without any PEs present. As expected, C10 and SNAC were found to have a profound effect on the release of insulin from the compacts, where complete release was observed across all media in the presence of the PEs, while media-dependent release was observed from the blank minitablets. The high C10 and SNAC contents in the minitablets were confirmed to increase the pH at the minitablet surface, and the difference in release behaviour was therefore attributed to an increase in insulin solubility at the elevated pH of the solid-solution interface in comparison to the bulk media. Moreover, insulin release behaviour did not appear to be impacted by the compaction pressure used during tableting. It is common for tablets compacted at higher pressures to exhibit reduced dissolution rates due to reduced penetration of the dissolution medium into the compacts (Maclean *et al.*, 2023). However, in Chapter 4 minimal differences were observed in the insulin release profiles of C10- and SNAC-containing minitablets compacted at 100 MPa and 200 MPa, suggesting that the high solubilities and potentially high wettability of the PEs enable unaltered release from the more densely packed compacts (Lu *et al.*, 2014).

Although the release of insulin in the presence of the PEs appeared to be unaffected by the composition of the biorelevant dissolution media and the compaction pressures used during tableting, this was not the case for the PEs themselves. Rapid dissolution of C10 and SNAC was observed to occur in simulated intestinal media, however, in

the case of SNAC, complete release was not achieved in this media. In addition, minimal release of C10 and SNAC was observed to occur in the simulated gastric media. On elevation of media pH to above 10 after completion of the dissolution experiments, complete recovery of C10 and SNAC was achieved in all solutions, suggesting that the partial release of C10 and SNAC observed was not due to poor dissolution, but rather inability to detect the molecules. It was therefore proposed that, in the low pH of the simulated gastric media, C10 and SNAC underwent conversion to their poorly soluble unionised forms, which have been shown previously to form a second phase that may have been removed during centrifugation prior to analysis (Namani and Walde, 2005). Similarly, the incomplete release of SNAC in the simulated intestinal fluids may be attributed to the formation of large colloidal self-assemblies or precipitates in solution that were likewise removed prior to analysis. Although the formation of these structures did not alter the release of insulin from the compacts, further indicating that insulin release was purely a function of the elevated pH at the minitabiet surface, it is possible that they may lead to a reduction in the *in vivo* efficacies of the PEs due to a low concentration of the monomeric form available to interact with the epithelia/ insulin molecules and have an enhancing effect. Another factor that may further reduce the amount of insulin that permeates across the GI epithelia is the staggered release of the PEs and insulin from the compacts.

Co-localisation of PEs and peptides at the site of absorption has been identified as an important factor for the successful improvement of peptide permeability, and tablet dissolution kinetics are key to achieving this (Bækdal *et al.*, 2021; Baluom, Friedman and Rubinstein, 1998; Sutton *et al.*, 1993). The disparity in dissolution times observed in our investigations, where C10 and SNAC dissolved more rapidly (10 – 20 min) than did insulin (30 min), would likely result in suboptimal insulin bioavailability due to

staggered presentation of the molecules at the epithelial walls. Several approaches have recently been adopted to combat this problem. In a recent study, Bapat *et al.* achieved synchronised release of SNAC and octreotide from an amorphous solid dispersion through the use of PVPVA as a gel-forming polymer (Bapat *et al.*, 2025). Similar release behaviour was observed from amorphous solid dispersions containing C10. However, it remains to be seen how these results will translate after compaction of the materials on a tablet press. In another approach, Tran *et al.* applied a thin layer of sodium alginate to the surface of tablets composed of SNAC and a GIP/GLP1 dual agonist peptide in an attempt to adhere the tablets to the gastric mucosa of monkeys and prevent dilution of the SNAC/ peptide molecules in the stomach (Tran *et al.*, 2025). Although these tablets exhibited excellent gastric retention, they did not offer improved oral bioavailability of the peptide compared to non-mucoadhesive SNAC tablets, likely due to slow dissolution *in vivo*. Clearly, there is a large scope for future investigations in this area.

6.4. Conclusions and Future Perspectives

This thesis has demonstrated the important impacts that stability and chemical PEs play in the development of an insulin tablet. Overall, the insulin raw material displayed acceptable chemical and conformational stability to the typical storage and processing conditions associated with a conventional tableting process. C10 and SNAC demonstrated distinct compaction behaviours during processing, where SNAC was found to be more suitable to processing via direct compression. Furthermore, C10 and SNAC were found to significantly impact the release of insulin from compacts through a modulation of the pH at the dissolution interface. To our knowledge, the compaction and dissolution studies performed in Chapter 4 and Chapter 5 of this thesis are the first in the literature to investigate the physical and mechanical properties of C10 and

SNAC, and their influence on the release behaviour of a peptide in different solution environments. As such, the novel findings reported here highlight the fundamental role that PEs play in the design and development of insulin tablets, and, indeed, oral peptide dosage forms more broadly.

There are several avenues of research that can be taken to build upon the work presented in this thesis. Firstly, although insulin was a suitable model for the purposes of our investigations, it has not proven to be a successful candidate for oral delivery. Initially, the potential to deliver insulin orally provided the drive behind the development of several of the bioavailability enhancement approaches used currently. However, given its failure to translate to the clinic, the field has since transitioned towards the use of smaller peptides with larger therapeutic windows, longer half-lives and higher potencies in search of greater success (Fattah *et al.*, 2020; Naylor *et al.*, 2017; Fonte *et al.*, 2013). Therefore, use of peptides such as GLP-1 analogues would provide a more realistic representation of the types of peptides currently under development and would prove insightful into how the characterisation of insulin stability in the solid-state could be translated to a structurally diverse group of peptides, while also indicating if the effects of C10 and SNAC on insulin release is similar for other therapeutic peptides.

Secondly, during the investigations in Chapter 3, it was not possible to elucidate whether there were changes in the physical state of insulin occurring during storage as its heterogeneous chemical structure prevented accurate determination of T_g using DSC. More in-depth physical characterisation using techniques such as dynamic mechanical thermal analysis (DMA) may prove more effective for investigating the conformational fluctuations and phase changes which occur in solid peptide materials (Newman and Zografi, 2019; Batens *et al.*, 2020). As the physical state of insulin has

been shown to be highly influential over its degradation profile in the solid-state, such information would be valuable in informing the processing conditions used to prepare peptides in the solid-state and possible methods of maintaining stability during storage.

Thirdly, as we have shown, C10 and SNAC have a major impact on the manufacturability of tablets produced by direct compression. This was found to be significantly improved on addition of commonly used direct compression excipients to the formulation. As such, it would be of interest to investigate the influence of a broader range of excipients on the compaction and flow behaviour of C10 and SNAC. Moreover, the introduction of additional processing steps, such as granulation, could further improve this behaviour to enable large scale production of peptide tablets. Consequently, these alternative approaches may alter the release kinetics of the dosage forms, while also introducing additional stressors that may impact the stability of peptides and, thus, are of interest for explorative investigations.

Finally, the complex environment of the gastrointestinal milieu presents a number of challenges to the successful transport of peptides across the GIT. For example, sequestration of PE molecules into mixed micelles with the bile salts and phospholipids present in intestinal fluids may unfavourably alter the activity of the PE through a reduction in free monomer concentration. Therefore, the use of techniques such as 2-dimensional NMR spectroscopy to probe the association state of PEs and possible interactions that may occur with the components of the gastrointestinal media may be beneficial to understand the behaviour of the PEs upon dissolution *in vivo*. Indeed, investigating the interactions between peptides, PEs and/ or the mixed micelles formed may shed further light on the mechanisms of action of PEs such as

SNAC and C10 (Vinarov *et al.*, 2018; Dening, Douglas and Hageman, 2021; Katev, Vinarov and Tcholakova, 2021).

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Appendices

S2. Appendix 1: Supplementary Information Chapter 2

S2.1. Additional chromatograms

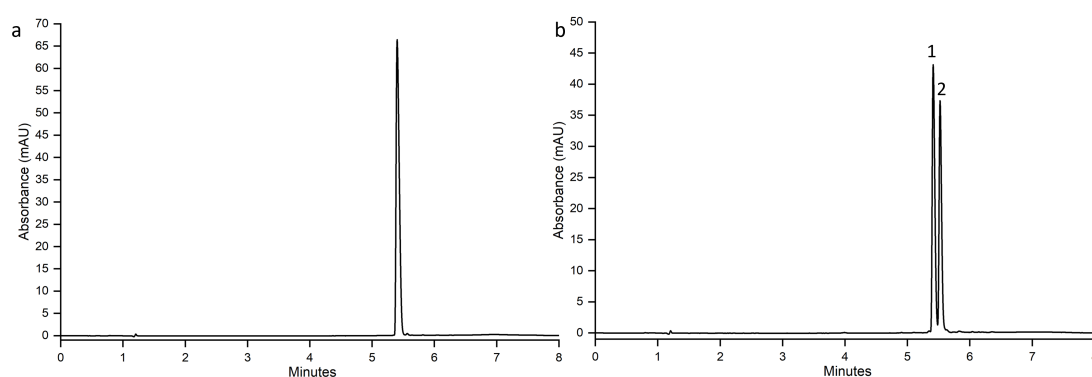


Figure S2.1. Representative RP-HPLC chromatogram of (a) 1 mg/ mL insulin standard sample and (b) 1 mg/ mL insulin sample stored in 0.1% FA for 1 month, where the native insulin peak is labelled 1 and the degradant peak is labelled 2. RP-HPLC was performed using an Agilent 1260 Series HPLC system, with a Poroshell 120 SB-C18, 2.7 μ m, 4.6 x 150 mm (Agilent Technologies) column. Mobile phase A (MPA) consisted of 95% H₂O, 5% acetonitrile (ACN) and 0.1% formic acid (FA), while mobile phase B (MPB) consisted of 5% H₂O, 95% ACN, 0.1% FA. MPB was linearly increased from an initial content of 20% to 35% over 4 min, returned to 20% MPB in 1 min and held for 3 min. The flow rate was 1 mL/ min and a total injection volume of 5 μ L was used. The HPLC column was heated at 60°C. 280 nm was used as a protein specific detection wavelength.

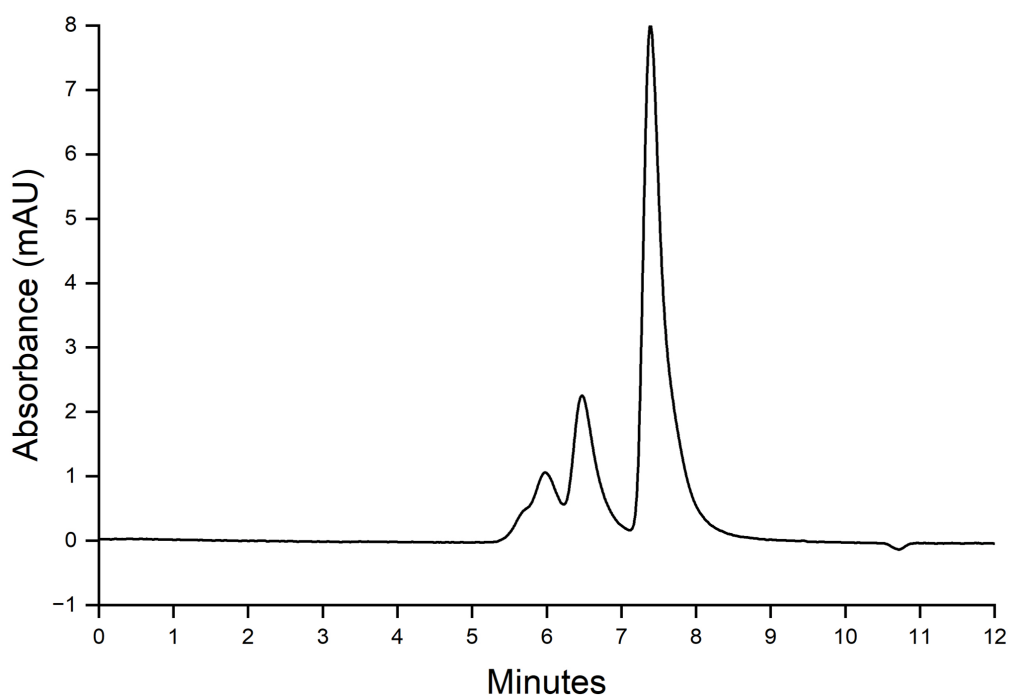


Figure S2.2. Representative SEC chromatogram of 1 mg/ mL insulin sample heated in the solid state at 60°C/ 75% relative humidity (RH) for 7 days. SEC was performed using an Agilent 1260 Series HPLC system with an AdvanceBio SEC 130Å, 2.7 μ m, 7.8 x 300 mm (Agilent Technologies) column. The mobile phase consisted of 65% L-arginine (1 g/ L)/ 20% acetonitrile (ACN)/ 15% acetic acid (AA). An isocratic elution method was used, with a total run time of 12 min required. A flow rate of 1.000 mL/ min and injection volume of 5 μ L were used. 280 nm was used as a protein specific detection wavelength.

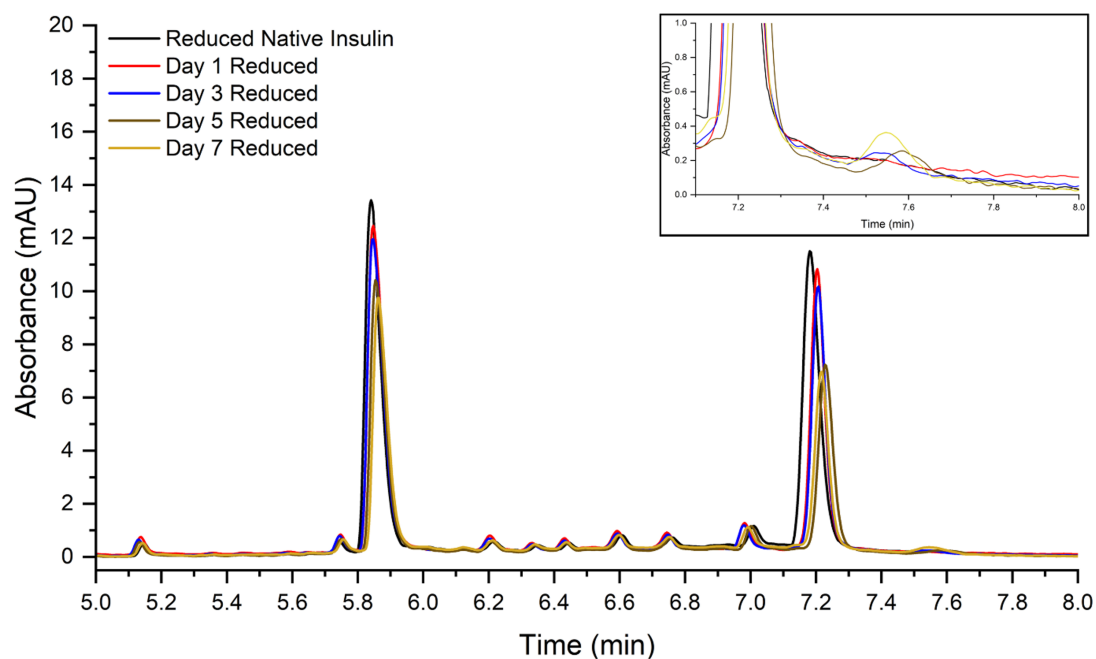


Figure S2.3. Representative RP chromatograms of a native insulin sample (black) and insulin samples heated in the solid state at 60°C at 75% RH for 1 (red), 3 (blue), 5 (green) and 7 (yellow) days, treated with 50 mM DTT and 6M urea and heated at 65°C and stirred at 300 rpm for 30 min. The inset shows an expansion of the deamidation peak at ~ 7.5 min. The chromatographic method was the same as that described in Figure S2.1.

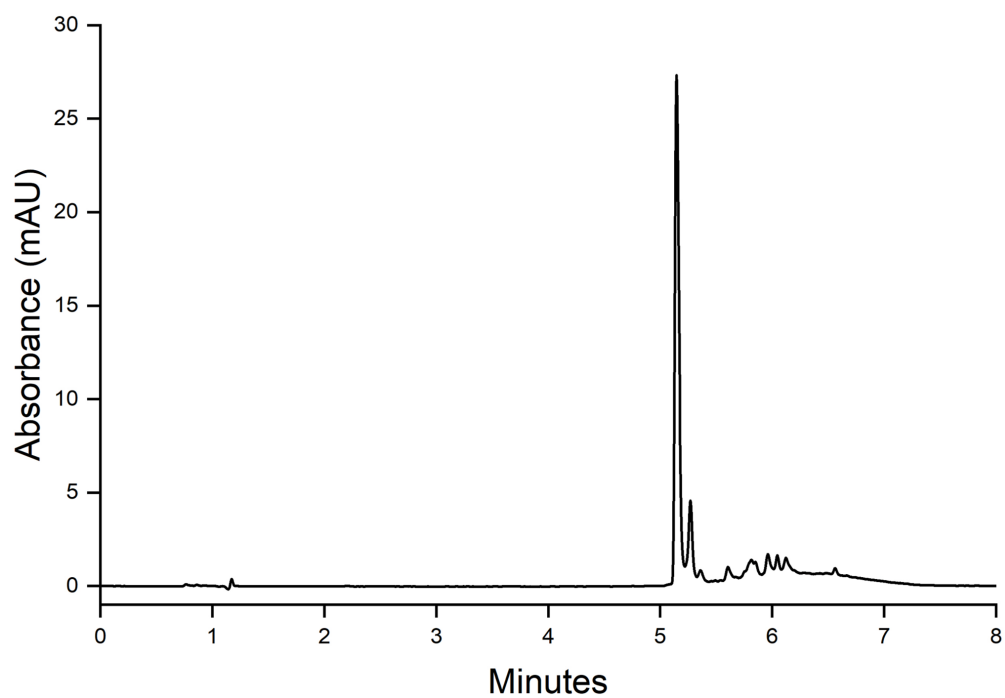


Figure S2.4. Representative RP-HPLC chromatogram of 1 mg/ mL insulin sample heated in the solid state at 60°C/ 75% relative humidity (RH) for 7 days. The chromatographic system and method described in Figure S2.1 was used for the analysis.

S2.1. Additional mass spectra

Table S2.1. Predominant ions present in mass spectra of 1 mg/ mL insulin deamidated samples, $n = 3$.

Sample	Species	Ion (m/z)	Charge	Mass (Da)
Rep 1	Native insulin	968.79	+6	5812.7
		1162.34	+5	5811.7
	Degradant	968.95	+6	5813.7
		1162.54	+5	5812.7
Rep 2	Native insulin	968.79	+6	5812.7
		1162.34	+5	5811.7
	Degradant	968.95	+6	5813.7
		1162.54	+5	5812.7
Rep 3	Native insulin	968.79	+6	5812.7
		1162.34	+5	5811.7
	Degradant	968.95	+6	5813.7
		1162.54	+5	5812.7

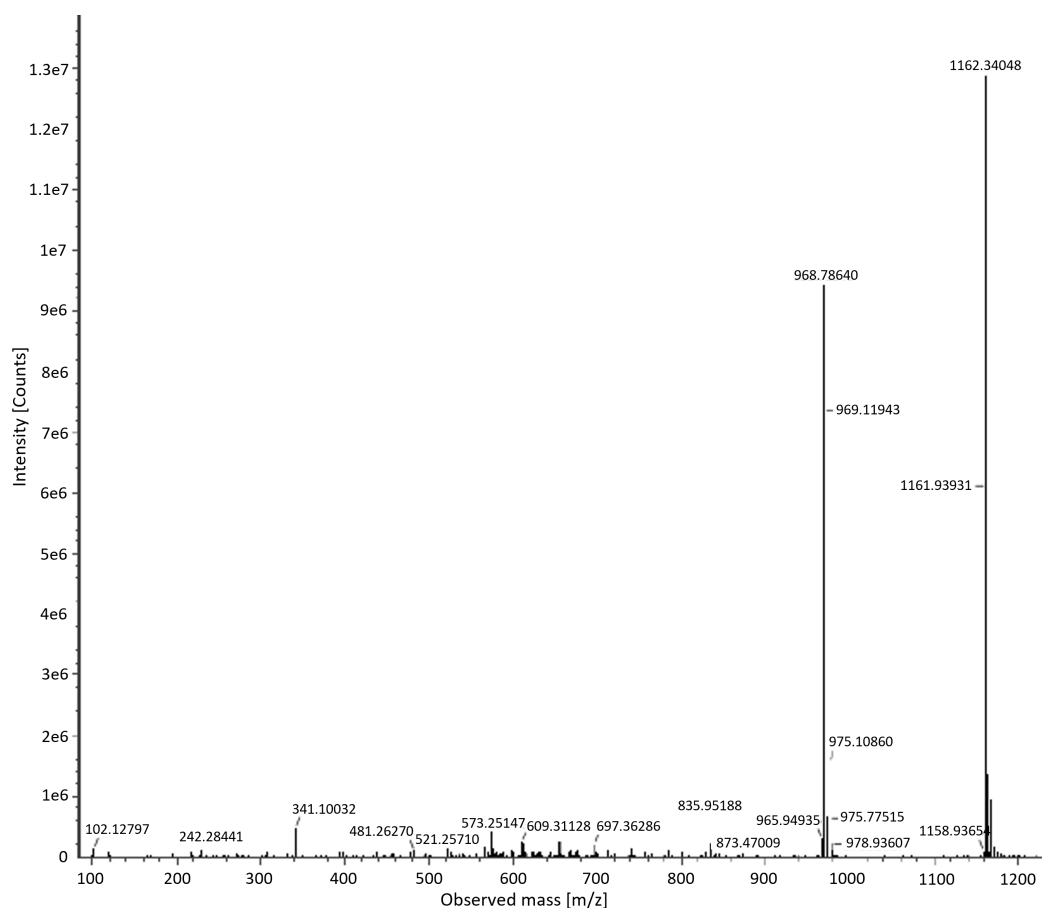


Figure S2.5. Representative mass spectrum of peak 1 in Figure S1b. A Waters Acquity I-class UPLC coupled to a Vion IMS Mass Spectrometer (Waters Corporation) was used for LC-MS analysis. The chromatographic method was the same as that described in Figure S2.1.

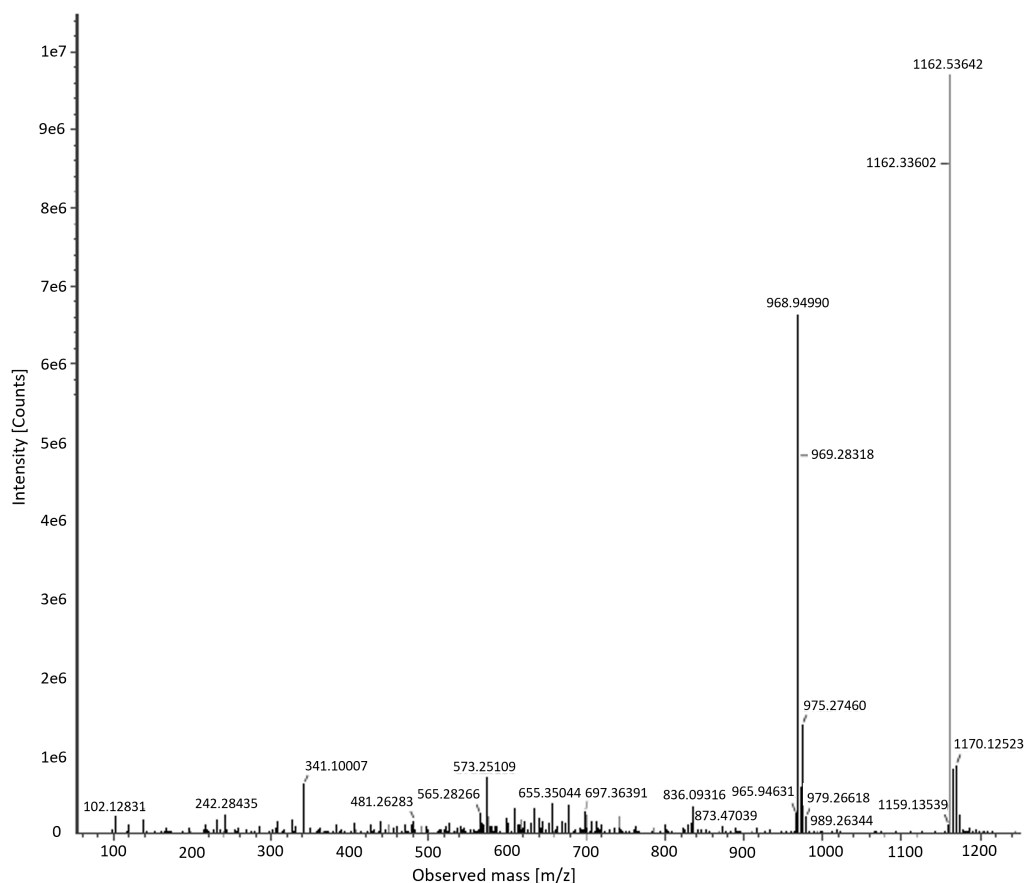


Figure S2.6. Representative mass spectrum of peak 2 in Figure S1b. A Waters Acquity I-class UPLC coupled to a Vion IMS Mass Spectrometer (Waters Corporation) was used for LC-MS analysis. The chromatographic method was the same as that described in Figure S2.1.

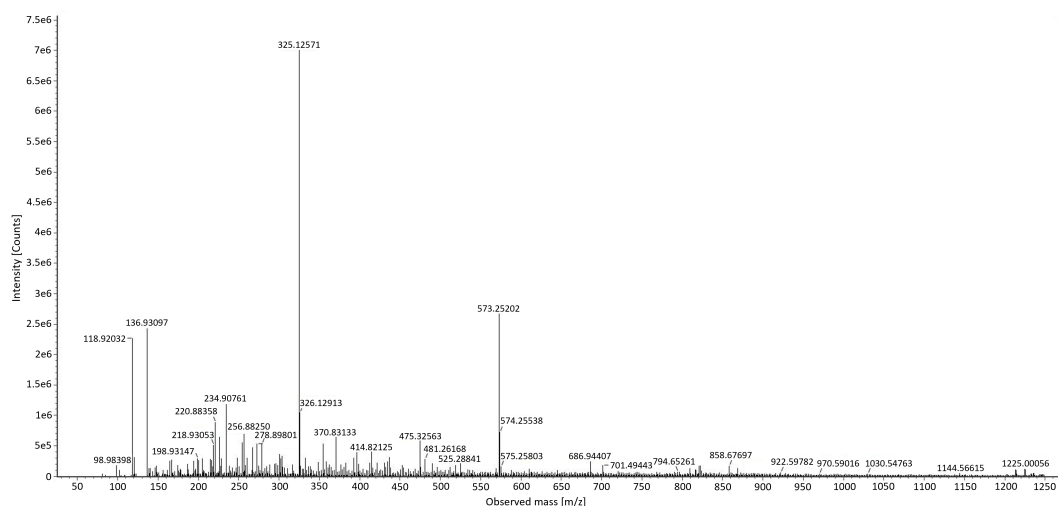


Figure S2.7. Representative mass spectrum showing the ions present in the region between the peaks present at 5.8 min and 7.2 min in Figure S2.5. A Waters Acquity I-class UPLC coupled to a Vion IMS Mass Spectrometer (Waters Corporation) was used for LC-MS analysis. The chromatographic method was the same as that described in Figure S2.1.

S3. Appendix 2: Supplementary Information Chapter 3

S3.1. Experimental set-up



Figure S3.1. Schematic representation of experimental set up. Kilner[®] jars were used as bespoke humidity chambers, into which insulin samples and saturated salt solutions were placed. Humidity chambers were then placed in ovens with set temperatures.

S3.2. Percent insulin dissolved over time

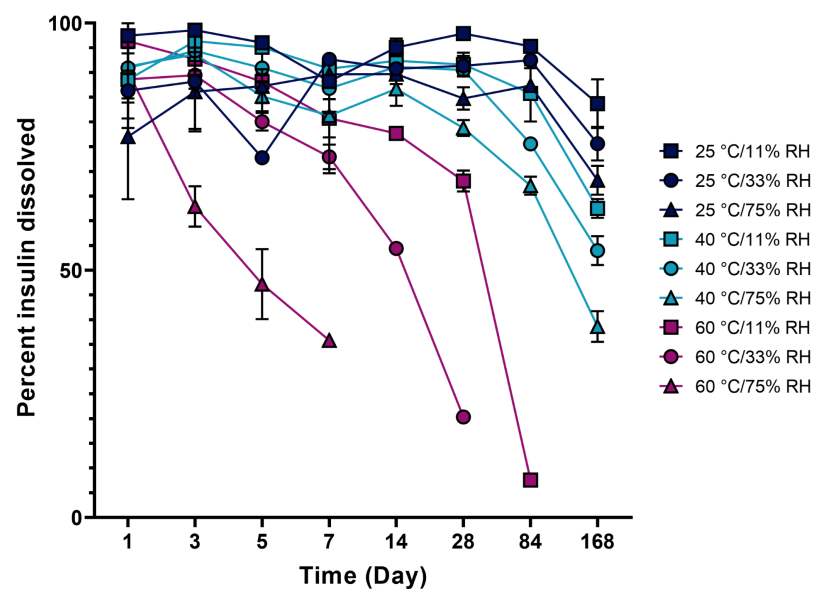


Figure S3.2. Change in percent of insulin samples dissolved over time on exposure to different temperature and humidity conditions. Percent insulin dissolved was calculated by comparing the average total peak area observed in the RP chromatograms of each degraded sample at a target concentration of 1 mg/mL with the average total peak area of an untreated 1 mg/mL control sample.

S3.3. Additional chromatogram

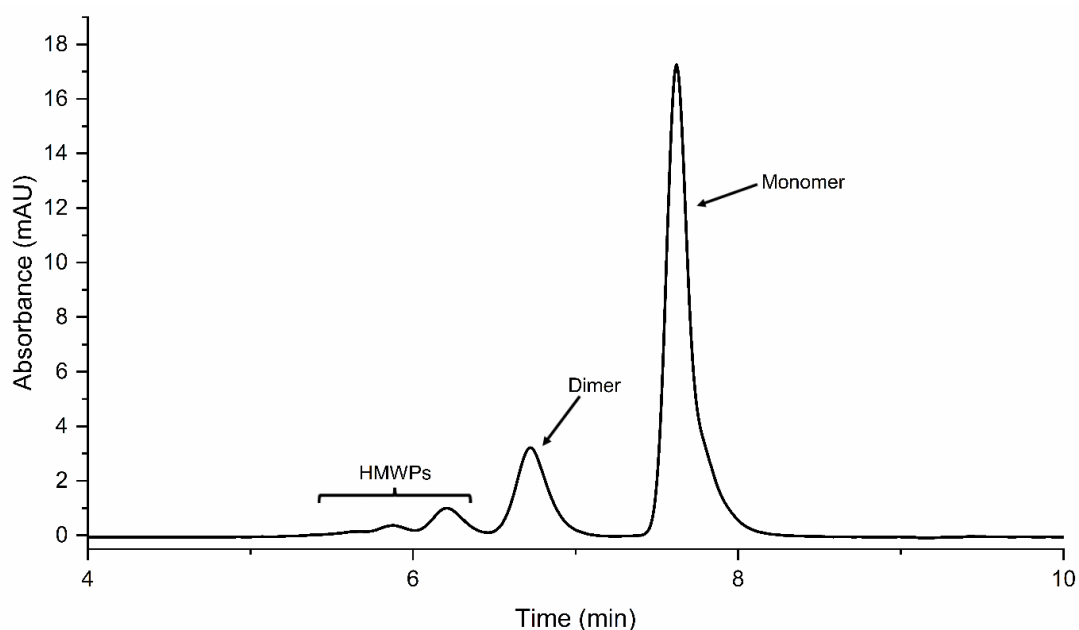


Figure S3.3. Representative SEC chromatogram of an insulin sample heated in the solid state at 60°C/11% relative humidity (RH) for 28 days. Samples were reconstituted in 0.1% formic acid at a target concentration of 1 mg/ mL. SEC was performed using an Agilent 1260 Series HPLC system with an AdvanceBio SEC 130Å, 2.7 μ m, 7.8 x 300 mm (Agilent Technologies) column. Chromatography was performed isocratically, where the mobile phase consisted of 65% L-arginine (1 g/ L)/ 20% acetonitrile (ACN)/ 15% acetic acid (AA). The flow rate was maintained at 1 mL/ min, a total run time of 12 min and injection volume of 5 μ L were used. 280 nm was used as a protein specific detection wavelength.

S3.4. Differential Thermogram

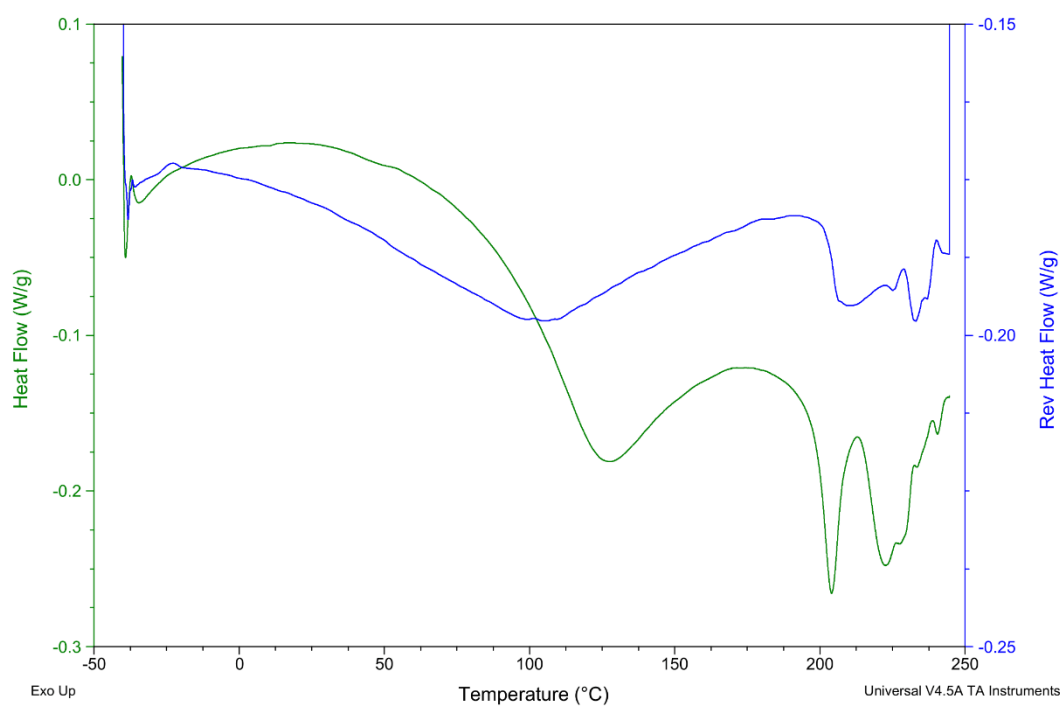


Figure S3.4. Representative differential thermogram showing total (green) and reversible (blue) heat flow vs temperature of recombinant human insulin over a temperature range of -40-250°C.

S4. Appendix 3: Supplementary Information Chapter 4

S4.1. C10 raw material images

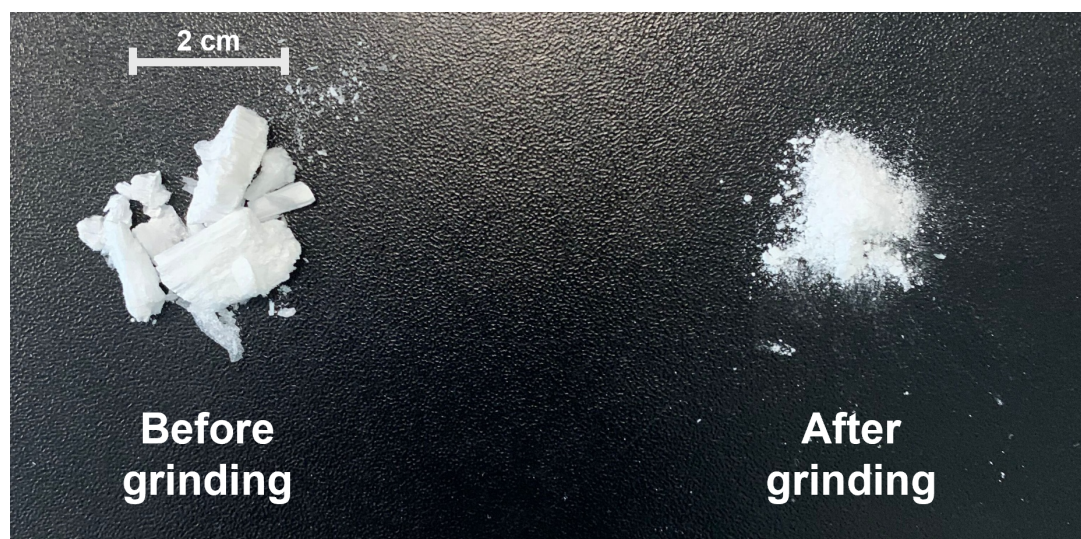


Figure S4.1. Representative image of C10 materials before and after grinding in pestle and mortar to reduce particle size.

S4.2. Particle size analysis and powder XRD

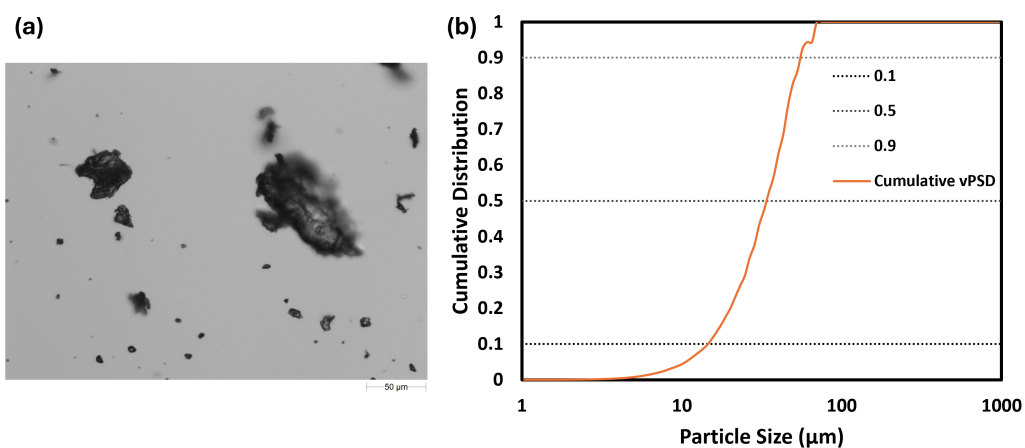


Figure S4.2. (a) representative microscope image of C10 particles at 20X magnification. (b) volume-weighted cumulative particle size distribution (vPSD) of C10 particles, where dashed lines represent d_{10} , d_{50} and d_{90} .

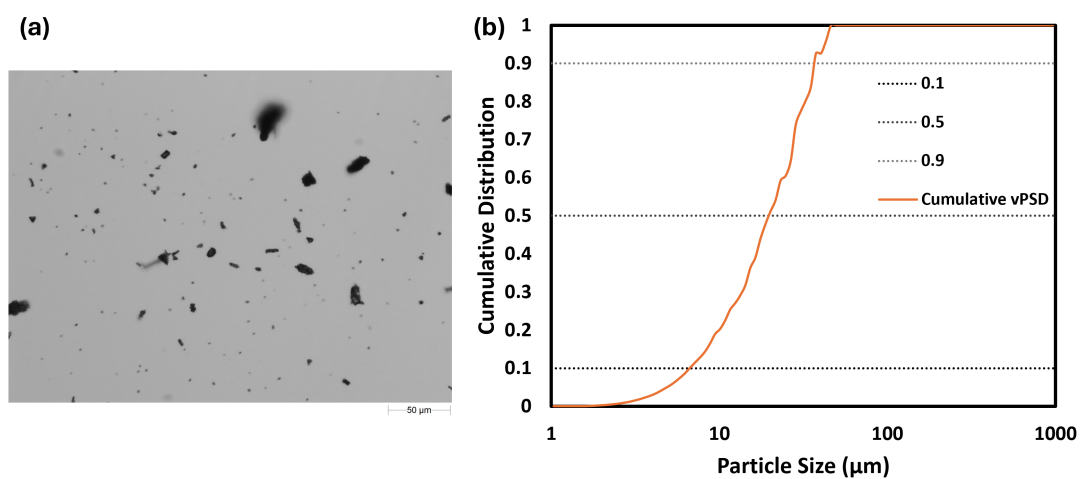


Figure S4.3. (a) representative microscope image of SNAC particles at 20X magnification. (b) volume-weighted cumulative particle size distribution (vPSD) of SNAC particles, where dashed lines represent d_{10} , d_{50} and d_{90} .

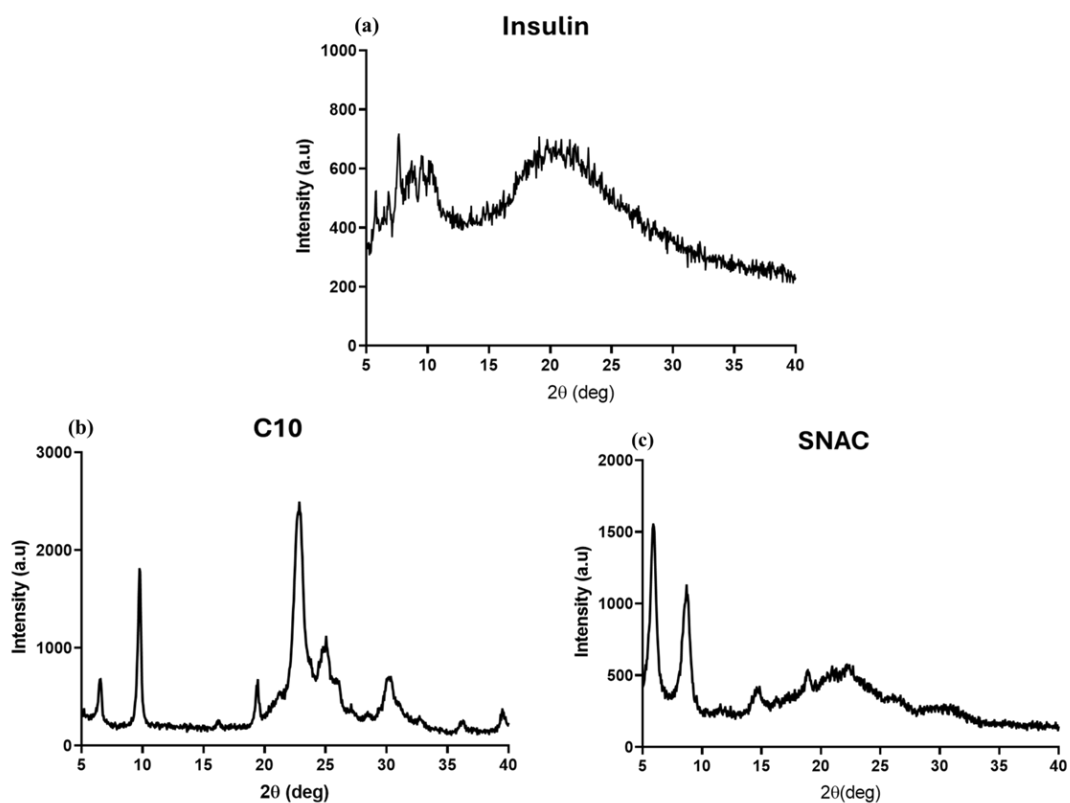


Figure S4.4. PXRD diffractograms of (a) Insulin, (b) C10 and (c) SNAC. C10 and SNAC showed characteristic diffraction peaks indicating partial crystallinity, whereas insulin displayed an amorphous profile. PXRD analysis was carried out using a Rigaku Miniflex desktop X-ray diffractometer equipped with a Cu K α radiation source ($\lambda = 1.54 \text{ \AA}$), operated at 30 kV and 15 mA. Diffraction patterns were recorded over a 2θ range of $2\text{--}40^\circ$ with a step size of 0.05° . All measurements were performed at ambient room temperature.

S4.3. Constrained mixture DoE

Table S4.1. Coefficients for statistically significant terms in stepwise regression model for C10 and SNAC formulations

Term	Coefficient	Coefficient SE	Model R ² (adj.)	Term	Coefficient	Coefficient SE	Model R ² (adj.)
C10	1.56	0.04	89.95 (%)	SNAC	2.65	0.04	99.09 (%)
MCC	10.73	2.55		MCC	10.71	2.70	
PVP K30	1.38	0.84		PVP K30	5.38	0.89	
C10*MCC	-9.39	3.28		SNAC*MCC	-7.12	3.48	
C10*Compaction Pressure	0.27	0.03		SNAC*Compaction Pressure	1.12	0.04	
MCC*Compaction Pressure	5.22	2.36		MCC*Compaction Pressure	1.73	0.19	
				SNAC*PVP K30*Compaction Pressure	2.57	0.97	

SE represents the standard error of the coefficients