DES modified protein-based materials and their use in pharmaceutical applications

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By

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Abstract

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This study is the first to investigate the modification of proteins using deep eutectic solvents. The production of plastic materials with suitable properties could then lead to their use in biomedical or pharmaceutical applications.

Three polypeptides were used, namely gelatin, zein and soy protein. All of them made plastics when mixed and pressed with the deep eutectic solvent, Glyceline, but all three had very different properties. The material made from gelatin showed variable properties which appeared to depend on how long the two components were mixed for. Conductivity measurements indicate the existence of a continuous phase which may be useful in later applications, particularly for the transdermal drug delivery systems.

Composites of gelatin with pharmaceutical DESs (PDES) were then made for oral ingestion. Dissolution rates of three PDESs were measured from a gelatin matrix. When the ingredients were left to mix for an extended time before the pill was pressed, more API could be incorporated compared to tablets pressed straight away showing that the DES was slow to diffuse into the gelatin structure.

Lastly, PDES modified gelatin was made into a patch for transdermal drug delivery. It was found that for the small number of compounds tested, drug delivery rates were much higher than those from comparable materials made with the same API in a solid form and not in a PDES. The rate of extraction of the PDES from the patch was dependent on the viscosity of the PDES as might be expected.

In conclusion, this study has shown that proteins can be modified with DESs to produce mechanically strong materials with a significant content in an amorphous form. The DESs form pools of liquid within the structure and this can be used to release active pharmaceutical agents either through the stomach or across the skin barrier.

Statement of originality

The accompanying thesis submitted for the degree of Ph.D entitled " DES modified protein-based materials and their use in pharmaceutical applications" is based upon work conducted by the author in the Department of Chemistry at the University of Leicester during the period between January 2016 and January 2019.

All the work described in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other University.

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29th January 2019

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Table of Contents

List of Abbreviations

APIs	Active pharmaceutical ingredients
ChCl	Choline chloride (2-hydroxyethyltrimethylammonium chloride).
DES	Deep Eutectic solvents
DMA	Dynamic Mechanical Analysis
DSC	Differential Scanning Calorimetry
HBD	Hydrogen Bond donor
HDPE	High Density Polyethylene
IL	Ionic Liquid
NMR	Nuclear Magnetic Resonance
PDESs	Pharmaceutical Deep Eutectic Solvents
QCM	Quartz Crystal Microbalance
SEM	Scanning Electron Microscopy
Tg	Glass Transition temperature
TGA	Thermogravimetric Analysis
TMA	Thermomechanical Analysis
UTS	Ultimate tensile strength
XRD	X-Ray diffraction.

Chapter 1: Introduction

1. Introduction

1.1 Biopolymers

Biopolymers can be divided into two categories: i. naturally produced by living organisms, and, ii. other synthesised from naturally occurring monomers. Polynucleotides (such as the nucleic acids DNA and RNA), polypeptides (proteins) and polysaccharides (polymeric carbohydrates) are the main three classes of biopolymers, in which the monomeric units are nucleotides, amino acids, and, sugars respectively.[1] Like other polymers, their monomeric units are covalently bonded to form larger structures.

One of the main differences between biopolymers and synthetic polymers is their structures. Many biopolymers spontaneously fold into characteristic compact shapes, which determine their biological functions and depend in a complicated way on their primary structures, while most synthetic polymers have much simpler and more random structures.[2] The synthesis of most natural polymers is controlled by a template-directed process so all biopolymers are alike with similar sequences and numbers of monomers and thus the same mass.[2] This phenomenon is called monodispersity. In contrast, most synthetic biopolymers have a broad polydispersed molecular weight which generally results in a less defined copolymer.[3]

For sustainability and environmental reasons, there is an interest in using biopolymers as bulk materials. Nature has designed degradation pathways for biopolymers through enzymes which can hydrolyse peptide and saccharide links. The use of polyolefin such as polystyrene (PS) or polyethylene (PE) for short-term applications is clearly causing issues with environmental build-up.[4] Some biopolymers such as poly-lactic acid and semi-synthetic polymers such as cellulose acetate are not only biodegradable but also compostable, as they can be put through an industrial composting process with 90% being broken down by six months.[5] Biopolymers offer important contributions by decreasing the dependence on fossil fuels and the related environmental impacts such as carbon dioxide emissions. As a result, there is a worldwide interest in replacing petroleum-based polymers with renewable resource based raw materials. However, in spite of all the advancements, there are still some shortcomings stopping a wider commercialization of bio-based polymers in many applications. This is mainly due to the performance and

commercial reasons when comparing with their conventional counterparts, which remains a significant challenge for bio-based polymers.

In recent years, as excipient development has become one of the core areas of research in pharmaceutical drug delivery, biopolymers have also gained attention owning to the fact that they have an advantage of being readily hydrolysed into removable and non-toxic products, which can be subsequently eliminated by metabolic pathways.[89] They are applied in pharmacy as the pharmacological substances, blood substitutes, drug delivery and therapeutic systems, in the synthesis of macromolecular prodrugs (modified therapeutic agent which is metabolized into active precursor in human body) and in the technology of prolonged release drug formulations.[6]

1.2 Bio-based materials

A bio-based material is a material made from biopolymers derived from natured resouces.[7] One of the fundamental issues with bio-based materials is the variability of the starting material and the generally protracted methods required to obtain pure materials. Although bio-based materials may appear to be green materials as they come from a renewable resource and are readily biodegradable, the inherent energy requirement needed to grow and process them combined with the associated waste produced in their manufacture makes the metrics for production poor from a viewpoint of energy efficiency, carbon footprint and carbon efficiency. Producing polyethylene by cracking hydrocarbons and then gas phase polymerisation produces a material with a much lower carbon footprint, a much higher energy efficiency and a lower E factor (ratio of waste to product) than any biopolymer. The main issue with synthetic polymers is their environmental legacy caused by their incorrect disposal.[8]

Examples of bio-based materials include:

Cellulose fibres: Cellulose ***(Figure 1.1)*** is a naturally occurring linear macromolecular chain which can be derived from a wide variety of plants including cotton, jute and flax as well as the more obvious tree-based species. It has long been harvested as commercial fibres to use as a common building material due to its great chemical variability and potential in a wide variety of applications. In which wood represents a composite material (40–55% cellulose) that has high-strength and durability,[9] and recently came again into

focus as a renewable energy resource. In the nineteenth century, methods were developed to separate wood cellulose from lignin and hemicelluloses and then used as fibres and modified into plastics.[10]

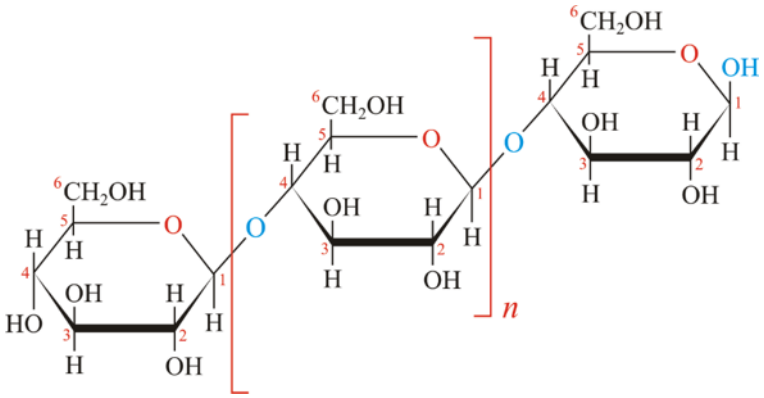

Figure 1.1 *Cellulose structure*

Cellulose has also been extensively used in pharmaceutical and cosmetic industries. Two main cellulose derivatives are, cellulose ethers and cellulose esters are broadly used in the formulation of dosage forms and healthcare products.[11] These compounds have significant roles in various types of pharmaceuticals such as compressibility enhancers (in compression tablets), thickening agents and stabilizers (in liquid dosage forms), binders (in granules and tablets), gelling agents (in semisolid preparations) and many other applications.[11]

Casein: Casein is a protein that is commonly extracted from milk during the process of creating low-fat milk. It has a wide variety of uses, including being food additives and food source, it is also known to process in various ways to make: plastic, dietary supplements for body builders, glue, cotton candy, protective coatings and paints.[12] It contains a large number of non-interactive proline residues and so has relatively little tertiary structure.[13] It is relatively hydrophobic and so has a low solubility in water.

Like cellulose, casein also possesses a number of interesting properties that make it a good candidate for conventional and novel drug delivery systems. As one of the food proteins, casein holds high nutritional value as well as excellent functional properties, including emulsification, gelation, foaming and water binding capacity.[14] It forms networks that have the ability to interact with a wide range of active compounds via functional groups on their polypeptide primary structure, thus offering a variety of

possibilities for reversible binding of active molecules and for protecting them until their release at the desired site within the body.[14] Casein may be used in pharmaceutical products either in the form of acid casein which has a low aqueous solubility or sodium caseinate which is freely soluble except near its isoelectric point.[14]

Polylactic acid: Polylactic acid, (PLA) ***(Figure 1.2)*** is a biodegradable thermoplastic aliphatic polyester derived from renewable resources, such as corn starch, tapioca roots, starch, or sugarcane. In 2010, PLA had the second highest consumption volume of any bio plastic.[15] It is useful for producing loose-fill packaging, compost bags, food packaging, and disposable tableware.[16]More recently, in the field of tissue engineering, PLA has been intensively studied to be used as a bicomponent for scaffold manufacturing. By combining with highly bioactive chitosan (CS), a more appropriate interface for cell adhesion and proliferation was provided.[90]

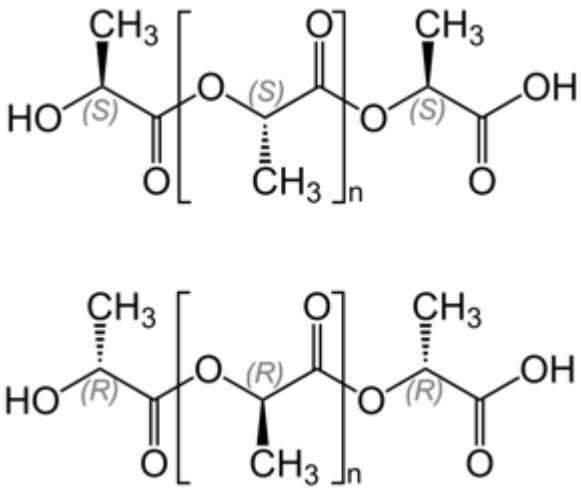

Figure 1.2 *Polylactic acid structure*

Apart from being applied in food and chemical industries, PLA have also been one of the main ingredient in wellness industries including both medical and pharmaceutical field. This due to its mechanical and biological unique properties such as biocompatibility, biodegradability, and thermoplastic process ability. In addition, the nontoxic and non-carcinogenic effects on the human body make Polylactic acid an acceptable candidate for biomedical application such as in sutures, clips and, like cellulose and casein, drug delivery systems.[17]

1.3 Proteinaceous material

Among natural polymers that can be used as a base for bio-based materials, proteins remain a strong candidate, which can be used for the development of new blend and/or composite material. Any material that has a protein base, like aforementioned casein, is known as a proteinaceous material,[18] other examples include animal glue, soy protein, and collagen etc. They are a group of complex organic macromolecules that are composed of one or more chains of amino acids.

Compared to synthetic polymers, they have the advantage of being water-soluble, biocompatible, biodegradable and non-toxic. Furthermore, many natural animal proteins such as keratin, collagen, elastin, and silk are relatively cost-effective and sustainable. They are easily derived from their natural sources and simple to process under mild conditions. Given their unique properties, proteins have been a growing interest for the past few decades, offering a variety of solutions for the development of innovative biomaterials, including films, foams, composites and gels, in particular for biomedical applications such as biosensors, scaffolds for tissue regeneration,[19,20] and drug delivery systems.[21] There have been many attempts on the synthesis of multifunctional protein-engineered materials with a wide range of customizable properties and activities, including protein-based composite from elastin, collagens, silks, keratins, and resilins.[22]

In this context, this study intends to review the potential of several protein-based materials within the sustainable polymers context, covering aspects ranging from protein types, additives, properties of protein-based materials and their potential applications.

1.4 Protein Structures

The characterization of the structure and function of the base protein is of critical importance for the mechanical, chemical, electrical, electromagnetic, and optical properties of the end-result proteinaceous materials. As such, their structure and composition are discussed at length as follows.

Although a protein's function is essentially determined by its structure rather than its sequence of amino acid, the sequence of the amino acids is still important as it determines the end structure of the protein. Protein generally has a tightly regulated structure held together by hydrophobic interactions, hydrogen bonds and the van der Waals forces between nearby amino acids, as well as di-sulphate bridges between cysteine residues. It

has up to four levels of complexity.

The primary structure describes the chain of amino acids in the polypeptide chain. One "end" of an amino acid has a carboxyl (COOH) functional group while the other end has an amino functional group. When two amino acids react, they form a peptide bond. The resulting molecule, called a dipeptide, still has one end that is acidic and another that is basic. With this fundamental reactive pattern, it is possible to string together many amino acids to form a polypeptide. For such a chain, the end that has the carboxyl group is referred to as the C-terminus; the amino end is referred to as the N-terminus ***(Figure 1.1).***

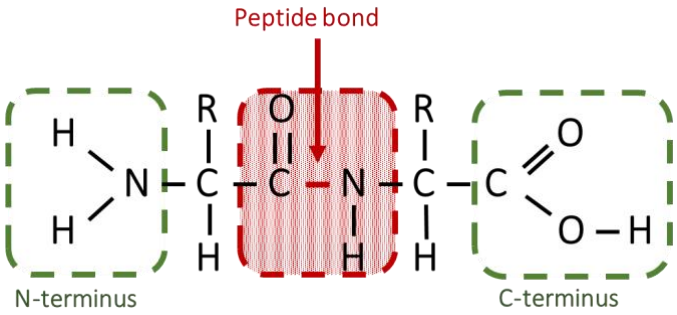

Figure 1.3*: Primary structure of protein*

The next level of protein structure is called the secondary structure. The side chains of the residues have various functional groups that can have different types of forces: some are hydrophobic, and others are hydrophilic; some participate in hydrogen bonding interactions while others do not. These forces lead to conformations (geometric arrangements of the residues) that result in lower energies. Two specific arrangements that are found regularly are shown in ***Figure 1.4***. The α-helix structure (which looks like a corkscrew) that is held by hydrogen bonds between the oxygen atom in a carbonyl group of one amino acid and the hydrogen atoms of the amino acid group that is just four amino acid units further along the chain. β-pleated sheet (that looks like a paper sheet that has been folded and opened) formed by similar hydrogen bonds between continuous sequences of carbonyl and amino groups that are further separated on the backbone of the polypeptide chain.

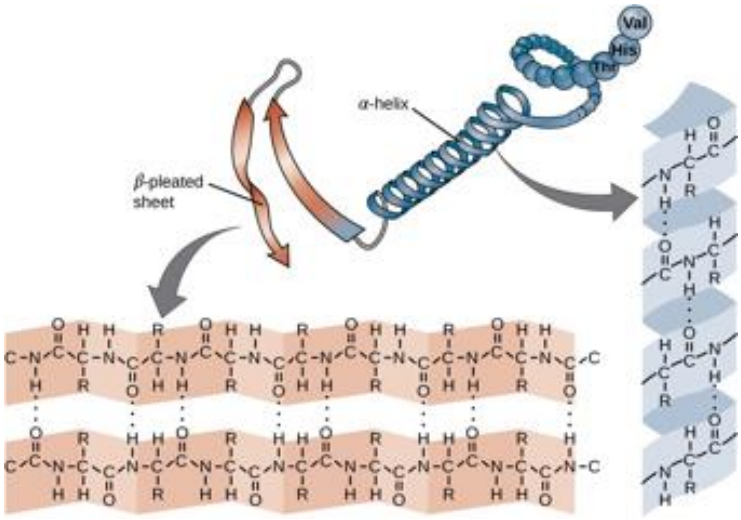

***Figure 1.4**: secondary structure of a protein (Image credit: OpenStax Biology.)*

These interactions result in a tertiary structure of the protein, which is the large-scale three-dimensional shape of a single polypeptide chain. It is determined by interactions between amino acid residues that are far apart in the chain. Some proteins can also form structures with multiple units (dimers, trimers, or tetramers), and these collections of units will provide yet another level of structure called the quaternary structure.

The secondary, tertiary, and quaternary structures of proteins result in various interactions and bindings differing in position, type, and energy. Therefore, numerous proteins such as corn zein, wheat gluten, soy, peanut, cottonseed, sunflower, rice bran, serum albumin, egg white, collagen, gelatin, myofibrils, casein, and whey proteins have been studied as the basis for potential materials.[23]

In a native protein, the majority of polar and apolar groups are unavailable due to the internal bonds resulting from van der Waals forces, hydrogen bonds and hydrophobic interactions.[24] The formation of globular proteins is a typical example of how hydrophobic interactions stabilises this type of tertiary structure. Another structural form of proteins is the fibrous form of proteins. Many familiar components of tissues are composed of fibrous proteins, including keratin (the protein present in hair), collagen (a structural protein found in tendons and cartilage), myosin (a protein found in most muscle tissue), and fibrin (the protein that allows blood to clot and form scabs). These proteins are built up from three main structures, namely the α-helix, the antiparallel β-pleated sheet and the triple helix.[25]

1.5 Protein folding

Protein folding is the physical process by which a protein chain folds into a 3-D structure, its mechanism is especially important when it comes to understanding the cooperative nature of the unfolding transition, which is a key factor on interactions within a modified protein-based material. During the protein folding process, a polypeptide acquires its three-dimensional structure from random coiling. Each protein exists as an unfolded polypeptides or random coil until it is translated to a linear chain of amino acids. Formation of a secondary structure (also known as alpha helices or beta sheets) is the first step in the folding process during which a protein starts to assume its native structure. This step often happens rapidly as they are stabilized by intramolecular hydrogen bonds, which provides another important contribution to protein stability. Every alpha helix has a hydrogen bond formed from the carbonyl oxygen to the amide hydrogen to produce form its spiral shape. The beta-pleated sheets exist in two forms, anti-parallel and parallel. Hydrogen bonds are formed between the amide hydrogen and carbonyl carbon, which is similar to alpha helices. The stability of the hydrogen bonds in the anti-parallel beta sheet is stronger, as it has the ideal 180-degree angle compared to the slanted hydrogen bonds formed by parallel sheets. The alpha helices and beta-pleated sheets tend to contain a hydrophilic portion and a hydrophobic portion. This property of these secondary structures helps with the folding of the protein as it aligns the helices and sheets in a way where the protein and the hydrophobic sides are facing the hydrophobic core of the protein while hydrophilic sides are facing the aqueous environment.

This folding process occurs even before a protein is fully formed, and the folded structure is known as the native state. The resulting 3-D structure is determined by the amino acid sequence or primary structure, and it is essential to its function. Failing to fold into a native structure generally produces inactive proteins, and in some circumstances, it might even result in toxic functionality.

Although the amino acid sequence of each protein contains the information that leads to its native structure and the pathway to attain that state, it does not mean that nearly identical amino acid sequences always fold similarly. Environmental factors play a big part as well; similar proteins fold differently based on where they are found. Folding is a

spontaneous process independent of energy inputs from nucleoside triphosphates. The passage of the folded state is mainly guided by hydrophobic interactions, formation of intramolecular hydrogen bonds, and van der Waals forces, and it is opposed by conformational entropy.

The process of protein folding also depends on the solution components, including the concentration of salts, the pH, the temperature, the possible presence of cofactors and the possible existence of molecular chaperones. The pH and added salts in solution will change the zeta potential around a protein. This will affect the repulsive and attractive forces between protein chains and hence their folding.

1.6 Globular and fibrous proteins

Depending on the tertiary structures the proteins are folding up to, they are classified as two main categories.

Globular proteins:

Globular proteins are one of the most common types of protein, and they are characterized into four classes: all-α, all-β, α+β, and α/β. The all-α and all-β classes are dominated by α-helices (α > 40% and β< 5%) and by β-strands (β > 40% and α < 5%), respectively. The α + β class contains both α-helices and β-strands that do not mix but tend to segregate along the polypeptide chain, while the α/β class proteins have alternating segments of α-helical and parallel β-strands.[26]

The globular nature of these proteins can be determined by using ultracentrifuge or dynamic light scattering techniques. The spherical structure is induced by the protein's tertiary structure. The apolar (hydrophobic) amino acids of a molecule are bounded towards the interior of the molecule whereas polar (hydrophilic) amino acids are bound outwards, allowing dipole-dipole interactions with the solvent, so the entire structure is held together by the pressure of the hydrogen-bonded water molecules outside it, which explains the molecule's solubility. However, the detailed packing of specific groups within the protein is organized and maintained through numerous medium-weak electrostatic bonds (hydrogen bonds and salt links) (***Figure 1.5***).

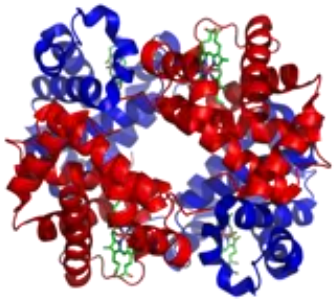

***Figure 1.5**: 3-dimensional structure of 11 aemoglobin, a globular protein.*

Globular proteins are only marginally stable as the Gibbs energy, ΔG, is relatively small when the protein folded into its native conformation due to the entropic cost of protein folding. The enthalpy of folding, ΔH, is small but negative due to the formation of hydrogen bonds and electrostatic interactions. Folding will make the chain more ordered and so the entropy change, ΔS, will be negative. This is one of the reasons that proteins denature at higher temperatures since

$$\Delta G = \Delta H - T\Delta S$$

According to the equation above, the increasing temperature will make $T\Delta S > \Delta H$ and accordingly ΔG will become positive and the protein will unfold. Though polypeptide chains can form numerous conformations, native globular structure restricts its conformation to only a few, which results in a decrease in randomness.

Some examples of globular proteins are listed as follows:

Zein:

Zein is a naturally occurring protein polymer acquired from the industrial maize production. α-Zein is the major component of zein, which is distinct from most globular proteins because of its water insolubility, which is determined by its sequence and structure.[27] According to the native structure model, more than half of the solvent-accessible surface of zein was occupied by hydrophobic residues.[28] The structure and conformation of α-zein in various solutions have been extensively studied, and it was found that zein has an elongated conformation enriched by an α-helix secondary structure (***Figure 1.6***).

***Figure 1.6**: A possible structural model for α-zeins. The helices are folded upon each other in antiparallel fashion, linked at each end by glutamine-rich turns or loops, and stabilized by hydrogen bonds.*

Thanks to their unique features, including the insolubility in water, resistance to grease and microbial attack, glossy appearance, and large availability, zein protein has been used in the food-packing, biomedical and pharmaceutical fields.[29] It has excellent film forming properties owing to the development of hydrophobic, hydrogen and limited disulphide bonds between zein chains, and can be used for fabrication of biodegradable films.[30] Edible films can be formed by drying aqueous ethanol solution of zein,[31] The resulting films appeared to have better water barrier properties compared to other edible films, and it can be improved by adding fatty acids or by using a cross-linking reagent. Zein coating have also shown an ability to reduce moisture and firmness loss and delay colour change (reduce oxygen and carbon dioxide transmission) in fresh tomatoes.[30] However, zein's chemical inertness and globular structure make it challenging for moulding, and therefore require plasticizer addition for increasing flexibility.[30]

Arcan and the group showed that zein-based film can be incorporated with different phenolic acids (gallic acid, p-hydroxy benzoic acid or ferulic acids) or flavonoids (catechin, flavone or quercetin), which lead to antioxidant and antibacterial properties.[32] The addition of these compounds also helped to eliminate the classical brittleness and flexibility problems associated with raw zein. Such achievements can be especially beneficial for using zein

in pharmaceutical applications.

Soy:

Unlike zein, whose primary structure is dominated by α-helices, soy protein mostly consists of β-sheets. Results have been obtained suggesting that this type of structure might be essential for the network formation in films, which leads to the theory that the intermolecular hydrogen bonding between segments of beta-sheet can be acting as junction zones in the film network.[33] Soy proteins consist of two globular proteins: conglycinin and glycinin.[34] It separates into fractions 2S, 7S, 11S or 15S based on the molecular weight and sedimentation coefficient, with the 7S globulin and 11S globulin comprise 37% and 31% of the soy proteins, respectively. Glycinin (11S) is the most important storage protein of soybeans and is a globular protein. In combination with other film-forming proteins, glycinin can act as a gelling agent, emulsifier and foaming agent. The currently most accepted model of glycinin is that of a hexamer with six monomeric subunits (where A represents an acidic polypeptide and B a basic polypeptide). The A and B chains are linked by a single SS bond. Native glycinin (11S) consists of six acidic (A) and six basic (B) subunits. According to Badley et al. the AB subunits associate into two hexagonal rings forming a hollow cylinder held together by hydrophilic interactions (electrostatic interactions/H-bridges).[35] B-conglycinin on the other hand, is less heat stable than glycinin and gets denatured at temperatures of 70°C and 80°C.[34]

As a major co-product of soybean oil industry, soy protein is one of the cheapest proteins currently produced. Despite the low oil content of the seed (20 wt% on a moisture free basis) soybeans are the largest single source of edible oil and account for 52% of the total oil seed production of the world.[36] With each ton of crude soybean oil, approximately 4.5 tons of soybean meal (protein content 44%) is produced,[24] and for each ton of soybean processed, the commercial value of the meal obtained usually exceeds that of the extracted oil. Despite considerable public and commercial interest in soybean products as food, the proportion of soy protein consumed directly in human nutrition and other industrial uses is small and there is a need to look for the new industrial uses of soy protein.

Owing to their good biodegradability, biocompatibility, and process ability, soy protein as a source for biodegradable materials has received much attention.[91-93] Various modification strategies, including bulk and surface modifications, have been utilized to improve its mechanical properties and water resistance. As for these modification methods, there are still some drawbacks including high moisture sensitivity and low interfacial adhesion between the protein and hydrophobic plasticizers or polymers,

toxicity of chemical cross-linkers containing aldehyde groups, high price of radiation treatment, and degradation by high-UV irradiation, etc. Therefore, novel approaches are still needed to improve performance of resultant soy protein based plastic materials as well as high feasibility of modification.

Amino acid	Composition (wt %)
Isoleucine	4.54
Leucine	7.78
Lysine	6.38
Methionine	1.26
Cysteine	1.33
Phenylalanine	4.94
Tyrosine	3.14
Threonine	3.86
Tryptophan	1.28
Valine	4.80
Arginine	7.23
Histidine	2.53
Alanine	4.26
Aspartic acid	11.70
Glutamic acid	18.70
Glycine	4.18
Proline	5.49
Serine	5.12

***Table 1.1**: Amino acid composition of soybeans*[37]

Soy proteins possess a balanced composition of polar, non-polar, and charged amino acids (***Table 1.1***)[24], allowing a variety of drugs to be incorporated. Furthermore, soy protein molecules can aggregate, and form a variety of structures such as microspheres, hydrogels and polymer blends upon addition of dissolvent or crosslinking agent, which makes the soy protein an ideal coating material for the encapsulation of bioactive compounds.

Fibrous protein:

Fibrous protein are found exclusively in animals, where they make up skin, bones, muscles, tendons, and hair cells. Compare to globular proteins, which generally have a more compact and rounded shape and have functional roles, fibrous proteins are generally composed of long and narrow strands and have a structural role. The amino acid sequences of fibrous proteins often contain repeating sets of amino acid residues. Such repeating sets tend to cause the protein to be both elongated and strong.[38]

Collagen, for example, is a fibrous protein that forms fibrils through a self-assembly process. This contributes to elastic fibres with high strength that can form connective tissue. The fibril-forming collagens usually consist of one or more characteristic triple helices containing three polypeptide chains, with dimensions of 300 nm in length and 1.5 nm in diameter (***Figure 1.7***).[39]

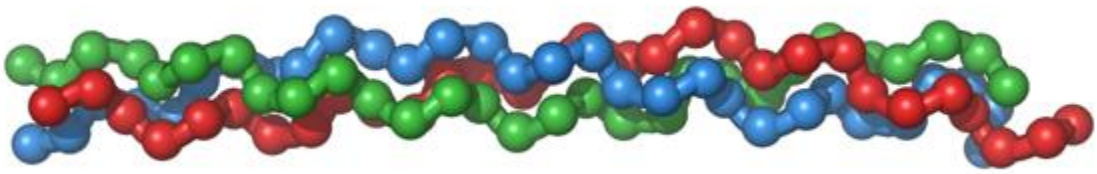

Figure 1.7*: Structure of collagen (an example for fibrous protein)*

The unique structure of fibrous proteins can provide important material options in a variety of fields, for example, controlled release, biomaterials and tissue engineering. Their relative stability, compared to globular proteins, in combination with their biocompatibility and unique mechanical properties, provide the foundation upon which to exploit these natural proteins.

Gelatin:

Gelatin is a translucent, colourless, brittle (when dry), flavourless material derived from collagen. It is a mixture of peptides and proteins produced by partial hydrolysis of collagen extracted from various animal-based raw materials including the skin, bones, and connective tissues of animals such as domesticated cattle, chicken, pigs, and fish. In addition, as an irreversibly hydrolysed form of collagen, during hydrolysis, the natural molecular bonds between individual collagen strands are broken down into a form that rearranges more easily, resulting in a broad molecular weight range associated with physical and chemical methods of denaturation, based on the process of hydrolysis. (**Figure 1.8**)

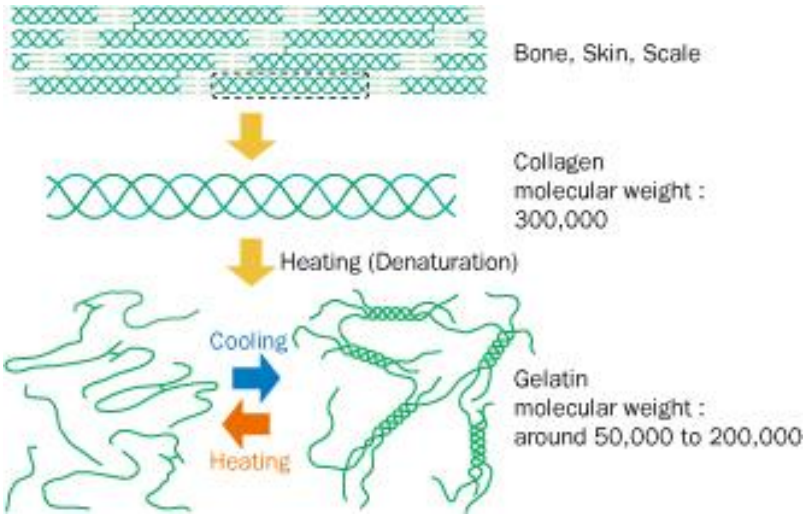

***Figure 1.8**: Denaturisation of gelatin*

Gelatin with its well-defined mechanical properties like high strength, reversible extensibility through only a small range, is commonly used in fields like food, pharmaceutical, photography, and cosmetic manufacturing. It is also it suited to special purposes like being put into the animal body as tendon, bone, tusk, skin, the cornea of the eye, and intestinal tissue.[40]

Gelatin has also been used in the manufacture of various pharmaceutical dosage forms since the early 19th century, including hard capsules,[41] soft gels[42] and tablets[43]. The manufacturing of gelatin based hard capsules involves dipping stainless steel molding pins into a warm gelatin solution, then stripping the gelatin off the pins after drying.[44] The caps and the bodies of capsules are later joined together with the correct dosage of product filled inside.[44] Soft gels on the other hand, begins with the formation of two gelatin sheets that have been cut into desired shapes and filled with a liquid dosage of the target pharmaceutical afterwards.[44] A further common use of gelatin in the medical fields is in tablets. As an essential ingredient, it can act as a binding agent for the dosage and coating to reduce unwanted scent and taste.[45] On top of which, its particular solubilising characteristic often enables the gradual and timely release of medication.

1.7 Protein-based hydrogels

Among all functional properties, protein holds a gel-forming property that is especially interesting. Gels of diverse mechanical and microstructural properties can be formed by

assembling protein molecular chains, thus offering the possibility of developing biocompatible carriers for pharmaceuticals.

This polymeric network is called a hydrogel. It can be considered a new class of recyclable plastic. In addition, they have much closer analogues in nature than most other forms of plastic. It refers to a three-dimensional solid network fabricated by physically or chemically cross-linked hydrophilic polymeric structures that can entangle a lot of water or other biological liquid inside their network as shown in **Figure 1.9**.[46] Hydrogels formed chemically generally undergo significant volume changes during the solid to gel transition. These chemical cross-links can be formed in many different ways, such as disulfide formation, polymerization or the reaction between thiols and acrylates or sulfones.[47] The density of crosslinking can easily vary which effects the mechanical properties of the final material. Physically cross-linked hydrogels, on the other hand, do not undergo significant volume changes during the solid−gel transition.[48] They can be prepared via the self-assembly of polymers, which occurs in response to external stimuli such as pH and temperature.[48] One advantage of physical cross-linking is that the property is not dependent on the addition of organic solvents or cross-linking reagents, which make it particularly promising for biomedical applications such as the tissue engineering and the controlled release of drugs delivery.[20]

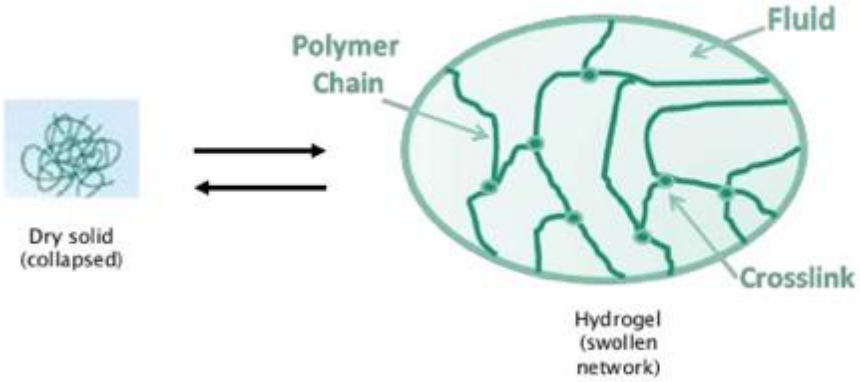

Figure 1.9 *Formation of hydrogels*

The ability of molecules of different sizes to diffuse into (drug loading) and out of (drug release) hydrogels allows the possible use of dry or swollen polymeric networks as drug

delivery systems for oral, nasal, buccal, rectal, vaginal, ocular and parenteral routes of administration.[48] The network structure and the thermodynamic nature of the components of these networks play a key role in their diffusional behavior, molecular mesh size changes (especially in environmentally responsive hydrogels), and the associated molecular stability of the incorporated bioactive agents.[48] Furthermore, the characterization of their physical, chemical, and mechanical properties need to be established in order to choose other ingredients (excipients).

Protein-based hydrogels, with their superior properties such as high fluid content, tuneable viscoelasticity, biocompatibility and biodegradability, have captured considerable attention in the biomedical field, especially emulsion-based delivery system.[20] Depending on the structure of the protein, the molecules aggregate and undergo a different process during the hydrogel formation. For example, globular proteins (i.e. zein and soy) form heat-set gels due to formation of hydrophobic bonds between non-polar patches exposed after the proteins undergo thermal denaturation, whereas fibrous proteins (i.e. gelatin) tend to form reversible cold-set gels due to formation of hydrogen bonds between helical regions on different biopolymer molecules.[20] The transition temperature and the reversibility/irreversibility of gel formation are essential for designing hydrogel particles that respond to temperature changes in specific ways.[20] For instance, gelatin is often used to form hydrogels that release encapsulated components upon oral administration as it forms reversible gels that melt around body temperature.[49]

The properties of the gel depend strongly on the interaction of the two components, the tangled polymer network and the liquid medium. The liquid prevents the polymer network from collapsing into a compact mass, while the network prevents the liquid from flowing away.[50] As a result, there has been significant interest recently in developing liquid drug formulations.

1.8 Pharmaceutical formulations

A pharmaceutical can contain a variety of active ingredients with advantageous pharmacological effects on the human body. Methods of drug administration includes external (by mouth), parenteral (into the blood stream), and other (which includes giving a drug through intranasal, topical, inhalation, and rectal means).[51] It can be performed in various dosage forms such as pills, serums, dispersions, tablets, or capsules.[52]

Pharmaceutical formulation is the process where different chemical substances, including the active drug, are combined to produce a final medicinal product.[53] Formulation studies involve developing a preparation of the drug which is both stable and acceptable to the patient. It is also important to understand that the components of the produced drugs should be compatible with each other, especially in the synthesis of capsules or tablets that contains a variety of other potentially inert substances apart from the drug itself.

Current drug formulations can suffer from several issues:

Polymorphism

Polymorphism is the ability of a drug compound to exist in more than one form or crystalline structures that have different arrangement and/or conformations of the molecules in the lattices.[54] The polymorphism in pharmaceutical substances affects their physical and chemical stability, dissolution, melting point, manufacturability of the drug product, density, solubility and bioavailability.[54] Dissolution rates are also affected as they depend on the crystal form of a polymorph and medicine is often administered orally as a crystalline solid.

Many drugs receive regulatory approval for only a single crystal form or polymorph. In the case of the antiviral drug Ritonavir, which is mainly used for Human Immunodeficiency Virus (HIV) treatment, not only was one polymorph virtually inactive, but it was found to convert the active polymorph into the inactive form on contact.[55] Its lower energy and greater stability make spontaneous interconversion energetically favorable.[55] This issue was ultimately solved by reformulating the medicine into gel caps and tablets, rather than the original capsules. Another example is Cefdinir, which is a drug appearing in 11 patents from 5 pharmaceutical companies, in which a total of 5 different polymorphs are described.[56] Polymorphic purity of drug samples can be tested using techniques such as powder X-ray diffraction or IR/Raman spectroscopy.

Solubility

Solubility is the dissolution of a solid, liquid, or gaseous chemical substance in a solid, liquid, or gaseous solvent, forming a homogenous system.[57] It is considered one of the most challenging aspects in the current pharmaceutical industry. The solubility of a

substance is essentially determined by the physical and chemical properties of the solute and solvent as well as the temperature, pressure and the pH of the solution.[57] The extent of solubility of a substance in a specific solvent is measured as the saturation concentration where adding more solute does not increase its concentration in the solution, it ranges widely from infinitely soluble (i.e. ethanol in water) to insoluble (i.e. silver chloride in water).[57]

Descriptive term	Part of solvent required per part of solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10,000
Practically insoluble	10,000 and over

***Table 1.2**: USP and BP solubility criteria.[57]*

Bioavailability

Bioavailability considers the rate and extent of medicine which enters the systemic circulation and the amount at which the active ingredient exists in a target organ.[58] It is one of the principal pharmacokinetic properties of drugs. By definition, when a medication is administered intravenously, it reaches 100% bioavailability. However, when it is administered via other routes (i.e. orally), its bioavailability generally decreases due to incomplete absorption and first-pass metabolism, hence it must be considered when calculating dosages for non-intravenous routes of administration.

Various physiological factors including aqueous solubility, drug permeability, dissolution rate, first-pass metabolism, pre-systemic metabolism, and susceptibility to efflux mechanisms will affect the availability of drugs prior to their entry into the systemic circulation, yet the most frequent causes of low oral bioavailability remain poor solubility and low permeability.

Though there have been numerous efforts on using drug formulations to improve the drug performance, many showed practical limitations. Salt formulation for example, is not

feasible for neutral compounds and may not be practical for weakly acidic or weakly basic drugs as well. Even when salts can be prepared, an increased dissolution rate in the digestive tract may not be achieved in many cases due to the reconversion of salts into aggregates of their respective acid or base forms. Using organic solvents or aqueous media to solubilize drugs and create liquid formations is usually undesirable either owing to the viewpoints of patient acceptability and marketing.

To overcome these issues there has been many efforts in developing alternative formulations for desirable drug delivery methods, among which, the topic of ionic liquids has been reviewed.

1.9 Ionic liquids

Ionic liquids are pure compounds which consist only of cations and anions (i.e., salts). They should melt at or below 100 °C and many are liquid at 25 °C, hence the name room temperature ionic liquids.[59] In the past 20 years, ionic liquids have been widely used in the fields of catalysis;[60] electrochemistry,[61] and material chemistry,[62]

Early research focused on the formation of ionic liquids by mixing metal salts, mostly zinc, aluminium, tin and iron chlorides, with quaternary ammonium salts.[63] Although both salts have very high melting points, their proper mixing leads to the formation of a liquid phase, the so-called eutectic mixture. These eutectic mixtures are generally characterized by a very large depression of freezing point, generally higher than 150 ^{o}C.[64] With the introduction of the concept of green chemistry in the early 1990's, the search for metal-free ionic liquids (ILs) has become of growing interest.[65] In this context, many studies were dedicated to the design of ILs by combining organic cations with inorganic anions. ILs have emerged as a new class of promising liquids and the possibility to chemically modify the cationic moiety almost infinitely has led to their description as designer solvents. The large choice of cations and anions allow tuning of the physical properties such as melting point, solubility, viscosity, density, conductivity, and refractivity, among others. Seddon suggested that there may be as many as 10^{18} different ILs that could be theoretically produced, and to date less than 10^{3} have been made and characterised.[66]

Owing to their low vapour pressure and high boiling point, ILs were qualified as green solvents. However, the ''green credentials'' of these neoteric solvents is now largely

contested in the current literature. Indeed, many reports pointed out the hazardous toxicity and the very poor biodegradability of most ILs.[66] ILs with high purity are also required since impurities such as water, Li^+ and OH^- even in trace amounts, can significantly affect their physical properties.[67] Additionally, their synthesis is generally complex so parameters such as yield, E factors and carbon efficiency can be poor. These drawbacks together with the high price of common ILs limits the industrial emergence of some technologies. Nevertheless, a few processes have been commercialised albeit using ILs on a relatively small scale. Plechkova and Seddon carried out the most comprehensive review of applications although these are now somewhat outdated and have not been comprehensively revised.[68]

In the pharmaceutical field, ionic liquids have been used as a basis for developing a range of pharmaceutical formulations with anionic and cationic active ingredients. One example is the combinations of cations such as lidocaine (used as a local anesthetic) together with anions such as docusate (a laxative).[69] The end result formulation showed curative effects.[69] Yet one of the issues remains that with each new combination of anion and cation, a novel material was made and as such needs new toxicological testing and registration.[70]

1.10 Deep eutectic solvents

A Deep Eutectic Solvent (DES) is a liquid formed from mixtures of a quaternary ammonium salt and either a metal salt or a hydrogen bond donor. Carboxylic acids, amides and alcohols are examples of appropriate hydrogen bond donors.[64] The first of these systems to be described was the choline chloride (ChCl)/ urea system mixed in a 1:2 mole ratio.[64] The hydrogen bond donor interacts with the chloride anion decreasing the lattice energy of both components and the melting point of the mixture is lower than that of individual constituents. In many cases, the DESs have melting points lower than 100 ^oC. The advantages of these solvents are that they are easier to make than ILs, they can be less toxic and many can be made from naturally occurring compounds such as creatine, urea, oxalic, citric and succinic acids, glycerol and glucose. DESs are now gaining interest in many fields of research including metal deposition, electro polishing, metal oxide processing and some of these applications have been scaled up to > 1 tonne.[71]

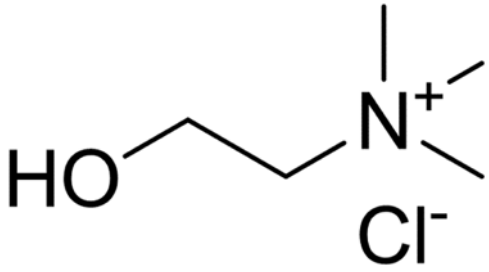

Figure 1.10 *chemical structure of choline chloride*

As one of the most widespread components used for the formation of these DESs, choline chloride (**Figure 1.10**) is a very cheap, biodegradable and non-toxic quaternary ammonium salt. It is classified as pro-vitamin B6 and is produced on the Mtonne p.a. scale as an additive to animal feed and a common additive for household products. It can be either extracted from biomass or readily synthesised through a very high atom economy process (ethylene oxide + HCl + trimethyl amine).

The topic of DESs has recently been reviewed in depth by Smith *et al*.[72]

In the field of catalysis and organic synthesis, DESs could contribute to the design of eco-efficient processes. In particular, the possibility to (1) selectively and conveniently extract products of the reaction from the DESs phase, (2) adjust the pH of DESs, (3) dissolve not only organic and inorganic salts but also transition metal-derived complexes or nanoparticles and (4) recycle these media is, among the most promising advantages of DESs.[66] The issue that remains separating the reagents/products from the DES.

In material chemistry, it is also apparent that ILs can be advantageously replaced by cheap and safe DESs for the ion thermal synthesis of a wide range of inorganic materials with different textures and structures. Although a proper selection of the exactly required DESs still remains a big challenge, the works described above have clearly demonstrated that very important materials, from microporous zeolites[12] to carbon materials, can be synthesized in DESs. In these syntheses, DESs may play different roles such as solvent, structure-directing agent, water inhibitor, reactant for structure crystallisation, etc.

DESs can be designed to have toxicity and biocompatibility. It is suspected that some living cells may have a composition close to a DES when dehydrated and some natural fluids such as maple syrup could be classed as DESs. They have also been considered as

a way of delivering pharmaceutical ingredients into the human body.[66]

1.11 Pharmaceutical Ionic liquids and DESs

More than 50% of medicines on the market are now sold as organic salts.[73] As a crucial step in drug development, salt formation using a drug substance can have a huge impact on the final product's solubility, dissolution rate, hygroscopicity, stability, impurity profile and particle characteristics.[74] The most commonly used cation is the sodium ion while the most encountered anion is chloride. The active pharmaceutical ingredients (APIs) were predominantly delivered as solid, crystalline forms, mainly for reasons of purity, thermal stability, manufacturability, and ease of handling, while liquid drug formulations are rarely found.[75] Given that many APIs are ionic, the idea of ionic liquids have gained significant attention as a potential drug formulation. With a carefully chosen counter ion, an API can be designed to possess a low, or even non-existent, melting point and hence be present as a liquid at room temperature or below.[75]

One of the most appealing features for pharmaceutical based ILs is that they are customizable which means that they can be specially made with pre-selected characteristics depending on the cations and anions of which they are comprised. Different combinations result in various ILs with wide ranges of hydrophobicity/ hydrophilicity, acidity/basicity, viscosities, etc. A number of examples have been published establishing numerous pharmaceuticals and other bioactive moieties being made into ILs combining APIs with suitable counter-ions. One approach is to pair an active ion with a counter-ion that is a known excipient. For example, the IL propantheline acesulfamate contains the active cation from the API propantheline bromide (an antimuscurinic used to treat a number of conditions such as excessive sweating, cramps, spasms of the stomach, etc.) with acesulfamate, a known artificial sweetener and a pharmaceutical additive.[76] The IL formed from these two ions has no observable melting point and passes into a glassy state at −20°C, on top of that, bulk samples of this material have known to be stable in the liquid state for more than one year.[76]

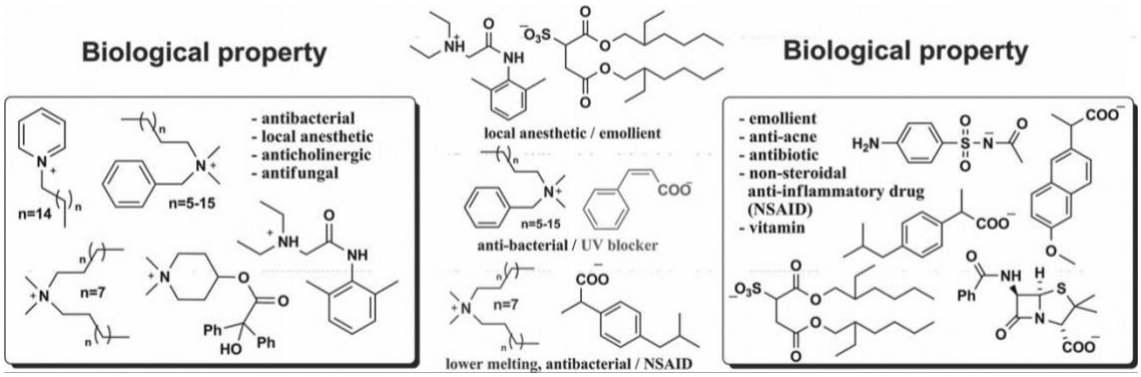

Figure 1.11 *ILs with targeted biological properties combined with chosen physical and chemical properties*[69]

Liquid APIs can overcome some of the issues associated with drugs in their solid state, including polymorphic conversion, low solubility, and low bioavailability for crystalline solids, and the tendency of amorphous forms to spontaneously crystallize.[75] The potential of pharmaceutical based eutectic mixtures has been explored to increase the pharmacological growth of a desired API. The high thermodynamic functions of eutectics, such as free energy, enthalpy and entropy, can confer solubility and dissolution advantage to poor solubility drugs, similar to the more popular amorphous solids and solid dispersions.[77] Solid dispersions sometimes also exhibit a eutectic like behavior with improved solubility, e.g. fenofibrate–polyethylene glycol.[77] On the other hand, the low melting point of organic eutectics can also pose stability issues. As have often been confused with solid dispersions, eutectics share the problems associated with the other category as well. The absence of a clear distinction between phases of a given drug can, in principle, be separately patented. Though it is accepted by regulatory bodies in the US that different solid forms of a drug are effectively different drugs (i.e., have different pharmacokinetics and pharmacodynamics), European regulators still view them to be the same.[75]

Recent work by Abbott and the group showed that highly concentrated liquid formulations could be produced from APIs in a DES formulation. Many active pharmaceutical ingredients are either hydrogen bond donors or quaternary ammonium salts, or, drugs with amine functionalities that can be converted to the quaternary ammonium salts with HCl aiding its solubility. It was shown that compounds which demonstrate poor solubility in water can be made to be miscible with water through a

DES formulation. The study also showed that drug molecules which exhibit polymorphism such as Adiphenine and Ranitidine can be made into liquids and the hydrogen bond donor can also be a pharmaceutical active ingredient e.g. aspirin.[70]

1.12 Ionic liquids and DESs as plastic modifiers

One benefit of using ILs or DESs as the basis for drug formulations is, apart from contributing to the performance of the pharmaceutical ingredients, as additives, they are also known to improve the properties of the carrier material, often as plasticizers.

Plastics, by definition, are products made from the essential polymer mixed with a complex blend of materials known collectively as modifiers (or additives).[78] For decades, they have been extensively utilized for producing plastics with more desirable qualities to be used in commodity, engineering and medical applications. Plasticizers are one of the most commonly used additives in plastics and can typically make up 2-3 wt% of the material. According to the council of International Union of Pure and Applied Chemistry (IUPAC), it is defined as "a substance or material incorporated in a material (usually a plastic or an elastomer) to increase its flexibility, workability or extensibility. A plasticizer may reduce the melt viscosity, lower the temperature of a second order transition, or lower the elastic modulus of the product".[79] Since 1862, when plasticizers were first introduced to the production of flexible plastics,[80] they have become an inherent part of current plastic industry. In 2014, worldwide plasticizer market was worth more than 53.34 million kg, with almost 90% consumed by polyvinyl chloride (PVC), to improve its flexibility and durability.[81] In Europe alone, the consumption was 8.26 million kg, mainly used in films and cables.[81] Over the last 60 years, more than 30,000 different substances have been evaluated for their plasticizing properties, adipates, azelates, benzoates, phthalates, trimellitates and phosphates are some of the most frequently used ones.[80-82] Phthalates, as the most dominant class of plasticizers, dominates approximately 87% of the entire plasticizer industry, while di(2-ethylhexyl) phthalate (DEHP) accounts for almost 50% of total plasticizer consumption.[79] Other materials can also be improved by blending with plasticizers, including concrete, clays and their related products.

Inside the polymer matrix, plasticizers act by embedding themselves between the polymer chains, spacing them apart (therefore increasing the free volume) and breaking up the inter-molecular interactions to form secondary polymer plasticizer bonds, thus rendering

mobility to polymer chains or chain segments, which results in a lower glass transition temperature.[83] With the weak interactions in between the polymer-plasticizer interaction, a dynamic process also exists where a plasticizer molecule attached to one site in the polymer network may be dislodged and replaced by another.[79] Hence the concept of plasticizing efficiency, which is thought be related to the plasticizer molecules' structure, chain length, molecular weight (MW), etc.[79] Different plasticizers, therefore, show different plasticization effects owing to their differences in strength in plasticizer-polymer and plasticizer-plasticizer interactions.

Plasticizers that are currently available on the market have been offering different polymers with a wide range of end-use properties. But the applications of many have presented a number of potential problems, including flammability, limited compatibility, and poor stability under UV rays or high temperature instability and reduced lubricity at low temperatures. However, leaching and migration of plasticizers, especially phthalates from medical and commodity plastics, remains one of the most hotly debated problem over the past few decades.[84] Mainly due to the fact that the plasticizers are not bound to the polymer matrix. Countless studies have linked phthalates to a host of maladies in human and other life forms. It has been shown to be an estrogen mimic and is proposed to cause fertility issues in a variety of species. It has also been proposed as a possible carcinogen and has been banned from a variety of products including children's toys and dinking bottles.[84] The European Union has already banned a number of phthalates from certain applications and the US Food and Drug Administration (FDA) has suggested manufacturers to consider the possibility of replacing DEHP with safer alternatives.[84]

Several alternatives have been currently under investigation, with ILs being one of the novel options which showed promising results in a number of earlier publications. Considering the diverse challenges associated with different traditional plasticizers, ILs have been proposed as alternative plasticizers in the rapidly growing plastic industry. The ability to modify the shape and properties of ILs means that they could be designed to specifically interact with moieties on the polymer chain.

As plasticizers, ionic liquids (1-butyl-3-methylimidazolium chloride and 1-octyl-3-methylimidazolium chloride) were proven to be effective for polyelectrolyte complexes (PEC).[85] Compared to a simple inorganic salt (i.e. NaCl), IL cations with their stronger

hydrophobicity and larger volume, enhanced their partitioning into PEC.[85] Therefore, more free volume was created for polymer segments to move. The T_g (glass transition) was thereby decreased as the salt breaks ionic bonds between the two oppositely charged polyelectrolytes.[85] Additionally, ILs with larger and more hydrophobic cations were proved to provide greater PEC plasticization, which supported the free volume theory.[85]

Common plastics, such as polyethylene terephthalate (PET), have revolutionised food storage and transport,[86] but they have had a lasting environmental impact, with industries struggling to recycle the growing mounds of plastics at landfill sites and found washed up along coastlines.[86]

Starch is one of the most common and easily obtained natural polymers, making it attractive as a potential bio-based alternative to synthetic polymers. ILs and DESs, have been used to plasticize starch. It was shown that the crystallinity of the starch can be controlled by formulating DES, allowing the stabilization of mechanical properties. The new systems showed even more compatibility with starch than conventional plasticizers like glycerol and urea. Moreover, in the case of corn starch/zein blends (a hydrophobic protein from corn), 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) was used as a co-plasticizer, and a homogenous outcome was achieved.

A similar study by Abbott et al showed that a simple quaternary ammonium salt combined with urea or glycols forms effective modifiers that produce flexible plastics with good mechanical properties that are comparable to some polyolefin plastics. The processing conditions were shown to significantly affect the structure of the polymer which had an effect on the mechanical and physical properties of the resulting plastic. Using a glycerol based DES a sustainable and biodegradable material was produced which could be injection moulded.[87]

Figure 1.12: *Sample of starch plasticised with glycerol and choline chloride injection moulded at 200 °C and 60 bar. Sample diameter 7 cm.*[87]

DESs have also been used to aid the blending of hydrophobic and hydrophilic polymers. One strategy to accelerate the mechanical degradation of polyolefin plastics in the environment is to blend them with carbohydrate-based polymers. Unfortunately, polyolefin are hydrophobic whereas carbohydrates tend to be hydrophilic so the two do not blend without chemical modification of the carbohydrate. In this study high density polyethylene, HDPE and thermoplastic starch, TPS are used as the polymers with deep eutectic solvents, DESs as the modifiers. Both TPS and DESs are biodegradable and the DESs are water miscible and biocompatible ensuring that the composite plastic contains a biodegradable flaw which should enable mechanical and chemical degradation. It is shown that DESs enable facile mixing of the two polymers. The composite has a strength similar to TPS but a ductility greater than either of the two components. The glass transition temperature of the composite plastic shows that they are homogeneously mixed and data suggests that the DESs act as lubricants rather than plasticizers.[88]

1.13 Research aims

Previously, it was shown that choline chloride based deep eutectic could be used as functional additives in thermoplastic starch with efficient plasticization and enhanced strength. This opened new perspectives in using DESs as environmentally friendly alternatives for other material additives. DESs are good plasticisers for starch as they interacted with the intramolecular hydrogen bonded network. In principle, proteins have

a similar network of intramolecular hydrogen bonding which produced crystalline, brittle materials without the addition of a plasticiser. The aim of this research is to develop protein-based plastics using DESs as modifiers.

By breaking the intermolecular hydrogen bond between protein chains, deep eutectic solvents will be used to improve the plasticisation of the materials. The newly formed hydrogen bond will prevent the recrystallizing hence lead to more flexible materials.

This study will be split into three sections:

i. Optimisation of the properties of proteinaceous materials based on three different protein types; zein, soy and gelatin. The study will investigate the effect of composition in terms of the DES: protein ratio. It will also optimize the processing conditions of temperature and pressing time to obtain the optimum strength and flexibility.

ii. One of the advantages of biopolymers over synthetic polymers is their biocompatibility, particularly when they are put into the human body or contacted with the skin. To this end biopolymers will be developed which can be used as drug delivery systems. In the second section, instead of common DES being used to modify protein, a variety of pharmaceutical deep eutectic solvents, PDES, will be used. It has been found that pharmaceutical DESs can be produced where the pharmaceutical ingredient is either a hydrogen bond donor or a quaternary ammonium salt (usually an amine hydrochloride). These liquids are quick to disperse and can overcome solubility issues of some pharmaceutically active molecules. In this part of the study PDESs were used to modify gelatin. The effect of the different PDESs on the mechanical properties will be determined and then the dissolution rate will be quantified in media which simulate the fluid found in the stomach.

iii. The final part of the study will use the same PDES modified gelatin and determine whether the drug molecules enter the skin more easily in a liquid state than they would from a solid. Their egress from the protein into the skin will be measured in a number of ways to see if transdermal pharmaceutical patch delivery is viable with these types of materials.

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Chapter 2: Experimental

2. Experimental

2.1 Formation and preparation of liquids and proteinaceous material

2.1.1 Chemicals

The protein powders used in this thesis are soy flour, fish skin gelatin and zein powder, DES components include glycerol, choline chloride, imipramine HCl, ascorbic acid, aspirin, catechol. All chemicals were used as received. Their purities and sources are given in Table 2.1 below.

***Table 2.1**: List of used chemicals in this work.*

Chemicals	Source	Purity %
Glycerol	Fischer	98
Resorcinol	Sigma-Aldrich	99
Choline chloride	Sigma-Aldrich	≥98
Aspirin	Sigma-Aldrich	≥ 99
Paracetamol	Sigma-Aldrich	98
Salicylic acid	Sigma-Aldrich	≥ 99
Catechol	Sigma-Aldrich	≥ 99
Urea	Alfa Aesar	≥98
Ascorbic acid	Sigma-Aldrich	99
Imipramine HCl	Sigma-Aldrich	≥ 99
Ethylene glycol	Sigma-Aldrich	≥99
Gelatin	Sigma-Aldrich	CAS 9000-70-8

2.1.2 Preparation of DESs

Glyceline was prepared using choline chloride (ChCl) (Sigma-Aldrich, >98%) and glycerol (Fischer Scientific, >98%), which were mixed together in a 1:2 ratio respectively. The four PDESs were Catechol: ChCl (1:1), Aspirin: ChCl (1:1), Imipramine HCl: glycerol (1:2), Ascorbic acid: ChCl (1:2). The method of synthesis was the same for all of the liquids.

After mixing, the liquid was then put into a Thermo Scientific Heraeus oven (50^{o}C, overnight). To finish off the melting process, the partially melted mixture was placed on a hotplate-stirrer (50^{o}C), the top of the beaker was sealed with Para film. After the melting process was complete the resulting Glyceline DES was stored in a sealed bottle at 50^{o}C.

2.2 Experimental techniques

2.2.1 Compression moulding

Samples for mechanical analysis were prepared by making sheets of TPS using a hydraulic press (Fontune Grotnes Laboratory Press TH400 (**Figure 2.3**). The powdered mixture was placed between two copper plates (**Figure 2.1**) lined with non-stick silicone sheets with a 1 mm copper separator as shown on the middle between the two copper plates (10 cm square aperture). The sandwich was then placed in the press and a force of 120 kN was placed on the sample for 10 minutes at different temperatures. Zein was pressed at 110 ^{o}C, soy at 130 ^{o}C and gelatin at 75 ^{o}C.

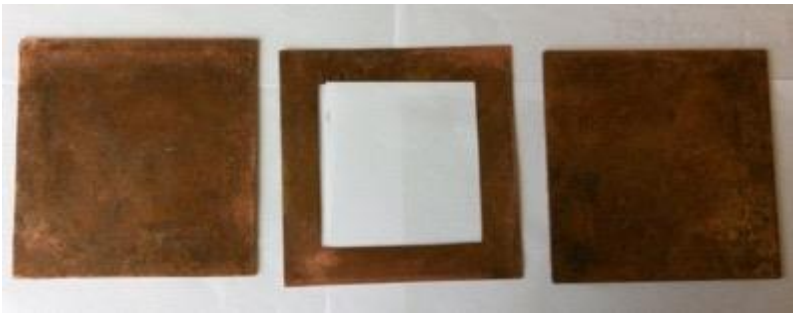


Figure 2.1*: The 1 mm copper separator and the two copper plates*

The sample was then cooled back to room temperature in 5-10 minutes in the press with the force still applied. Subsequently, the sample was removed from the press and then the mould. Three sheets were made for each mixture with dimensions as appeared in **Figure 2.1**.

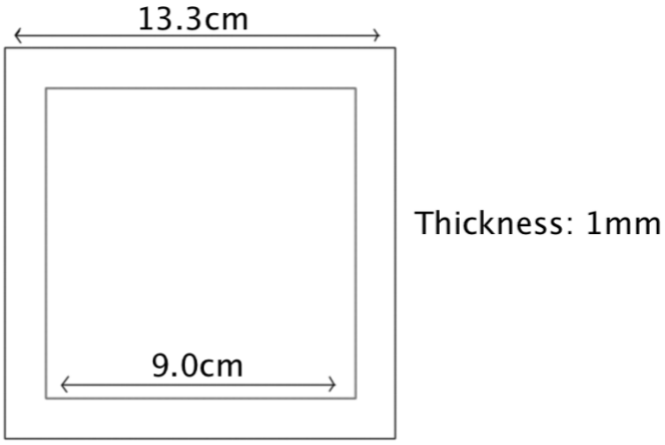


***Figure 2.2**: The dimensions for sample sheets*

***Figure 2.3**: Fontune Grotnes Laboratory Press TH400.*

2.2.2 Tensiometer

All samples were pressed into small sheets using the procedure discussed above, then using a mechanical 'dog bone' press (Ceast Hollow Die Punch, Type 6051) to cut test shapes (test area size: L 1⁄4 30 mm, W 1⁄4 4 mm, D 1⁄4 1 mm) (**Figure 2.4**).

Then each specimen thickness of the samples prepared above was measured by a micrometer mechanically, and tested using an Instron 3343 tensiometer fitted with a 500 N load cell at a rate of 2 mm/min.

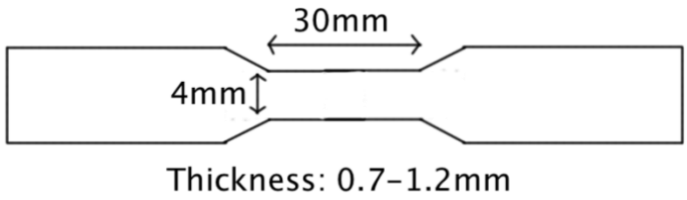


Figure 2.4: *The dimensions for test shapes*

The material strain and stress was controlled by Instron Bluehill 2 software and average values were taken from eight or more samples, the error reported as the standard deviation

2.2.3 Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) is a technique that makes possible to analyse physical and chemical changes involving heat absorption or release, while recording at the same time the temperatures and amounts of heat associated with the process under study. With regard to proteins, denaturation curves result from thermal changes associated with the breakdown of those bonds involved in stabilizing protein structure (e.g. hydrogen bonds, hydrophobic interactions, electrostatic interactions, etc.), and, in some cases, an additional aggregation process.

Differential Scanning Calorimetry (DSC) was used to evaluate the thermal behaviour of the plastics. The Mettler Toledo DSC1 was used to test the material pellets in 2 stages, starting from room temperature (25^{o}C) until it passed the glass temperature (*Tg*). The results were analysed with STRe software. Samples (5-15 mg) were placed on the standard aluminium DSC pan and weighed accurately. The lid of the pan was pierced with a pin before it was pressed together with the pan, which is required to prevent the gas pressure build up inside. The sample was placed within the furnace next to an empty pan (with pierced lid) and used as a reference. Testing conditions were as follows:

Stages	Increase (at 10^{o}C/ min)	Held (for 5 min)
Zein	25 ^{o}C to 200 ^{o}C	200 ^{o}C
Soy	25 ^{o}C to 250 ^{o}C	250 ^{o}C
Gelatin	25 ^{o}C to 150 ^{o}C	150 ^{o}C

Table 2.2*: The temperature profile for different samples during DSC test*

2.2.4 X-ray diffraction (XRD)

X-ray diffraction was carried out using a powder diffractometer, Bruker D8 Advance that was equipped with a LynxEye linear position sensitive detector and a 90-position autosampler.

The diffraction patterns were collected using the following conditions: 2θ range: 4°-60°, step size: 0.02°, step time: 15 s taken over 11.5 hours. All samples were measured on a conventional sample holder using blue tack as adhesive substance.

2.2.5 Dynamic Mechanical Analysis (DMA)

Dynamic Mechanical Analysis measurements were carried out using a Mettler Toledo DMA1 STARe system, operating in the dual cantilever bending mode using titanium clamps. Tests were performed at 1, 2, and 5 Hz and the temperature was ramped from 0 °C to 100 °C at a rate of 2 °C/min.

The dimensions of each single sample were measured accurately by a micrometre before every experiment.

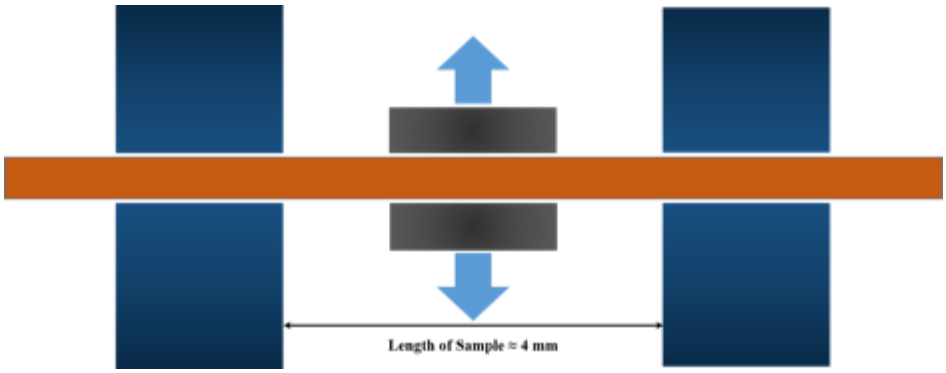


***Figure 2.5**: A diagram showing the dual cantilever mode in DMA*

2.2.6 Thermogravimetric analysis (TGA) with humidity attachment

Thermogravimetric analysis was carried out using a Mettler Toledo TGA/DSC1 STARe system using open aluminum pans. The mass of each sample in all TGA experiments was around 15 mg.

Water uptake experiments were carried out at 80 °C throughout the experiment. The

relative humidity was kept at 0% for 60 minutes, then jumped to 100 % and then held constant for 60 minutes before it went back to 0% humidity and kept for another 60 mins. The relative humidity was controlled by connecting a humidity generator, type MHG-23 with a pro umide MHG control system, version 3.03 and the software was used MHG method editor, version 3.02, software. The percentage of mass change was measured by the provided STARe system software.

2.2.7 Contact angle

Contact angle experiments were carried out using a Cam 100 optical Angle Meter KSV Instruments Ltd., Finland) with the provided software.

Using the 1 mm thick sample sheets formed by compression moulding mentioned above, a drop of pure water was put on the surface. Images were recorded three times during 10 min. Once immediately after the drop, once after 5 min, and the last one was taken at the end. The procedure was repeated for at least 10 times for each sample and the average value was calculated.

2.2.8 Density measurements

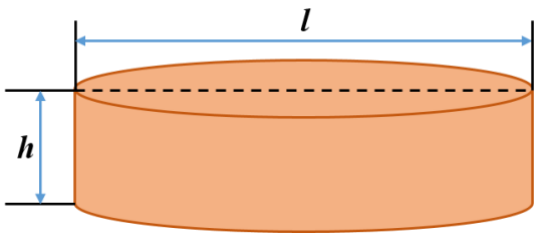
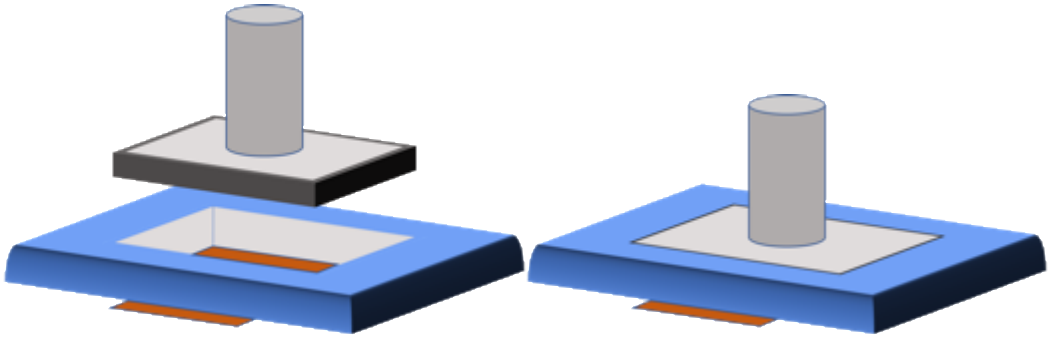


***Figure 2.6**: measurements for drug tablets*

The TPS sheet mass ($\boldsymbol{m}$) were measured before their volume ($\boldsymbol{v}$) were determined by measure the dimensions (surface area diameter $\boldsymbol{l}$ and height $\boldsymbol{h}$) of gelatin tablets (tablets were seen as cylinder), then the density ($\boldsymbol{\rho}$) was calculated using the equation down below**.**

$$\boldsymbol{\rho = 4m/l^2 \pi h}$$

2.2.9 Adhesiveness test



Step 1 Step 2

***Figure 2.7**: surface adhesiveness test*

To test the adhesiveness of the sample surface, a piece of sample sheet was placed under a block frame to stabilize, with a stamp pressing down to the sample surface and release. The force required to retract the stamp was measured (Stable Micro System Texture analyser TA-XT plus).

2.2.10 UV-visible Spectroscopy

The absorption of the samples was measured in a UV spectrophotometer (Shimadzu model UV-1601 spectrophotometer) within the range of 200-400 nm and the concentrations were determined by using calibration curves. The results were analysed by Origin 2015 software.

The absorption spectra of the four drugs in use (aspirin, ascorbic acid, catechol and imipramine hydrochloride) exhibit absorption maxima at about 276 nm, 244 nm, 277 nm, and 251 nm respectively.

2.2.11 Quartz Crystal Microbalance (QCM)

Viscosity measurements were conducted for the prepared DESs using a Quartz Crystal Microbalance (QCM) Agilent Technologies E5061A300 KHz ENA Series Network Analyzer at 25 °C. The thin wafer (~ 0.2 mm of thickness) of the 10 MHz quartz crystals has the surface finish of 2 μm for the smooth crystals.

2.2.12 Nuclear magnetic resonance (NMR)

For the gelatin with Glyceline, all the NMR experiments were performed in a Bruker Biospin DMX 300 operating at a ^{1}H frequency of 300.13 MHz. NMR nuclear spin relaxation times T_1 and T_2 were measured using standard inversion recovery (Fukushima, E., Roeder, S.W., Addison-Weslkey, Reading, US, 1981.) and CPMG (Carr, H. Y., Purcell, E. M., Physical review 1954, 94, 630-638.) techniques, respectively. Experimental data were fitted using single exponential functions. The T_1 relaxation time constant was obtained by fitting the experimental data to the equation (Fukushima, E., Roeder, S.W., Addison-Weslkey, Reading, US, 1981.):

$$S(t) = S_0\left[1 - 2\exp\left(-\frac{t}{T_1}\right)\right] \quad (1)$$

The T_2 relaxation time constant was obtained by fitting the experimental data to the equation (Fukushima, E., Roeder, S.W., Addison-Weslkey, Reading, US, 1981.):

$$S(t) = S_0\exp\left(-\frac{t}{T_2}\right) \quad (2)$$

Chapter 3: DES modified globular and fibrous proteins

3. DES modified globular and fibrous proteins

3.1 Introduction

Proteinaceous materials have numerous structures, many of which aid in the roles they perform. Some need to impart strength while others need elasticity or toughness. The protein structure is an important aspect to consider when it comes to a better understanding of the interactions between plasticizer and the protein matrix and therefore the long-term properties of the protein material.

With the primary structure being the sequence of amino acids linked by polypeptide chains, the secondary structure refers to the local folding of the polypeptide structures and includes two structures that are held in shape by hydrogen bonds, α helix and β pleated sheet. The overall three-dimensional structure of a polypeptide is called its tertiary structure, which can be classified into two categories, globular and fibrous protein. (**Figure 3.1**)

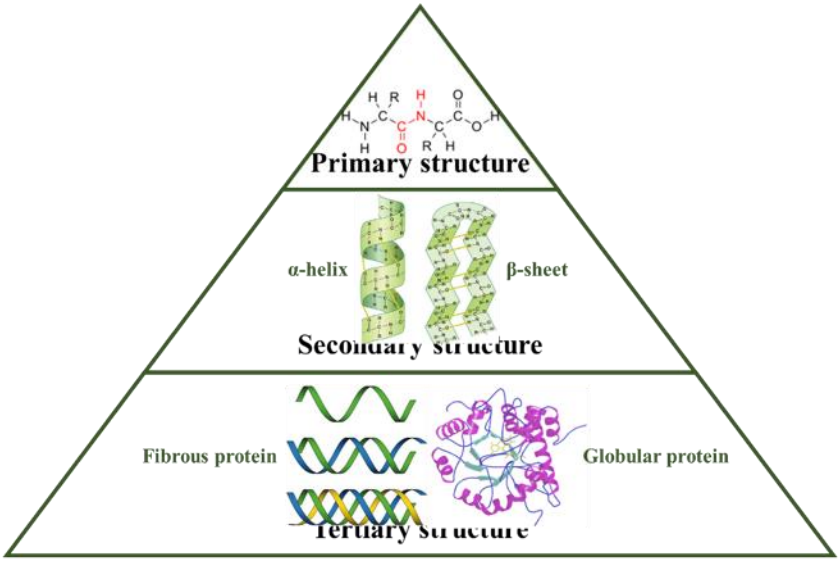


Figure 3.1 *Levels of protein structure*

Fibrous proteins can also have a quaternary structure when the fibrils become entangled to form microscopic fibres. Although the precise relationship between the amino acids composites and the secondary structure of protein is yet to be confirmed, the sequence of the amino acids plays an important role in predicting the protein structure. This is due to the fact that the secondary structure is made up of hydrogen bonding interactions and it

is determined by the position of where the bonding occurs, which is based on the primary sequence.

The properties of the proteinaceous materials depend on the composition of the non-protein-based components. In their pure, dry state most materials are crystalline and brittle if formed into sheets. The addition of modifiers can change the properties significantly. An example of this is leather. When animal skin is processed, it first has the hair removed using sodium sulphide and then has the fats removed using calcium hydroxide (lime). If, this material is dried then it becomes brittle. Vellum is made by this process using calf skin. If, the limed material is kept damp then it can be crossed linked with either vegetable of mineral agents making a stronger material which is classified as a leather or suede. While strong, it is still relatively stiff and brittle unless lubricants are added. These are usually in the form of vegetable or fish oils called fat liquors which impart softness and flexibility to the material. These sit between the quaternary structures and enable the fibres to move past each other.

It has previously been shown that DESs can plasticize starches by breaking up the hydrogen bonding structure and enabling movement of amylose and amylopectin chains past each other.[1] When going through certain processing conditions (pressure and temperature), in the presence of a plasticizer such as DES, starch loses much of its original granular structure and is transformed into a molten plastic state called thermoplastic starch.[2] Unfortunately, thermoplastic starch has its disadvantages in comparison with most plastics currently in use, namely its high moisture absorbance ability and poor mechanical properties.

Native starch also has been previously incorporated into the synthetic plastic as a filler,[3] however, due to poor interfacial adhesion these materials have poor mechanical properties. The hydrophilicity of starch results in failing to form a continuous phase with the synthetic polymer, which can lead to stress concentration points, resulting in mechanical failure even at low tensile stresses. One way to increase compatibility in starch blends is to use a compatibilizer containing groups capable of hydrogen bonding with starch hydroxyl groups.[2,4]

Subsequently, a study was done on polyolefin-based plastics to determine whether DESs would mix with a non-polar polymer such as HDPE.[5] Not only samples of DES-modified

HDPE were successfully made, they could be extruded with DES-modified starch to produce a homogeneously blended material, under similar conditions to those for DES-modified starch.[5] This was significant in the sense that not only it helps with the strategy to accelerate the mechanical degradation of polyolefin plastics in the environment by blending them with carbohydrate-based polymers, given the fact that polyolefin are hydrophobic whereas carbohydrates tend to be hydrophilic, this proved DESs can be used to incorporate itself with both hydrophilic and hydrophobic materials with improved properties. This is particularly useful since protein as a complex system presents both hydrophobic and hydrophilic characters. Interestingly, it was concluded that the DES just filled the void volume of the HDPE and did not specifically interact with the polymer chain. Additionally, the study also shows that when boiled, the DES components will leach from the HDPE: TPS blends and enable it to be mechanically degraded. This shows that by blending starch with HDPE using DESs as modifiers, it not only increases the amount of sustainable material included in plastic but also improves the mechanical degradation once immersed in water. Since as starch, proteins also have superior biodegradability, similar attempts can be made to incorporate hydrophobic materials like HDPE with proteins as a way of improving the sustainability of the product.

Fundamentally DES-modified starches and DES-modified proteins should behave in the same way i.e. a proportion of the DES will strongly bind to the polymer chain whereas the remainder will be less strongly bound and act as a lubricant/ plasticizer. It may be expected that the properties of the DES will affect the properties of the modified material because they will have different interactions with the functional groups of the polymer and the DESs will have different fluid properties and hence lubricate in different ways.

The properties of the materials may also be expected to be different as the proteins will be more hydrophobic and the intermolecular interactions are between different functionalities, not just amides. In starch the interactions are all between –OH functionalities. Another fundamental difference between starches and proteins is that the former has repeating monomer units of the same monomer whereas the latter has a more random sequence of monomers and the secondary structure is more ordered. Starches will always have elastomeric properties due to the coiled structure of amylopectin whereas the proteins will behave differently depending on whether they are predominantly α-helices or β-sheets.

Amino Acid	Zein (%)	Soy (%)	Gelatin (%)	Side chain polarity
Alanine	8.06	3.8	10.88	Nonpolar
Arginine	1.95	6.7	9.9	Polar
Aspartic Acid	4.53	10.2	7.46	Polar
Cystine	0.78	1.1	-	Nonpolar
Glutamic Acid	19.97	16.8	11.28	Polar
Glycine	1	3.7	32.63	Nonpolar
Histidine	1.1	2.3	0.77	Polar
Isoleucine	3.47	4.3	1.44	Nonpolar
Leucine	15.94	7.2	3.00	Nonpolar
Lysine	0.17	5.5	3.46	Polar
Methionine	1.41	1.1	0.42	Nonpolar
Phenylalanine	6.18	4.6	1.99	Nonpolar
Proline	8.7	4.5	12.52	Nonpolar
Serine	4.13	4.6	3.62	Polar
Threonine	2.26	3.3	2.11	Polar
Tryptophan	0.13	1.1	0.4	Nonpolar
Tyrosine	4.32	3.3	0.4	Polar
Valine	3.07	4.5	2.18	Nonpolar

Table 3.1 *Amino acid composites of zein, soy and gelatin protein*[6–8]

With proteins dominated by α-helices, the amino acid composition analysis shows high levels of hydrophobic amino acids such as leucine, proline, and alanine. Meanwhile, valine and isoleucine showed high assignments in β-strands. Three proteins were chosen for their ready availability and because they represented different types of structures. The proteins chosen were zein, soy and gelatin. The amino acid composition of the three chosen proteins are listed in Table 3.1. With the help of analytical tools such as the Structural Bioinformatics Protein Data Bank, it was determined that though both have globular tertiary structures, zein protein is mainly composed of α-helices while soy protein is dominated by β-sheets. Gelatin, as a fibrous protein, is also consisted of helix structures made by α-helices.

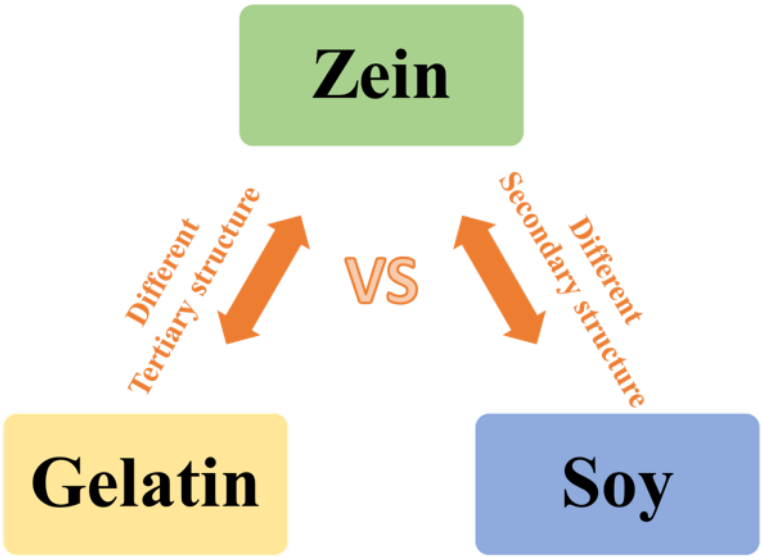


Figure 3.2 *A diagram of the structural relationship of the three chosen proteins*

In this chapter a method for making DES modified protein sheets is investigated together with the compositional effects of different amounts of DES in the formulation. This will be carried out for all three proteins and the effect of the modifier on the mechanical properties of sheets formed will be presented together with the effect of crystallinity and the glass transition temperature, *Tg*.

3.2 Methodology

A standard system has been developed for modifying thermoplastic starch using DESs by Abolibda.[1] A variety of DESs were tested with starches with varying amylose and amylopectin contents. The properties of the material formed were found to be depend at on the amount and type of the DES together with the type of starch used, as these had different amylose: amylopectin ratios. It was also revealed that the choline chloride/glycerol DES as an additive gives the strongest TPS since glycerol has more hydroxyl groups than the rest which improve the plasticization. Consequently, in this chapter Glyceline is used as a choice of DES to see if different types of proteins can be modified in the same way.

The pressing conditions to form homogeneous sheets were in the region of 120 kN force at 120 to 160 ^{o}C. The properties of the material were found to depend upon the proportions of the components in the mixture and the pressing conditions. The DES phase is known

to be partially continuous i.e. some DES is still fluid in the material. This is very important for the topic of drug delivery as discussed later in this thesis. No significant difference was observed between the glass transition temperatures of all non-extruded samples and the extruded ones, as shown in **Table 3.2**. This indicates that the properties of the modified TPS are dependent upon the amount and type of plasticizer rather than the extruding process, as the salt can modify the structure of the starch hence affecting the ways that the polymer chains interact with each other.

Composition Starch: ChCl: HBD	*Tg* / °C	
	Non-Extruded	Extruded
HBD = urea	72. 9 ± 6.5	69.9 ± 8.4
HBD = ethylene glycol	72.0 ± 7.7	71.2 ± 11.8
HBD = propylene glycol	68.6 ± 9.1	68.1 ± 7.8
HBD = glycerol	65.4 ± 6.5	57.0 ± 9.9

***Table 3.2**: Effect of hydrogen bond donor on the glass transition temperature of various extruded thermoplastic starch samples.*[1]

The difference required to process proteins compared with starch is the pressing temperature. The issue with most proteins is that they de-nature when pressed at higher temperatures i.e. they lose their secondary structure. The pressing temperature was lower than the protein cooperated with starch, as shown in Table 3.3. Protein samples were prepared by mixing the corresponding protein powder with the pre-prepared Glyceline in different weight ratio. The resultant mixture was then thoroughly mixed manually without extruding. The mixture was then placed between two copper plates lined with non-stick silicone sheets and equipped with a 1 mm copper separating unit with a 100 mm^2 aperture. The resultant sandwich was then placed in a hydraulic press (Fontune Grotnes Laboratory Press TH400) and a force of 120 kN was applied to the sample at different temperature (**Table 3.3**) for 10 min. The sample was subsequently cooled to ambient temperature whilst remaining under pressure prior to removal from the hydraulic press. The cooled sample was subjected to a mechanical ‘dog-bone’ press (Ceast Hollow Die Punch, Type 6051) to cut into test shapes (test area size: L = 30 mm, W = 4 mm, D = 0.7–1.2 mm).

Protein	Pressing temperature (°C)
Zein	110
Soy	130
Gelatin	75

Table 3.3 *Pressing temperatures for different kinds of protein*

Tensile testing was then performed on the dog-bone samples using an Instron 3343 tensile apparatus (Instron Ltd., Assembles, USA) with a load cell of 500 N. The material stress and strain were controlled by Instron Bluehill 2 software and average numerical values were taken from eight or more samples. In each case, the thickness of the sample was measured using a micrometer and subjected to a strain rate between 2–10 mm min^{-1}.

Deformation of an object occurs when a force or stress is applied to that object. Tensile stress and strain can be used to determine the degree of that deformation, and they have a relationship called the Hooke's law.[10]

$$\sigma = E\epsilon \qquad \textit{Equation 3.1}$$

Where $\boldsymbol{\sigma}$ is stress, is $\boldsymbol{\epsilon}$ strain and $\boldsymbol{E}$ is modulus

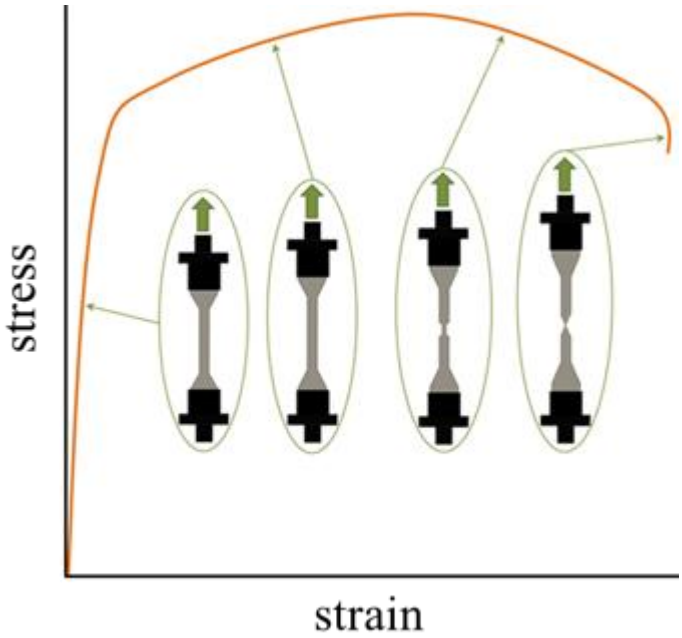


Figure 3.3: *the four stages of a stress-strain curve during plastic deformation when a load is applied to a dog-bone*[1]

When the stress and strain follow a linear relationship, the deformation is elastic, and the slope is referred to as the elasticity modulus (***E***). The elastic material returns to the initial shape when the applied stress is released. However, when the deformation is non-recoverable, and it is permanent, it is identified as plastic deformation. In plastic deformation the tensile stress increases with the continuous load till it reaches the maximum and then decreases till the material breaks as shown in **Figure 3.3.**

To better understand the degree of the protein unfolding caused by Glyceline as a plasticiser, a series of thermal tests were carried out using differential scanning calorimetry (DSC) in order to identify the thermal behaviour of materials, especially the glass transition temperatures (*Tg*). *Tg* is the temperature when a reversible transition occurs when a material changes from a hard and relatively brittle "glassy" state into a more flexible or rubber-like state, as the temperature is increased. This is an important parameter all synthetic and natural polymers not only from a mechanical perspective but also because it enable the permeation of gas molecules through a material. It also affects the conditions under which the materials are moulded or extruded. Generally, *Tg* is increased by increasing the amount of stiff chains and bonds, bulky side groups, crosslinking between chains, and the degree of crystallinity, whereas *Tg* is decreased by the addition of a plasticizer which increases the free volume and thereby allows increased backbone chain segmental mobility, which in turn produces structural relaxation at a decreased temperature. The depression of *Tg* may have a significant effect on the shelf-life and stability of the materials. In conclusion, dynamic mechanical properties of modified protein-based plastic can provide information about the relaxation and movement of molecule, which reflect the changes in structure of the final product.

Stages	Increase (at 10^{o}C/ min)	Held (for 5 min)
Zein	25 ^{o}C to 200 ^{o}C	200 ^{o}C
Soy	25 ^{o}C to 250 ^{o}C	250 ^{o}C
Gelatin	25 ^{o}C to 150 ^{o}C	150 ^{o}C

Table 3.4*: The temperature profile for different samples during DSC test*

Differential Scanning Calorimetry (DSC) analysis was performed to analyze thermal events with the samples. A Mettler Toledo DSC1 was used to determine glass transition temperatures and the results analyzed with STARe software. Samples (5–10 mg) were placed on in a standard aluminum DSC pan before the lid of the pan was pierced. The sample was placed within the furnace next to an, otherwise identical, empty reference pan. The samples were heated according to profiles in **Table 3.4**.

3.3 Zein

Zein is a protein present in corn in the seeds of maize in large amounts. As a biopolymer derived from agricultural crops, the research on its development has gained momentum in recent years. Recent attempts of modification, according to its utilization, include many methods. For example, in the medical field, a method was proposed to produce a peptide fraction from zein by enzymatic hydrolysis to improve the molar ratio of the branched chain amino acids (leucine, isoleucine and valine) vs. the aromatic amino acids (phenylalanine and tyrosine), in order to produce a Non-bitter Peptide Fraction for Patients with Hepatic Encephalopathy.[54] Zein-based plastics also showed a potential as a commercial material for industries like food packaging, as it has suitable thermoplastic properties and film-forming behavior. It can be used as a barrier to control the transfer of moisture, oxygen, carbon dioxide, and can prevent quality deterioration and increase the shelf-life of food products. As a way of thermally modifying starch-based films, zein can be mixed with starch by a simple dry heating procedure, which can induce both a higher water contact angle and tensile strength thus improving the functionality of edible films from starches.[55] Similarly, a series of chitosan-modified zein composite films were fabricated from zein and chitosan with superior physical properties. However, the process involves a more complicated procedure including blending, solution casting and evaporation.[56]

However, zein's chemical inertness and globular structure create difficulties during molding, thus the addition of plasticizing agents to the zein material is often required to overcome its brittleness caused by extensive intermolecular forces. Plasticizers can prevent cracking of the materials during subsequent handling and storage. Low-molecular weight compounds or diluents, acting as external plasticizers, are an integral part of polymeric systems. The plasticizers must be compatible with the polymer matrix.

Attempts have been made by multiple groups using different plasticizers, including polyols like glycerol [11,12]. They serve to increase the flexibility and workability of the otherwise rigid neat polymers. Results showed increasing most plasticizers content will decrease the ultimate tensile strength (UTS) while increase the strain at break (SB) of zein films, except when the mannitol content increased from 0.7 to 1 g/g zein. This was suspected to be due to the use of low levels of plasticizers for film making and the increase of chain association with low amounts of plasticizers.[13]

However, as glycerol can only provide a temporary hydrogen bonding system, with time, the material could recrystallize. By using Glyceline instead of just glycerol, the chloride ions (a strong Lewis basis) is able to break up the intra molecular hydrogen bonding hence forming a network between themselves and the glycerol, therefore keep a stronger interaction for a longer time. So it stops the protein from hydrogen bonding back with itself and recrystallizing Samples of starch plasticized with Glyceline have lasted more than 5 years without signs of recrystallization.

The compatibly of ionic iiquids and zein protein have been studied by Biswas and the group, by solubilizing zein protein in 1-butyl-3-methylimidazolium chloride (BMIMCl) and 1-butyl-3-methylimidazolium dicyanamide (BMIMdca) in concentrations up to 10% (w/w).[14] It was demonstrated that IL could be used as a solvent for the chemical modifications of zein.

Zein as a protein contains a high content of hydrophobic amino acids (leucine, proline and alanine). If both zein and starch can be processed in the presence of DES as plasticizers, the possibility of combining thermoplastic starch and plasticized zein will have great potential in the field of biodegradable plastics. Additionally, the low solubility of zein in water could increase the hydrophobic character of the corresponding thermoplastic starch compositions. On top of which, as zein and starch coexist in corn, a material combining both will eliminate unnecessary procedure during the initial stage of the production.

3.3.1 Thermomechanical Analysis

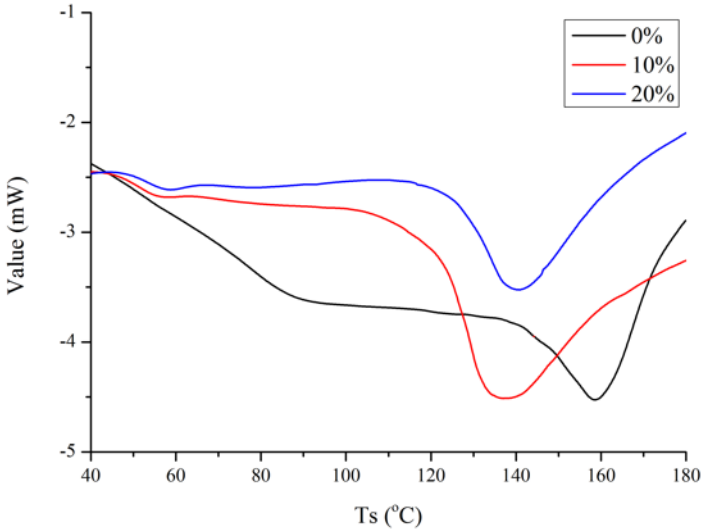


Figure 3.4 *transition temperatures for zein samples with 0%, 10% and 20% Glyceline measured by DSC*

	0%	10%	20%
Transition temperature T_1 (°C)	89	57	58
Transition temperature T_2 (°C)	159	139	140

Table 3.5 *The transition temperatures of zein samples with different Glyceline content*

Table 3.5 shows the transition temperature values obtained from **Figure 3.4** for three samples with different Glyceline contents. Kokini and co-workers have used DSC measurements as a way of studying thermal properties of cereal proteins including gliadin, glutenin, and zein.[15] According to literature, the glass transition temperature of anhydrous zein is 162-165 °C, which is similar to the T_2 value in **Table 3.5**.

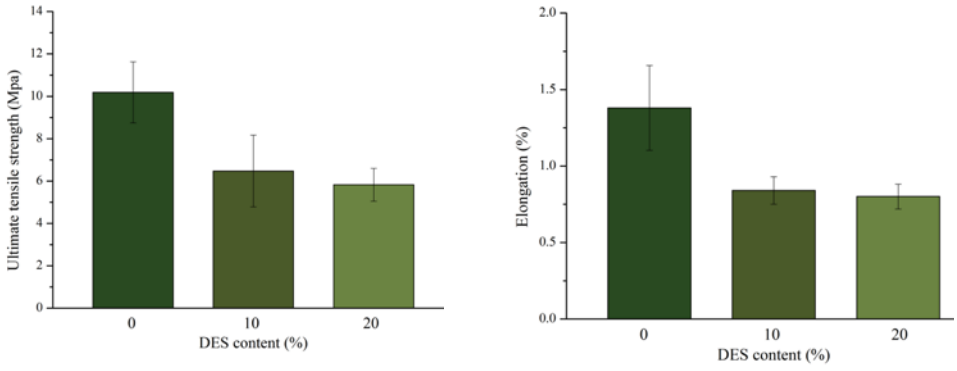
Results show a lack of linearity in the relationship between the DES contents and the T_g (T_1) values; adding 10% DES has a significant effect on T_1 but this does not change when the amount of DES is doubled.

This result is consistent with the conclusion gathered by di Gioia and Guilbert who studied the plasticization of corn gluten meal (a by-product of corn starch), with various polar (water and glycerol) and amphiphilic (octanoic and palmitic acids, dibutyl tartrate

and phthalate, and diacetyl tartaric acid esters of mono-diglycerides) plasticizers.[16] It was established that the first amounts of added plasticizer (<10%) were the most effective at lowering *Tg*. However, at higher plasticizer content (between10 and 30%) plasticization effectiveness slowed down, more markedly so for amphiphilic than for polar plasticizers. Changes in plasticization effectiveness were attributed to an increasing difficulty for the plasticizers to diffuse into the polymer matrix.[16] Similar results have also been published by Lawton where the more hygroscopic plasticizers had a most significant effect on T_g with only a small amount. However, once the content reached 20% by weight, greater amounts did not greatly lower *Tg*.[17]

3.3.2 Mechanical properties

The tensile strength and elongation at break of samples were measured in the dry state after fabrication, which showed that the manufacture method using a hydraulic press and Glyceline as the additive in this study allowed for the fabrication of homogeneous samples with decent mechanical properties (**Figure 3.5**).



(a) (b)

Figure 3.5 *(a) tensile strength and (b) elongation for zein samples with different Glyceline contents*

The samples with no additives had both the highest tensile strength of 10.19 MPa and the largest elongation of 1.38 %, and the lowest values for the samples with 20% of DES were 5.82 MPa and 0.8%, respectively. The material with 10% and 20% of modifier showed results with similar values. This shows that once the initial intermolecular interactions have been overcome by the DES swelling the material addition of further

DES does not significantly change the inter-chain interactions. It is important to compare these values with those for TPS to understand what the polymer backbone is doing to the properties. TPS has a higher UTS (16.8 MP with 25 wt% DES) than all of the zein protein samples. While it may be expected that the interactions between protein chains would be stronger than between carbohydrate chains the most probable explanation is that starch chains tend to be much longer than protein chains so there is more chance of entanglement with starch. The elongation at break was minimal with proteins. For TPS the elongation at break is about 15% which is due to the amylopectin content which contains a lot of coiled polymer.

These results suggest that the DES does not act like a plasticizer otherwise there should be increased elongation with decreased tensile strength. On the contrary, both elongation and tensile strength showed an overall descending trend suggesting the property change is primarily caused by the presence of additives which lowered the amount of zein protein within each sample. It was also suggested by Lawton that the moisture brought by the nature of the plasticizer can affect the tensile properties of plasticized zein samples too, as plasticizers that were more hygroscopic tend to absorb more water.[17]

However, DES may serve as mechanical anti-plasticizers when present at low concentrations, resulting instead in stiffer polymer diluent blends than the neat polymer. The phenomena that plasticizers like glycerol can act as both a plasticizer and an anti-plasticizer have been shown in food applications in recent years.[18] It has been reported that glycerol can exert different effects depending on its concentration. For example Lourdin and colleagues reported that glycerol at a content below 12% could increase the ductility of potato starch film while the ductility decreased when the amount of glycerol exceeded 12%.[19] At low content, Glyceline can decrease the local dipolar relaxation of the amorphous zein matrix and thus appear to act as an anti-plasticizer on the molecular level.[20]

3.3.3 Summary

Based on previous model (**Figure 1.6**), further possible formations were proposed,[21] suggesting nine antiparallel and sequential helices with three polar lines or segments on the helical surface can be arranged within a distorted cylinder of oval cross-section. The distortion is necessitated by the ability of side groups to pack in the cylindrical interior

(**Figure 3.6**). The helical axes are parallel to the cylinder and two of the three polar segments would be utilized in intramolecular hydrogen-bonding contacts. The third polar segment is available for intermolecular contacts such that a molecular plane of zein proteins could be formed. The glutamine residues which compose the turning regions between the anti-parallel helices would exist at the caps or ends of the cylindrical clusters. Their hydrogen bond interactions would allow stacking of the zein molecular planes (**Figure 3.6**).

Since no significant changes were observed on either the glass transition temperature or physical properties upon DES addition, it seems likely that the DES can only break apart a small portion of the hydrogen bonds existing within the system, i.e. the intramolecular hydrogen-bonding contacts between cylinders. The reason behind this is suspected to be the hydrogen bonding between the two components of Glyceline are stronger than the potential hydrogen bonding that would be formed between Glyceline and the exposed secondary structure of the zein protein. Also, the presence of Glyceline can decrease the local dipolar relaxation of the amorphous zein matrix and thus appear to act as an anti-plasticizer on the molecular level.

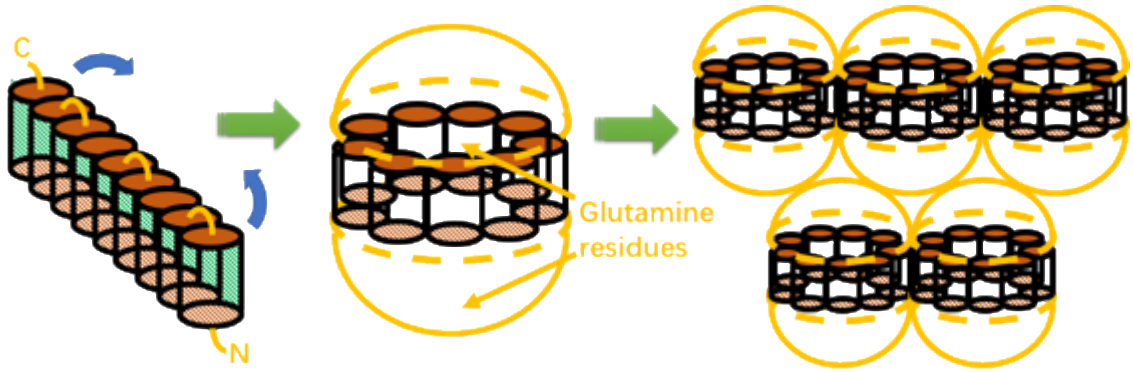


Figure 3.6 *A possible model for the arrangement of zein proteins within a plane as well as for the hexagonal stacking of molecular planes.*

Where the DES is located is an interesting topic. While there are several models that could be considered, the general two possibilities will be discussed here: 1) the DES breaks up the inter-chain interactions between cylinders and sits inside the system swelling the polymer or 2) the DES fits into the existing spaces in the lattice as shown schematically in **Figure 3.7**.

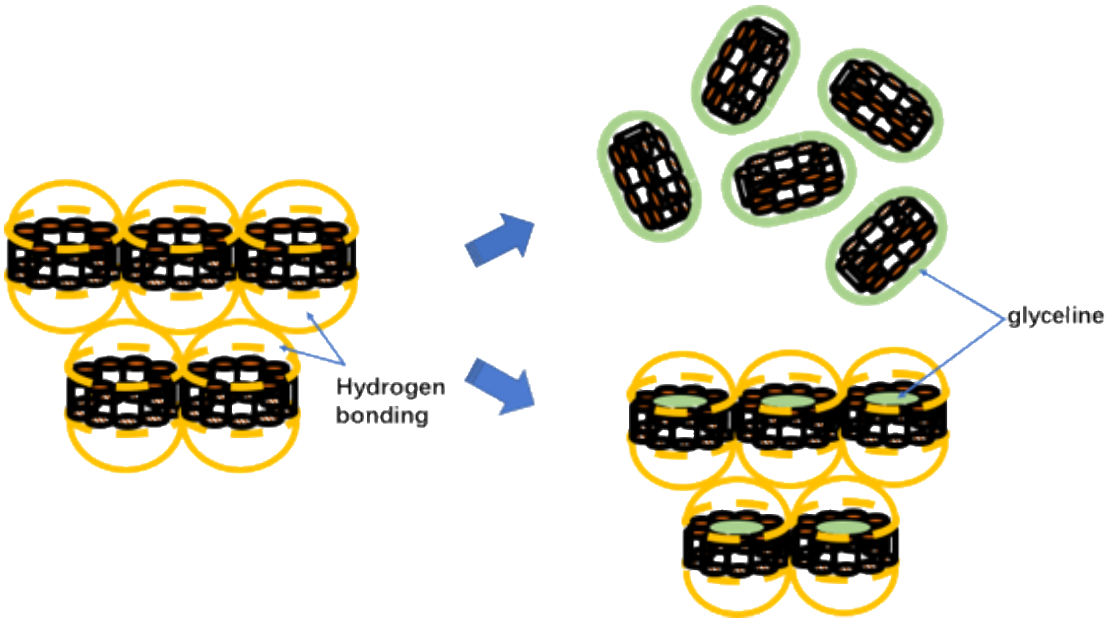


Figure 3.7 *Two possible mechanisms of zein protein modification with DESs*

Density measurements were made in order to test the two models. If the second theory is right, the density of the sample should increase as the Glyceline was added on top of the already existing zein protein network (**Figure 3.6**). For reference, the density of Glyceline is 1.193 g/cm^3.[22] The density is, however, decreased when Glyceline is added which means that the protein system is expanded and the DES must be bonded to the exposed hydrophilic side (causing by the glutamine turns) on the tertiary structure of zein protein, as predicted previously.

	0%	20%
Volume (mm^3)	9254	8383
Weight (g)	11.72	10.48
Density (g/cm^3)	1.266	1.250

Table 3.6 *Density comparison between plain (0%) and DES modified (20%) zein samples*

In this section, the efficacy of our new approach to modify zein-based materials using DES (Glyceline) was demonstrated. The combination of adding DES and high-pressure heat treatment led to materials with properties including decreased glass transition temperature and reduced elongation and tensile strength. This investigation has shown that Glyceline can significantly modulate the physical properties of zein films in a dose-

dependent manner. It was established that the first amounts of added plasticizer (<10%) were the most effective at lowering T_g while a higher plasticizer content will have less of a plasticizing effect. Changes in plasticization effectiveness can be attributed to an increasing difficulty for the plasticizers to diffuse into the polymer matrix. It was also proposed that DES acting as the anti-plasticizer caused a decrease in the interactions with zein molecules. The poor physical properties of DES modified zein protein (it is too weak, too brittle and too rigid) do not really make it a suitable candidate for potential applications in drug delivery without further improvements.

3.4 Soy Protein

Though both being globular proteins, unlike zein, soy protein is more similar to starch, with its hydrophilic nature. Many efforts have been made to its modification so as to maximize its potential, especially in the field of adhesives, which gained much attention in recent years. In 2017, commercial SPI powder was thermally modified into five different soy protein isolate to combine with sanded thermo-hydro-mechanically (THM) densified beech wood by Vnučec et al., results showed promising results where significantly lower tensile shear strength of bonded THM specimens was achieved when comparing with that of bonded control specimens.[57]

Moreover, as one of the alternative materials to petroleum plastics, soy with its advantages like abundancy, renewability, while being inexpensive and eco-friendly, has great potential to be used in many applications. Moreover, as a natural and non-toxic compound, it can achieve improved properties by mixing with plasticized starch.[23]

Unfortunately, the low strength and high moisture absorption of soy protein plastics limit the scope of its application. Therefore, to process it as a thermoplastic with the aid of plasticizers such as glycerol and sorbitol, among others,[24,25] has been the focus of many researchers. Effects of polyhydric alcohols on mechanical properties of soy protein-based plastics have also been studied and reported by Wang.[26] Otaigbe and Adams studied the composite of soy protein with polyphosphates, and their results showed enhanced strength, stiffness and improved water resistance.[27] However, not all attempts were successful, Ly et al. grafted vinyl monomers such as methyl methacrylate, ethyl acrylate, butyl acrylate and hexyl acrylate to soy protein using a free radical mechanism to form vinyl polymer modified soy protein, but did not get significant improvement in

mechanical properties.[28]

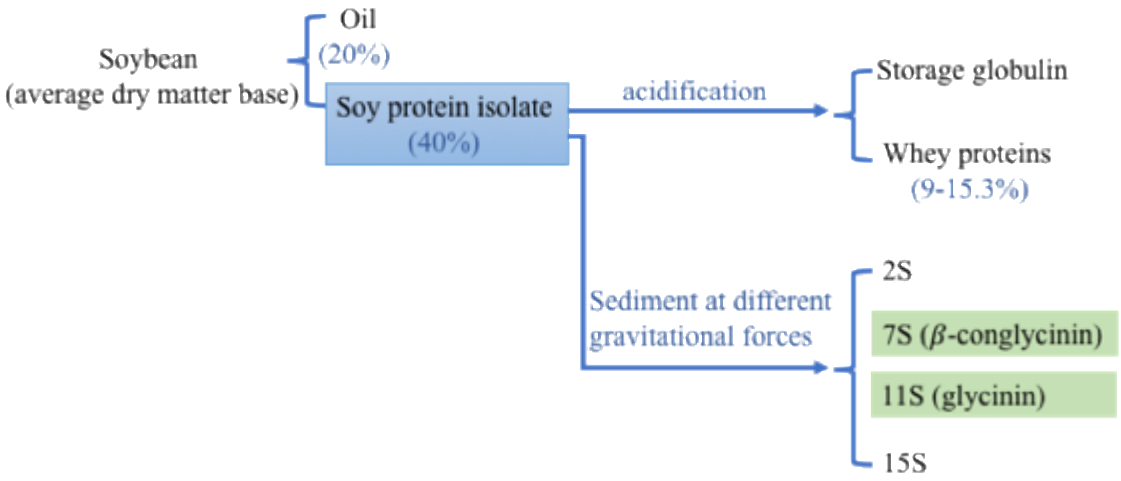


Figure 3.8 *Soybean separation process*

Soybean has a high protein content containing four major molecular fractions, 15S, 11S, 7S, and 2S **(Figure 3.8)**, which sediment at different gravitational forces when the solution is subjected to a centrifugal field.[29] Through the years, research on structure-function relationships has been carried out mainly on 11S and 7S, as they represent more than 80%[30–33] Although the quaternary structures of the 7S and 11S globulins are different from each other (the former being a trimeric protein and the latter a hexameric protein), they are believed to be derived from a common ancestor because of the partial homologies in their amino acid sequences and limited proteolysis patterns. This assumption has been confirmed by X-ray crystallography of 7S and 11S globulins derived from soybeans.[34,35]

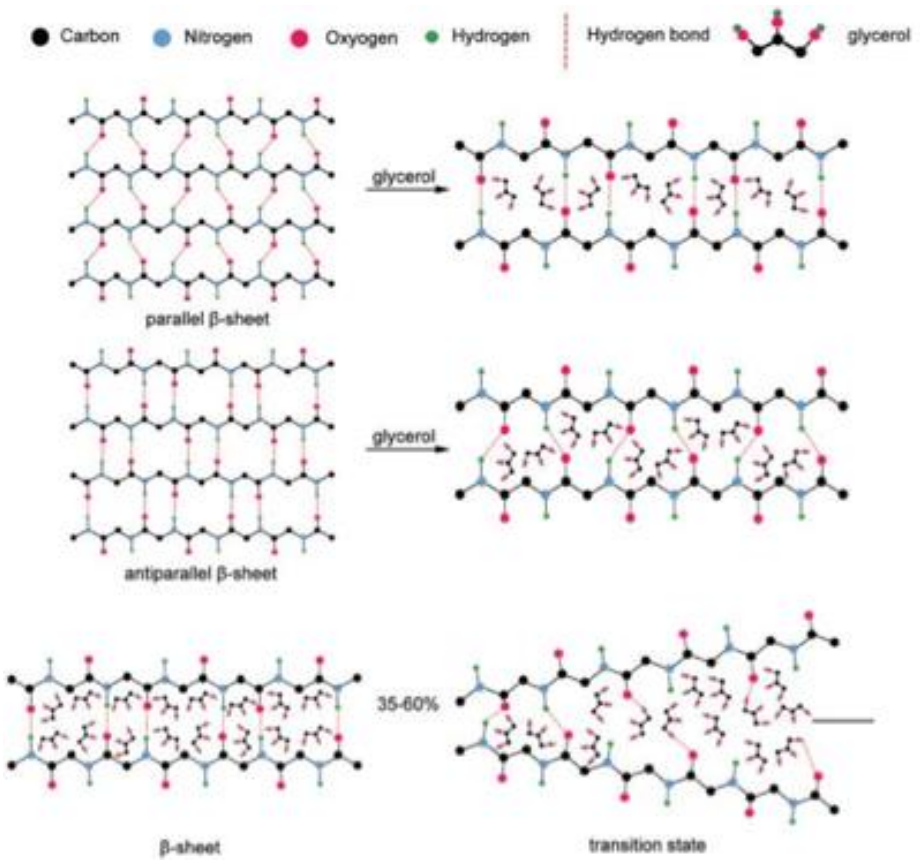


Figure 3.9 *Schematic conformation transformation of SPI motivated by the hydrogen bond between SPI and glycerol.*[36]

Yan et al. have shown that using glycerol as the modifier[36] in order to improve the properties of soy-based film. At the initial state, the film is rigid and brittle because the predominant secondary structures are ordered. It was established that the glycerol was inserted into the β-sheet and weakening the H-bonding among this conformation. With an increase in glycerol content, the H-bond between soy protein and glycerol (C=O…H–O–) will replace the one between the β-sheets (C=O…H–N–), and results in an increase of the free volume of the peptide backbone **(Figure 3.9)**.

3.4.1 Thermomechanical Analysis

The 0% in **Figure 3.10** shows a typical DSC thermal history for soy protein by itself, in which the two characteristic denaturation peaks for 7S and 11S globulins are as shown. Protein denaturation is commonly defined as any non-covalent change in the structure of a protein, and may involve the alteration of the secondary, tertiary or quaternary structure of the molecules. Additionally, the denaturation of soy protein includes unfolding and

breaking of hydrogen bonds, hydrophobic interactions and salt linkages at elevated temperature as well as decomposition. The denaturation temperature of soy protein is also thought to be related with moisture content since water is a plasticizer for soy protein. These two peaks are consistent with similar DSC curves that have been obtained by a number of researchers.[37] The same pattern was identified in both the 7S-enriched fraction and the 11S-enriched fraction, suggesting that neither of the two fractions was pure. Two different transition temperatures were identified as 95 °C and 206 °C, respectively.

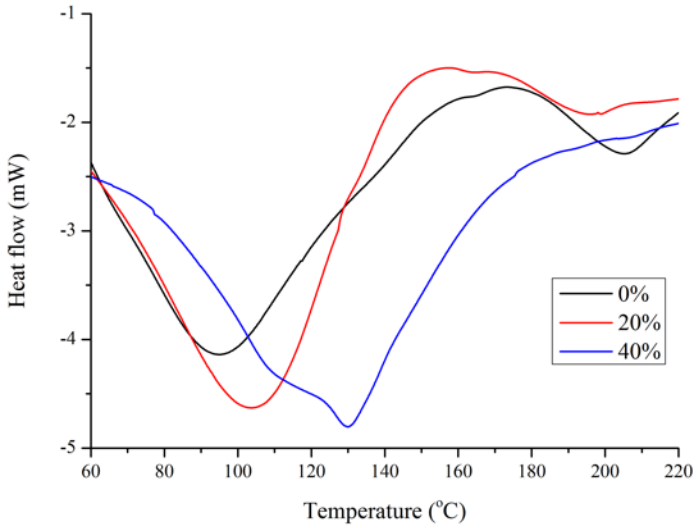


Figure 3.10 *transition temperatures for soy samples with 0%, 20%, and 40% DES measured by DSC*

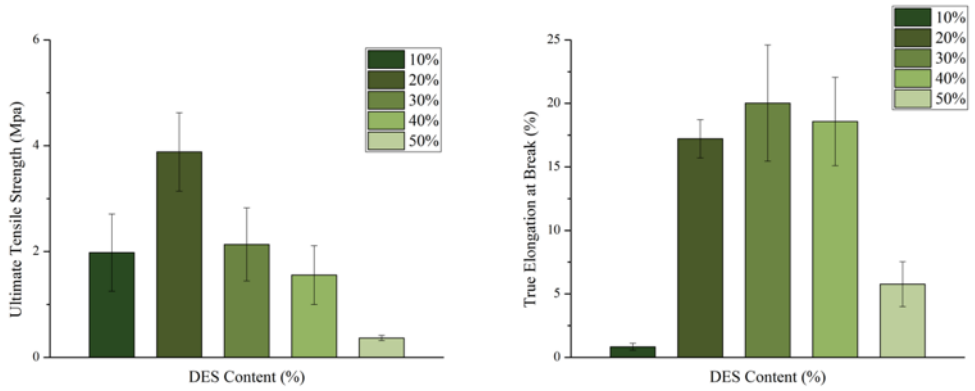
	0%	10%	20%	30%	40%	50%
T_1 (°C)	95	108	105	108	106	102
T_2 (°C)	**N/A**	**N/A**	**N/A**	127	130	122
T_3 (°C)	205	199	195	191	**N/A**	**N/A**

Table 3.7 *The transition temperatures of soy samples with different DES composites*

It can clearly be seen from **Figure 3.10** that the added Glyceline significantly changed the structure of the soy protein. This can be seen as the result of interactions between Glyceline and the reactive functional groups (such as hydroxyl groups) present on the soy protein molecule. The protein attempts to minimize its free energy by burying as many

hydrophobic groups inside the material while exposing as many polar groups as possible to the plasticizer, which greatly weakens the short range interaction and hence protein folding structures so as to change the property of the native protein. From **Table 3.7**, it can be seen that as the DES percentages increase, the second transition temperature (identified as the 11S-enriched fractions) becomes less obvious, with the first peak (identified as the 7S-enriched fractions) shows a similar trend. Meanwhile, another transition peak appeared in the middle temperature region with the 30%, 40% and 50% DESs. This indicates the two transition temperatures are merging together in a way that made the materials become more amorphous, which confirms that the combination of high temperature and high pressure, along with the added DES, is affecting the physical properties of the overall products.

3.4.2 Mechanical properties



(a) (b)

Figure 3.11 *(a) tensile strength and (b) elongation for soy samples with different DES composites*

There are many factors that will affect the strength of the soy materials, including but not exclusive to the pre-treatment, composition, water content, pressing time and pressing temperature. The sample without DES was extremely brittle and its mechanical properties were very difficult to measure as many samples shattered when attempts were made to cut them into a dog-bone shaped test piece.

As it can be seen from **Figure 3.11**, there are significant variations in the tensile strength within each type of the materials hence the large error bars. It is notable that among all

the samples, the sample with 20% DES achieved the best results in terms of the highest strength with the highest ductility. This also means that the sample will be comparatively tough (the area under the stress-strain curve). Moreover, modifier helps to enhance the ductility of the samples with 20%, 30% and 40% DES compare to the one with 10%. These improvements are significantly greater than those reported for chemically modified soy plastics.[38]

However, when 50% DES was added where it appeared to have less effect, an elongation approximately 10 times bigger (compare to the 10% DES sample) is still observed. The overall decreasing trend after 30% might suggests that higher DES content leads to inhomogeneous mixtures.

3.4.3 Contact angle

Hydrophobicity is a very important way of describing the physical and chemical properties of a substance. Contact angle is a measure of the hydrophobicity of a solid surface which measures its wettability. Other factors can also affect the contact angle due to its roughness or heterogeneity.

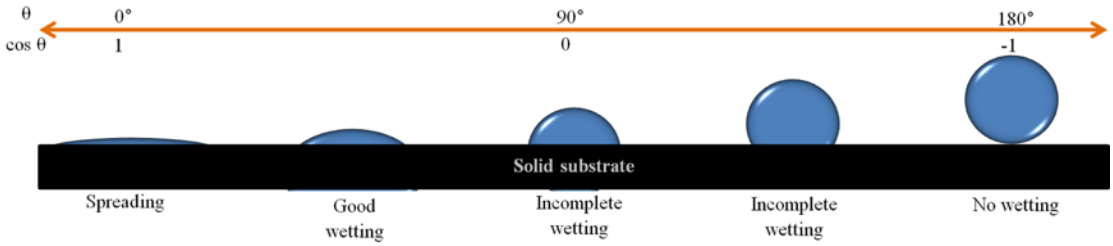


Figure 3.12 *Contact angle changes with increasing hydrophobicity*

Time	0 %	10 %	20 %	30 %	40 %	50 %
5 min	17.7	17	10.6	6.4	7.5	4.4
10 min	17.1	16.2	12.0	8.4	6.4	0

Table 3.8 *The contact angle for soy samples with different DES composites*

The contact angle range can vary from 0° to 180° depending on the solid material

wettability, the more hygroscopic the material the smaller the contact angle. Thus, the contact angle of a very hydrophobic material will be close to 180° with an almost spherical drop on the surface, while the very hydrophilic substrate will give a contact angle of nearly 0° with the drop spreading almost flat on the surface **(Figure 3.12)**.

There are generally three different phases when a water droplet comes into contact with soy protein. During the first phase of water absorption by soy protein, water binds to the ionic and high energy sites on the polypeptides to produce a highly structured monolayer. Beyond the monolayer, an additional amount of water is hydrogen bonded to the polar groups, accompanied by changes in conformation and initial swelling of the protein matrix. In the last phase, more water is absorbed into the crevices of soy protein matrix and the water is only loosely bound or free, accompanied by further swelling and partial solvation.

Table 3.8 shows the effects of DES concentration on the static contact angles of soy sheets prepared using a hydraulic press. As a general trend, the amount of bound water should decrease as the sample's protein concentration decreases, but as can be seen from the data provided, the contact angle decreased as the DES content increases, which means despite the reduced protein percentage, the modified material still manage to bind more water than the untreated sheets. This means the modifier makes the surface more hydrophilic which would be due to the dissociation and unfolding of soy protein exposing additional binding sites and increase water binding. Water binding can also be directly related to the concentration of ionized amino acid groups on the protein as ionized amino acid groups are capable of binding more water than unionized groups.

3.4.4 Summary

As an alternative to common synthetic plastics, an ideal soy-based protein materials should be strong, elastic and have very low moisture permeability. In this study, a material with both high strength and high ductility was achieved.

From the DSC results, together with the aforementioned study by Yan,[36] it can be concluded that the size of both β-conglycinin and glycinin decreased with higher DES content, introducing a new form of structure created by the mixture glycinin/ β-conglycinin, which increased with the decreasing β-conglycinin and glycinin content. The

follow-up studies using a Tensiometer confirms the newly formed structure showing superior qualities on both tensile strength and elongation. Combining both, it can be seen that the DES plays the role of breaking up the original beta-sheets formed by 11S and 7S separately, and help forming a new polymer network that has more strength and flexibility. From the bond strength point of view, this indicates that both the hydrogen bonding between the two components of DES, and the hydrogen bonding within the original 11S and 7S network, are weaker than the newly formed hydrogen bonding that's holding the new structure together.

This corresponds to the results acquired from modified zein products where a small portion of the hydrogen bonds were broken while a general network structure was maintained.

The contact angle however, has decreased as the DES content increased, which means despite the reduced protein percentage, the modified material still managed to bind more water than the untreated sheets. This means the modifier makes the surface more hydrophilic which would be due to the dissociation and unfolding of soy protein exposing additional binding sites and increase water binding. Although this property in particular, might indicate that the functional properties of the DES-modified soy protein materials are still not comparable to those of synthetic ones, results over all still shows promising potential of the methodology in improving the mechanical properties of soy-based protein materials. While these properties may not enable the use of these materials for packaging, they have desirable properties for biomaterials, particularly for the delivery of pharmaceutical reagents.

3.5 Gelatin

Gelatin is a high molecular weight polypeptide composing of amino acids mainly glycine, hydroxyproline and proline. Because its molecules are tightly bound with hydrogen bonds, pure gelatin materials are normally brittle. Furthermore, the polar groups present in its structure cause the gelatin to have high moisture absorption. Therefore, to make it suitable for applications, a plasticizer is needed during production. This will also aid the incompatibility between hydrophilic gelatin and hydrophobic synthetic polymers when mixed together.

Polyols have been reported to be particularly efficient in plasticizing hydrophilic polymers.[39] For this reason, many recent researchers have focused on using polyols such as glycerol,[40] sorbitol,[41] and mannitol[42]. Studies have shown that the addition of these compounds as plasticizers have improved the mechanical properties of gelatin films. Fakhoury et al.,[43] for instance, showed that adding 10% glycerol produced a material with a UTS of 108.28 ± 6.38 MPa while the addition of 20%, caused it to decrease to 1.75 MPa.[44] The blend of different plasticizers in film formulation have also been used to improve the functional properties of the gelatin film. For example, the use of the glycerol with sorbitol mixture[44] and glycerol with polyethylene glycol (PEG)[45] both showed the improvement on tensile strength with increased concentration. As a small hydrophilic molecule, glycerol can be inserted between protein chains, increasing the distance between the protein chains and reducing their direct interactions, hence acting as a plasticizer interspaced in a protein network.

Room temperature ionic liquids (ILs) have also been used as surfactants for gelatin by Singh, T., Boral, S., Bohidar, H. B. and Kumar, due to the amphiphilic nature of their cation or anion. A detailed physicochemical investigation was done on the interaction of gelatin with ILs like [C_8mim][Cl] and [C_4mim][C_8OSO_3] in the phosphate buffer medium.[46] Results show that initially, the IL monomers interact with gelatin at the interface to form a monomer complex after which hydrophobic micro domains have been observed. Alaysuy in our group has shown the compatibility of collagen and DES by which used DESs in the leather post-tanning process. Results show that when it comes to a macroscopic material, all the DES is present inside the system as a liquid which can be squeezed out. DES is suspected to sit in between the protein fibers themselves, and not change the crystalline structure of the collagen.[47] This is different to the study of DESs with starch by Abolibda which showed that the polymer was largely amorphous but the DES remained as a continuous liquid phase inside the material.[1] To investigate the role Glyceline is playing in the modified gelatin material, a series of experiments were carried out.

3.5.1 Thermomechanical Analysis

Figure 3.13 shows the comparison between DSC traces for native gelatin without the addition of DES before and after the heat press treatment. The *Tg* values are at 75 °C and 82 °C, repectively, both are lower than either zein or soy protein and reflects the weaker inter peptide chain interactions in gelatin. The slight increase in *Tg* caused by heat treatment can be explained by the greater interchain interaction of heat-treated gelatin strands, mostly likely via hydrophobic and hydrogen bond interactions. Similar results have also been documented by Hoque. while studying the effect of heat treatment on cuttlefish based gelatin films.[47]

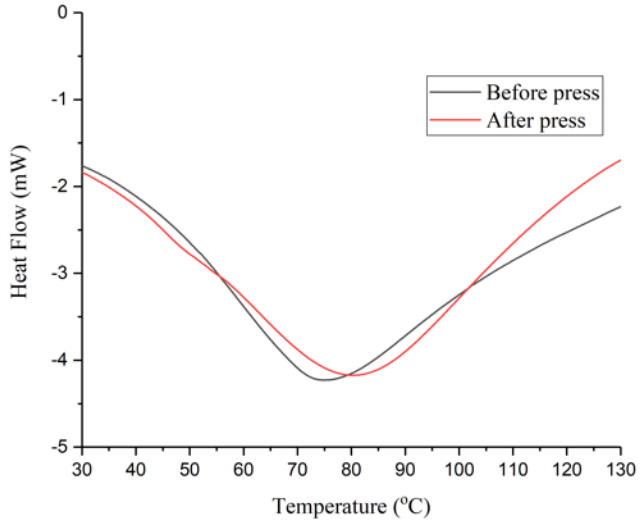


Figure 3.13 *Thermal profile of plain gelatin before and after heat press treatment measured by DSC*

As proteins derived from animals are known to differ by the species they come from (e.g. gelatin can be derived from bovine or fish skin). It was immediately apparent when gelatin was modified with Glyceline that the materials produced were significantly different in behavior to those for zein or soy proteins. To identify the nature of the transition temperatures, also clarify other factors like the evaporation of moisture from the gelatin sample upon heating, apart from DES, some dynamic mechanical analysis (DMA) measurements were carried out. This was not possible with the modified zein or soy proteins as they were too brittle.

As previously mentioned, amorphous polymers have different glass transition temperatures, above which the material will have rubbery properties instead of glassy behavior. Another thing to be noted is that during the glass transition process, the stiffness of the material will drop dramatically with an increase in viscosity. DMA is often used to characterize the glass transition temperature of a material based on such fact.

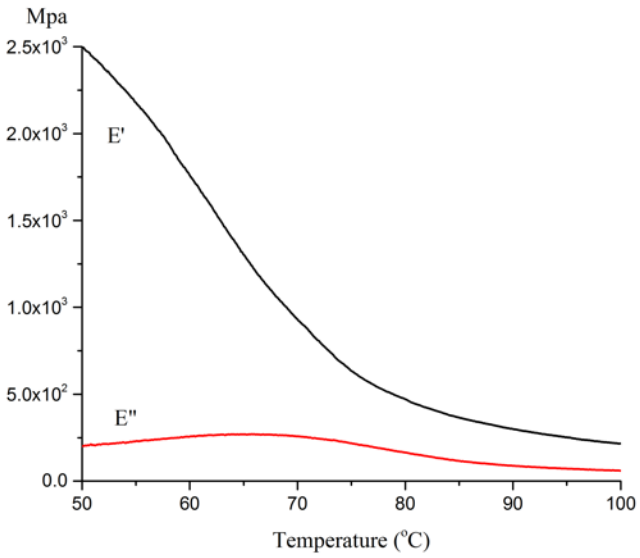


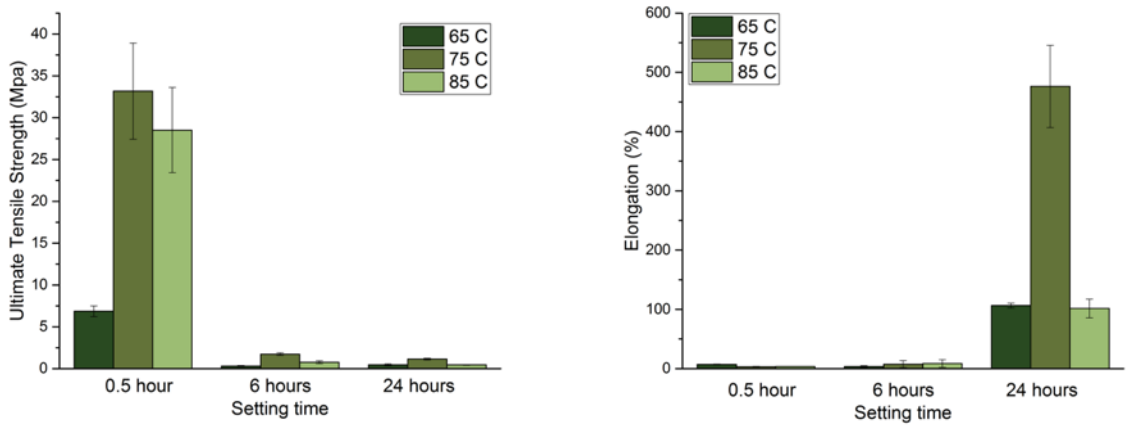
Figure 3.14 *Thermal profile of an unmodified gelatin sheet measured by DMA*

The storage modulus (*E′*) of a material is related to its ability to return energy, while the loss modulus (*E″*) is related with its propensity to lose energy. The onset of the drop in storage modulus can represent the *Tg* as *Tg* is the temperature in which short range relaxations can occur which reduce the resistance against deformation. However, detecting the onset of the drop in E' may be tricky especially in materials with wide molecular weight distribution. Because of that the peak in *E″* where the rotational and vibrational frequencies of small segments of polymer chains match and result in a maximum loss or peak in the loss modulus can also be used for *Tg* determination. In the DMA spectra of gelatin as shown in **Figure 3.14**, temperature increase leads to a well-defined phenomenon in the *E'* curves of the gelatin: a stepwise decrease in correspondence to the glass transition observed on DSC (around 75°C), with *E''* showing a peak in the same region. It is comparable with the literature value, ranging from 80 to

90 °C,[48] higher than those of films made from tuna skin gelatin (7.4 °C),[49] but was lower than T_g values reported for unplasticized cod gelatin films (99 °C).[50]

Interestingly, the preliminary investigations also discovered that the mechanical properties of DES modified gelatin is strongly influenced by a variety of factors including, but not limited to, the pressing temperature, composition, and most importantly, the setting time of the mixture before pressure-heat treatment. As a result, to achieve a production system for the most desirable results, more measurements were carried out to quantify these effects.

3.5.2 The effect of compression temperature



(a) *(b)*

Figure 3.15 *(a) tensile strength and (b) elongation for gelatin samples with 20% DES under different compression temperatures*

The pressure of the hydraulic press was kept constant at 120 kN for each experiment and three experiments were run between 65 and 85 °C based on the glass transition temperature obtained earlier. For each experiment three sheets were pressed after different setting time (From 0-24 h). **Figure 3.14** shows the effect of pressing temperature on the tensile stress and elongation of the samples. It can be seen that the pressing temperature affects both physical properties of samples, with elongation more significantly than the UTS. Results for samples pressed at 65 °C can be explained by partially melting of the mixture which results in inhomogeneous sheet, while the one pressed in higher temperature might result in DES leakage causing less plasticizing effects. As a result, 75 °C was proven to be the temperature at which the material with superior quality can be

achieved. With the pressing temperature of the optimum processing conditions determined, more variables were tested in the next section.

3.5.3 The effect of setting time before heat treatment

As can be seen from **Figure 3.15** and **3.16**, with a longer setting time, the DES decreases the tensile strength of the materials while significantly increasing the elongation. To put these numbers into a practical description, Samples which are pressed immediately after mixing are strong and brittle and snap when bent. Samples which are left for 24 h are flexible like skin and can be stretched by hand. Accordingly, the former material may be more useful for tablet formulation whereas the latter is more suitable for transdermal delivery of pharmaceutical DESs.

The more brittle and more flexible materials can be explained by the plasticizing effect of the DES. This proves that longer mixing time leads to a higher degree of polar interactions between the protein and the modifier and forming a more homogenous and amorphous material. In addition, the fact that the properties of the material remained the same after pressing shows that it is possible to control the degree of hydrogen bonding in the material.

These results suggest that the DES interrupts protein–protein interactions and causes an increase in chain mobility in gelatin. The protein: DES composition of 80: 20 wt% was chosen for follow up experiments as it made samples with the most plasticizing effect without DES leakage.

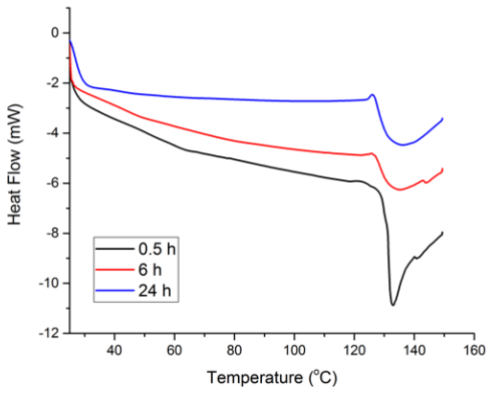


Figure 3.16 *DSC curves for gelatin samples with 20% after different setting times*

Setting time (hour)	0.5	6	24
Transition temperature (T_p1) (°C)	63	50	47
Transition temperature (T_p2) (°C)	133	135	136

Table 3.9 *transition temperatures of gelatin samples with 20% DES as a function of setting time measured by DSC*

The temperatures of the different transitions observed from Figure 3.16 are tabulated in Table 3.9. It was established that over a 24 h period, an increased setting time caused a decrease in the first transition temperature, which is probably the glass transition temperature. This corresponds to the idea that the longer the DES/gelatin mixing time the more the DES can solvate the gelatin chains and the greater mobility these chains have thus decreasing the temperature at which movement in the chain can occur. This must be associated with a decrease in density freeing up polymer chain movement. The resulting T_g values are comparable with the reported value for the plasticized films made from bovine-hide gelatin which was 41.5 °C.[51]

One possible reason for this time dependency in mechanical properties is that the interactions between DES and gelatin molecules may require a longer setting time to reach equilibrium to form a more uniform material was made. This finding is especially interesting as it opens the possibly of controlling the degree of gelatin cross-linking by simply controlling the setting time, which is essential to gelatin's dissolution behavior hence its drug releasing ability.

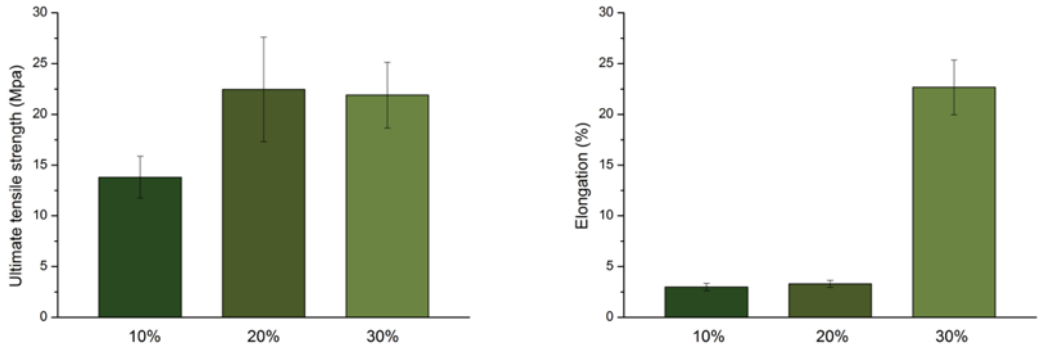
Generally, the thermal stability of gelatin gels is closely related to the length of the triple helices, and the melting enthalpy is proportional to the triple helix content. The second transition temperature in ***Figure 3.16*** can be seen as the effect of setting time on the melting behavior of gelatin gels. The melting temperature of gelatin gels was nearly invariable after different period of time. However, the melting enthalpy showed a tendency to decrease with additional time. These results indicate that the time for hydrogen bonding interactions are important for the nucleation of triple helices but have little effect on its growth.

(a) ***(b)*** ***(c)*** ***(b)***

Figure 3.17 *Photographs of gelatin samples with 20% DES after setting time of (a) 0.5, (b) 6 h, (c) 12 h and (d) 24 h*

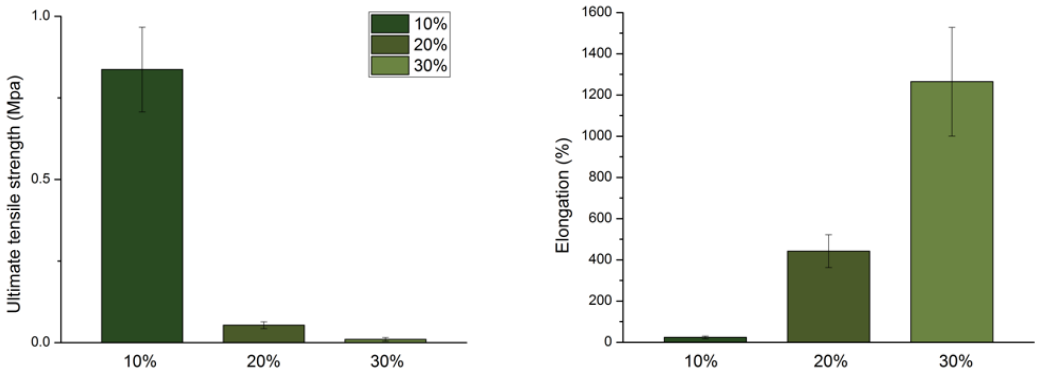
Optical properties are essential to define the ability of films and coatings to be applied over a food surface, since these affect the appearance of the coated product, which is an important quality factor.[52] **Figure 3.17** shows that by altering the setting time of the mixture DES had a marked impact upon the physical appearance of the samples. The degree of transparency is shown to increase as the length of setting time increases. This fits in with the earlier idea; a more crystalline material will scatter light due to the size of the crystallites whereas an amorphous material will be more transparent.

3.5.4 The effect of DES composition



(a) *(b)*

Figure 3.18 *(a) tensile strength and (b) elongation for gelatin samples with different DES composites with 0.5 h of setting time*



(a) *(b)*

Figure 3.19 *(a) tensile strength and (b) elongation for gelatin samples with different DES composites after 24 h of setting time*

These data above show that upon addition of DES, the ultimate tensile strength is reduced for samples in both setting time groups, while analysis of the elongation at break shows a significant increase. By increasing the weight percentage of DES, a material with lower strength but higher ductility was attained. It is indicated that both reaction time and the amount of DES show significant effects on the properties of the prepared modified gelatin. They suggest that longer reaction time is needed in order to achieve the completion of modification. This time dependent quality of modified gelatin is also published in other groups' work when different plasticizers were used.[53] With a longer setting time, the DES is suspected to have a role in homogenizing the protein by filling the void spaces in the polymer matrix. While this offers increased tunability in the properties of the material it does also mean that in the manufacture of a product, mixing time is another variable that needs to be standardized.

3.5.5 Summary

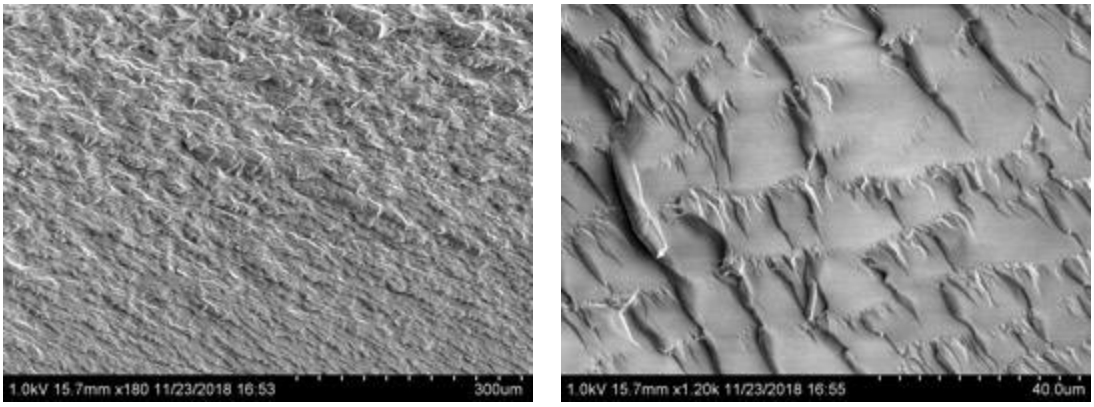
The study above has been devoted to the effect of DES on the mechanical and thermal properties of gelatin gels using cold water fish gelatin. It was found that the reaction time and the temperature, as well as the DES/gelatin weight ratio in the mixture have effects on the degree of modification of the gelatin materials.

As a water-soluble protein, gelatin is derived from the hydrolytic processing of collagen, where the triple helix of collagen is broken up and single strand macromolecules are

obtained. This biomaterial has an amphoteric behavior, because of the presence of basic and acidic amino acids functional groups (**Table 3.1**). Gelatin molecules are composed of repeating sequences of glycine-X-Y triplets, where proline for X and hydroxyproline for Y positions are the most common amino acids. During the hydrolysis collagen tertiary structure triple helix is broken down into single chains (α-chains), covalently cross-linked double α-chains (β-chains) and triple α-chains species (γ-chains) then rearranges to form gelatin. Though sharing the same amino acids composition, collagen and gelatin have different chemical properties, due to the simplicity of the gelatin structure. This caused the DES in gelatin system acting more like DES/starch rather than DES/collagen, with DES changing the structure of gelatin by causing the plasticizing effect. Even though it has shorter chains in comparison to collagen, gelatin still possess the same triple helix structure where three helix formed protein chains were bonded by hydrogen bonding and forming another helix structure.

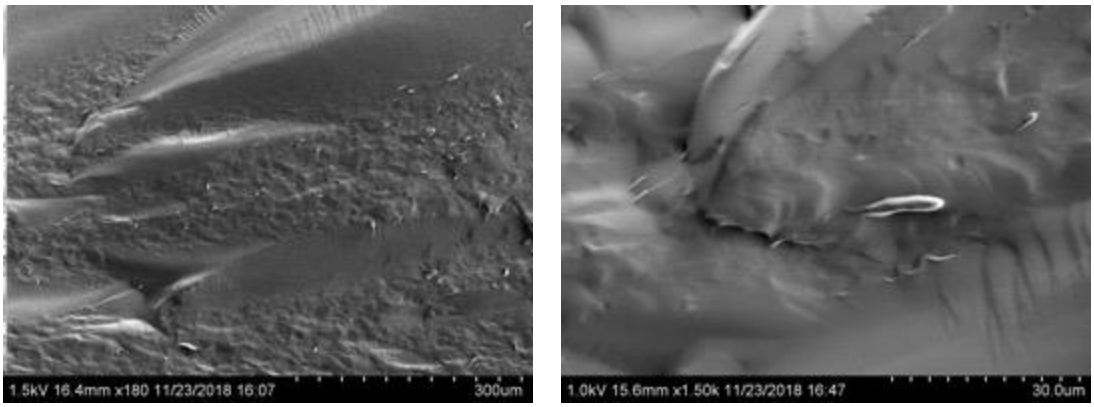
Among all the factors that caused the structural change of gelatin, setting time remains the most obvious. SEM images were taken to obtain additional data about the structure and to some extent the composition of the material (**Figure 3.20**).

Gelatin with shorter setting time (0 h)



(a) (b)

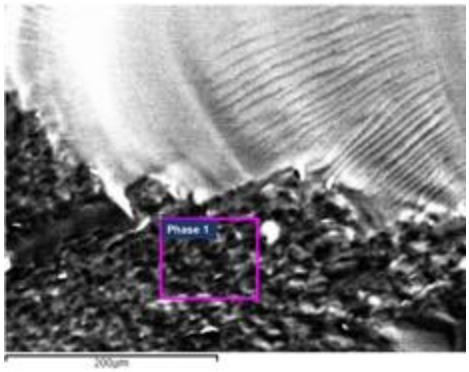
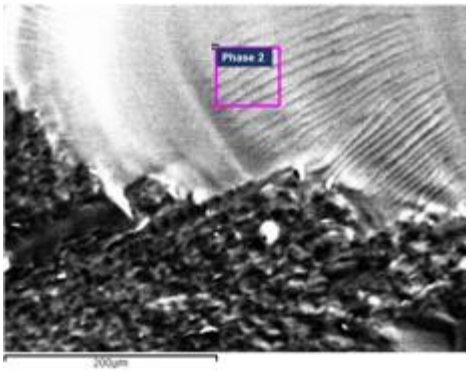
Gelatin with longer setting time (48 h)



(c) (d)

Figure 3.20 *Morphology of gelatin samples after different setting time*

Images (a) and (b) showed the gelatin sample with no setting time being a homogeneous material with high crystallinity, indicates the effect of DES on the structure has only reached the outer layer of the gelatin matrix, which combining with the literature, is suspected to be the tertiary structure of the protein chains. As the setting time elongates, as shown in (c) and (d), two distinctive phases within the structure appeared.

		
Elements	Weight percentage (%)	
C	68	69
N	1	0.5
O	22	24
P	0.4	0.3
S	1	1
Cl	7.6	6.1

With the help of elemental analysis (Cl percentage in particular), it was concluded that the two phases have slightly different DES contents, where the phase 1 showed signs of more DES in the structure. The early stage of interactions between DES and gelatin is like zein and soy, with a small percentage of the hydrogen bonds broken while maintaining the general network structure. However, unlike those two globular proteins, because of its simpler structure, as the reaction continues, the DES within the system proved to be able to cause the plasticizing effect, with added mobility among sections of the protein network.

This result is innovative in many ways. It is the first documented attempt to modify gelatin material using ILs in a solid state instead of carrying out in solutions. Moreover, even this is the first study that has shown that there is a time dependency in the mixing of the components. With extended mixing time the material becomes more amorphous and less brittle. This seems to suggest that there are DES rich domains that slowly diffuse into the crystalline gelatin regions. Once the sample is hot pressed the changes in properties are locked into the material. Mostly, a better understanding of the role DES is playing in the

gelatin system was achieved. Previously, it was shown that choline chloride based deep eutectic solvents could be good plasticisers for starch as they interacted with the intramolecular hydrogen bonded network. Therefore, the interaction between gelatin and DESs were suspected to show similarities as they both have a similar network of intramolecular hydrogen bonding which produced crystalline, brittle materials without the addition of a plasticiser. Results above shown that gelatin becomes less crystalline as the Glyceline content increased, which corresponds to the conclusions of Abolibda using starch with DESs. However, the results with starch also showed signs of the continuous phase existing inside the modified starch materials, yet no conductivity was detected when using conductivity meter on gelatin samples. Nevertheless, this doesn't eliminate the possibility of the liquid phase of DES in-between gelatin polymer chains, as a result, more experiments needed to be carried out.

3.6 Conclusions

This study has assessed the idea of using both globular and fibrous protein in DES blends to form bioplastics which may have application in drug delivery systems. The effect of DES content on the thermal/mechanical properties of the material were determined together with the water sensitivity measured using contact-angle.

The mechanical and physical properties of the three materials produced are summarized by the representative stress strain diagrams shown in **Figure 3.21**. The globular proteins chosen in this study, zein and soy, both showed a significant change in the properties by the addition of DES.

In each case a homogeneous material was produced. That formed from zein was weak and brittle and of very little mechanical integrity. The material formed from soy protein was slightly stronger and has a larger elongation, particularly when 20 wt% Glyceline was added. Both of these materials were, however, weaker and less ductile than the starch-based materials previously reported in the literature.

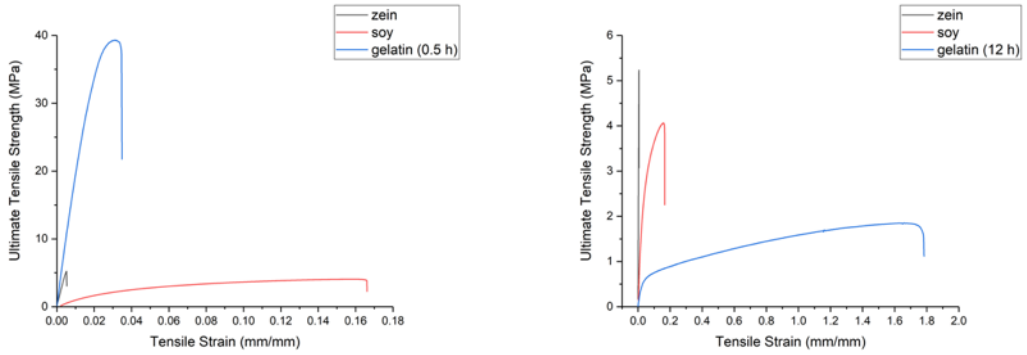


Figure 3.21 *The stress strain curves of three proteins modified by 20% Glyceline after different setting time*

When gelatin was plasticized by DES a very different material was obtained. Initially a very strong material was produced which displayed a strength similar to HDPE or polypropylene. Enabling the DES to contact with gelatin for different periods of time resulted in materials with different properties. Pressure molded samples did not, however change their properties depending on how long they were stored. The mechanical results showed that the setting time helps increasing the flexibility of the materials yet decreased its strength.

In terms of the further study in this project the aim is to develop a biopolymer with good biocompatibility, particularly when they are put into the human body or contacted with the skin. The aim of the next section will be to use gelatin as a drug delivery system for both orally ingested tablets and as a medium for trans-dermal drug delivery. Both of these will need materials which as soft, tough and flexible. For these reasons the future studies will focus on the gelatin DES systems particularly using DESs formed from HBAs and HBDs which are pharmaceutical active compounds. This area pioneered by Qader has been shown to be a method of maintaining poorly soluble pharmaceutical active agents in the liquid form. This gets around problems associated with polymorphs.

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Chapter 4: Design and evaluation of gelatin tablets as controlled release drug delivery systems

4. Design and evaluation of gelatin tablets as controlled release drug delivery systems

4.1 Introduction

Many pharmaceutical active ingredients are polar or ionic as they were designed for binding with the active site of the substrate, which often results in materials with high lattice energies and low solubility in water. On top of which, polymorphs of pharmaceutical compounds can cause different chemical and physical reactivity. These differences are caused by their difference in molecular mobility and thermodynamic properties.

To overcome this issue, numerous investigations have been made on developing liquid formulations, including ionic liquids. For example, the IL propantheline acesulfamate (**Figure 4.1**) consists of the active cation from the propantheline bromide (an antimuscurinic used to treat a number of conditions such as excessive sweating, cramps, spasms of the stomach, etc.) and acesulfamate, a known artificial sweetener and a pharmaceutical additive.[1]

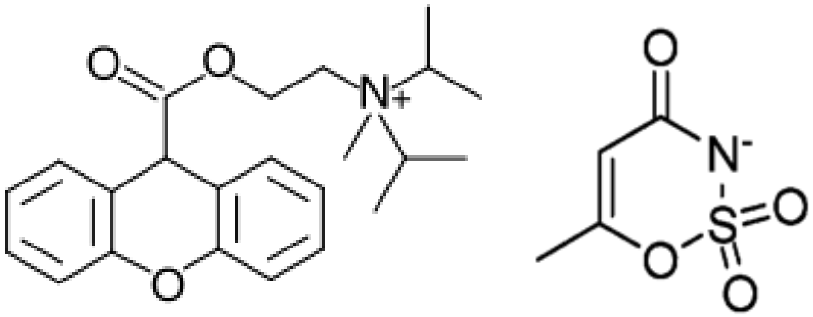


Figure 4.1 *structure of propantheline acesulfamate*

Although promising results have been achieved in this area, the popularity of ILs in biosciences have been delayed by the debate on the toxicity of some ILs.[2] As a result, other developments have been approaching similar territory where liquid drug formulations were prepared as eutectic mixtures. From a chemistry perspective, many pharmaceutical active ingredients (API) are either hydrogen bond donors or amine functionalities that can be converted into quaternary ammonium salts, which are two of the main components to form a DES.[3] The increased surface area of the DES components

can be responsible for an increased dissolution rate of a sparingly water-soluble drug.[4] These mixtures can overcome solubility issues in water as the eutectics prevent recrystallization of the active ingredient when dispersed in water.

Eutectic mixtures with an API were used for the first time by Stott and co-workers in 1998 for transdermal drug delivery.[5] It was reported that the mixtures of different terpenes with an API can increase skin permeation. It is described that the eutectic mixture can enhance the solubility, absorption and permeation of the API. A similar study was done by Morrison and co-workers who used malonic acid-choline chloride and urea- choline chloride as DESs to solubilize griseofulvin, benzoic acid, danazol and itraconazole (**Figure 4.2**).[6] They reported that these molecules have better solubility in DESs rather than in water.

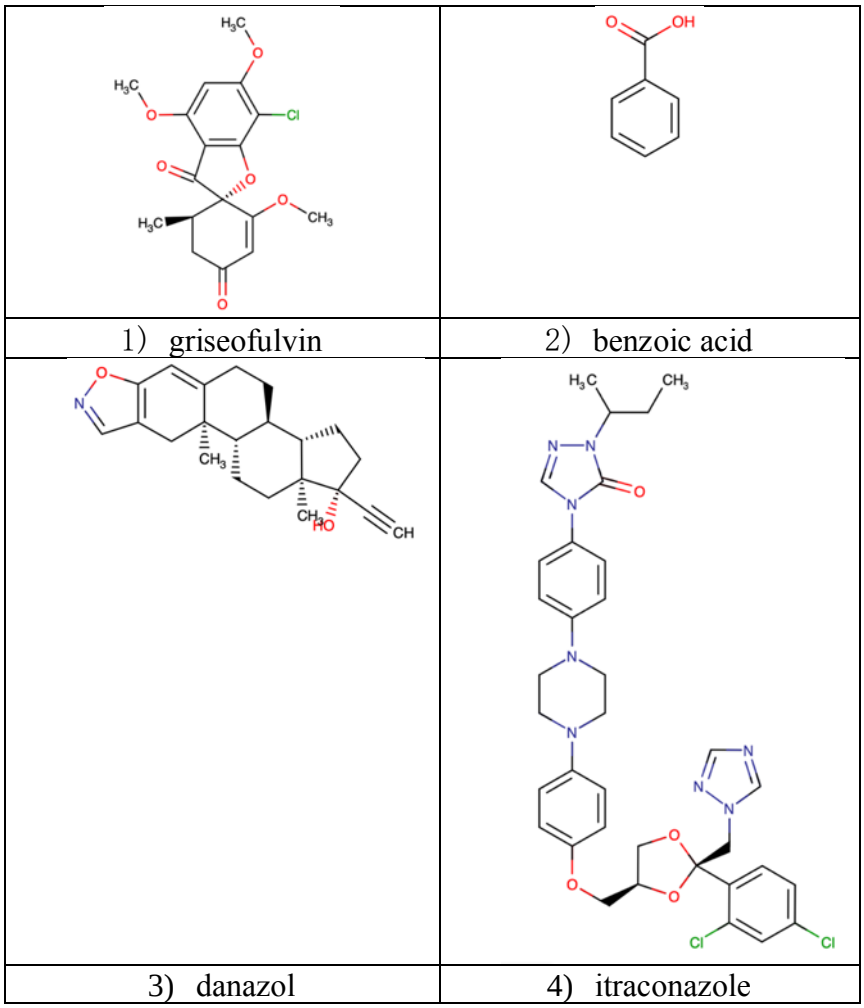


Figure 4.2 Chemical structure of griseofulvin, benzoic acid, danazol and itraconazole

In this study, two types of DESs were used, with the active ingredient being the hydrogen bond donor (HBD) or the hydrogen bond acceptor (HBA), in this case in the form of a quaternary ammonium salt, formulated using the method recently reported by Abbott, A. P. et al..[3] Choline chloride is chosen in this study as it is a pro-vitamin which is already extensively used in food and other consumer products. The result formulations are liquid and can lead to highly concentrated formulations.

Oral administration, being more natural and less invasive than other conventional methods such as intramuscular injection, is seen as the preferred mode of most drug administration. However, the physiological availability of many drugs administered via this route is poor for several reasons, including a lack of stability in the gastrointestinal environment, resulting in degradation of the active compound prior to its absorption. In order to circumvent this problem, many polymers have been used to modulate drug delivery.

Among which, gelatin as a traditional water-soluble functional biopolymer of high interest and value, has been widely used in pharmaceutical application, due to its ability of forming transparent gels under specific conditions. Although there are some differences in the manufacturing technology, gelatin is remarkable known for its unique gel-forming ability, which makes it a valuable material for investigating the fundamental functional properties in colloid studies. However, despite the wide utilization of this protein as capsule casing, very little information could be found in literature about its usage in tablet forming. Tablet has many advantages over capsules, including being able to be customized into different shape and appearances, easy for dose splitting, lower cost, and can contain larger amounts of API per serving depending on compressibility. Moreover, controlled release agents can be used in tablets which can aid specific nutrient uptake, by dissolution control, quick, delayed, or extended release can be achieved. The polymer chosen for this purpose needs to be fairly dry, powdered or granular, and somewhat uniform in particle size as different particle size will cause different densities, which can result in tablets with poor drug or active pharmaceutical ingredient (API) content uniformity. Content uniformity ensures that the same API dose is delivered with each tablet. Some commonly used polymers ones include lactose, dibasic calcium phosphate, sucrose, modified cellulose and corn (maize) starch.

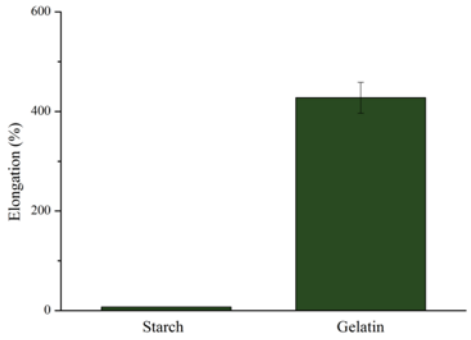


Figure 4.3 *Elongation comparison between starch and gelatin (both modified by 20% Glyceline)*

As previously mentioned, starch and DES have been successfully combined and form homogeneous materials,[7] so as a potential candidate for drug carrier, what makes protein superior? A recent study by Abolibda et al. shows starch-based material being less flexible in comparison with modified gelatin (**Figure 4.3**), which lead back to the potential risk of the segments scratching internal lining if shattered. Structurally, carbohydrates have the same repeating units all the way through, which results in an order wanting to become solvated (**Figure 4.4**). Protein on the other hand, with its complexity on primary structure that contain different amino acids each time, a different form of solvation was required. Ultimately, this leads to proteins (i.e. gelatin) having a better capability to have the maxim amount of DES with the minimum amount of material, which makes gelatin a better gelling agent than starch. Starch-based material, can contain up to 36% DES and still remain as a rigid solid,[7] gelatin can successfully combine 50% wt DES as previously stated. This means with the same dosage, a smaller tablet can be made for the patient, which increased the manageability hence eased the possibility of a potential choking hazard.

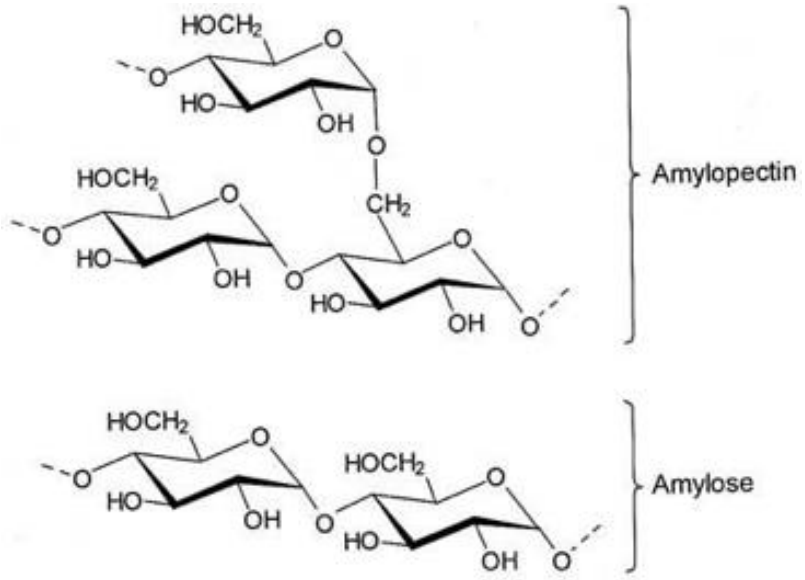


Figure 4.4 Chemical structures of amylopectin and amylose

The aim of the present study is to investigate the different solubility and dissolution rate of the chosen drug before and after being formulated into a eutectic mixture, as a part of a pre-formulation study to develop an oral formulation using protein-based plastics as the matrix. The physical characteristics and dissolution rates of the resulting products are presented in this chapter.

4.2 Formation of PDESs

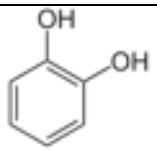
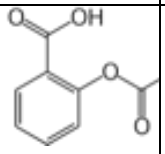
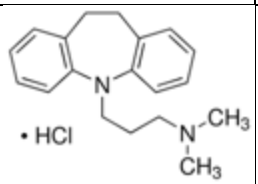
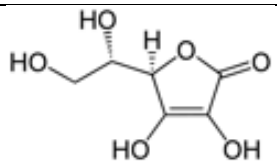
API	Catechol	Aspirin	Imipramine HCl	Ascorbic acid
Structure				
Salt	ChCl (HBD)	ChCl (HBD)	Glycerol (HBA)	ChCl(HBD)
Ratio	1:1	1:1 or 2:1	1:2	1:2

Table 4.1 *Pharmaceutical deep eutectic solvents and their composition*

Four Pharmaceutical Deep Eutectic Solvents (PDESs) were chosen for this study. All 4 contain a chromophore which enables their concentration in solution to be determined using UV-spectroscopy (**Table 4.1**).[3]

Aspirin is a common antipyretic, analgesic, anti-inflammatory drug and it has significant potential for reducing mortality associated with unstable angina, myocardial infarction and thrombotic stroke.[8,9] From a chemical perspective, Aspirin, also known as 2-acetoxybenzoic acid, has two polar groups (CH_3COO- and -COOH), ortho-binding on the benzene ring (**Table 4.1**). A eutectic form of aspirin was prepared by Cherukuvada, S. and Nangia, using aspirin with glycerin or propylene glycol (w/v), was proven to be useful as an ointment for topical applications and also to increase the shelf-life of aspirin by controlling its hydrolysis.[10]

Ascorbic acid, commonly identified as vitamin C, is one of the most essential water-soluble vitamins in the human diet. Though it is synthesized from glucose in the liver of most mammalian species, it cannot be synthesized by the human body,[11] so the main sources of this vitamin are fruits, vegetables and in numerous food formulation supplements and pharmaceuticals. The importance of ascorbic acid for the metabolism of connective tissue substances, especially collagen, has been established by numerous investigators,[12] and it is also important for the treatment and inhibition of scurvy.[13] Vitamin C is called an antioxidant because, by donating two electrons from a double bond between the second and third carbons of the 6-carbon molecule (**Table 4.1**), it prevents other compounds from being oxidized. However, by the very nature of this reaction, vitamin C itself is oxidized in the process. The possibilities of ascorbic acid based eutectic mixture have been explored by Keji and Noboru, and a two-component system were made using sulfathiazole (an organosulfur compound used as a short-acting sulfa drug) and *l*-ascorbic acid,[14] a melting point below 170 ^oC was achieved.

Imipramine HCl (IMP) have been used extensively in the treatment of psychiatric patients suffering from depression. Clinically, it acts neither as a sedative nor like a tranquillizer.[15] Furthermore, its effects are very different from the amphetamine group of stimulants and psychoenergizers.[15] Imipramine hydrochloride is a phenothiazine compound, which possess a hydrophobic nitrogen-containing heterocycle bound to a short chain carrying a charged amino group (**Table 4.1**). Because of IMP's amphiphilic properties, it is inclined to self-associate in a surfactant-like manner in aqueous environment to form small aggregates,[16] which is strongly dependent on the solution conditions, such as pH, ionic strength, nature of additives, temperature, etc. [17–20]

Catechol, also known as 1,2-dihydroxybenzene (**Table 4.1**), is a common ingredient in consumer products. It was first discovered by destructive distillation of the plant extract catechin. About 20 million kg is now synthetically produced annually as a commodity organic chemical, mainly as a precursor to pesticides, flavors, and fragrances.[21] There is also considerable evidence that intramolecular H-bonding in the catechol ring system has a pronounced effect on antioxidant activity (**Table 4.1**).[22]

To understand the solubility of a molecule in a solvent the thermodynamics of solvation must be considered. For a solute to dissolve in a solvent, the lattice energy of the solute must be overcome and the enthalpy of creating a cavity in the solvent must be overcome. Both of these terms require energy to be put into the system and this is usually recovered through the solvation enthalpy. The solubility of compounds such as aspirin are relatively low in water because the lattice energy of polar molecules is relatively high. In general, the larger the molecule the more energy needs to be inserted to create a cavity. In this section the concept is investigated that if a PDES is a liquid, its lattice energy will be negligible and hence its solubility will be greater.

Among the various analytical techniques, UV spectrophotometric method remains one of the most popular methods for quantitive analysis. It is a simple, rapid, accurate and sensitive method for the determination of API in pharmaceutical dosage forms, and has been used by many researchers.[23] UV–vis spectra usually contain non-specific data, which can be converted into useful information by derivative or multivariate calibration methods. The absorption spectra of aspirin, ascorbic acid, catechol and imipramine hydrochloride exhibits absorption maxima at about 276 nm, 244 nm, 277 nm, and 251 nm respectively.

Initial tests revealed that the absorption peaks of all drugs remained the same except for aspirin, which indicates that catechol, imipramine HCl and ascorbic acid remained in their original form in DES formulation while aspirin was suspected to decompose through hydrolysis, yielding salicylic and acetic acids, which corresponds to the observed peak shift from 277nm (aspirin) to 303nm (salicylic acid) (**Figure 4.5**).

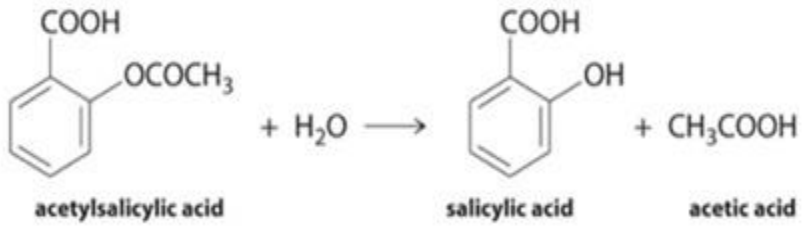


Figure 4.5 *Reaction mechanism of aspirin (acetylsalicylic acid)*

In conclusion, three drugs were chosen for further tests, and the prepared PDESs from these drugs are liquid at room temperature as shown in **Figure 4.6**.

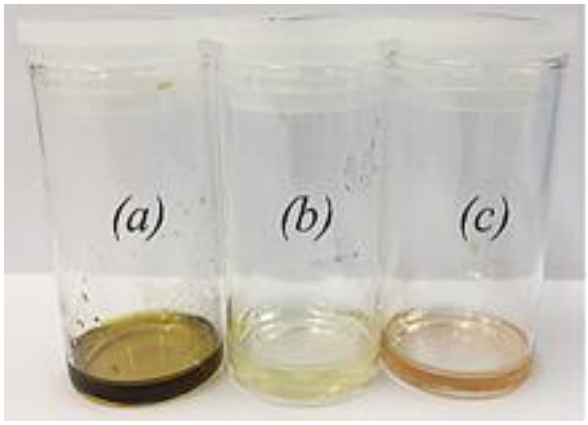


Figure 4.6 *(a) Imipramine HCl PDES, (b) ascorbic acid PDES and (c) catechol PDES*

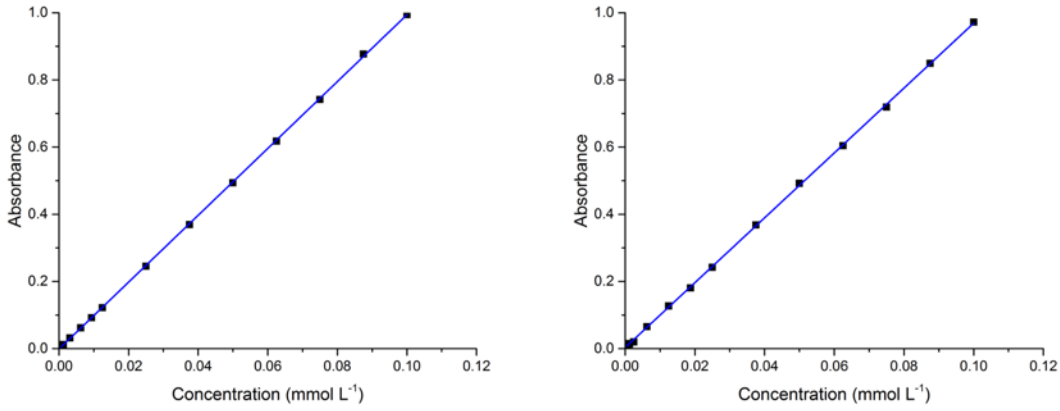
4.3 Calibration curves

In analytical chemistry, a calibration curve, is a general method for determining the concentration of a substance in an unknown sample by comparing the unknown to a set of standard samples of known concentration. By preparing a series of standards across a range of concentrations, and measuring the absorbance a linear calibration plot can be made fitting the data to the Beer-Lambert law.

Absorbance = $\log_{10} I_o/I = \boldsymbol{\varepsilon}\, \boldsymbol{l}\, \boldsymbol{c}$

Where $\boldsymbol{\varepsilon}$ is the molar absorbance (extinction) coefficient, $\boldsymbol{l}$ is the path length and $\boldsymbol{c}$ is the concentration. In most cases, the calibration curve will show a linear relationship, and the concentration of analyte can then be calculated by interpolating.

A set of standard solutions were prepared from pure substances in the dissolving media (0.1 M HCl to approximate the contents of the stomach) and calibration curves were produced as a function of concentration. Example is shown in **Figure 4.7** for ascorbic acid and the corresponding PDES, results for imipramine hydrochloride and catechol are displayed in the appendix **(Figure 4.21, 4.22)**.



(a) *(b)*

***Figure 4.7**: UV-Vis spectroscopy calibration curves for standard concentrations of a) ascorbic acid b) ascorbic acid: ChCl in 0.1M HCl.*

In all cases, straight line correlations between absorbance and concentration were observed in accordance with the Beer-Lambert law. Noted that the error bars for all plots are within the dimensions of the plot symbol, which shows the high reproducibility of replicate determinations.

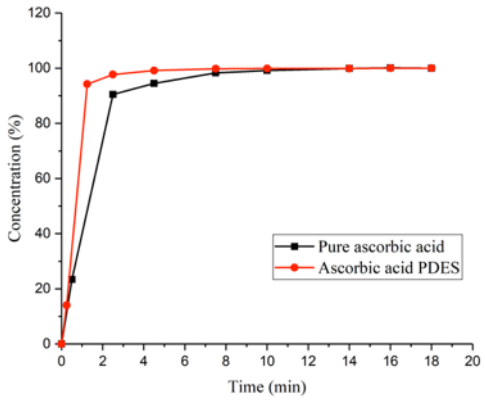
4.4 Dissolution studies for pharmaceuticals/PDES

Dissolution is defined as the process by which a known amount of drug substance goes into solution per unit of time under standardized conditions. Dissolution testing is a required test currently used to demonstrate the performance of all solid oral dosage forms in which absorption of the drug is necessary for the product to perform its therapeutic effect, it is a fundamental part of drug product development and as it is also employed as a quality control tool to monitor batch-to-batch consistency of the drug release from a product.

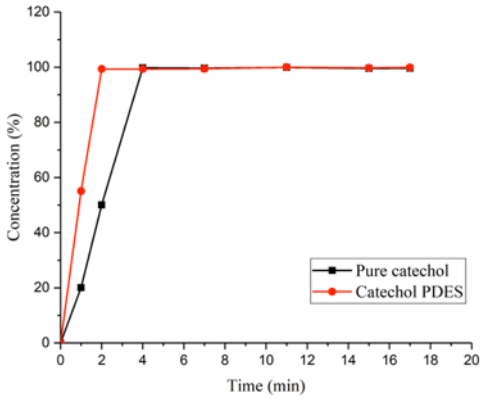
Pharmaceutical ingredient alone and its eutectic mixtures were directly added to 500 ml of 0.1 mol/l HCl solution at 37℃, respectively. Dissolution studies were performed according to CP (2005 Edition). The rotation speed of the paddle was adjusted to 100 rpm. Dissolution samples were collected every minute and analyzed by direct UV spectrophotometry. The cumulative amount of drug released was calculated and plotted versus time.

The dissolution curves of different pharmaceuticals and their eutectic formulations examined are shown in **Figure 4.8**. The release rate profiles were plotted as the percentage of the pure drug in the tablet as a function of time.

The dissolution rate of all three PDESs appeared to be faster than the drugs in their pure form. For all three drugs, the concentration of the drug in solution reaches the maxim before the pure drug. Especially for catechol, 1 second into the experiment, the dissolution rate of the eutectic mixture already exhibits more than twice as fast as the pure drug.



(a)



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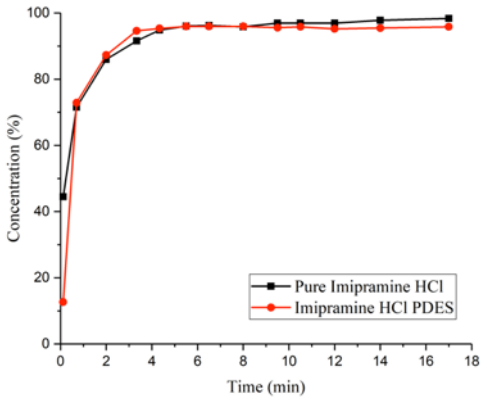


(c)

Figure 4.8 *Dissolution study for ascorbic acid, catechol and imipramine HCl in their eutectic mixtures*

It should be noted that these experiments were operated with very slow magnetic stirring to avoid further evaporation of the solution, which also lead to the PDESs not being immediately miscible. While the PDESs are miscible with water, their high viscosity does necessitate a small but finite time for the two liquids to mix homogeneously.

The ease of distributing the PDESs into water can be due to the lack of needing to solvate the eutectic system. It has been demonstrated that by reducing the melting point of a drug, the solubility and transdermal permeation can be increased.[24] Thus, eutectic formation can be used as a method to reduce the melting point of drugs without compromising their pharmaceutical activity. Another factor can be the wetting and solubilizing effect of the carrier, which could reduce the interfacial tension between the active pharmaceutical

ingredient and the dissolution medium, thus leading to a higher dissolution rate. Take for example the imipramine HCl PDES, when a eutectic mixture consisting of a drug with lower solubility and a highly water-soluble carrier, in this case glycerol, was dissolved in an aqueous medium. It could be thought that the carrier would dissolve rapidly, leaving the insoluble drug in an extremely fine state of subdivision.

4.5 Investigation of the state of DES in gelatin using ^{1}H NMR

To test the possibility of the gelatin-based drug tablet, first a better understanding of the material structure needs to be achieved. As stated previously, the gelatin-DES system forms elastic gels at room temperature, where a three-dimensional network of polymer chains is produced during the gelation process. Experimental methods such as thermal and physical analysis have been used to investigate the properties of these polymer networks (chapter 3).

However, the above results only indicate Glyceline is combined onto the polymer structure and disrupts the polymer-polymer interactions, but there do not give an indication of what is happening to the DES liquid phase. Is it behaving like starch, with DES lubricating the system as a continuous phase, or like leather, with DES only existing in the vacancies created by collagen network, but can be released under extreme pressure? To better understand the role DES is playing in a modified gelatin material, NMR experiments were carried out.

^{1}H NMR (also known as proton nuclear magnetic resonance) is the application of nuclear magnetic resonance with respect to hydrogen-1 nuclei within the molecules of a substance, in order to determine the molecular structure or interactions. The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the ground energy to a higher energy level (generally a single energy gap). The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its ground level, energy is emitted at the same frequency. The signal that matches this transfer is measured and processed to yield an NMR spectrum for the nucleus concerned.

The application of NMR to the study of solid polymers has developed significantly in

recent years. In NMR spectroscopy, the term relaxation describes how signals change with time. In general, signals deteriorate with time, becoming weaker and broader. The deterioration reflects the fact that the NMR signal, which results from nuclear magnetization, arises from the over-population of an excited state. In this work ^{1}H NMR study is used to investigate the role of DES in the gelation process. The aim of this study is to see whether any signal can be detected for the DES in the gelatin. The study focused on the signal for the hydrogen-1 proton on DES modified gelatin samples.

Figure 4.9 shows that an ^{1}H proton can clearly be seen, confirming that there is a continuous liquid DES phase in the gelatin. If there had been no signal then the DES would be assumed to be immobile. This could be because the DES was adsorbed on the polymer surface or "frozen" due to specific interactions with the polymer.

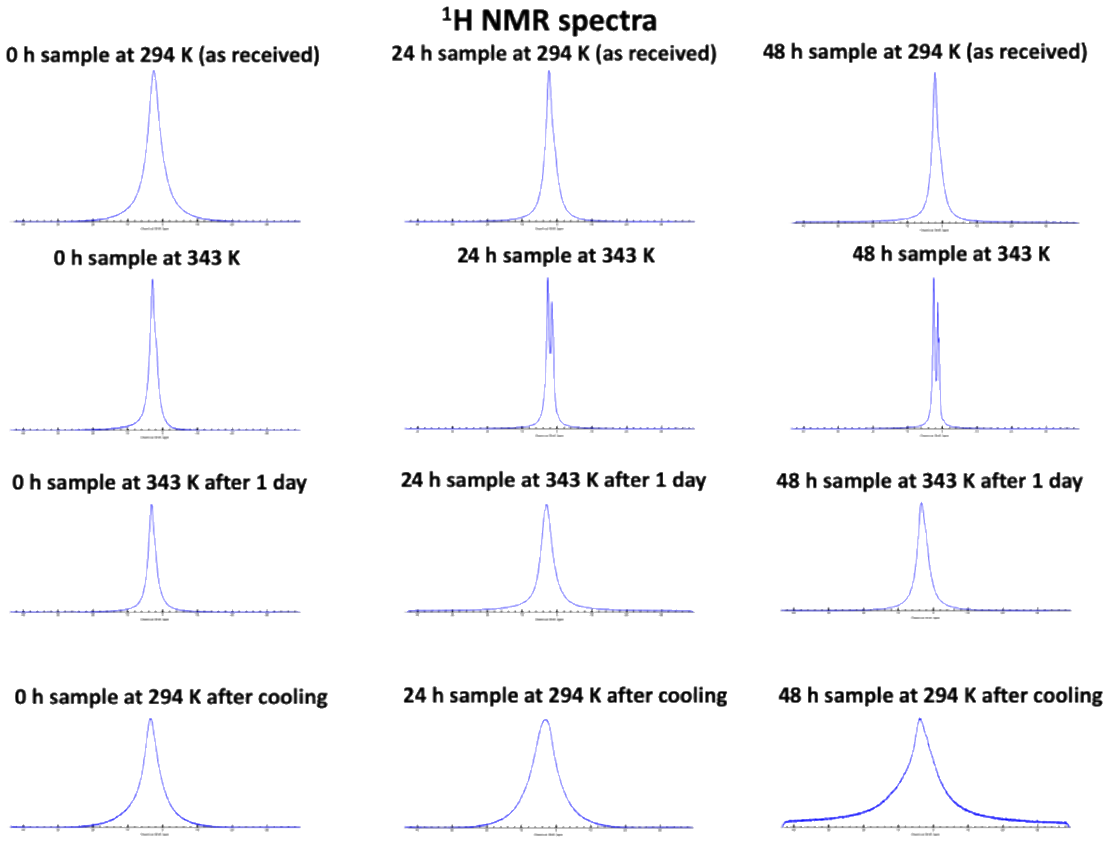


***Figure 4.9** ^{1}H spectra for gelatin with 20% DES after different setting time*

At room temperature, 294 K (around 21^{o}C), broad peaks can be observed for all samples, with the widest being the 0 h sample. Generally, liquid samples (provided that they are homogeneous and diamagnetic) yield sharp signals in NMR spectroscopy. This is despite

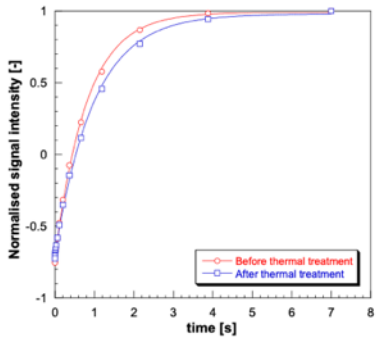
the existence of large dipolar interactions and chemical shift anisotropy that, were it not for rapid isotropic molecular tumbling, would broaden the spectrum to the extent that it would be unobservable with a high-resolution NMR spectrometer. The ^{1}H-NMR spectra of solids under regular acquisition conditions, show signals usually wider than their chemical shift range and too broad to observe with a regular high-resolution NMR spectrometer. In the intermediate case between liquids and solids when the liquid is viscous or a gel and/or the molecule is large (such as a protein) then less broadening is observed. The 0h having the broadest peak is consistent with the fact that it is the most rigid. The sample which was left for 48 h before pressing appears to produce a signal which suggests that the DES is more mobile i.e. it has a sharper peak.

As the temperature of the sample in the NMR tube is increased to 343 K (70^{o}C, just below the T_g of gelatin which is 75^{o}C), the spectra become sharper (indicating an increase in mobility) and for the samples pressed after 24 h and 48 h of mixing some spectra show two peaks. The features are likely to come from both Glyceline, this supports the theory that there are micro domains of liquid-in-solid existing in gelatin samples with longer setting time.

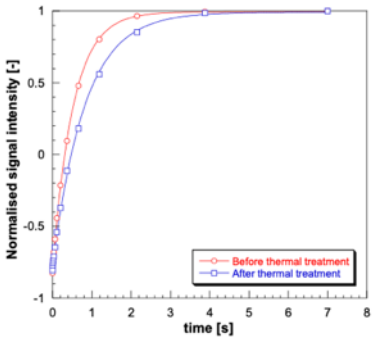
The interesting thing is that the samples of 343 K are not very stable and change spectral features over time. After approximately one day at this temperature the spectral features cannot be distinguished anymore, and the peaks become broader, even broader than the 0 h in the same conditions. This suggests that the sample over time becomes less mobile, possibly due to the leakage of the DES out of the sample and re-solidification (crystallisation) of the gelatin. It is clear that gelatin samples with longer setting time are highly affected by thermal treatment. **Figure 4.10** shows the sample after holding at 70 ^{o}C for 24 h. The sample is more crystalline and becomes more rigid and brittle. This also shows that the DES is mobile at elevated temperature and can form larger pools and leave the polymer structure.

Figure 4.10 *gelatin sheets before and after 24 hours of heat treatment (mixing time of 24 hours)*

The deterioration of an NMR signal is then analyzed in terms of two separate processes, spin-lattice and spin-spin, each with their own time constants. Spin-lattice relaxation, associated with T_1, can be measured from the buildup of magnetization along the static applied magnetic field. It is responsible for the loss of signal intensity. The other process, associated with T_2, describes the decay of the excited magnetization perpendicular to the applied magnetic field, and it is responsible for the broadening of the signal. The proton nuclear spin relaxation times T_1 and T_2 can provide information about the dynamics of DES and their local environments.



(a)



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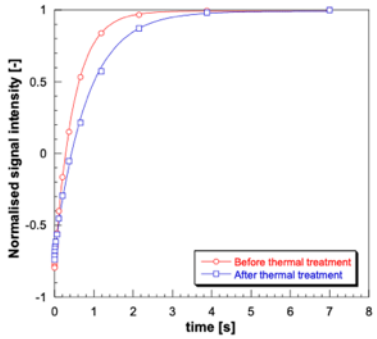


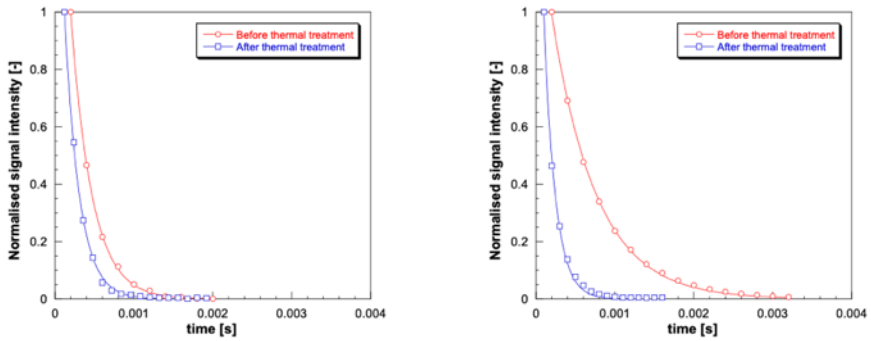
(c)

Figure 4.11 *T_1 relaxation for (a) 0 h sample at 294 K, (b)24 h sample at 294 K and (c) 48 h sample at 294 K**

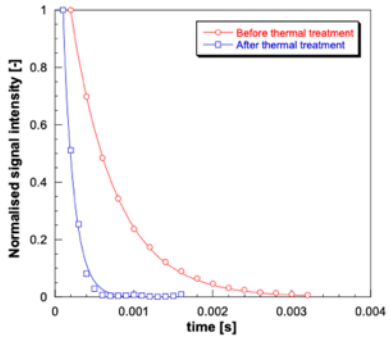
Sample	T_1 (Before) ms	T_1 (After) ms
0 h	800	982
24 h	522	816
48 h	485	811

Table 4.2 *T_1 value of samples after setting time of 0h, 24h, 48h at 294 K*

T_1 values for polymers lie within the range ~10^{-2}-10 seconds which represents the characteristic time when inverted magnetization decays through zero to return to its equilibrium state. The decomposition of T_1 decays is suitable for the experimental assessment for physical mixtures of two polymers, in a manner similar to the analysis of composite T_2 traces described in detail below.



(a) (b)



(c)

Figure 4.12 *T_2 relaxation for (a) 0 h sample at 294 K, (b)24 h sample at 294 K and (c) 48 h sample at 294 K**

** Before treatment means as received. After treatment refers to the samples kept at 343 K for 24 hours.*

Sample	T_2 (Before) ms	T_2 (After) ms
0 h	0.27	0.17
24 h	0.56	0.15
48 h	0.57	0.14

Table 4.3 *T_2 value of samples after setting time of 0h, 24h, 48h at 294 K*

Typically, nuclei in the amorphous regions of a semi-crystalline polymer which is above or near its glass transition temperature, undergo rapid motion, evidenced by a long T_2 value while the crystalline regions may still be well below their melting point for which a much shorter T_2 is appropriate. Such measurements in fact provide the 'mobile fraction' which has obvious temperature dependence. T_2 values range from about 10^{-5} sec for the rigid lattice to values greater than 10^{-3} sec for the viscous or rubbery state. As shown in **Figure 4.12**, all three samples are in the rubbery state, with the 0 h sample closer to a rigid state. It is also evident that after heat treatment, the 24 h and 48 h samples show a bigger difference compared to the sample before treatment, and a longer T_2 value were observed in both cases. This corresponds with the XRD results below where the materials with shorter setting time appear to have a higher crystallinity and coincide with the conclusion in chapter 3 where the one with longer setting time has a higher T_g.

Combining both T_1 and T_2 values, it can be concluded that, as the setting time increases,

molecular dynamics of the sample becomes faster and move towards a more liquid-like structure. This can be seen from the increase in T_2 and decrease in T_1. A clear distinction between 0 h and the 24 h, 48 h can be observed. This is consistent with the physical properties analysed in chapter 3 where the 0h gelatin possessing more of a rigid nature than the one with longer setting time. It can also be seen that soon after the thermal treatment, T_2 decreases drastically, particularly for 24 and 48 h, whereas T_1 increases in a similar manner, which suggests that the molecular dynamics of the samples is becoming much slower.

4.6 Determine the degree of crystallinity using X-ray diffraction

The degree of crystallisation can be determined using X-ray diffraction measurements. This has previously been used to study the structure of DES modified starch.

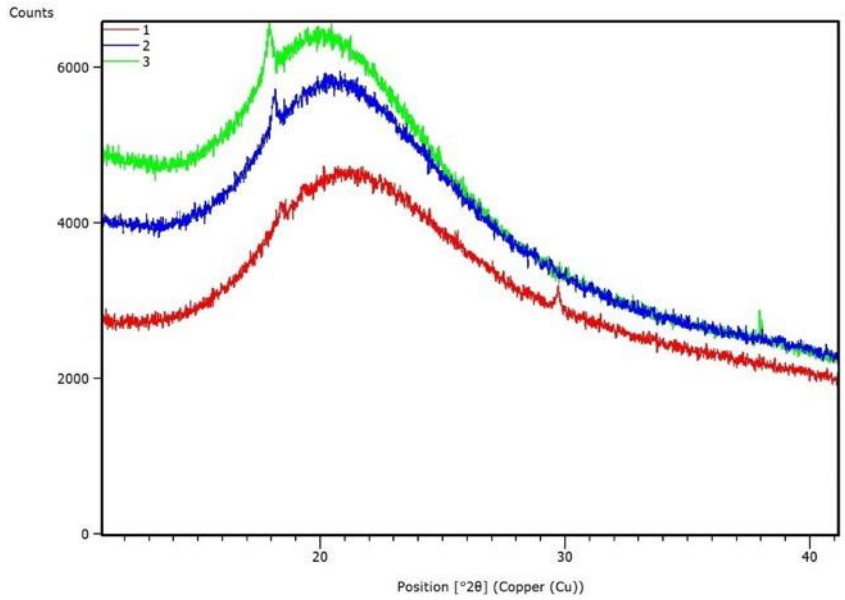


Figure 4.13 *X-ray diffraction pattern of gelatin with 20% DES after different setting time (**1.** 0 hours **2.** 12 hours **3.** 24hours)*

A crystal structure is composed of periodically arranged atoms in a 3D space. On the other hand, amorphous materials do not possess that periodicity and atoms are randomly distributed in 3D space. When there is periodic arrangement of atoms, the X-rays will be scattered only in certain directions when they hit the formed lattice planes (formed by atoms). This will cause high intensity peaks. Amorphous phase on the other hand, is when X-rays are scattered in many directions leading to a broad distribution of diffraction angles.

Figure 4.13 compares the XRD patterns of three samples with the same composition (gelatin with 20% Glyceline) after setting hours of 0 hour, 12 hours and 24 hours.

As mentioned above, the calculation of crystallinity by XRD is based on the presumption that the broad peak comes from the amorphous phase, and the sharp peak comes from crystal phase. According to Tanioka, Miyasaka, and Ishikawa,[31] the board peaks indicate

the reconstitution of the collagen like triple-helix structure. Likewise, Bigi, Panzavolta, and Rubini[32] working on gelatin films also stressed that the broad peak is related to the diameter of the triple helix and its intensity would be associated with the triple-helix content of the films. As a result, the intensity of the broad peaks can be an indication of the percentage of the amorphous phase in each material. Since there's a decreasing trend with the board peaks as the setting time gets longer, the tested material is suspected to have an increasing DES/gelatin ratio. Which corresponds to the UV-vis observation with the PDES-gelatin pills.

If, crystals of triple helices form stable structures in the gels, then one might expect that the scattering patterns for the crystalline junctions in the dry and humid samples would remain identical. It was established that crystals of triple helices are not likely to form in the gel state, and the only ordered units in gelatin gels are the sequences in triple helices. Additionally, not only all three samples show similarity in the position of the board peaks, they all display sharp peaks at 2θ of 19^{o} while demonstrating a decreasing trend in their intensity, thus, there appears to be a reduction in crystallinity of the material as the setting time elongates. This result is consistent with the observation on the appearance of the samples in chapter 3 where the one with shorter setting time appears to be less transparent.

Results above confirmed the gelatin being an amorphous material. This is important as it gives more information when it comes to establishing the role DES is playing in the gelatin materials. It was previously confirmed that when adding DES to a macroscopic material, like leather, it exists as a liquid phase which can be squeezed out. On the contrary, studies on starch proved the existence of a continuous phase inside the system through conductivity measurements. So, when it comes to a macroscopic protein like leather, DES is not going into the primary, secondary, or tertiary structure, it is just sitting in between the fibers themselves, and not changing the crystalline structure of the collagen. However, in starch, conductivity showed DES being possibly continuous, yet it also changed the structure of the starch. The NMR results showed gelatin behaving in a different way than either the proteinaceous material, i.e. leather, or the carbohydrate material, i.e. starch. When considering the bulk structure of the DES liquid, results clearly indicates a liquid phase being in the system, it still flows and diffuses, but conductivity shows it is not continuous. This implies the modified gelatin being a structure where a dispersed phase which is droplets of IL are contained within the material, giving clear NMR signals.

This is particularly important as it makes the PDES-gelatin a controlled release system. Unlike most tablets, where APIs are mixed with the binding materials in its original solid form, it is acting like a drug capsule and tablet hybrid, a controlled release material with tiny pools of pharmaceuticals in deep eutectic form. As the material surrounding it dissolve slowly, individual drug droplets will be released to the surroundings, in this case, the stomach. As a result, when the stomach digests the gelatin, the liquid containing active ingredient will flow out rapidly.

4.7 Dissolution studies for protein-based PDES tablets

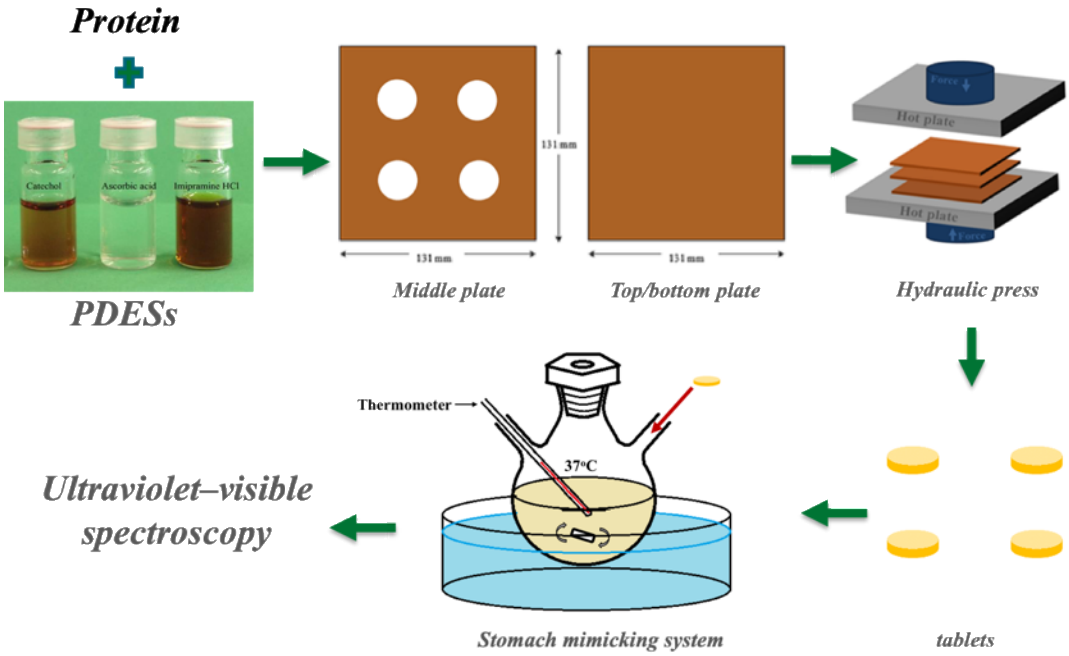
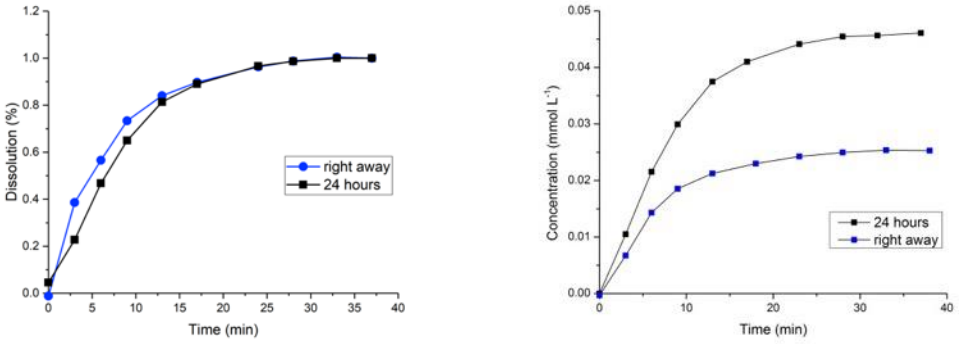


Figure 4.14: *Schematic diagram of tablets production and dissolution rate studies.*

Gelatin as a traditional water-soluble functional protein of high interest and value, it has the ability of forming transparent gels under specific conditions, which is ideal when it comes to the manufacture of pharmaceutical capsules, ointments, cosmetics, tablet coating, and emulsions are concerned.[33]

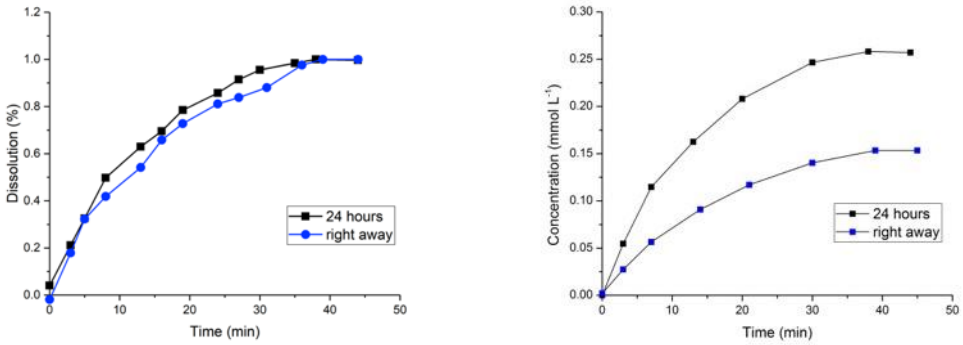
Apart from being a suitable candidate for pharmaceutical use, gelatin was also found earlier in chapter 3 that the material property is highly dependent on the procedure of the production, more specifically, it was suspected that the degree of crystallinity in the gelatin is affected by the length of time that the gelatin and PDES mix before they are pressed.

The theory behind the design of the experiments demonstrated above is that not only combining DES and gelatin can result in materials with desirable qualities, because the way in which the gelatin-PDES material is made will affect the properties of the material, it was suspected that it may affect the releasing behavior of the drugs in the form of PDES as well. Hence the possibly of controlling dissolution rate of the tablets by simply change the time factor of the manufacturing.



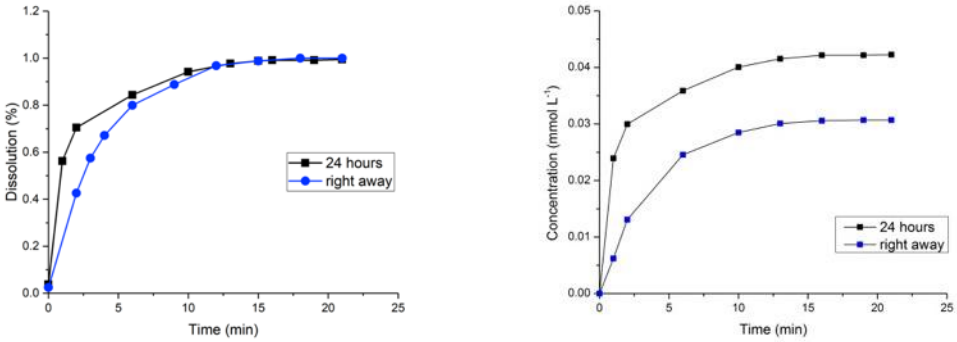
(a) (b)

Figure 4.15 *Dissolution rate study for gelatin-based ascorbic acid based PDES tablet in the form of (a) overall concentration percentage and (b)concentration released to the dissolution medium*



(c) (d)

Figure 4.16 *Dissolution rate study for gelatin-based catechol based PDES tablet in the form of (c) overall concentration percentage and (d)concentration released to the dissolution medium*



(e) (f)

Figure 4.17 *Dissolution rate study for gelatin-based Imipramine HCl based PDES tablet in the form of (e) overall concentration percentage and (f)concentration released to the dissolution medium*

To verify, two different formulation methodologies were tested. Firstly, gelatin was mixed with the PDESs and the mixture was pressed straight away. In the second the mixtures, gelatin and PDES was mixed and left for 24 hours to form a more gel-like texture before it was then pressed into tablets. The tablets produced by these two methods present similar appearance but had a different feel i.e. the sample which was pressed after 24 hours of mixing was more flexible and plastic-like than the sample pressed straight away. The results resemble the one obtained in chapter 3 where gelatin was modified by Glyceline. Here, the amount of the API uptake was estimated based on the concentration increase detected by UV-vis, similar to section 4.4. Simultaneous dissolution of the gelatin tablet was observed, and they disappeared at the same time that the drug release was completed. It was suggested that the gel structure was destroyed while releasing the API in PDES form.

Dissolution study of each drug indicate that the gelatin with different setting time shares the same release profile. However, when it comes to the absolute number of the amount of the drug being released, it can clearly be seen form (b), (d), (e) in **Figure 4.15-4.17** that the gelatin pill with longer setting time managed to contain more active drug ingredient than the ones pressed right away.

As previously stated, it was concluded that by leaving the gelatin and PDES mixture for a certain amount of time before pressing, a different structure was formed which managed to keep more API inside the system in a liquid phase. Studies on the structure of gelatin modified by water, were carried out by Pezron et al., who studied polymer fibers drawn from gelatin gels using wide-angle X-ray scattering. They concluded that significant swelling of the fibers was induced by the water's ability to drive apart the triple-helices in the gels.[34]

Figure 4.18 *Different stages of hydration for gelatin fibers: a) Fiber b) swollen fiber c) gel*

More specifically, the side-by-side distance of the triple helices was found to increase continuously as more water is added to the fibers. PDES was suspected to have a similar role in the gels, by breaking the side-by-side alignment associate by hydrogen bonding (**Figure 4.18**), inducing significant swelling of the fibers.

If the structure of the gelatin-DES mixture was purely dependent upon the dissolution of gelatin then it would be expected that all tablets would have the same dissolution rates. However, it is clear by comparing all three PDESs from **Figure 4.15-4.17** that all the formulations have different dissolution rates. This may be due to the way in which the PDES is distributed throughout the gelatin. By dissolving the carrier material, which was gelatin, the system was opened up releasing PDES in droplets, which cause the dissolution rate being effected by both the structure of the gelatin and the composition of the drug formulation stored inside as well. From (a), (c), (e) in **Figure 4.15-4.17**, it can

be seen that with both ascorbic acid and catechol based PDES, the setting time showed no noticeable difference on the disillusion behavior of the gelatin tables, with similar dissolution rates observed irrespective of the processing conditions. However, when it comes to DES composed of Imipramine HCl, the less crystalized structure of the tablet has an effect on the releasing speed of the API inside the tablet. The sample of gelatin-Imipramine HCl-ChCl pressed immediately showed a faster drug release than that left for 24 hours. This may be due to the reaction between Imipramine and the dissolving medium, i.e. HCl solution.

This theory has been backed up by other researchers by studying the release profile of Imipramine HCl using other material as carriers. For example, Tomida et al studied the release rate of Imipramine HCl from Ca-alginate as a vehicle for the controlled release of in different dissolution mediums.[35] It was found that in a more acidic solution, in this case 0.1 mol/L HCl, a more rapid release was observed. It was suggested that by interacting with the H^+ in the release medium, the drug is more quickly leached out of the beads.

4.8 Comparison between fibrous protein with globular protein as tablet candidates

Soy protein was tested as the matrix because of its high availability and biodegradability,[36] good melt processability,[37] high thermal stability,[37] and non-toxicity. Its feasibility has been shown in a variety of applications in the polymer, food, and agriculture fields, and recently, the use of soy for the production of melt-extruded or injection-molded drug/matrix compounds has also been explored.[38]

Experiments were carried out to explore the possibility to encapsulate a drug into a soy matrix by forming PDES and subsequently studying its suitability to be used as a drug delivery system. Soy protein is composed almost exclusively of two globular protein fractions differentiated by sedimentation coefficient: 7S (β-conglycinin) and 11S (glycinin). In chapter 3 it was suggested that the size of both β -conglycinin and glycinin decreased with higher DES content, introducing a new form of structure created by the mixture glycinin/ β -conglycinin, which increased with the decreasing β -conglycinin and glycinin content, and results in an increase of the free volume of the peptide backbone, hence trapping more PDES in the form of pools.

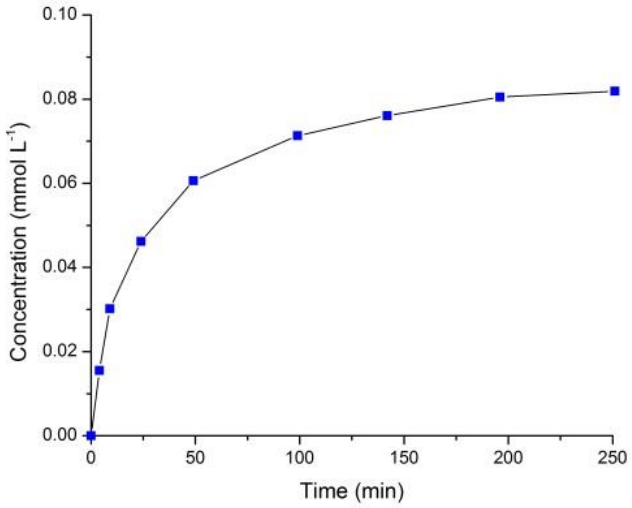


Figure 4.19 *Dissolution study for soy-based Imipramine HCl PDES tablet*

Soy matrix systems with an encapsulated drug (theophylline, commonly used in therapy for respiratory diseases) were previously compounded by extrusion and further injection-molded into the desired shapes.[38] It was found that melt-based processing techniques can successfully allow the encapsulation of a drug in a protein based system without any subsequent finishing operations.[38] Results also show that the degree of cross-linking has an effect on the drug releasing rate. This can be attributed to differences in the matrix structure.

However, the soy material in this study prove to be not fully soluble in the chosen dissolving media, as there was still visible tablet residue even after 200 mins into experiments. The limited protein release in simulated gastrointestinal fluid can be attributed to diffusion of loosely cross-linked protein molecules into the dissolution medium as a consequence of swelling. **Figure 4.19** shows that the drug continues to be released after 3 h in HCl. Similar results were observed by Chen, L. et al. when the release of drugs continued for 6 hours in simulated gastrointestinal fluids with pH of 1.2, without digestive enzymes.[39]

Consequently, unlike gelatin, the release rate profiles were plotted as the concentration of the drug (mmol/L) in the tablet as a function of time, since the determination of the total amount of drug existing in the tablet proved to be difficult to determine causing by incomplete solubility.

The result indicates that the soy-based PDES tablet cannot be compared in the above experiment due to the lack of digestive enzymes necessary to fully digest the soy protein. Therefore, a better stomach mimicking system would need to be used for further results. On the other hand, this proves that for water-soluble proteins, the PDESs will be released as the network system is opened up, while the ones with low solubility, or has no solubility at all, will leach out to the water environment without damaging the material structure.

4.9 Summary

The different solubility and dissolution rate of four chosen drugs were measured before and after being formulated into a eutectic mixture, as a part of a pre-formulation study to develop an oral formulation using protein-based plastics as matrix. With initial results revealing acetylsalicylic acid losing its pharmaceutical property during PDES formulation, three other PDES were chosen for further experiments.

It is clear that the dissolution rate study of ascorbic acid and catechol based PDES showed a slight increase in dissolution from the physical mixtures, this was suspected to be caused by the wetting and solubilizing effect of the carrier, which could reduce the interfacial tension thus leading to a higher dissolution rate. It also shows that better solubility can be achieved when drug was acting as the hydrogen bond donor in DES formulations.

Gelatin based PDES tablets were formed using the same method mentioned in chapter 3. It can clearly be seen that the gelatin pill with longer setting time managed to release more active drug ingredient than the ones pressed right away. The micro-domain theory was then proposed, which describes the phenomenon where the network structure formed by protein chains connecting to each other by hydrogen bonding was expanded as the setting time lengthens and storing PDES inside in small droplets in liquid form. The reason behind was that the energy of both the hydrogen bond formed by the protein chains itself and the ones formed inside PDESs are stronger than the potential bonding between the PDES and the protein chains. The protein component is not denatured by the DES but remains in its original structure encapsulating small pools of PDES. Enthalpically speaking, both the PDES and protein chains are in their structured form, in the sense of molecular dynamics. There is no entropic driving force encouraging the disruption of the system by forming hydrogen bonding between PDESs and protein chains. With longer setting time, the system was further lubricated and expanded hence absorbing more

PDESs into the system, while the ones with shorter setting time tend to leach out the remaining PDES during the heat-pressing procedure. As a result, the samples with longer setting time have higher drug content was released after the carrier is solubilized in HCl solution.

The XRD patterns of all gelatin samples supports the theory mentioned above where the material with longer time have a higher DES/gelatin ratio. The crystallinity of the material appears to be slightly affected by the procedure by which the tablet is made.

^{1}H NMR was carried out revealing that at room temperature, the sample with longer setting time appear to be the most mobile. As the temperature increase to near the glass transition temperature of gelatin, all spectra become sharper indicate increase in mobility, and for the 24h and 48h two peaks can be distinguished (**Figure 4.21)**.

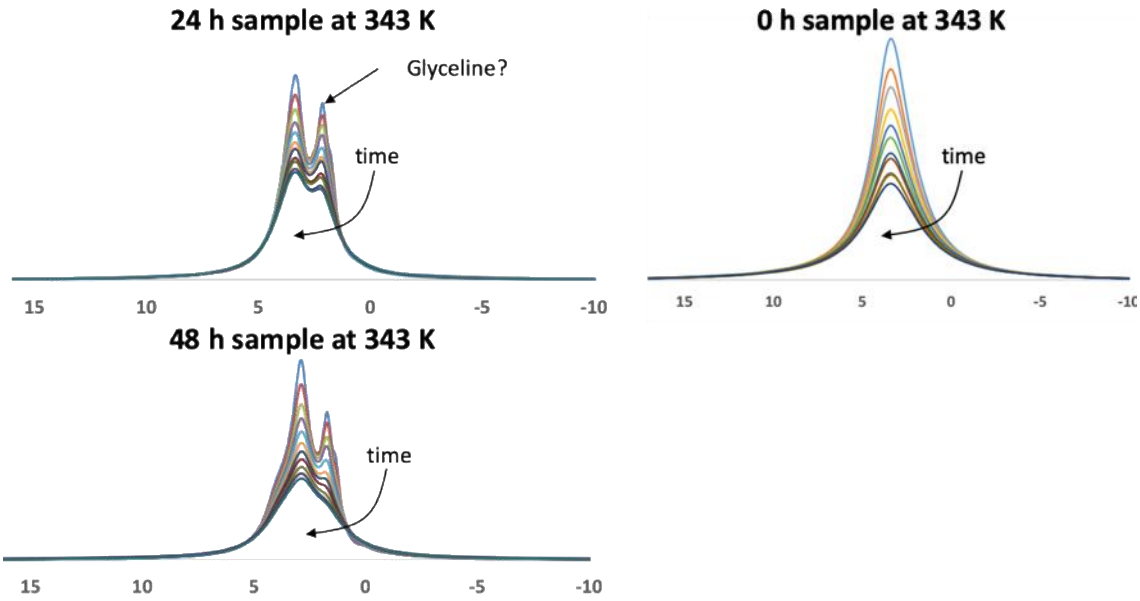


Figure 4.20 *^{1}H spectra for gelatin with 20% DES after different setting time at its T_g over time*

The features are likely to come from both Glyceline and the gelatin. This supports the theory that there are micro domains of liquid-in-solid existing in the gelatin samples with longer setting times. Results above confirmed the gelatin is an amorphous material when modified with DESs. This is important as it gives more information when it comes to establishing the role DES is playing in the gelatin materials. It was previously confirmed

that when adding DES to a macroscopic material, like leather, it exists as a liquid phase which can be squeezed out. On the contrary, studies on starch proved the existence of a continuous phase inside the system through conductivity measurements. So, when it comes to a macroscopic protein like leather, DES is not going into the primary, secondary, or tertiary structure, it is just sitting in between the fibers themselves, and not changing the crystalline structure of the collagen. However, in starch, conductivity showed DES being possibly continuous, yet it also changed the structure of the starch. The NMR results showed gelatin behaving in a different way than either starch or leather. When considering the bulk structure of the DES liquid, results clearly indicates a liquid phase being in the system, it still flows and diffuses.

This is particularly important as it makes the PDES-gelatin a controlled release system. Unlike most tablets, where APIs are mixed with the binding materials in its original solid form, it is acting like a drug capsule and tablet hybrid, a controlled release material with tiny pools of pharmaceuticals in deep eutectic form. As the material surrounding it dissolve slowly, individual drug droplets will be released to the surroundings, in this case, the stomach. As a result, when the stomach digests the gelatin, the liquid containing active ingredient will flow out rapidly.

So, what is the mechanism of drug releasing from gelatin if it is made into a drug patch instead? As a tablet, the gelatin will distribute to the surroundings while gelatin is being digested, and each pools of liquid is individually released. However, as a patch, gelatin is not dissolving, the skin absorption of the API can only happen when there's an exchange happening between the liquid on each side of the skin barrier. By confirming the existence of the liquid phase inside the PDES-gelatin system, it opened up the possibly of its potential in the transdermal drug delivery field. The fact that the material conducts electricity shows that the DES is continuous and so there should be a network by which the PDES can transfer from the starch patch into the skin. If the drug is successfully delivered without compromising the gelatin carrier itself, then it can be seen as evidence for inter connectivity between those tubules.

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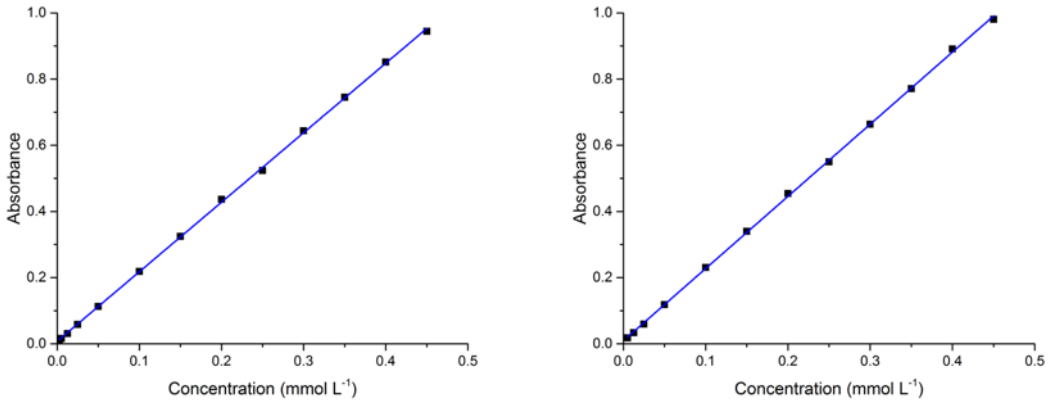
4.11 Appendix

4.11.1 Absorbance range and R^2 values for all calibration curves

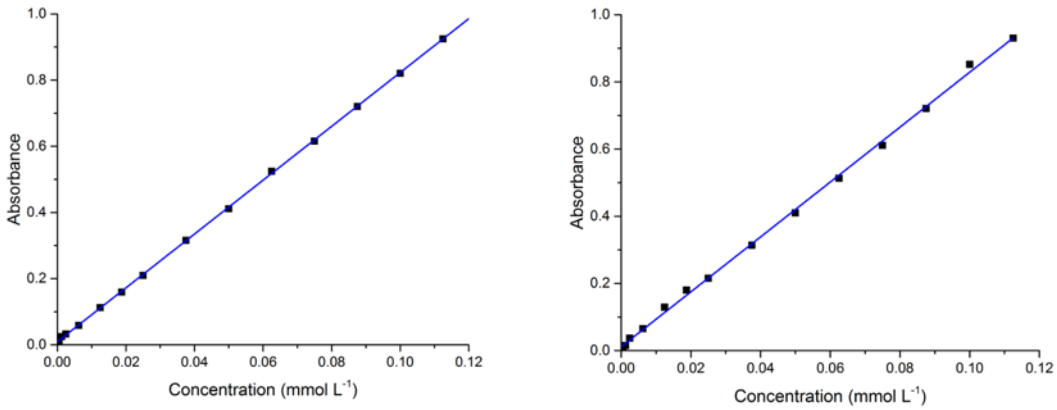
	Ascorbic Acid	R^2	Catechol	R^2	Imipramine HCl	R^2
Drug	0.007-0.994	0.9999	0.013-0.944	0.9997	0.011-1.03	0.9996
DES	0.009-0.972	0.9998	0.018-0.980	0.9997	0.007-0.93	0.9993

4.11.2 Calibration curves for all three drugs



(a) *(b)*

***Figure 4.21**: UV-Vis spectroscopy calibration curves for standard concentrations of a) Catechol b) Catechol: ChCl in 0.1M HCl.*



(a) *(b)*

***Figure 4.22**: UV-Vis spectroscopy calibration curves for standard concentrations of a) Imipramine HCl b) Imipramine HCl:glycerol in 0.1M HCl.*

Chapter 5: Design and evaluation of gelatin patches as transdermal drug delivery systems

5. Design and evaluation of gelatin patches as transdermal drug delivery systems

5.1 Introduction

In the previous chapter gelatin modified with PDESs was studied as a means for a drug to be delivered into a body through the stomach. While this is probably the most common mechanism of drug delivery there are significant drawbacks to this approach. In this chapter a transdermal approach is tested using the same gelatin-PDES materials. This should enable a concentrated and localized transdermal drug delivery system to be tested.

In comparison to other synthetic biomaterials, gelatin hydrogels resemble living tissues closely in their physical properties because of their relatively high moisture content and soft and rubbery consistency. They show minimal tendency to adsorb proteins from body fluids because of their low interfacial tension. Furthermore, the ability of molecules of different sizes to diffuse into (drug loading) and out of (drug release) hydrogels allows the possible use of dry or swollen polymeric networks as drug delivery systems for multiple routes of administration.

Transdermal delivery as an alternative for drug administration can overcome many of the problems associated with oral delivery such as high first-pass metabolism, variation in gastrointestinal absorption and patient non-compliance, since it: (a) by-passes first pass metabolism; (b) enables control of input; (c) avoids problems of stomach emptying, pH effects and enzymatic deactivation associated with gastrointestinal tract passage. Transdermal drug delivery not only provides controlled, constant administration of the drug, but also allows continuous input of drugs and eliminates pulsed entry into systemic circulation. Transdermal drug delivery can also improve the therapeutic efficacy and safety of drugs by more site specific way but spatial and temporal placement on the body. The first transdermal patch was approved in 1981 for the relief of motion sickness, nausea and vomiting.[1] The most common method of transdermal drug delivery on the market is based on passive patch technology that relied on sample diffusion across the skin.[2] This offer a wide array of capabilities allowing the delivery of compounds under 500Da.[3] However, the success of the dermatological drug to be used for systemic drug delivery is highly dependent on the ability of the drug to penetrate through skin in sufficient

quantities to achieve the desired therapeutic effect. **Table 5.1** lists some of the main transdermal drug delivery systems currently available on the market.

Brand name	Drug	Manufacture	Indication
Alora	Estradiol	Thera tech/Protocol and Gamble	Postmenstrual syndrome
Androderm	Testosterone	Thera tech/GlaxoSmith Kline	Hypogonadism in males
Catapress	Clonidine	Alza/Boehinger Ingelheim	Hypertension
Climaderm	Estradiol	Ethical holding/WyethAyerest	Postmenstrual syndrome
Climra	Estradiol	3M Pharmaceutical Labs	Postmenstrual syndrome
Deponit	Nitroglycerine	Schwarz-pharma	Angina Pectoris
Duragesic	Fentanyl	Alza/Janssen Pharmaceutica	Moderate/severe Pain
Estraderm	Estradiol	Alza/Novertis	Postmenstrual Syndrome
Fematrix	Estrogen	Ethical Holding	Postmenstrual Syndrome
FemPatch	Estradiol	Parke Davis	Postmenstrual Syndrome
Habitraol	Nicotine	Novartis	Smoking Cessation
Minitran	Nitroglycerine	3M Pharmaceutical	Angina Pectoris
Nicoderm	Nicotine	Alza/Glaxo Smithkline	Smoking Cessation
Nitrodisc	Nitroglycerine	Roberts Pharmaceutical	Angina Pectoris
Nitro-dur	Nitroglycerine	Key Pharmaceutical	Angina Pectoris
Nuvelle TS	Estrogen/Progesterone	Ethical Holding/Schering	Angina Pectoris
Testoderm	Testosterone	Alza	Hypogonadism in males

Table 5.1 *Transdermal patches currently available on the market*[4]

Many approaches have been taken to increase the efficiency of transdermal delivery systems. The most common one is with the help of skin enhancers, which are compounds that can be used to improve the partitioning of drugs passing through the skin barrier. There are of three types of these：lipophilic solvents, surface active agents， and ； two component systems.[5] Other techniques have also been used for this purpose, ultrasound

for example, has been applied to boost the transdermal flux and decrease the lag time of the transport.[6] Electroporation is another method of penetrating drugs into lipid bilayers, which involves the creation of transient aqueous pores and the application of an electric pulse.[7] However, as the stratum comeum has a much higher electrical resistance than other parts of the skin, an electric field applied to the skin will mostly concentrate in the stratum corneum, resulting in other viable tissues being exposed to much lower fields, which as a result, can cause insufficient electroporation.

Another approach is through the chemical modification of the drug itself, as the way an active pharmaceutical ingredient is formulated can also have a major impact on the release of the drug from the device. The newly formulated drug should penetrate the skin well and have the ability of being converted back to its original pharmaceutical form once it reaches the human body, for example, with the aid of epidermal enzymes. The API matrix can be prepared by dispersion of the drug in a liquid or solid state synthetic polymer base. Polymers used in the formulation of the transdermal drug delivery should have biocompatibility and chemical compatibility with the API, but the drug delivery system also often contains other components such as penetration enhancers. The manufacturer needs to ensure that the formulation provides consistent and effective delivery of a drug throughout the product's intended shelf life. It is generally believed that drug formulation candidates for passive adhesive transdermal patches should possess certain properties including, but not limited to, low molecular weight (less than 500 Da), relatively sufficient solubility in both oil and water, high potency (dose in mg per day) and a low melting point (less than 200°C).

Eutectics have been used as a way of formulation to boost the efficiency of transdermal drug delivery, owing to the fact that they are water-compatible, non-flammable, nontoxic, biocompatible and biodegradable. Furthermore, according to regular solution theory, the lower the melting point, the greater the solubility of a material in a given solvent, including skin lipids. For example, a eutectic composition of the local anesthetic drugs lidocaine and prilocaine has been used to enhance the transdermal permeation of lidocaine. This provides an effective local anesthetic for pain-free venipuncture and other procedures.[8] When administered individually, these drugs have a slower skin penetration because their melting points (lidocaine, 68 °C; prilocaine, 38 °C) are higher than the body temperature. As a eutectic mixture, however, it has a melting point as low as 22 °C which

triggers faster skin permeation and rapid pharmacological action. On top of which, as mentioned in chapter 4, the high thermodynamic functions of eutectics, such as free energy, enthalpy and entropy, can confer solubility and dissolution advantage to poor solubility drugs. Moreover, its superior compatibility with hydrophobic substance can ease the challenging transdermal transport of negatively charged hydrophilic molecules through the overall hydrophobic stratum comeum. However, it is not without its flaws, as the low melting point of eutectics can also pose stability issues.

In addition, some DES components have also been documented as effective skin enhancers. Glycerol for example, can change the water activity across the skin membrane. It has been shown that glycerol can alter the degree of skin hydration and hence regulate the skin permeability to model drugs with different lipophilic characteristics.[9] One possible explanation is that changes in the water gradient can induce reversible structural alterations in stratum corneum lipids (SC) or protein components, which can lead to significant changes in the transport characteristics.[10] It has been confirmed by previous studies that osmolytes like glycerol and urea can stabilize fluid structures in phospholipid bilayer systems where the lipids would otherwise form solid bilayer structures.[11]

To fully understand the transport mechanism of the API across the barrier, the structure of the skin needs to be understood. Skin is a structurally complex and thick membrane, and its barrier properties are primarily determined by the outer layer of the skin, called the stratum corneum. It is a dead tissue composed of flattened cells filled with cross-linked keratin and an extracellular matrix made up of lipids arranged largely in bilayers, which is primarily consist of ceramides, cholesterol, and fatty acids.[12] SC, as the outermost layer of skin, constitutes the main barrier towards both inward and outward diffusional transport.[13] When a molecule reaches the skin, it contacts cellular debris, microorganisms, sebum and other materials. The diffusion then has three potential entry routes, including through the hair follicles with their associated sebaceous glands, via the sweat ducts and across the continuous stratum corneum between these appendages (**Figure 5.1**).

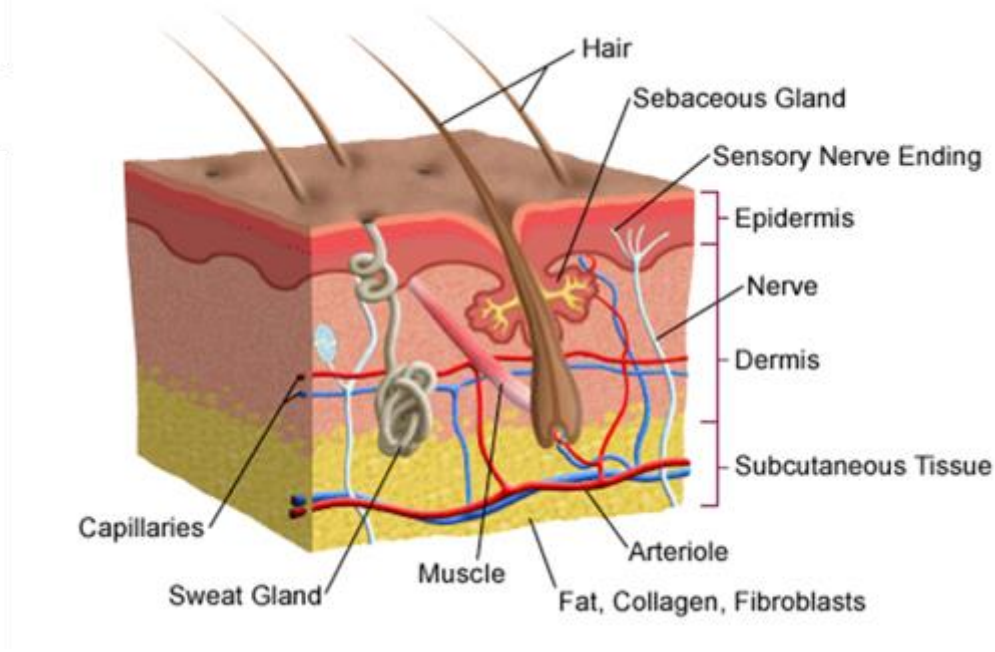
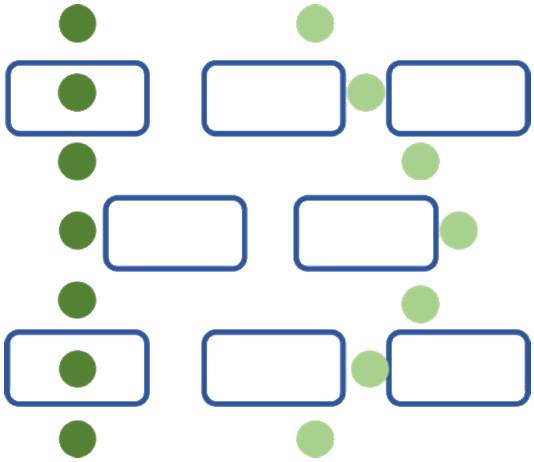


Figure 5.1 *Pathways through the human skin*

For a systemically-active drug to reach a target tissue via the last route, it has to possess some physicochemical properties which will facilitate the drug passing though the stratum corneum, either by the way of transcellular (across the cells) or the way of intercellular (between the cells) (**Figure 5.2**).



(a) (b)

Figure 5.2 *Schematic diagram of the routes of drug penetration through stratum corneum, (a) transcellular route and (b) intercellular route*

It has been shown using electron photo-microscopic examination that intracellular region in stratum corneum is an amorphous material filled with lipid.[14] During cornification the lipid composition shifts from polar to neutral constituents. Though the intercellular pathway avoids the cell contents, it appears to be less direct. As a result, transcellular can be the preferred pathway for penetration of small polar molecules to avoid long path lengths, however, it is considered an unlikely avenue for larger molecules.

The trans-follicular pathway by comparison, is the shortest way that provides a relatively large area for molecule diffusion. It involves passage or diffusion of drug molecule through the hair shaft openings which presumably are filled with sebum. It will then penetrate the viable epidermis, the papillary dermis and the capillary walls into the blood stream or lymph channels, whereupon they are delivered to the viable tissue by flow of blood or lymph.

5.2 Design of a drug patch

Controlled release can currently be categorised into two main categories, rate-programmed transdermal drug delivery systems and physical stimuli-activated transdermal drug delivery systems.[14] The former method has been the choice of many researchers owning to its ability to provide control of the release rate and enable the transdermal permeation of drugs without the aid of outside technologies.

The basic components of such transdermal delivery system include an outer layer of drug-impermeable metallic plastic, a polymer matrix with drug reservoirs, and a pressure-sensitive adhesive that anchors the patch to the skin as shown in **Figure 5.3**.

In the drug reservoir compartment, the drug solids are either dispersed homogenously in a solid polymer matrix (e.g. polyisobutylene adhesive) or suspended in an unbleachable, viscous liquid medium (e.g. silicone fluids) to form a paste like suspension.[14] The adhesive is covered by a release liner which needs to be peeled off before applying the patch to the skin. The constant release rate of the drug is the major advantage of this drug release system. However, poor choice of the adhesive layer might increase the risk of accidental breakage which can result in dose dumping or rapid release of entire drug content. Furthermore, an incidental contact between the adhesive and the drug and penetration enhancer may also cause instability of the drug, penetration enhancer or the

adhesive.

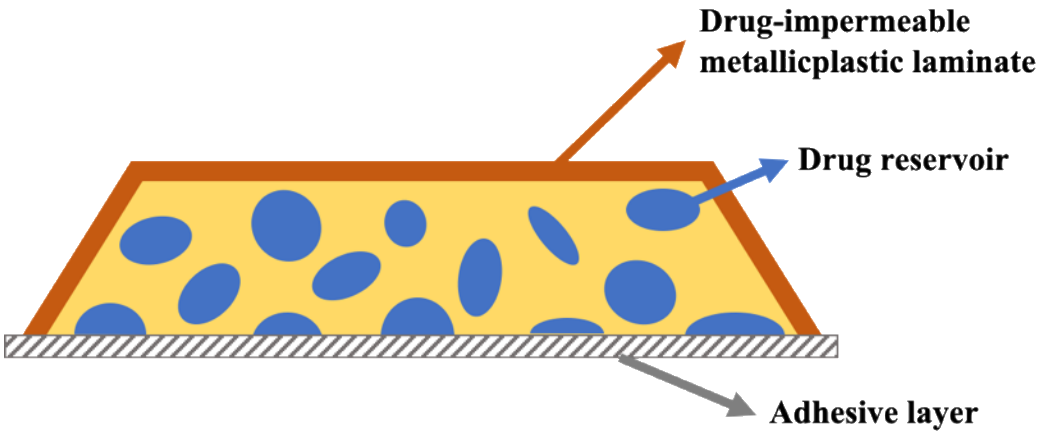


Figure 5.3 *Basic components of a transdermal drug delivery system*

A simplified form is then proposed, called the Single-layer Drug-in-Adhesive system. It is characterized by the inclusion of the drug directly within the skin-contacting adhesive, where the drug reservoir is formulated by directly dispersing the drug in an adhesive polymer e.g. poly (isobutylene) or poly (acrylate) adhesive and then spreading the medicated adhesive, by solvent casting or hot melting to produce an adhesive diffusion-controlled delivery system.[14] In this transdermal system design, the adhesive not only serves to attach the system to the skin, but also as the formulation foundation containing the drug.

As established in the previous chapter, gelatin can be moulded with the DES containing pharmaceutical ingredient to form an amorphous material with suitable physical properties. In this chapter, the possibility of using such products as a Drug-in-Adhesive matrix for transdermal drug delivery is explored.

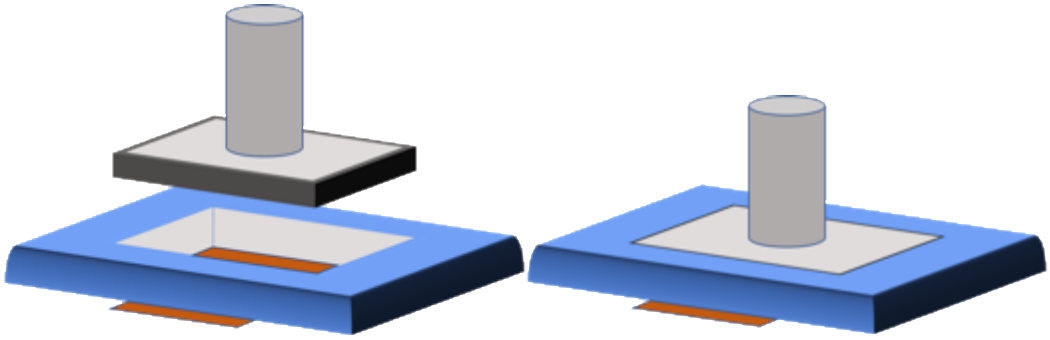
5.3 Evaluation of gelatin as patch material

5.3.1 Adhesion test

As mentioned before, most transdermal drug patches require additional adhesives to increase the permeability of stratum corneum in order to attain higher therapeutic levels of the drug. However Drug-in-Adhesive systems eliminate such problem by incorporating the polymer matrix with the adhesive layer using an adhesive polymer as the patch

material.

To maintain an intimate contact between the transdermal system and the skin surface, the adhesion should not more than can be applied by finger pressure. It should also be permanently tacky, and exert a strong holding force. Additionally, it should be removable from the smooth surface without leaving a residue. On top of which, the adhesion should not be affected by the diffusing drug. It can be influenced by the molecular weight, the degree of crosslinking and the composition or type of the polymer and the modifier content.



Step 1 Step 2

***Figure 5.4**: Surface adhesion test*

Many methods have been used to test the adhesiveness of a patch material, including shear adhesion test where a strip of an adhesive sample is applied onto a stainless-steel plate, and a specified weight is hung from the strip pulling in a direction parallel to the plate. Shear adhesion strength is determined by measuring the time it takes to pull the tape off the plate.

In this study, the adhesion is measured using the compression method which is commonly used in the food industry for the determination of the stickiness of pasta. It measures the maximum force incurred during the test. The procedure involves a known force (mass) (in this case, 1kg) being applied to the product for a certain amount of time (10s), followed by the compression plate being moved upward (**Figure 5.4**). The stickier the product the greater the negative force will be exerted on the compression plate. The maximum forces required to pull off different samples are calculated and the results are shown in **Figure 5.5**.

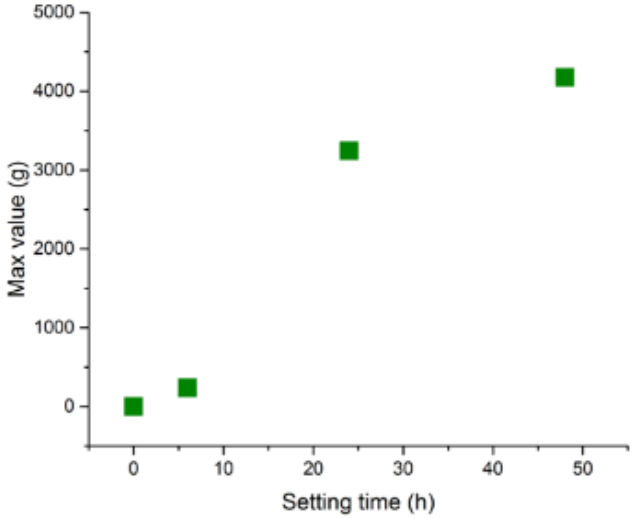


Figure 5.5 *Adhesion comparison for gelatin patches modified by 20 % of ChCl: 2 ascorbic acid by weight after different setting times*

Ascorbic acid based PDES was chosen for this experiment, for the reason that by observation, it has a relatively high fluidity comparing to the other two, which will be further investigated later in this study. This makes it easier to be used in larger quantity and fully mixed with gelatin pellets before pressing into sheets.

The sample with no setting time showed an adhesion of nearly 0 N while when the setting time increased to 6 hours, a force of just over 2 N was required. The material carried on becoming tackier with time and at 24 h, the force required was 32 N which grew to 41 N after 48 h. From the results it can be concluded that not only the gelatin patches appeared to have relatively high adhesion, it can be modified by the additional setting time, which is important in the sense that the surface property can then be designed by controlling the production procedure, to tailor for different patient needs.

Similar properties have been observed by many other researchers and been used in other biomaterial fields, one example is the use of alginate/gelatin sticky gel in the fixations of the implant and implant-bone integration after joint arthroplasty to prevent inflammation and promote bone regeneration.[15] The reason behind this is suspected to be the high content of tyrosine residues in gelatin compared to other proteins, and these residues can be converted to a catechol-containing amino acid called L-3,4-dihydroxyphenylalanine (L-DOPA), which

is also known to be found in the structure of the adhesive proteins secreted by the foot organs of mussels.[15] DOPA can strongly bind to surfaces, even under wet conditions, *via* chemical interactions including hydrogen bonding, coupling with metals, and covalent crosslinking (**Figure 5.6**).

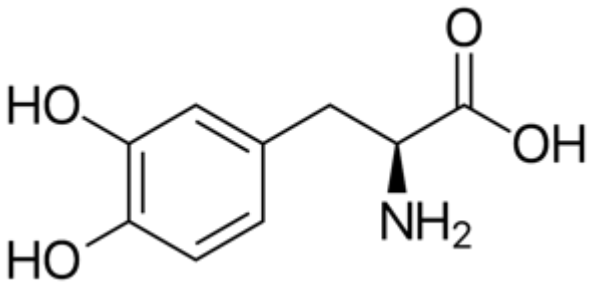
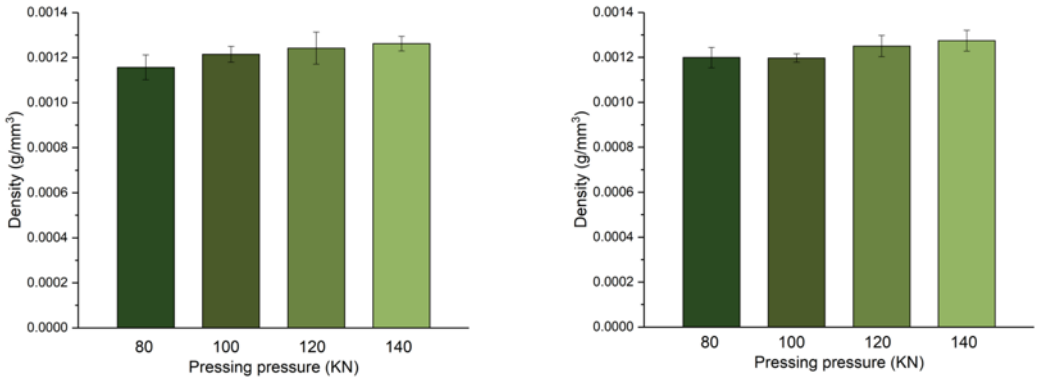


Figure 5.6 *Chemical structure of L-3,4-dihydroxyphenylalanine*

5.3.2 Density uniformity under different pressure:

As the polymer matrix for a transdermal patch, apart from the surface adhesiveness, its mechanical properties also need to be taken into consideration. Thickness of the patch especially, will affect each and every physical parameter (folding endurance, moisture content, moisture uptake, etc.), it will also affect the release profile of a drug.



(a) (b)

Figure 5.7 Density uniformity under different pressure (a) Gelatin with no modifier (b) Gelatin with 20% wt ChCl:2 ascorbic acid

From the **Figure 5.7**, it can be seen that both the modified and the unmodified samples of the gelatin patches went through the same trend where the density of the sheet increase as the pressure of the hydraulic press rises. On top of which, the density of each set of the

samples under the same pressure has similar density, for example, the one with no additive pressed under 80 KN has a density of 1.2 g cm^{-3} which is the same as the plasticised one under the same pressure. This is important in the sense that not only the density of the drug patches can be designed and tailored using the pressing conditions.

On top of which, the standard deviation of each and every group were relatively small, which means the maximum differences between the thicknesses of patches were kept in a small range, indicates that the prepared patches were of uniform thickness.

5.3.3 Hardness test

It was noted form Chapter 3 that when used as a plasticiser, DES can cause a gradual loss of plasticity over a longer storage period. Apart from the reduced strength and the increased ductility and tackiness on the surface, it was also noticed that the softness of the material grows as the setting time elongates.

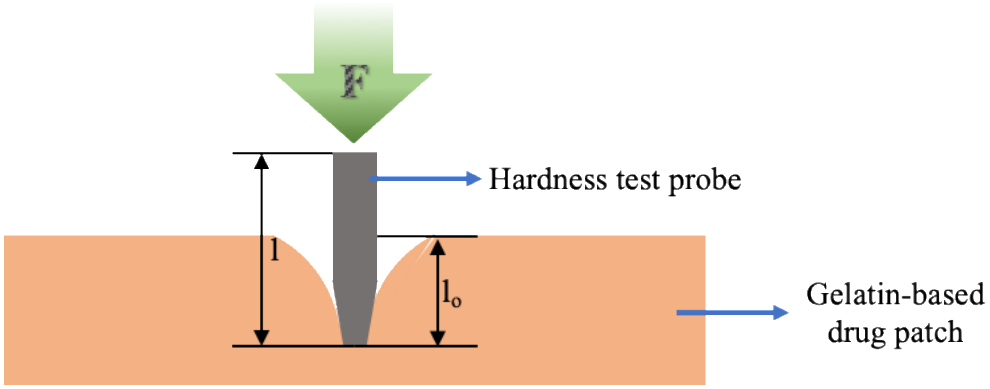


Figure 5.8 *Working mechanism of a hardness tester (l-length of the probe, l_o-depth of the penetration)*

Hardness (resistance to deformation) test were done using a Sauter HBA 100-0 Durometer by measuring the penetration depth, through a given force on a standardized pressure probe (**Figure 5.8**). The hardness of the material is presented the percentage of the hardness of the hardest material for comparison sake. **Figure 5.9** shows the hardness of gelatin patch modified with 20% wt ChCl: 2 ascorbic acid but left with different setting times. The sample with no setting time had the hardest surface while the hardness decreased as the setting time increased.

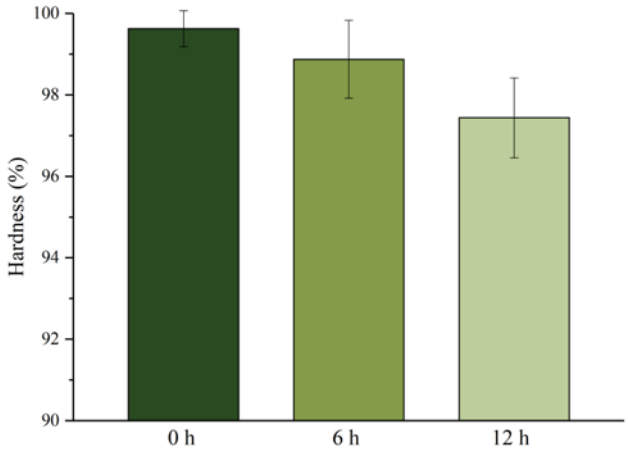


Figure 5.9 *Hardness comparison for gelatin samples plasticised by 20% wt ChCl:2 ascorbic acid after different lengths of setting time*

The hardness of a material is an indication of the way its deformation is influenced by its micro durability. It is also essential for the determining the suitability of it being considered as a drug patch candidate, as it effects many aspects of the final product, including its foldability, comfortability, and compatibility with the human skin. The above results showed that like the surface adhesion, this too can be controlled by varying the length of the setting time. From **sections 5.3.1-5.3.3**, it can be concluded that gelatin is a suitable candidate as a self-adhesive matrix for the transdermal drug delivery.

5.4 Evaluation of PDESs as transdermal formulation

5.4.1 Skin irritation evaluation

The transdermal route is an extremely attractive option for drugs with appropriate pharmacology and physical chemistry. However, to be delivered through this method, the drug formulation should be both non-toxic and should not act as an irritant to the human skin.

Irritation may be defined as a local, reversible inflammatory response of the skin to the application of an agent without the involvement of an immunological mechanism.[16] It can be due to many reasons, one of which is related to the dryness of the skin. Despite that

the SC normally experiences low relative humidity, the exposure to very dry environments can still result in defective skin conditions. The continuous hydration of the SC is therefore crucial for maintaining a healthy skin in order to play its role as a water regulator.

Choline chloride and quaternary ammonium chlorides in general, are a major component of PDESs. Apart from its ability of acting as the hydrogen bond donor to form eutectic solvents with many APIs, it has been documented to be used as a humectant in some skin care,[17] similar to humectants naturally existing in the SC. More commonly, moisturizers containing glycerol have been used for treatment or prevention of defective dry skin conditions to make the SC softer and more pliable. The biological effects of glycerol are traditionally attributed to its chemical structure. It has three hydrophilic hydroxyl groups, as shown in **Figure 5.10**, that are responsible for its water solubility and hygroscopicity, which is the ability to attract and hold water molecules from the surrounding environment, usually at normal or room temperature. Pure glycerol can absorb up to its own weight in water.[18]

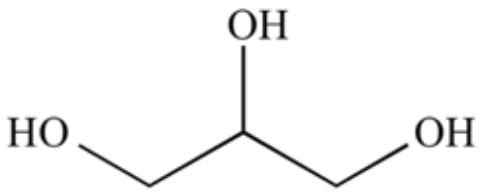


Figure 5.10 *Chemical structure of glycerol*

When being used in large amount or undiluted, both choline chloride and glycerol can draw moisture not only from the environment, but from the lower layers of skin due to their relatively high hydrophilicity. When applied topically, this could causes the skin to dry from the inside out, making them potentially irritating to the skin.

All salts are categorized as being irritants as they all absorb moisture to some extent. The amount of water which can be absorbed depend on the Lewis basicity of the medium and it would be expected that Glyceline would be less Lewis basic than pure ChCl as the chloride is already hydrogen bonding with the glycerol molecules. To quantify the hygroscopicity it is helpful to measure the mass increase of both media as a function of humidity and time.

Experiments were carried out using thermogravimetric analysis (TGA) with the humidity attachment, the percentage of the water uptake were calculated at 80 °C throughout the experiment as shown in **Figure 5.11**. The relative humidity was kept at 0% for 60 minutes, then jumped to 100 % and then held constant for 60 minutes before it went back to 0% humidity and kept at these conditions for another 60 minutes.

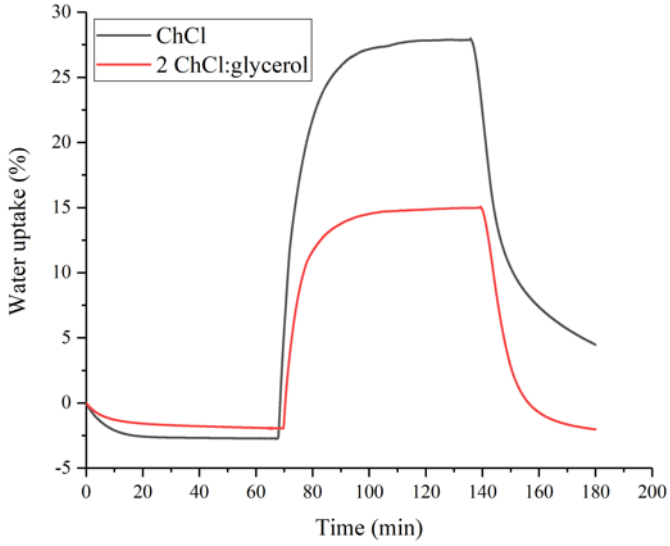


***Figure 5.11**. Comparison of water uptake by pure ChCl and a 1ChCl:2 glycerol mixture measured by TGA with the humidity attachment*

In the first 60 min of the experiment there is a small decrease in mass which corresponds to the loss of moisture which was originally in the PDES (and to a lesser extent in the gelatin) when the composite was made. During the second period, the water uptake under 100% humidity indicates the water absorption capacity is reduced after being made into to a eutectic mixture, from 25% to 14%. It could be argued that this is obvious since there is less salt in the eutectic mixture but glycerol is still hygroscopic but less so than ChCl. Furthermore, after removing the humid environment, the ChCl/glycerol manage to quickly return to its original weight within 60 mins whilst the ChCl by itself does not return to its original mass within the time window of the experiment.

The experiments above shows that by formulating into a DES reduces the risk of components with high hydrophilicity drying out the skin thus causing irritation. By forming hydrogen bonding with each other, forming DES lowers the risk of them forming

hydrogen bonding with water dawn from the lower skin.

5.4.2 Viscosity measurements

The viscosity of a fluid is measured as its resistance to gradual deformation by shear stress or tensile stress. Visually, it corresponds to the informal concept of "thickness" of the liquid. Viscosity is the property of a fluid which opposes the relative motion between two surfaces of the fluid that are moving at different velocities.

The ILs are known to have relatively high viscosities compared to molecular organic solvents[19,20] and can be judiciously varied through the choice of components, as shown in **Table 5.2**.The reason behind the relatively high viscosity is the low free volume of liquid caused by electrostatic and hydrogen bond interactions between the components. On top of which, the ions composing ILs are large in comparison to the molecules that make up most molecular solvents. These two factors combine to limit the mobility of the components of a DES.

Ionic liquids	Viscosity η /cp
EMIM BF_4	32
EMIM $N(CF_3SO_2)_2$	28
BMIM BF_4	180
BuMePy $N(CF_3SO_2)_2$	85
ChCl: urea = 2:1	632
ChCl: propanediol = 2:1	89
Acetylcholine Cl: propanediol = 2:1	117
ChCl: malonic acid = 1:1	3340
ChCl: ethylene glycol = 2:1	36

Table 5.2 *Viscosities of a variety of ionic liquids at 298 K*[21]

The viscosity of ILs was found to be proportional to the free volume of the liquid, which is also related to the surface tension in a way that the lower the surface tension the greater the free volume. This is the reason that fluorinated ionic liquids tend to have higher fluidities and lower surface tensions[22,23]

In the case of DESs, the type of HBDs and ammonium salts, HBD / organic salt molar

ratio, the water content and the temperature are all factors for the viscosities of the ionic liquids.[23]

The viscosity of PDESs in this study was measured using Quartz Crystal Microbalance (QCM). One advantage of using QCM to measure the viscosity is that it only requires a very small volume < 5 ml. A QCM sensor is made from a thin AT-cut quartz crystal inserted between two circular inert electrodes which have the same diameter as shown in **Figure 5.12**. The crystal will oscillate upon application of an external potential between the electrodes caused by the piezoelectric properties of the quartz crystal and shear deformation of the crystal will occur. The vibration of the crystal is exceptionally sensitive down to nanogram mass changes. Frequency changes of piezoelectric crystals in contact with a liquid or deposited solid are related to the ratio of kinetic energies of the uncovered crystal and the free crystal.[24]

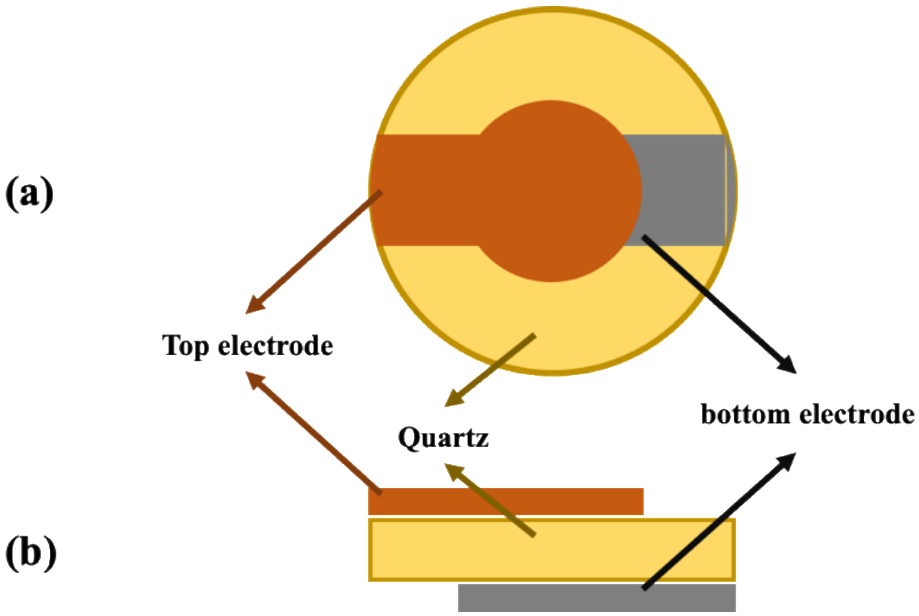


***Figure 5.12**: Top (a) and side (b) view of a QCM sensor.*

During this experiment, a polished gold crystal was first put on the top of a glass tube with only one face of a crystal being exposed to the liquid. A tube holding the crystal was then immersed in a liquid until the preferred temperature was obtained. The oscillating frequency of the crystal was determined for each liquid. The frequency of a free crystal was measured at the same temperature as the liquid. Afterwards, the frequency of the free crystal was subtracted from the crystal in a liquid to achieve the net frequency of the

crystal.

The relationship between the frequency and viscosity is given by **equation 5.1 (the Sauerbrey equation)**:

$$\Delta f = -f_0^{\frac{3}{2}} \sqrt{\left(\frac{\eta p}{\pi \mu_q p_q}\right)} \qquad \textbf{5.1}$$

Where: p and η are the density and viscosity of the solvent, while μ_q and p_q correspond to the shear modulus and density of the quartz crystal, respectively.

Study on PDES has shown that the viscosity of PDESs increases with the increasing molecular weight or size of the anion. The functional groups and their positions in HBDs will also have an effect upon the viscosity. It is suspected that the viscosity of binary eutectic mixtures is controlled by hydrogen bonds, van der Waals and electrostatic interactions.[25]

PDES type	Density /g cm^{-3}	Viscosity /cp	Glass transition temperature /^{o}C
ChCl: 2 Ascorbic Acid	1.17	686	-64.8
ChCl: Catechol	1.17	759	-76
2 Imipramine HCl: Glycerol	1.17	5312	-91

Table 5.3 *Densities, viscosities and T_g for the three PDES used in transdermal drug patches*

The liquid PDESs have glass transition temperatures rather than true melting points. This is quite common for many ionic liquids and DESs. The melting point and/ or glass transitions were measured for the PDESs using DSC. In the case of density, all three PDESs seemed to have the same density (to two decimal places) and this is not surprising as most DESs for this type are in the density range 1.10 to 1.25.

Table 5.3 indicates that the viscosities of PDESs are strongly influenced by the molecular weight of the components, hence the relatively high viscosity of Imipramine HCl based-PDES. The lower, yet still relatively high viscosity of the catechol-based PDES may be due to the intermolecular and intramolecular interactions of catechol. However contrary

to what was expected, the viscosity of the ascorbic acid/ChCl is higher than the catechol/ChCl mixture. The reason for this could be due to the additional functional groups increasing the amount of intermolecular interactions in the pure system as shown in **Figure 5.13**. Results also show that the glass transition point of a PDES is related to its viscosity.

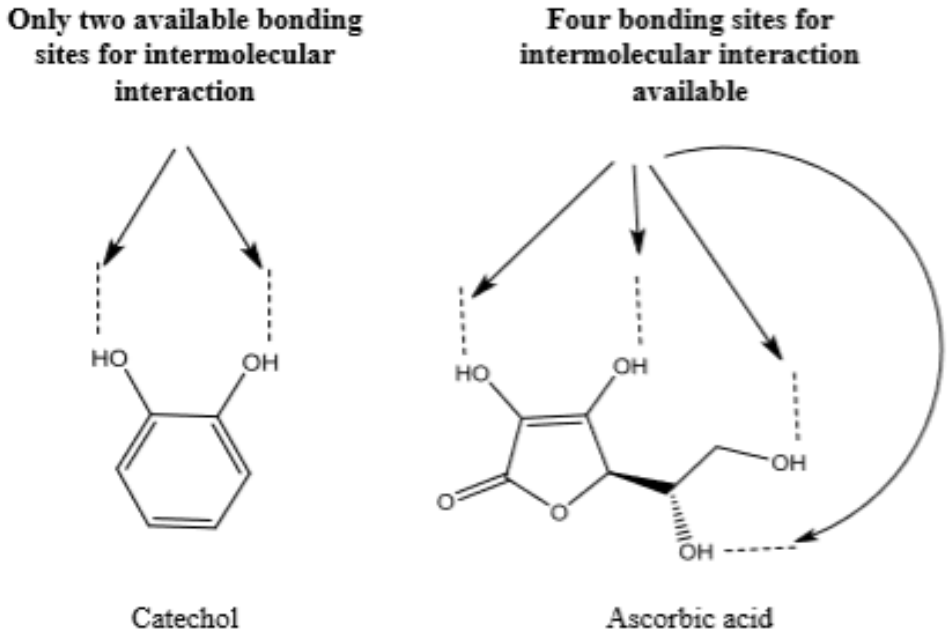


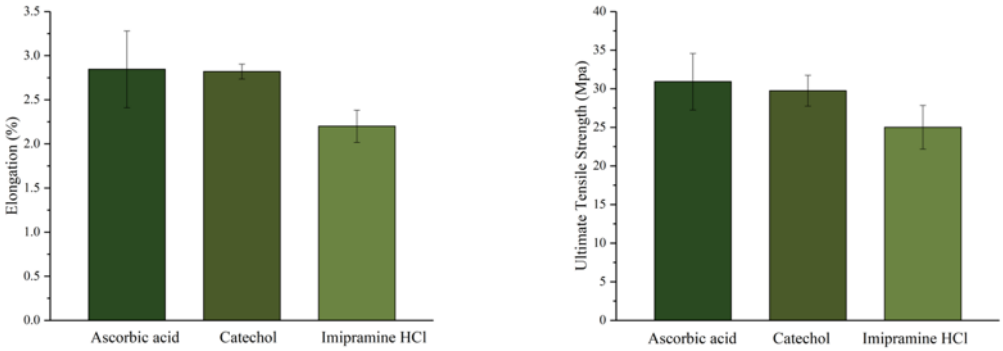
***Figure 5.13**: Schematic diagram of the interactions occurring in catechol and ascorbic acid.*[10]

Ascorbic acid includes four hydrogen bond donor sites while catechol only has two, this means that in ascorbic acid there is a greater degree of hydrogen bonding compared with other HBDs. The addition of ChCl interrupts this structure by interacting with some of the functional groups that were no longer interacting with each other. This results in a system with a greater degree of freedom, hence a reduction in viscosity. This agrees with the study described by Harris for formations of DESs for both (1,2-ethanediol with ChCl) and (glycerol with ChCl).[26]

5.5 Mechanical properties of gelatin-based PDES patches

From chapter 4 it was concluded that the DES modified gelatin is a matrix with small reservoirs of liquid, as a result, it is important to investigate the influence the physical properties such as melting point and viscosity of the DES possibly has on the overall physical property of the material.

Accordingly, experiments were done comparing the physical properties between gelatin samples prepared using different PDESs.



(a) (b)

Figure 5.14 *(a) Ultimate tensile strength and (b) elongation for gelatin samples with 20% of different PDESs after 12 hours of setting time*

Contrary to what was expected, **Figure 5.14** showed no indication that the mechanical properties being influenced by the viscosity of the modifier, in this case PDESs. Taking the standard deviations into consideration, the samples showed high similarities in both the ultimate tensile strength and the elongation, which are indications of the strength and flexibility of the drug patches, respectfully. This implies that the gelatin structure changes caused by plasticization dominates the mechanical behavior of the liquid infused system rather than the fluidity of the reservoirs. Consequently, the physical property of DES-modified gelatin materials doesn't differentiate from one DES to another.

The continuance in physical properties regardless of drug formulations is important as it resolves the concern of possible batch variations caused by different PDESs, which is an essential factor for the product quality control and the ease of application.

5.6 Transdermal drug delivery experiment design

With the combination of an appropriate choice of polymers and drug formulation, the matrix for transdermal drug delivery should allow a controlled release of the active pharmaceutical ingredient. The manner by which drug release in most of the controlled/sustained release devices including transdermal patches is governed by diffusion. Several diffusion pathways will be created to generate the overall desired steady and sustained drug release from the patches.

The transdermal patches were prepared by the same method described in chapter 3 using the hydraulic pressing technique. For the ease of the experiment, all patches were molded into the round shaped tablet form in order to keep a relatively small surface area. This is to keep patches not too far apart from each other when placing on the skin which in turn will eliminate the possible variables caused by the difference in tissue structure of the different regions. The transdermal patches were made in two batches where one of which contains API in its pure form and the other contain 20% wt/wt of Imipramine HCl-based PDESs which will also be acting as plasticizers. To achieve a higher weight uniformity of different patches without cutting the samples, as in this particular case it will also compromise the surface area of the patches, new approaches need to be taken to keep the deviation to a minimum. Multiple patches were made and only a few were selected to keep the weight of the sheets within 0.20 ± 0.01, hence achieving the same amount of drug content throughout all samples.

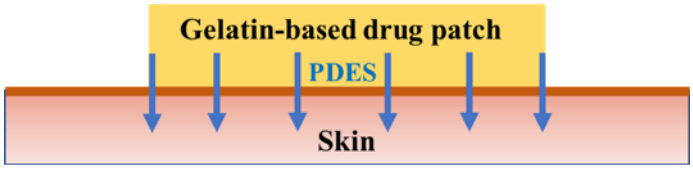


Figure 5.15 *A model for the transdermal drug delivery system showing the flow of API in blue and the flow of aqueous electrolyte in green.*

The assessment of percutaneous permeation of molecules is a key step in the evaluation of dermal or transdermal delivery systems. Experiments were designed as shown in **Figure 5.15** to determine the effect of PDES formulations on the release pattern of drug from the transdermal drug delivery system. It is predicted the transient exchange of active

ingredient across the skin is done while body fluid is being absorbed from the skin.

As a semi-permeable membrane, the skin allows only a small quantity of any drug molecule to passively infiltrate it. The penetration across the skin layer is a slow process due to the effect of the barrier properties. Consequentially, it makes skin penetration one of the biggest challenges when it comes to transdermal drug deliveries and the choice of skin model crucial to the experiment design.

As the drugs are intended for delivery to humans, the most appropriate setting to do the assessment is with *in vivo* human skin. However, this may not be possible for ethical, practical, or economic reasons, particularly in the early phases of development. It is thus necessary to find alternative methods using accessible and reproducible surrogates for in vivo human skin. Some popular skin models are as shown in **Table 5.6**.

Model	Advantages	Disadvantages
Human skin (In vivo)	Gold standard	Often precluded for ethical and practical reasons
Human skin (Ex vivo skin)	Best surrogate for in vivo humans	Not readily available, variability
Animal skin (In vivo)	Reasonably easy to obtain animals, can be scaled up to humans, hairless species available	Pigs: similar barrier to humans, but difficult to handle Rodents: different barrier properties from humans
Animal skin (In vivo)	Reasonably easy to obtain animals, can be scaled up to humans, hairless species available	Pigs: similar barrier to humans, but difficult to handle Rodents: different barrier properties from humans
Animal skin (In vivo chimeric model)	Human skin xenografts on mice allows testing on living human skin	Technically difficult
Animal skin (Ex vivo skin)	Easy to obtain	Different barrier properties, variability
Simple polymeric models	Useful for studying basic diffusion mechanisms, consistent and homogenous	Not representative of human skin
Lipid-based models	Useful for screening	Not representative of human skin
Reconstructed human epidermis	Built-in barrier properties	Usually more permeable than human skin
Living skin equivalents	Can be engineered to include a range of normal or disease features	Usually more permeable than human skin

Table 5.6 *Skin models for transdermal drug testing*[27]

In this investigation, two models have been developed from the prospective of ease of obtainability, compatibility and similarity to human skin, using *ex-vivo* animal skins from two different animal sources. There were hydrated bovine leather and pig skin.

5.6.1 Tests on bovine hides

For the preparation, a chromium tanned bovine leather sample was soaked in saline

solution for 30 minutes, kept refrigerated in sealed bags. This is to rehydrate the leather to achieve a similar moisture level to the human skin, which varies between 15 to 30 %. The hydration level for the sample was measured using a moisture meter (Testo 606-1 Moisture Meter,), ten measurements were taken each time and the average hydration levels for the leather before and after soaking were calculated as shown in **Figure 5.15**.

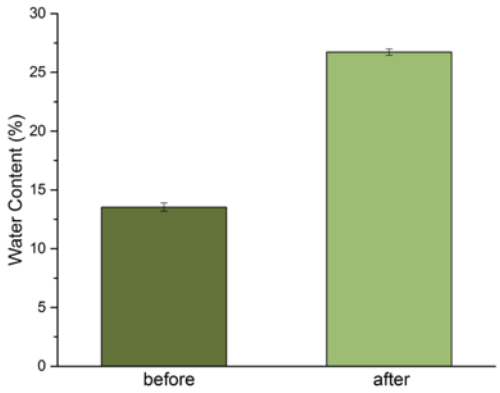


Figure 5.16 *surface hydration level change of bovine hide before and after hydration*

Experiments were then carried out by placing patches on the hydrated bovine hide sample according to the model presented in **Figure 5.15**. Two kinds of drugs were chosen (ascorbic acid and Imipramine HCl) according to their eutectic mixtures' viscosities, and each time two sets of patches (20% wt pure drug in powder form in gelatin and 20% wt PDES formulation in gelatin) pressed after a setting time of 12 hours were positioned within the distance where they are close enough to be in the same area on the leather without being contaminated by each other, as shown in **Figure 5.17**.

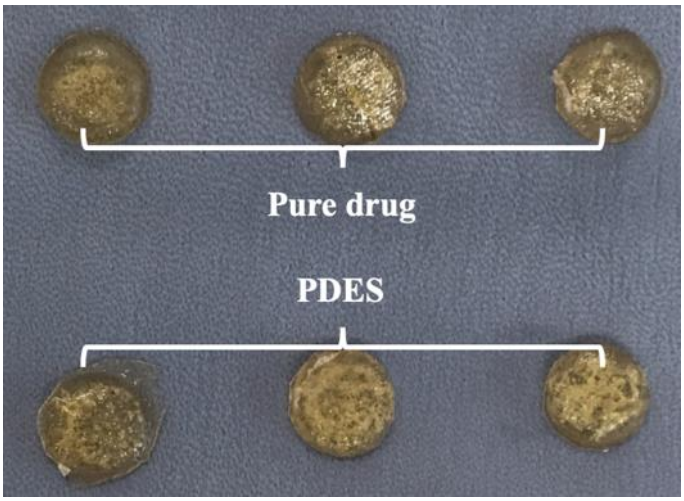
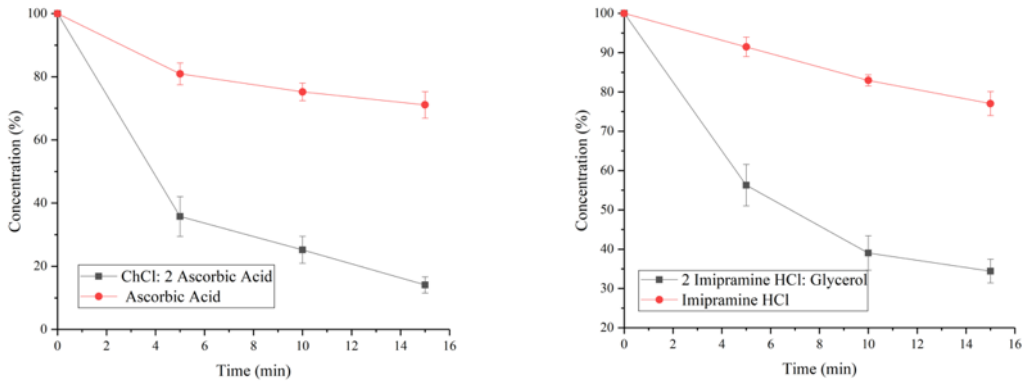


Figure 5.17 *Gelatin-based drug patches on bovine hide for transdermal drug delivery test. Above pure API in gelatin, below PDES formulation of API in gelatin*

In each experiment, two drug patches from each group were removed from the hide piece every 5 minutes and dissolved in 500 ml of saline solution at 37 ^{o}C (**Figure 5.18**), with the rotation speed of the paddle adjusted to 100 rpm. The experiment lasts for 15 minutes and were repeated 3 times.

After all the patches were fully dissolved, the resulted solutions were then analyzed by direct UV spectrophotometry to determine the remaining drug content. The collected data was calculated and plotted versus time (**Figure 5.18**).



(a) (b)

Figure 5.18 *Remaining PDES in patches after different amount of contact time with bovine hide (a) ascorbic acid and ChCl: 2 ascorbic acid (b) Imipramine HCl and 2 Imipramine HCl: Glycerol*

In the previous study, PDES as a drug formulation has been used to enhance aqueous solubility of certain APIs. It is predicted that it will also show improvements on the skin permeation through the skin barrier. The result in **Figure 5.18** showed a significant increase in the permeation rate from the patch into the hydrated leather sample. The percentage of drug release from the PDES patches after 15 minutes was found to be 65 % compared with just Imipramine HCl which released only 20 %. The result obtained from ChCl: 2 ascorbic acid showed a similar trend where the drug in PDES form was released at a much faster rate than in its pure form. The reason behind is because with the pure drug, for it to be absorbed it first needs to be dissolved in water. By comparing Imipramine HCl and ascorbic acid based PDESs, **Figure 5.19** confirms that the more viscous PDES will be slower to penetrate the skin barrier.

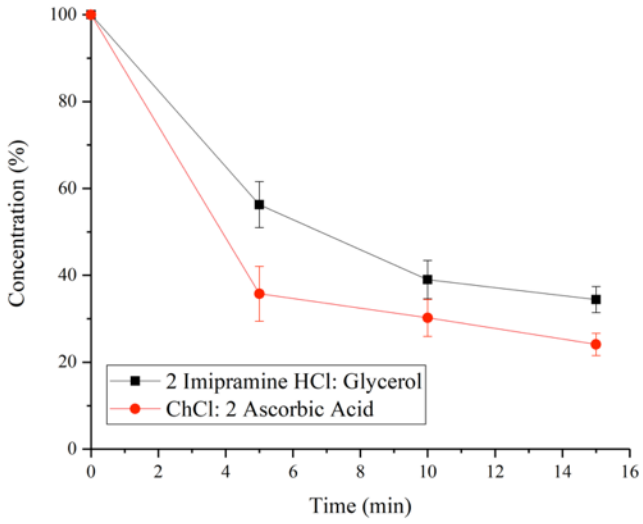


Figure 5.19 *Remaining PDES in patches after different amount of contact time with bovine hide (ChCl: 2 ascorbic acid and 2 Imipramine HCl: Glycerol)*

Research studies undertaken by Abbott et al. have shown the DESs demonstrate high solubility for polar compounds, which can be deployed to the application of leather processing.[28] It was found that the ingress of species into leather is dominated by interfacial processes and the use of DES can ensure the species partition into the solid, largely ionic matrix with less loss of the active ingredients. Additionally, the entrapment of the ionic component can act as an in-built plasticizer for the leather. This means the interaction between the bovine hide and PDESs can also be a contributor for the high

speed of the drug delivery.

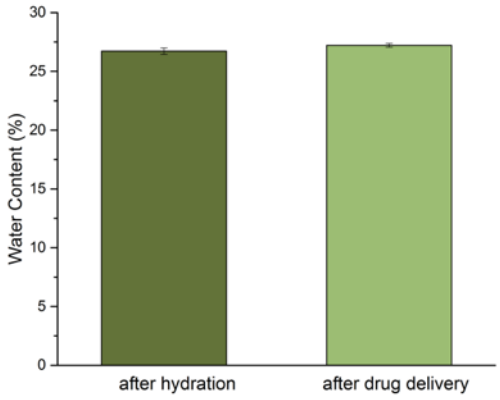


Figure 5.20 *Moisture change of bovine hides after 15 mins of contact with Imipramine HCl based PDES gelatin patch*

Moisture content was also measured before and after the experiments, results indicate no noticeable decrease in concentration (**Figure 5.20**). This confirms the PDESs being non-irritating to the skin.

The rate of diffusion (the average translational diffusion coefficient) can be estimated using the Stokes–Einstein equation (**Equation 5.2**).

$$D = \frac{kT}{6\pi\eta R} \qquad \textbf{5.2}$$

Where:

k = Boltzman constant 1.38×10^{-23} JK^{-1}

η = solvent viscosity (kg/ms)

T is temperature (K);

R is the radius of the spherical particle.

Assuming an approximate radius of the API of 5 Å and using the viscosity listed above ranging from 686 to 5312 cP then the approximate diffusion coefficient will be in the order of 6×10^{-13} m^2s^{-1}

The mean free path, $\langle x \rangle$ of a molecule diffusing is given by **equation 5.3**

$$\langle x \rangle = (Dt/\pi)^{1/2} \qquad \textbf{5.3}$$

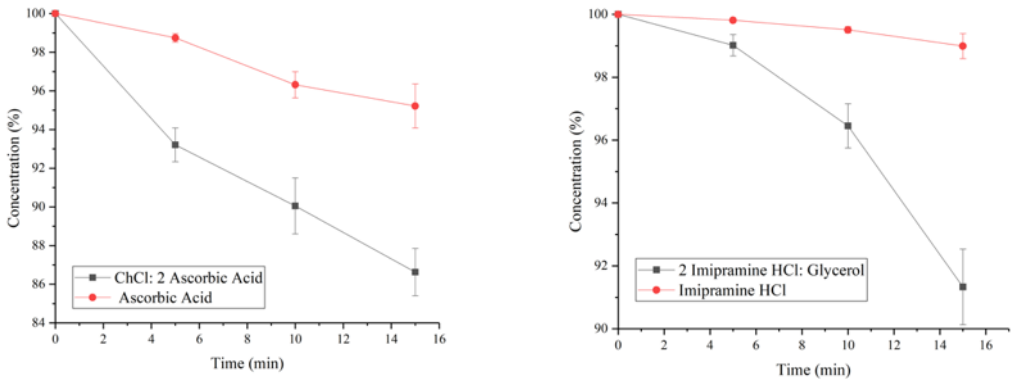
The mean distance travelled by a molecule in 16 minute experiment of **Figure 5.19** is approximately 14 μm. Given that the sample is 1 mm thick it is not surprising that no more than 40 % of the PDES has been leached in that time. Clearly this calculation makes a significant number of assumptions but it shows that the extraction rate is consistent with being limited by the viscosity of the PDES.

4.11.1 Tests on porcine cut

Pigs are often considered to be the ideal preclinical model systems thanks to the fact that they have a number of anatomic and physiologic similarities to humans in different systems. On top of which, as bovine hide was prepared by removing all fur, meat and fat, and those are essential components for a more perfected skin system, similar procedures on a pig loin cut has been studied.

A porcine loin cut was used within 2 hours of purchase. Hairs from the skin were removed with the help of a razor. Skin surface was washed with water and kept for saturation in phosphate buffer of pH 7.4 (Sigma-Aldrich) for about 15 minutes before it was used for permeation studies. Hydration level was measured as 27.1% (comparable to the one obtained from the bovine leather) before the same procedure was carried out as the one on leather (**Figure 5.21**).

Figure 5.21 *Gelatin-based drug patches on pig loin for transdermal drug delivery test*



(a) (b)

Figure 5.22 *Remaining drug in patches after different amount of contact time with pig skin (a) Ascorbic acid and ChCl: 2 ascorbic acid (b) Imipramine HCl and 2 Imipramine HCl: Glycerol*

Figure 5.22 showed a similar enhancing effect of the PDES formulation on the drug delivery behaviour as the one done on bovine hide. However, the delivery speed is much slower for both groups. With the ascorbic acid group, only a total of 13% of the drug was released after being formulated into PDES, which is still higher than pure drug by 9%. For the Imipramine HCl group, the drug in its original form was barely delivered across the skin, but 2 Imipramine HCl: glycerol managed to release 9%, which further confirms the enhancing effect of the eutectic formulations.

Noted that the experiment was kept within 15 mins because after a longer length of time, some skin tissue started to be peeled with the patches. Figure 5.23 shows that, as before with the bovine leather, ascorbic acid based PDES is extracted faster that the Imipramine HCl based PDES which is thought to be due to viscosity differences.

Neither of these skin mimics is ideal; the leather sample contains no hair follicles and no fat whereas the pork loin contains too much fat. Both show that PDESs diffuse faster than the pure APIs in the same formulation. The diffusion rates are also consistent with a model based on the viscosity of the PDES.

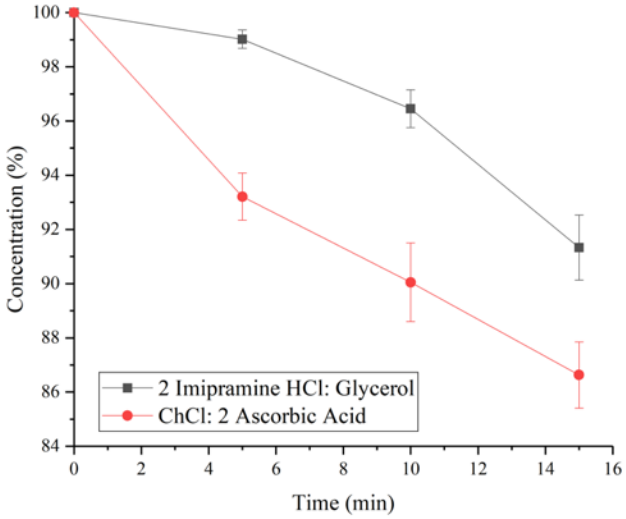


Figure 5.23 *Remaining PDES in patches after different amount of contact time with pig skin (ChCl: 2 Ascorbic Acid and and 2 Imipramine HCl: Glycerol)*

5.7 Summary

Investigations have demonstrated the efficacy of using PDES modified gelatin as a material for transdermal drug delivery system. Attempts were made to investigate the feasibility of using gelatin as the patch material which includes the surface adhesiveness test that showed interesting results as the gelatin surface became more sticky as the setting time goes up, despite the fact that it was formulated by the same components. This opens the possibility of developing a drug patch with no additional adhesive. Uniform densities were also proved to be achievable using different pressure.

PDESs were then studied for its skin compatibility. ChCl is proven to be an irritant for human skin as it is highly hydrophilic and will absorb moisture from the epidermis. The irritation potential of these compounds was investigated by measuring the water uptake abilities of different formulations of ChCl-glycerol DESs using TGA. The results showed that by formulating DES, the water absorbance was decreased compared to pure ChCl, hence lowing the risk of skin irritation.

The properties of the patches are somewhat dependent on the PDESs in terms of their strength and ductility, so they feel slightly different in terms of their flexibility, but the differences are relatively small, and all perform well enough to be useful for practical

patches.

Two sets of transdermal patch model were then designed based on different skin alternatives. On both the bovine hide and the porcine loin cut, PDES as a formulation showed enhancing effect on the delivery speed of both drugs (ascorbic acid and Imipramine HCl). However, results showed a much slower diffusion cross pig loin which can be resulted from the combination of the plasticising effect of PDESs on leather and rawhides' more porous structure which will facilitate a faster drug delivery.

Although the uptake on pig skin is relatively slow, it can still be predicted that all drug can go in within a period of hours, which is still ideal for applications. From the viscosity results, it can be seen that all three PDESs are relatively viscous. Especially for Imipramine HCl/glycerol mixture, which has a viscosity as high as 5000 cP. Notwithstanding the drug still managed to go across the skin barrier showing the great potential DES has on drug formulations. By lowering the viscosity of the PDES, i.e. adding other additives, a significant improvement can still be made in its releasing speed.

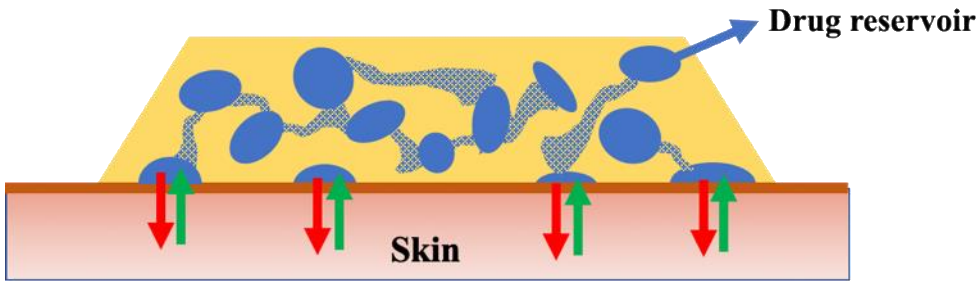


Figure 5.24 *Schematic diagram of the gelatin-PDES transdermal drug delivery system (not to scale)*

More importantly, the fact that the drug can be delivered across the skin continuously using the gelatin-PDES matrix indicates that not only there are small pools of PDES inside the system in liquid forms, they are connected in a way that they can be delivered by diffusion, possibly through small tubules, as shown in **Figure 5.24**.

In conclusion, gelatin-PDES can successfully make a transdermal drug delivery matrix, but the releasing speed is dependent on the PDES's viscosity. Previous work has shown fluidity being dependent on the drug size, there for this matrix should work for drugs with smaller molecule sizes. Aspirin discussed in chapter 3 should have worked if it did not

react with the DES itself, so for this method to move forward, one need to make sure that the active drug molecule does not react with the DES formulation.

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6. Conclusions and future work

This study is the first to investigate the modification of proteins using deep eutectic solvents. While starch based composites have been previously studied the interaction of DESs with proteins is still unstudied. If the work of carbohydrates is transferrable to polypeptides then it could be expected that novel plastics can be produced. The initial aim of the study was to characterise the properties of three polypeptides with some standard DESs. The production of plastic materials with suitable properties would then lead to their use in biomedical or pharmaceutical applications. The cost of proteins compared to carbohydrates would always mean that the materials would not necessarily lend themselves to bulk plastics such as packaging but it would be more likely to be reasonable for applications where biocompatibility was an important property.

In the first results section three polypepties were used namely gelatin, zein and soy protein. These were chosen as they are readily available at low cost and in a relatively pure form and constant composition. They were also chosen to demonstrate the difference between fibrous and globular proteins. In this section the DES Glyceline was used as it is non-toxic has an intermediate viscosity similar to some of the pharmaceutical DESs use in later chapters and it was the liquid studied in most detail for starch modification.

All of the polypeptides studied made plastics when mixed and pressed with Glyceline but all three had very different properties. The materials had to be pressed at a far lower temperature than those used for starch so as to avoid thermal decomposition. The globular proteins chosen in this study, zein and soy, both showed a significant change in the properties by the addition of DES. As with starch, the optimum amount of DES was found to be about 20 wt%. Previous studies showed that the DES was not very soluble in polyethylene due to the lack of hydrogen bond interactions but proteins can clearly absorb more liquid due to the extensive network of amide linkages and sidechains capable of forming hydrogen bonds. Conductivity measurements showed that the modified proteins were conducting in the same way that DES-modified starch was and this shows that there is a continuous film of DES throughout the material. While unsurprising, this may be useful in later applications, particularly for the transdermal drug delivery systems.

The plastic formed from zein protein and Glyceline was weak and brittle and of very little

mechanical integrity. The material formed from soy protein was slightly stronger and has a larger elongation at break, particularly when 20 wt% Glyceline was added. Both of these materials were, however, weaker and less ductile than the starch based materials previously reported in the literature. The material made from gelatin showed variable properties which appeared to depend on how long the two components were mixed for. If the components were mixed straight away then a strong but brittle plastic formed which displayed a strength similar to HDPE or polypropylene. If the components were mixed for 24 h before pressing then a weak but very flexible material resulted. Samples which were pressure molded did not, however change their properties depending on how long they were stored. This shows that diffusion of the liquid into the gelatin was slow at ambient temperature and once pressed the gelatin retained its shape and the DES no longer diffused in the structure i.e. the temperature fixed the gelatin structure.

Biocompatibility, particularly when they are put into the human body or contacted with the skin was the most important parameter for the remainder of the thesis and so chapters 4 and 5 focussed solely on gelatin modified with PDESs. The aim was to use gelatin as a drug delivery system for both orally ingested tablets and as a medium for trans-dermal drug delivery. Both applications require a material which is soft, tough and flexible. For these reasons the future studies will focus on the gelatin DES systems particularly using DESs formed from HBAs and HBDs which are pharmaceutical active compounds.

In Chapter 4 of the thesis, composites were made of gelatin with pharmaceutical DESs for oral ingestion. Four PDESs were chosen and their dissolution rates were measured from a gelatin matrix. It was found that acetylsalicylic acid lost its pharmaceutical property in a PDES formulation due to unexpected side reactions. PDESs based on ascorbic acid and catechol showed enhanced solubilisation rates compared to the pure API.

When the PDES was incorporated into gelatin based tablets it was found that the release time was dependent on how the tablet was made. When the ingredients were left to mix for an extended time before the pill was pressed more API could be incorporated compared to tablets pressed straight away showing that the DES was slow to diffuse into the gelatin structure.

XRD analysis of the gelatin based materials show similar patterns. The material is largely

amorphous when modified with DESs and the degree of crystallinity decreases the longer the materials are mixed prior to pressing. This supports the idea that the DES is slow to diffuse into the gelatin structure.

^{1}H NMR measurements were carried out on the DES modified gelatin samples and it was found that signals for the DES component could clearly be observed. This shows that the DES exists in small pools in the material rather than acting as an adsorbed modifier. It was found that samples with a longer setting time had the most mobile DES. As the temperature increase to near the glass transition temperature of gelatin, all spectra become sharper indicating an increase in mobility, and for the 24 and 48 h samples two peaks can be distinguished. This supports the theory that there are micro domains of liquid in the gelatin samples with longer setting time.

In Chapter 5, PDES modified gelatin was made into a patch for transdermal drug delivery. A useful property of these patches is that they are naturally sticky and will adhere to skin without the need for an added adhesive. The properties of the patches are somewhat dependent on the PDESs in terms of their strength and ductility so they feel slightly different in terms of their flexibility, but the differences are relatively small and all perform well enough to be useful for practical patches. It was found that for the small number of compounds tested, drug delivery rates were much higher than those from comparable materials made with the same API in a solid form and not in a PDES. The rate of extraction of the PDES from the patch was dependent on the viscosity of the PDES as might be expected. In all cases, however a significant drug delivery could be achieved in 15 minutes. Practical delivery systems for topical drug delivery would want to administer the API in 10s of minutes. Two skin mimics were tested and both showed that this timescale was achievable.

In conclusion, this study has shown for the first time that proteins can be modified with DESs to produce mechanically strong materials with a significant content in an amorphous form. The DESs form poor of liquid within the structure and this can be used to release active pharmaceutical agents either through the stomach or across the skin barrier. These could form the basis for controlled drug release systems which are biocompatible.

However, this is the first study to modify proteins with DESs and as such there are many new areas that could be studied.

In the first part of this study the properties of three proteins with one DES were studied. This is clearly an area that needs to be expanded upon. Previous studies have shown that starch-DES mixtures can significantly change their properties depending upon the type and amount of DES used. Interestingly it was found that the viscosity of the DES did not directly correlate with the strength or ductility of the plastic produced. It would be useful to test the effect of the DES on the properties of other proteins to understand how the secondary and tertiary structure of the proteins affects the mechanical properties of the plastics.

It would also be interesting to do this with proteins such as collagen. Previous, unpublished results from the group have extruded collagen with a chromium-based DES and found that it could cross-link the collagen to form a leather-like material. It would be interesting to extend this study further to understand the mechanism of interaction and structure of the material.

Previous studies with starch-DES mixtures used conductivity to monitor the setting process. This study could be extended to protein-based plastics to understand the flow of charge through the materials. It would be useful to characterise how the conductivity is affected by mixing time as this would provide more information about the nature of the channels through the material.

Extrusion was used in previous studies with starch-DES mixtures where it was found to significantly increase the quality of the materials in terms of their strength and ductility. It was also found that the extrusion conditions significantly improved the properties of the materials. Extrusion was not attempted in this project as the materials appeared to form without mechanical assistance but it would be interesting to compare the properties. It would also be important to understand the stability of some proteins with the operating conditions of extrusion.

This study has shown that the viscosity of PDESs is important as it affects the diffusion of the API in the transdermal drug delivery systems. Recent work has shown that water DES mixtures are not totally miscible but they can maintain the API in an ionic

formulation while significantly reducing the viscosity. It would be interesting to determine whether this approach could be used to regulate the rate of drug delivery.

Electrical signals can be applied across the skin to enhance the rate of transdermal drug delivery. This can be done by two methods, iontophoresis and electroporation. Iontophoresis applies a small voltage at a continuous constant current (c.a. 10 V and 0.5 mA cm^{-2}) to enhance the mobility of charged drug molecules. Electroporation, however, uses short pulses of higher voltage (c.a. 100 V of ms duration) to aid transport. The former is already used clinically with a variety of drug patches. The conductivity of the DES would be a significant advantage for this method.

In previous work it was shown that polyolefins such as HDPE could be coextruded with starch using DESs as homogenising agents/ lubricants. It would be interesting to see whether structural polymers such as Nylon or polypropylene could be coextruded with proteins. These could be used for implants to improve biocompatibility or potentially done with a patient's own collagen to decrease the rejection. It would be useful to determine the rate of biodegradation of these types of materials both in the pure state and as composites.