Ionically Cross-Linked Alginate Hydrogels as Drug Delivery Systems for Analgesics in Broiler Chickens

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Thesis Abstract

Treating birds with analgesic drugs requires continuous injections of near lethal concentrations to maintain the therapeutic dose in the blood plasma. This is due to birds having higher metabolic rates than mammals. Therefore, there is a need to develop drug delivery systems that can control and slow down the release of analgesics in birds. This study was designed to analyse the sustained release of the model analgesics, sodium salicylate and sodium aspirin, from ionically cross-linked alginate hydrogels, in *in vitro* and *in vivo* experiments using broiler chickens as the model bird. Analgesic loaded hydrogels separated into two layers, unlike the homogeneous blank hydrogels. This was labelled as the separation effect. Swelling studies indicated the absence of the insoluble cross-linked alginate material in the hydrogels where the separation effect occurred, with most of the hydrogels dissolving back into the medium. The highest equilibrium swelling percentage achieved in the loaded hydrogels was 68 %. In comparison, the highest equilibrium swelling percentage in the blank hydrogels was 622 %. *In vitro* drug release profiles showed that the hydrogels released up to 100 % of the sodium salicylate within 3.33 hours. In contrast, the hydrogels containing sodium aspirin released only 35 % of the encapsulated drug. Hydrogels containing a drug concentration of 150 mg/mL were injected into the model birds at a dose rate of 150 mg/Kg. No chicken reacted negatively to the hydrogel injection. *In vivo* results indicate sustained release of the model analgesic from the hydrogels compared to the release from the aqueous solutions of the drug. The effective concentration for an analgesic effect of sodium salicylate was maintained by the group injected with an aqueous solution of sodium salicylate 18 hours after the injection. The groups injected with the hydrogel with the maximum calcium chloride content saw the largest sustained release, with the plasma concentration of sodium salicylate remaining over the effective concentration for up to 36 hours after the injection.

Keywords: Sodium salicylate, sodium aspirin, hydrogel, analgesia, sustained release, broiler chicken.

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Chapter 1: Introduction

Every year, thousands of tons of pharmaceutical drugs are used worldwide in veterinary medicines (Meng, Zhang, Zheng, & Ai, 2014). One of the more successful parts of this industry is the use of analgesics, with the U.S. market making more than 13 billion dollars in over the counter medicines (including analgesics) in 2014 and this increased to over 14 billion dollars in 2015 (APPA, 2016). The reason behind this is simple: all animals at some point need relief from pain. Various species of birds and small mammals are becoming commonly kept as pets and increasingly presented to vets for painful conditions, with some needing surgical procedures. The use of analgesics is continuing to rise, emphasizing the importance of this class of drugs.

The metabolic rate in birds is much higher as compared to mammals due to the analgesic drugs clearing much faster in birds. Singh *et al* (2010, 2011) administered opioids in broiler chickens at the mammalian dose which lasted for 10 minutes. Salicylic acid also, when administered at a higher dose, was effective for 1 hour (Singh *et al*, 2011). The other studies on analgesic effects of carprofen on lame broiler chickens and butorphanol in lame turkeys (Danbury *et al*, 2000; McGeown, Banbury, Waterman-Pearson, & Kestin, 1999; Buchwalder, 2005) depict the similar problem of short duration of analgesia and a need for repeated doses to prolong the efficacy of a drug. The repeated administration could be lethal for the bird or cause swelling and inflammation at the site of injection, hence inadvertently putting the birds under considerable distress. As a result, finding new drug delivery systems to treat birds with pain is critical and necessary to help achieve maximum comfort for the bird and reduce the current limitations present in the administering of analgesics.

One field that has been successful in the delivery of analgesics is hydrogel chemistry (Coviello, Matricardi, Marianecci, & Alhaique, 2007) as some hydrogels are seen as non-toxic, biodegradable, cheap to make, and can release the analgesic in a sustained manner (Nguyen, Huynh, Park, & Lee, 2015; Ahmed, 2013).

This research is focused on developing a cheap, non-toxic, bio-degradable and sustained releasing hydrogel loaded with an analgesic to treat pain in the avian species over a

period. The objective is to load the hydrogel with an analgesic drug, inject it subcutaneously into chickens (as the model animal), and measure the concentration of the analgesic in the blood plasma at specific times over a period of time to demonstrate a suitable hydrogel has been prepared. In order to achieve this goal, the following three goals were set:

1) To develop an ionically cross-linked alginate hydrogel where the extent of swelling in water and biological media was manipulated by controlling the degree of cross-linking.

2) To load the hydrogel with a model analgesic and measure the rate of drug release in vitro to identify the degree of cross-linking required to achieve sustained release of the encapsulated drug.

3) To inject the drug-loaded hydrogels into chickens in a safe manner and achieve a concentration of 50 μg/mL analgesic present in the blood plasma of the chickens for as many hours as is achieved by an aqueous dose of the analgesic.

1.1. Definitions and Categorisations of a Gel

An important part of this research is distinguishing between the terms gel and hydrogel. To understand this, the different definitions of a gel need to be further explained.

1.1.1. Definition of a Gel According to IUPAC

The IUPAC recommendations 2007, defines a gel as "Non-fluid colloidal network or polymer network that is expanded throughout its whole volume by a fluid". A gel has a finite yield stress and can contain either a (1) covalent polymer network, (2) polymer network formed through physical aggregation of polymer chains, (3) polymer network formed through glassy junction points, lamellar structures, or (4) particulate disordered structures (Alemán et al., 2007).

1.1.2. Definition of a Gel According to the Academic World

Defining a gel has been in question for many years in the academic world, due to the increasing research conducted on them. In its simplest form, a gel is a "soft material"

which comprises at least two basic components; a polymeric network permeated by a solvent, where the polymeric network is solid and the medium (solvent) is a liquid, though this is not always the case (Rogovina, Vasil'ev, & Braudo, 2008; Carretti, Dei, & Weiss, 2005). The polymer volume fraction can vary between gels and this usually ranges from 0.1 – 10 % of the total volume which means that gels consist of up to 90 % solvent. Furthermore, the solvent can be water or an organic solvent such as oil. With such a large volume of liquid solvent, gels should behave like a liquid, yet they behave like a solid because of the three-dimensional cross-linked network which forms between the polymer molecules within the liquid (Rogovina, Vasil'ev, & Braudo, 2008; Carretti, Dei, & Weiss, 2005; Sangeetha, & Maitra, 2005).

1.1.3. Classification of Gels

A gel can be classified by its medium, which can range from organic liquids, such as oils or alcohols, to water. Furthermore, the medium is not required to be in its liquid phase. There are some examples of gels where the medium is gaseous (Alemán et al., 2007). Table 1 shows some of the classifications of gels based on their media. Note that for this project, the focus will be on hydrogels from here onwards.

Table 1. Classification of gels

Medium	Special requirements	Gel classification
Oils (e.g. olive oil)	-	Organo-
Alcohols	-	Alco-
Gaseous medium	-	Aero-
Water	Network component is polymeric	Hydro-
	Network component is colloidal	Aqua-
No Medium	Medium removed from gel using various methods	Xero-

1.2. Hydrogels

Hydrogels are hydrophilic three-dimensional polymeric networks which are held together through physical bonds or chemically cross-linked through covalent bonds as shown in Figure 1. They have a highly porous structure which gives them the unique ability to absorb large amounts of water or biological fluids and swell without the loss of structure or dissolution (Nguyen, Huynh, Park, & Lee, 2015; Ahmed, 2013; Li, Rodrigues, & Tomás, 2012). This hydrophilicity arises from the presence of polar, hydrophilic groups along the backbone of the polymer strands (Li, Rodrigues, & Tomás, 2012) which includes amino, carboxyl, and hydroxyl groups.

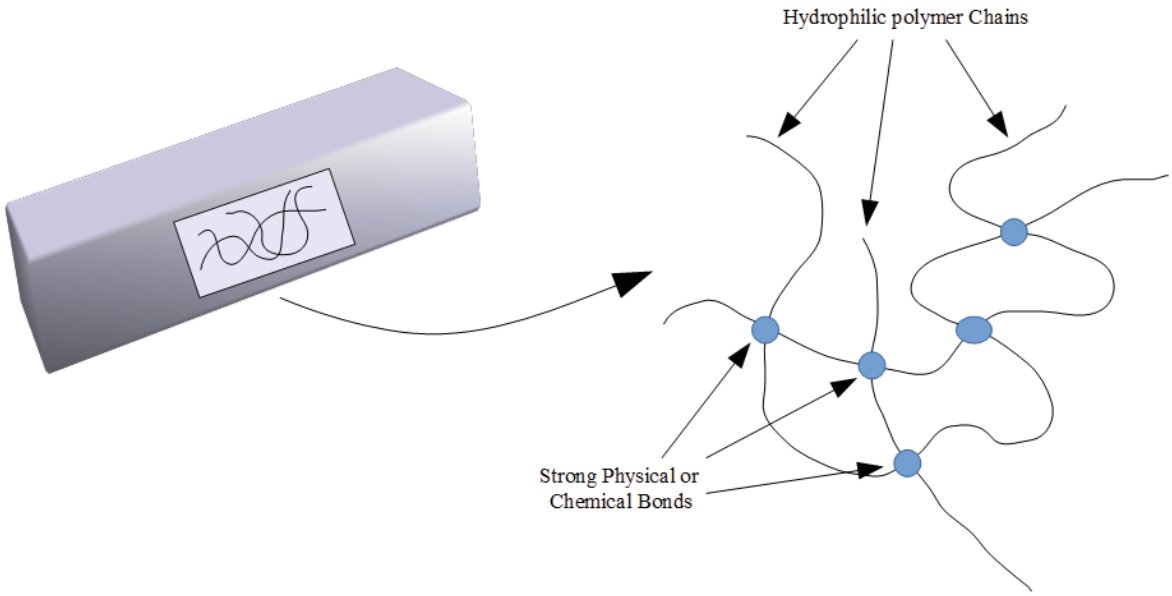

Figure 1. Simple schematic of a hydrogel showing the hydrophilic polymer chains and the chemical or physical cross-links (or bonds).

1.3. Classification of Hydrogels

Throughout the years of research, numerous classifications have been applied to hydrogels and hydrogel products. These classifications are described below (Ahmed, 2013) along with the use of the term "smart" hydrogels (Rafi, & Mahkam, 2015).

1.3.1. Six-Step Classification of Hydrogels

There are six classifications that hydrogel products can be grouped into based on their compositions, with most products being grouped into all six classifications. These classifications are described below.

1. The source of the polymer can be either natural or synthetic.

2. The polymeric composition of the hydrogel product can either consist of one polymer (homo-polymeric), two different polymers (co-polymeric) or a multi-polymer interpenetrating polymeric hydrogel (IPN).

3. The configuration or the physical structure and chemical composition of the hydrogel product can be classified as amorphous, semi-crystalline, or crystalline.

4. The type of cross-linking which holds the hydrogel product together can be classified as chemical or physical cross-linking.

5. The physical appearance of the hydrogel product.

6. The network electrical charge of the polymer backbone. It can be either neutral, ionic, an amphoteric electrolyte or zwitterionic.

1.3.2. Stimuli-Sensitive Hydrogels

One unique class of hydrogel that has been increasingly appearing in the academic field is the stimuli-sensitive hydrogels. Given the name “smart hydrogels”, these hydrogel products are similar to conventional hydrogels, except they have different swelling behaviours when introduced to different stimuli such as pH and temperature.

These smart hydrogels have become extremely popular in research due to their extensive applications in many fields including pharmaceuticals. Rafi and Mahkam (2015) took advantage of the pH difference in the colon and prepared a magnetic pH-sensitive film with alginate for colon specific drug delivery. By combining magnetic particles with a pH-sensitive hydrogel, they were able to prepare a smart hydrogel

which was sensitive to external magnetic field as well as behaving differently in acidic and basic solutions. Islam *et al.* (2012) also took advantage of smart hydrogels by preparing a pH-sensitive chitosan/poly(vinyl alcohol) hydrogel cross-linked with tetraethoxysilane to achieve controlled release of aspirin. These are just two of the many examples of articles where the authors took advantage of smart hydrogels and applied them to their drug delivery systems.

1.4. Characterisation of Hydrogels

Hydrogels can be characterised in a number of ways. The following section introduces characterisations of hydrogels.

1.4.1. Swelling Capabilities

Perhaps the most important characteristic of hydrogels is their swelling capability. Once the hydrogel is introduced to water, it goes through a number of steps of hydration until equilibrium is achieved between the forces encouraging the absorption of water and the forces resisting the absorption of water are balanced. When water is introduced to the hydrogel, it immediately hydrates the polar hydrophilic groups which relax the polymer backbone, allowing the hydrophobic groups to be exposed and hydrated. This leads to the formation of primary and secondary water respectively. Once the two water groups are formed, the hydrogel continues to absorb water which is referred to as free water due to the osmotic driving force of the polymer chain, and it is believed to fill the spaces between the polymer chains and/or the centre of the pores. However, there is an opposing cohesive force exerted by the polymer chains which resists the swelling of the hydrogel beyond a certain limit and over time, a balance between these two opposing forces will be achieved and hence equilibrium swelling is achieved (Nguyen, Huynh, Park, & Lee, 2015). Figure 2 summarises the hydration step and interactions between water and groups on the polymer chain.

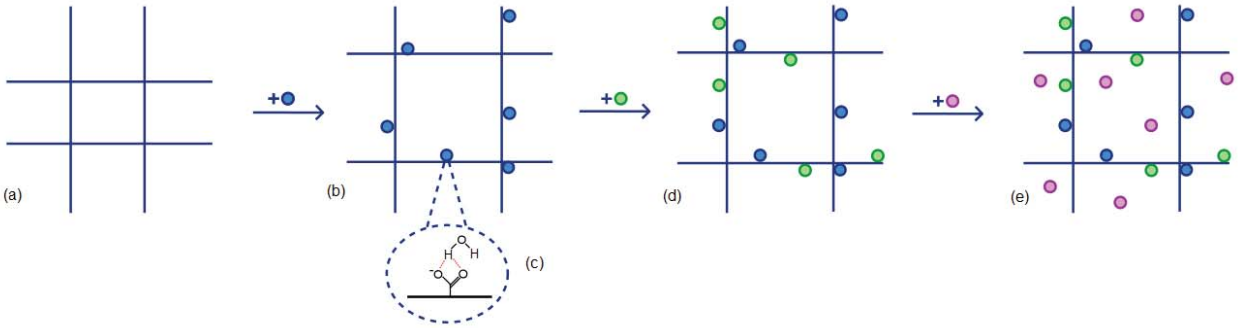

Figure 2. Hydration of a hydrogel. (a) Dried, water free hydrogel, (b) Hydrogel introduced to water resulting in the formation of primary water (blue), (c) Interaction between primary water and hydrophilic group of polymer chain, (d) Formation of secondary water (green), and (e) Formation of free water (purple).

1.4.2. Toxicity and Biodegradability

High on the list of important characteristics of hydrogels are their toxicity and biodegradability. Depending on the type of polymer and cross-linker, many hydrogels exhibit low toxicity and are highly biodegradable which makes them ideal for biological applications (Nguyen, Huynh, Park, & Lee, 2015; Ahmed, 2013; Li, Rodrigues, & Tomás, 2012; Gulrez, Al-Assaf, & O Phillips, 2011). This low toxicity and biodegradability mainly arises from the use of natural polymers. For example, a popular natural polysaccharide polymer used in hydrogel production is sodium alginate (Ahmed, 2013) which can be isolated and extracted from marine brown algae. It exhibits low toxicity and is biodegradable in biological environments which make it ideal for biological applications.

The cross-linker can also affect the toxicity and biodegradability of hydrogels as some can be toxic or have low to nil biodegradability. This is much more common in chemical cross-linked hydrogels.

1.4.3. Flexibility

Given a hydrogel's large water content, many have a soft consistency which is able to mimic natural tissue due to its specific viscoelastic properties (Saarai, Kasparkova, Sedlacek, Kitano, & Saha, 2012). This means that hydrogels have a large degree of

flexibility and can move like natural tissue, which is advantageous in the bio-medical field for using hydrogels as wound dressings, drug delivery systems, and scaffolding in tissue engineering (Saarai, Kasparkova, Sedlacek, & Saha, 2011).

Saarai *et al* (2011) prepared a sodium alginate/gelatin hydrogel cross-linked with either calcium ions (from $CaCl_2$) or glutaraldehyde. They demonstrated the swelling and the flexibility of a 50:50 sodium alginate:gelatin hydrogel cross-linked with calcium ions was optimal for producing a wound dressing capable of protecting the wound in a moist environment to promote healthy healing.

1.4.4. Biocompatibility

This is another extremely important characteristic of hydrogels. Ideally, hydrogels introduced into organic tissue, along with the degradation products formed during decomposition of the hydrogel, need to be biocompatible with the immune system to prevent side effects, such as inflammation and rejection from the host.

In most cases, this is not a problem as their hydrophilic surfaces have a low interfacial free energy when introduced into organic tissue, resulting in proteins and cells rarely adhering to these surfaces (Gulrez, Al-Assaf, & O Phillips, 2011).

1.4.5. Drug Entrapment and Release

Hydrogels have the ability to be loaded with different drugs depending on the desired application and can release these drugs in a sustained manner depending on the diffusion coefficient of the drugs through the hydrogel network. These abilities arise from the porosity of hydrogels and can be easily controlled by either 1) altering the porosity of the hydrogel or 2) employing physical and chemical strategies to slow release (Hoare & Kohane, 2008). By altering the degree of cross-linking present in the hydrogel structure, the sustained release of a drug can be slowed down or sped up, depending on the desired application. In theory, the higher the degree of cross-linking, the less space available between the polymer chains, the longer it takes for the drug to diffuse out of the hydrogel. This concept is dependent on the size and properties of the drug molecule (Peppas, Bures, Leobandung, and Ichikawa, 2000).

There are two main methods in loading a drug into a hydrogel; 1) prepare the hydrogel in the presence of the drug, or 2) prepare the hydrogel in advance and swell the hydrogel in a drug solution. The method that is chosen mainly depends on the drug being loaded into the hydrogel or the hydrogel being prepared.

1.5. Preparation of Hydrogels

Hydrogels can be prepared using different methods depending on the required structure and application. Two of the methods are discussed below

1.5.1. Chemical Cross-linking

This process is one of the most common methods in the preparation of hydrogels. It takes advantage of using a bi-functional cross-linking agent, such as glutaraldehyde, to form new intermolecular or intramolecular covalent bonds within the polymeric network (Nguyen, Huynh, Park, & Lee, 2015; Adbelhalim, 2006). It is important that the polymeric network chosen contains the appropriate side chain groups which can chemically react with the cross-linking agent to form the covalent bonds. The gelation process is influenced by the method chosen. Some methods require the use of preparation processes, catalysts, attention to hydrolytic stability and other specific properties of hydrogels (Nguyen, Huynh, Park, & Lee, 2015), while other methods require fewer steps as the cross-linking agent is strong enough to act like the catalyst. The resulting hydrogels are geberally strong, permanent, and the resulting three-dimensional structure allows entrapment of water, therapeutic agents, or living cells which is highly advantageous for applications in the biomedical field (Nguyen, Huynh, Park, & Lee, 2015; Gulrez, Al-Assaf, & O Phillips, 2011). Due to these properties, these types of hydrogels are usually used for implantable application. However, injectable hydrogels have received considerable attention, resulting in more chemically cross-linked hydrogels being formed *in situ.*

There are many examples of chemically cross-linked hydrogels in the academic world. For example, many researches take advantage of the Schiff's base reaction between the natural polymer, chitosan, and the cross-linking agent, glutaraldehyde. In this case, the nucleophilic amine groups present on the chitosan react with the electrophilic carbon atoms on the di-aldehyde, glutaraldehyde (Nguyen, Huynh, Park, & Lee, 2015). Lu *et al*

(2015) used this method to prepare rectorite/chitosan gels where the rectorite was "entrapped" within the 3D network formed by the crosslinking of chitosan and glutaraldehyde. The gel showed excellent absorption and retention of water and the dye, methylene blue. From these observations, Lu *et al* concluded that this gel could effectively remove cationic dyes from aqueous solutions. Figure 3 shows an example of the Schiff's base reaction mechanism between chitosan and glutaraldehyde.

Another common chemical cross-linking reaction is the reaction between polymers and heterocyclic compounds. These reactions typically involve the addition of nucleophilic groups on the polymers to the electrophilic carbons on the heterocyclic compound, which results in either (a) the opening of the heterocyclic compound, or (b) opening of the heterocyclic compound, conversion of one of the active groups, and re-closing of the heterocyclic compound. There are many of these reactions recorded in the literature, but one of the most common heterocyclic compounds is genipin, which is a naturally occurring compound present in gardenia fruit extract. It is highly effective in cross-linking natural polymers such as chitosan and is especially used for its non-toxicity. However, one limitation is the price as it is obtained via enzymatic hydrolysis with β-glucosidase (Xu, Huang, Zhu, & Ye, 2015). Nevertheless, it has been used in the literature to do a number of things. Lins *et al* (2014) used genipin to prepare composite poly(3-hydroxybutyrate) (PHB) /chitosan microparticles. The authors prepared microparticles of PHB containing the drug, ketoprofen. They coated the microparticles with chitosan and then modified the surface with genipin or glutaraldehyde, which resulted in the slow and sustained release of the drug. The authors finally concluded that this microparticle with cross-linked chitosan polymers on the surface had the potential to be a very promising polymeric carrier for drug delivery. Figure 4 shows an example of the mechanism between chitosan and genipin.

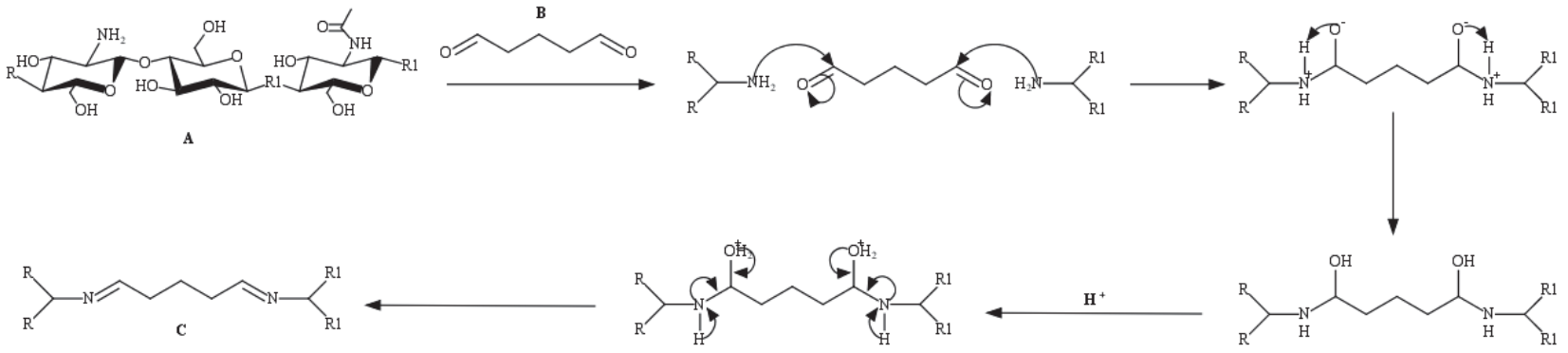

Figure 3. Example mechanism of Schiff's base reaction between chitosan (A) and glutaraldehyde (B) to form crosslinked hydrogel (C)

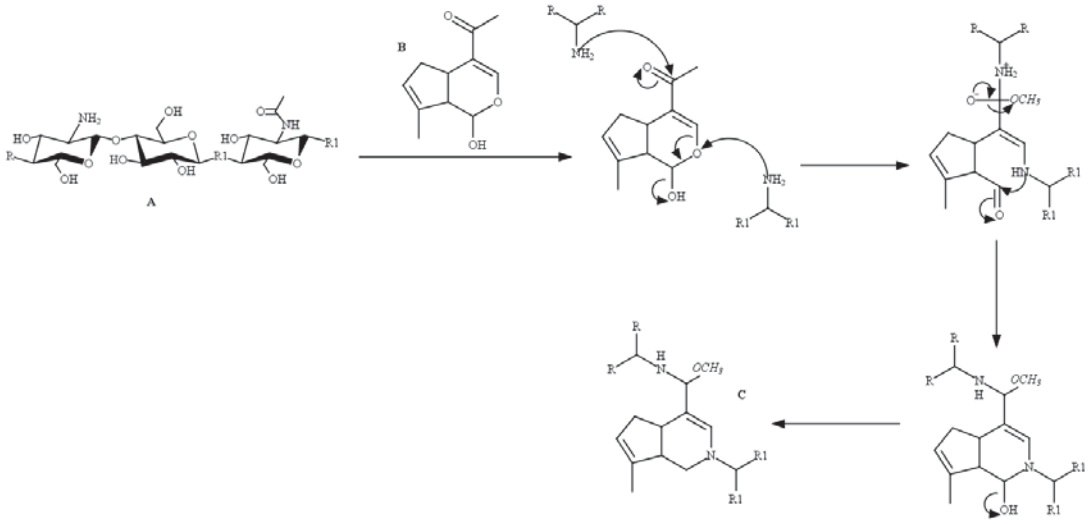

Figure 4. Mechanism of cross-linking between chitosan (A) and genipin (B) to form the hydrogel (C)

1.5.2. Physical Cross-linking

Physical cross-linking can be considered the complete opposite to chemical cross-linking as physically cross-linked hydrogels achieve the gel state using non-covalent forces/changes to the intermolecular forces within the polymeric network including hydrogen bonding, hydrophobic interactions, electrostatic ionic forces or the use of intermolecular assemblies (guest-host inclusion, stereo-complexation, and complementary binding) (Nguyen, Huynh, Park, & Lee, 2015). These forces can be achieved by the internal arrangement of the polymer molecules themselves or with the help of external stimuli such as pH, temperature, or the presence of other compounds (Nguyen, Huynh, Park, & Lee, 2015). What makes physical cross-linking appealing is the use of non-toxic, biodegradable, and biocompatible cross-linkers to achieve the gel state all while avoiding the harsh conditions and catalysts observed in some chemical cross-linking (Nguyen, Huynh, Park, & Lee, 2015). However, the down side to these hydrogels is their reversibility and low mechanical strength. The bonds are reversible leading to faster release of entrapped drug molecules, shorter shelf-life, and lower mechanical strength, yet strong enough to inhibit the dissolution of the hydrogel itself. (Coviello, Matricardi, Marianecci, & Alhaique, 2007).

1.6. Alginate and Alginate Based Hydrogels

As mentioned in above sections, the characteristics of hydrogels have proven to be beneficial in biomedical applications such as drug delivery systems. In the following section, alginate will be described along with its use for the development of hydrogels for drug delivery systems.

1.6.1. Composition of Alginate

Alginate (alginic acid) was first reported by British chemist E.C.C. Stanford in 1881 (Zia, Zia, Zuber, Rehman, & Ahmad, 2015). It is family of anionic polysaccharides obtained from brown algae or produced by bacteria. The chemical composition of alginate consists of (1→4) linked β-D-mannuronic acid and α-L-guluronic acid residues in random compositions and sequences, resulting in it being regarded as a copolymer with its blocks being composed of three different forms of polymer segments: G-, M-, and GM residue blocks (Coviello, Matricardi, Marianecci, & Alhaique, 2007; Zia, Zia, Zuber, Rehman, & Ahmad, 2015). In most cases, alginate is transported in its sodium salt form which is usually a brown coloured powder, and soluble in water. Figure 5 shows the structures of β-D-mannuronic acid, α-L-guluronic acid, and alginate with G-blocks, M-blocks, and GM-blocks.

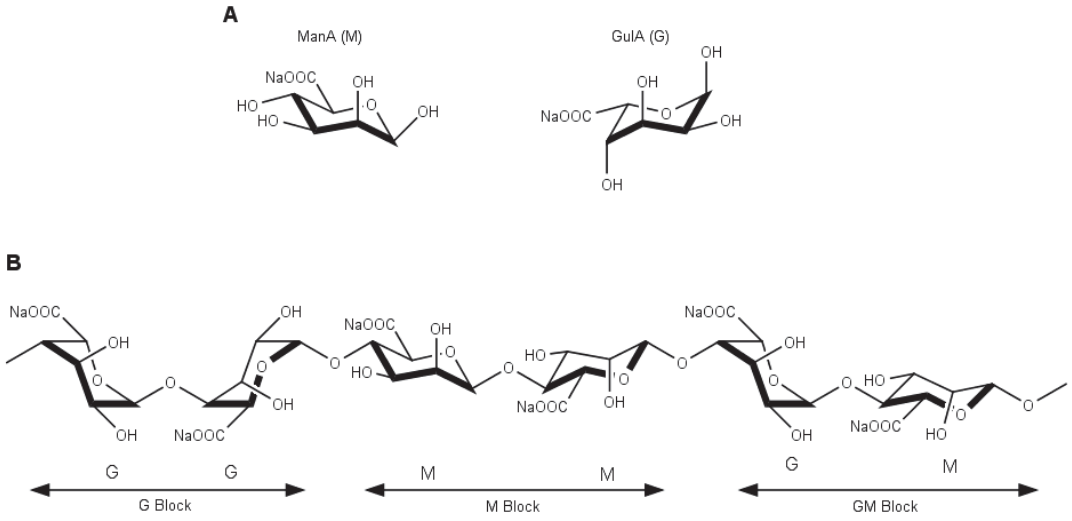

Figure 5. Chemical composition of alginate. A) Composition of β-D-mannuronic acid and α-L-guluronic acid residues. B) Composition of alginate with G-blocks, M-blocks, and MG-blocks.

1.6.2. Production of Alginate

Alginate is one of the most abundant biosynthesised natural materials in the world, with over 30,000 metric tons produced worldwide annually (Zia, Zia, Zuber, Rehman, & Ahmad, 2015; Donati & Paoletti, 2009). It represents a green, renewable, and biodegradable option for hydrogel production compared to synthetic polymers. There are two sources where alginate is collected from: marine plants such as brown algae or brown seaweed, and bacteria. When it comes to commercial use of alginate, it is mainly derived from brown algae including *Laminaria hyperborean*, *Ascophyllum nodosum* and *Macrocystic pyrifera*. Alginate is also extracted from bacteria including *Aztobacter* and *Pseudomonas* species, however, this method is expensive and only results in small recovery rates, hence not used in commercial labs (Zia, Zia, Zuber, Rehman, & Ahmad, 2015).

1.6.3. Properties of Alginate

Before alginate is used to prepare hydrogels, it exhibits different properties which effect how the hydrogel is prepared and performs. Below is a list of the physical and chemical properties sodium alginate exhibits (Porrelli et al., 2015; Donati & Paoletti, 2009)

- Water soluble
- An anionic polysaccharide
- Has gel-forming capabilities
- Forms hydrogels with divalent cations
- Has reactive carboxylic groups
- Has a negative charge at neutral pH
- Forms viscous solutions
- Is a high molecular weight polyelectrolyte
- Degrades before melting
- Can undergo chemical modification

Along with the physical and chemical properties, alginate is highly desired in research due to its added biological properties. Not only is it a natural polysaccharide, it is also non-toxic, biodegradable, and biocompatible with natural tissue. Yet alginate has its biological limitations. Below is a list of its biological properties and limitations (Donati & Paoletti, 2009).

- Stabilises aqueous mixtures, dispersions, and emulsions
- Has a degree of flexibility
    - Flexibility is dependent on chemical composition of alginate. It decreases in order of increasing blocks: MG<MM<GG.
- Can trigger macrophage activity
- Increases cytokine levels in wounds
- Is a bio-inert material
    - When introduced to biological tissue, it will neither respond nor interact with the tissue.
- Lack of bioadehesivity

1.6.4. Alginate Based Hydrogels

The major advantage of using alginate in research is its remarkable ability to form physically cross-linked hydrogels in the presence of divalent cations, due to its higher affinities for these ions compared to monovalent cations. This affinity has been theoretically explained as "a near-neighbour auto cooperative process which predicts that affinity towards a specific ion increases with increasing content of the same ion in the medium" (Donati & Paoletti, 2009). When sodium alginate is in the presence of Ca^{2+}, two sodium ions are removed from interaction with the COO^- groups on the alginate backbone and replaced by Ca^{2+} forming the hydrogel, as shown in Figure 6. It is important to note that the flexibility of the alginate greatly decreases when Na^+ is replaced with Ca^{2+} as Ca^{2+} coordinates to two alginates $–COO^-$ opposed to Na^+ which only coordinates to one (Zia, Zia, Zuber, Rehman, & Ahmad, 2015). The degree of cross-linking present within a hydrogel structure is dependent on the concentration of calcium ions added to prepare the hydrogel. The higher the concentration of calcium ions added, the higher the degree of cross-linking present within the structure. This simple factor effects many attributes of the hydrogel from the degree of swelling to the release of drug molecules encapsulated within the hydrogel (Peppas, Bures, Leobandung, and Ichikawa, 2000). These hydrogels are highly favoured for biological applications as they can form quite rapidly by simply mixing alginate and calcium chloride together, and these hydrogels remain non-toxic, biocompatible, and biodegradable in organic tissue (Chung, Naficy, Wallance, Naficy, & O'Leary, 2016).

Furthermore, the physical state of the hydrogels can exist in multiple forms including films, beads, nanoparticles, and emulsions.

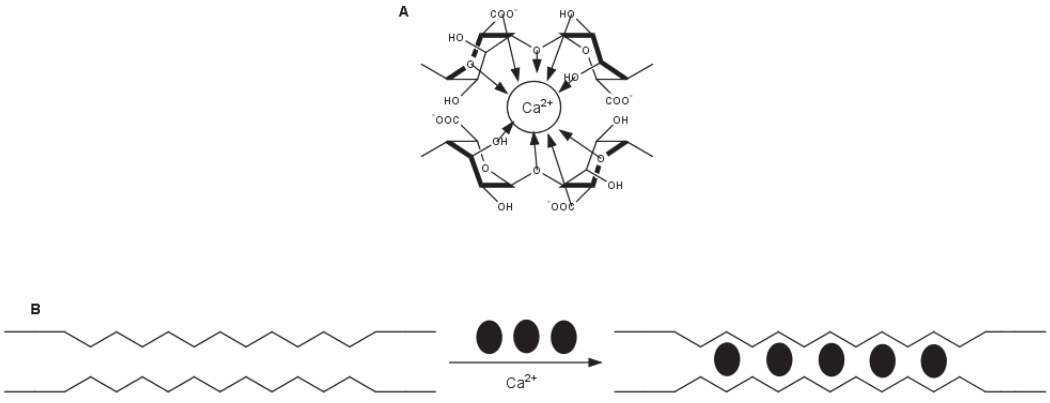

Figure 6. Binding of Ca^{2+} by alginate. A) Binding of Ca^{2+} to G-Block. B) Interchain formation.

Mahdi *et al* (2015) used the interaction between alginate and Ca^{2+} to produce a method to measure the *in situ* rheological behaviour of rapid forming hydrogel. They achieved this by attaching a petri dish on the lower plate of a rheometer, and then placing a piece of filter paper impregnated with $CaCl_2$, followed by a semi-permeable membrane to prevent the alginate coming in to contact with the filter paper and finally, a 4 w/w % alginate solution was loaded onto the membrane. The authors collected the G' and G" as a function of time to measure gelation time. The gelation was identified by the sharp increase in G' in the first three minutes, followed by the plateaued G' for the rest of the test. The authors went one step further to measure the degradation of the alginate hydrogels by replacing the $CaCl_2$ filter paper with filter paper impregnated with a calcium chelator (EDTA).

1.7. The Use of Analgesics in Poultry

Various species of birds are becoming household pets and as a result that are increasingly presented to vets for painful conditions, with some needing surgical procedures. Yet when the birds are introduced to analgesics, the dose needs to be high or constantly repeated due to the fast rate of avian metabolism, leading to multiple side effects such as inflammation. It is therefore important that new delivery systems are

created to minimise the harm to birds. For this work, the model analgesics chosen were sodium salicylate and sodium aspirin. This section will focus on the use of the model analgesics, present drug delivery techniques, composition of blood and the metabolism of birds.

1.7.1. Salicylic Acid (SA)

SA was first isolated in 1829, from the bark of the willow tree as salicyl alcohol glucoside (salicin) by Leroux and was later hydrolysed to produce glucose and salicylic alcohol which is metabolised to salicylic acid (Silverman, 1992). It is a phenolic derivative that possesses an aromatic ring with a hydroxyl group. It is transported as free SA, which is a white crystalline powder that melts at 157-159 ^{o}C (Hayat, Ali, & Ahmad, 2007).

SA is not very soluble in water, but is freely soluble in many polar solvents. Since it is not water soluble, for the current work, sodium salicylate was used because of its water solubility. Figure 7 shows the structures of salicyl alcohol glucoside, salicylic acid, and sodium salicylate.

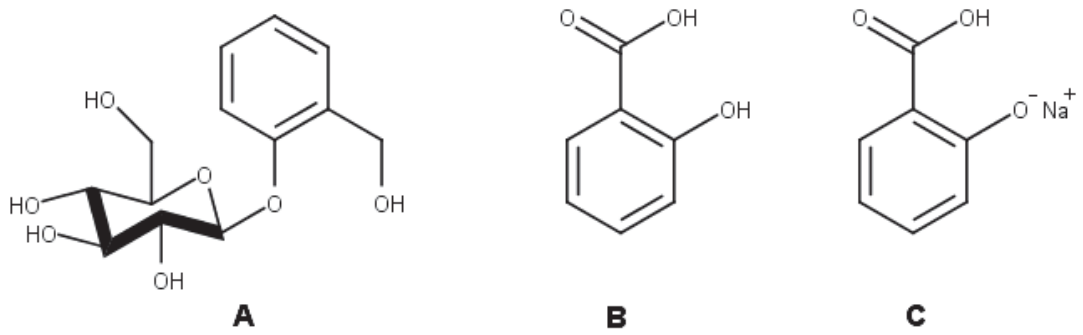

Figure 7. Chemical structures of salicyl alcohol glucoside (A), salicylic acid (B), and sodium salicylate (C).

1.7.1.1. Salicylic Acid Mechanism of Action

The first metabolic mechanism of salicylic acid was reported by Vane (1971). He stated that aspirin and aspirin-like drug molecules inhibit the production of cyclooxygenase (COX), which is the key enzyme in the biosynthesis of prostaglandins. Prostaglandins are biomolecules that are released when cells become damaged and they are found in increased concentration in inflamed cells. They are the main cause of fevers, headaches, and inflammation (Silverman, 1992). When the host is introduced to aspirin, Vane noted that the concentration of prostaglandins decreased, and this lead to the conclusion of aspirin inhibiting the COX enzyme, by acetylating a serine residue present in the active site of the enzyme. Figure 8 shows the mechanism of aspirin and Figure 9 shows molecular structures of several prostaglandin molecules.

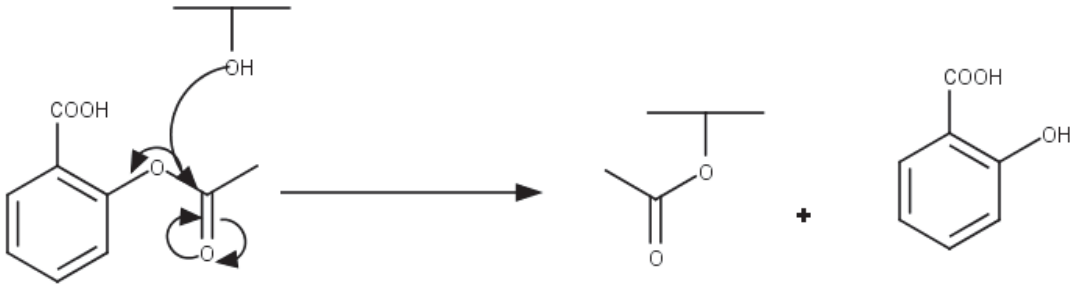

Figure 8. Proposed mechanism of inhibition of COX by aspirin.

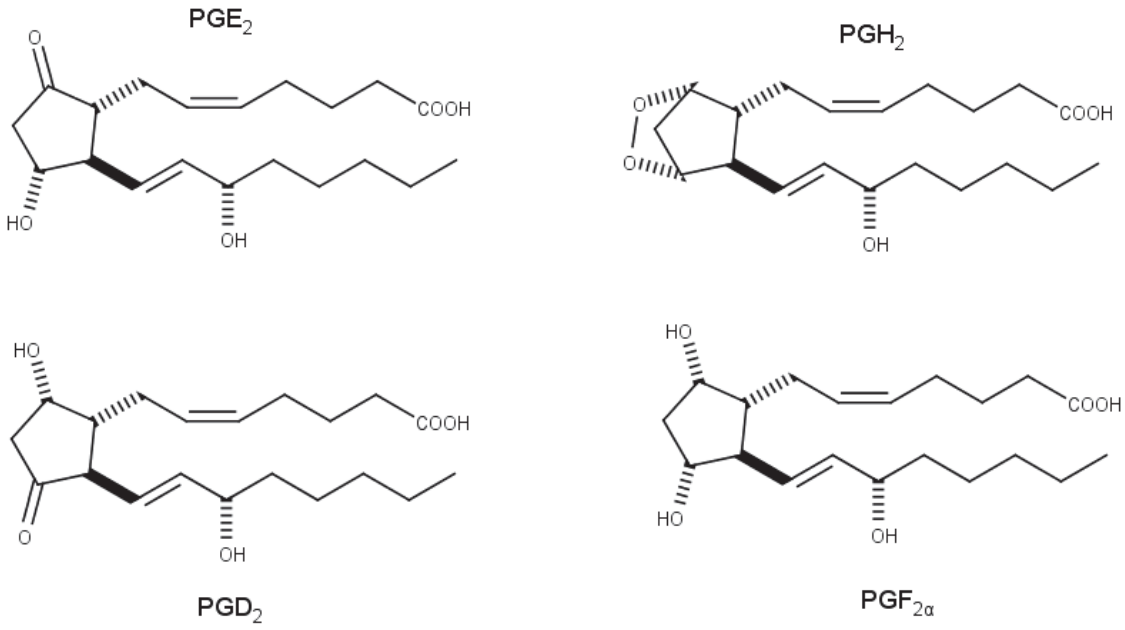

Figure 9. Selected chemical structures of prostaglandins released when cells are damaged.

One limitation to Vane's work was that it was still unclear as to what the true mechanism of salicylic acid was. Xu *el a*l (1998) expanded on the work of Vane and stated that salicylic acid supressed the transcription of the COX-2 gene. The authors introduced aspirin and sodium salicylate to mice pre-treated with lipopolysaccharides, and observed the prostaglandin concentration decreased as the aspirin and salicylate inhibited the COX-2 enzyme. However, when sodium salicylate was introduced to purified COX-2, no inhibiting occurred and prostaglandins were synthesised. From these results, Xu *et al* concluded that Vane's work was indeed valid, they just expanded onto it by introducing the idea of salicylic acid supressing the transcription of COX-2.

1.7.2. Present Drug Delivery Techniques

Drug delivery techniques of analgesics in birds are an important consideration as different drugs need different delivery techniques. Administering drugs to birds orally is not only highly difficult but can also be dangerous to the bird handler due to the sharp beaks. Add this to low bioavailability compared to that of drugs injected into the blood (100 %), injecting the drug into them is a reasonable option. There are two suitable injection methods used in poultry, intramuscular and subcutaneous. Intramuscular

involves injecting the analgesic straight into the muscle and possible sites include the breast, thigh, leg, wing, and tail head. For subcutaneous injections, the analgesic is injected under the skin and possible sites are the neck and the inguinal fold. When it comes to selecting the better option, a few factors need to be considered (Cobb-Vantress, 2013):

1) Ease of application
2) Analgesic reaction at injection site
3) Human safety

In terms of this research, the model analgesics will be injected subcutaneously in the neck of the chicken in order to reach the blood stream faster.

1.7.3. Present Sustained Release Drug Delivery Techniques

Given the higher metabolic rates of birds and the difficulties with administering the analgesics, one present sustained release drug delivery technique is the use of miniosmotic pumps. Clancy, KuKanich, and Skyes IV (2015) describes these pumps as miniature cylindrical devices that require no external power source and can be implanted into the birds. They work on the osmotic pressure differences between the bird's intestinal fluids and the osmotic agent present in the pump. As the extracellular fluid diffuses through the outer semipermeable membrane, the osmotic agent compartment expands, which presses on the inner flexible reservoir that contains the desired drug, hence releasing it at a steady rate. Unlike hydrogels, the use of osmotic pumps requires the bird to be anesthetised before and after implantation. Added to the high cost of buying the pump itself, this method for sustained drug release is an expensive procedure. Hydrogels can potentially overcome the limitations of osmotic pumps in favour of a less costly alternative, which can be decomposed by the biological system.

Clancy, KuKanich, and Skyes IV (2015) used osmotic pumps to sustain a drug release of butorphanol over a week in the common peafowl. The plasma concentration (106.4 ug/mL) of butorphanal peaked at 39.0 hours and once the pump was removed from the birds, the butorphanol was rapidly eliminated from the system, with a half-life of 1.45 hours.

1.7.4. Composition of Blood

After the model analgesics have been injected into the bird, the drug is introduced to the blood stream, where it is transported to the site of action. Blood is made up of multiple components which have different functions. These components are easily identified when blood is centrifuged, with two layers forming, both containing different components. Figure 10 illustrates these components of blood (Reece *et al*, 2014).

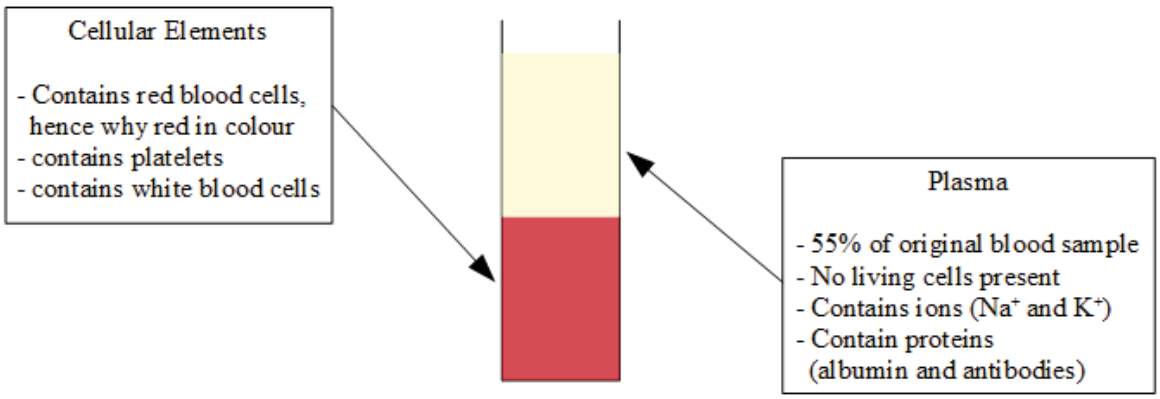

Figure 10. Illustration of the component of blood samples.

1.7.4.1. Salicylic Acid Affinity for Plasma Proteins

One limitation to using salicylic acid and its derivatives as the model drug is its high affinity to the plasma proteins, mainly albumin. There are many articles in the literature which demonstrates this limitation.

Davison and Smith (1961) studied the binding of salicylic acid and related substances to purified proteins. One of their experiments looked at the binding of 0.67 mM salicylic acid to varying concentrations of albumin. They noted that as the albumin concentration increased from 0.1 mM to 0.8 mM, the percentage of salicylic acid bound to the albumin increased from 8 % to 70 %. They concluded that salicylic acid exhibited extensive binding to the purified proteins due to the carboxylic acid group, as this is the group that confers a primary binding capacity upon the compound.

In 1992, Viani et al conducted a similar experiment on the protein binding of salicylic acid and other drugs in plasma. Again, they found that the lowest percentage of unbound salicylic acid was just under 40 % meaning that 60 % of the salicylic acid was

bound to the proteins in the plasma. Furthermore, increasing the albumin concentration decreased the percentages of unbound salicylic acid by 79 %. They concluded that the majority of salicylic acid binds to the albumin and that this can be disrupted by different competing molecules.

In the long run, this can be a potential problem for the analysis of salicylic acid in the plasma because processing the samples will lead to the removal of plasma proteins, which could also remove a substantial portion of the salicylic acid present. Therefore, techniques for removing salicylic acid from the proteins are required and could include simple techniques such as deproteinisation or more advanced techniques such as solid phase extraction (SPE).

1.7.5. Previous Studies of Analgesic Effects of Salicylic Acid in Birds

Previous studies in the literature have shown the higher clearance of drugs in birds (Baert & De Backer, 2002; Singh et al.,2010). In most cases, the authors have speculated the reason behind this is due to their faster metabolic rates as compared to mammals. Baert & De Backer (2002) injected salicylic acid intravenously into broiler chickens at a dose rate of 50 mg/Kg and found the half-life of salicylic acid was 4 hours. Pozniak et al. (2013) conducted a similar study. The authors found the half-life of salicylic acid at 50 mg/kg dose in broiler chickens to be 3.35 and 2.92 hours, after intravenous and oral administrations. In both examples, the concentration of salicylic remained above the effective concentration required for antipyretic and analgesic (50 μg/mL) for 5 to 6 hours. For effective pain relief in birds, new techniques are needed to prolong the release of analgesics to minimise the negative impacts of continuous injections.

Thesis Outline

Alginate is a promising polymer for the preparation of ionically cross-linked hydrogels. It possesses many beneficial attributes including non-toxicity, flexibility, solubility in water, and fast gelation rates in the presence of divalent cations such as calcium ions. Furthermore, alginate hydrogels possess the ability to release selected analgesics over a prolonged time period. Couple this to the numerous injections of analgesic required to maintain a therapeutic dose in birds, the main objective of this project is to reduce the number of injections by replacing the analgesic with a hydrogel loaded with the analgesic to achieve a sustained release over 24 hours minimum. It is hypothesised alginate hydrogels would make a suitable drug delivery system for the sustained release of a model analgesic, *in vitro* and *in vivo*, due to the high swelling capabilities and hence, the rate of drug release could be manipulated by varying the concentration of the calcium ions used to cross-link the hydrogel.

The approach of this thesis will be divided into two steps. In the first approach, alginate hydrogels cross-linked with calcium ions and loaded with the model analgesic will be prepared and *in vitro* studies including swelling profiles and drug release profiles will be collected to identify which hydrogels will be the most suitable for the *in vivo* testing. The results obtained will be compared to blank hydrogels prepared in the same manner as the loaded hydrogel, minus the model analgesic. Furthermore, alginate hydrogels cross-linked with alternative cross-linkers, such as copper ions and calcium salicylate, will be briefly studied and compared to the hydrogels cross-linked with the calcium ions to gain a better understanding at what appears to be a separation effect. In the second approach, the most suitable hydrogels from the *in vitro* testing will undergo *in vivo* testing in poultry. At this stage, the hydrogels will be prepared in the same manner as the *in vitro* testing, and injected subcutaneously into broiler chickens, with the intention of measuring the analgesic concentration in plasma samples collected from the chickens. These results will be compared to the *in vitro* tests to identify which hydrogel achieved the final objective the fastest, in the hope that sustained release of the analgesic is achieved.

Chapter 2: Materials and Methods

2.1. Materials

Low viscosity sodium alginate 99.5 % from brown algae, *ReagentPlus* 99.5 % sodium salicylate, calcium chloride, aspirin, sodium hydrogen carbonate, acetonitrile, sodium hydroxide, and boric acid were purchased from Sigma Chemical Co (St. Louis, USA). Acetic acid and ortho-phosphoric acid were of analytical grade and used as received. Simulated intestinal fluid (SIF) was prepared by mixing boric acid (40 mmol), acetic acid (42 mmol), and ortho-phosphoric acid (54 mmol) in water and adjusting the pH of the solution to 7.4 with 1N sodium hydroxide.

2.2. Instrumentation and Equipment

All plasma samples were processed using a ThermoFisher Scientific FRESCO 17 microcentrifuge equipped with a 24 x 2 mL rotor with a click seal lid. For chromatographic separation, the Thermo Scientific Dionex Ultimate 3000 HPLC system equipped with a photodiode array detector (PAD) was used and all measurements were recorded and analysed using the Chromeleon 7.2 computer program. The high performance liquid chromatography (HPLC) system consisted of SRD-3400 solvent rack, HPG-3400A pumps, WPS-3000TSI auto-injector, TCC-3000 column compartment, and PDA-3000 photodiode array detector (Thermo Scientific, America). The separation was achieved using a Phenomenex Synergi Hydro-RP (150 x 4.6 mm i.d, 4 um particle size) column equipped with a Phenomenex SecurityGuard cartridge holder loaded with a Phenomenex SecurityGuard cartridge (AQC18 4 x 30 mm i.d) at 40 ^{o}C. All pH measurements were made using a Sartorius professional meter PP-15, accuracy ±0.002 pH. All drug release studies were conducted on a Shimadzu UV-1800 240V IVDD UV spectrophotometer and all results were recorded and analysed using the UVProbe 2.5 computer program.

2.3. Methods for Preparation of Alginate Hydrogel Films

All hydrogel films were prepared and characterised using the same procedure unless stated otherwise. The method for drug loaded film and blank film preparation was adapted from Kuo and Ma (2001). Table 2 illustrates the compositions of the hydrogels, with B1-5 referring to the blank hydrogels and A-M referring to the loaded hydrogels.

2.3.1. Synthesis of Sodium Aspirin

5.4 g (0.0675 moles) sodium hydrogen carbonate was dissolved in 60 mL water at 4 ^{o}C. 10.8 g (0.0599 moles) aspirin was added to the sodium hydrogen carbonate solution and the mixture was left to mix at 4 ^{o}C until the reaction was completed. The solution was filtered to remove the unreacted aspirin and flash frozen at -10 ^{o}C overnight. The solution was lyophilised overnight and the resulting sodium aspirin was ground down to produce a fine powder (U.S. Patent No 3,064,038, 1962).

2.3.2. Stability of the Model Analgesics

The stability of sodium salicylate and sodium aspirin were measured in SIF using UV-Vis spectroscopy. Stock solutions of sodium salicylate and sodium aspirin were prepared and diluted down further to the desired concentrations, $2.0 \times 10^{-4}\ M$ and $1.5 \times 10^{-3}\ M$ respectively. The solutions were stored at 4, 21, or 37.5 ^{o}C for the duration of a week. At 0, 24, 48, and 72 hours, 5mL of each solution was removed and measured for drug content using UV-Vis spectroscopy at λ_{max} of 300 nm to determine the stability of the drug.

2.3.3. Synthesis of Calcium Salicylate

12.5 g (0.0781 moles) sodium salicylate was dissolved in 25 mL water. 4.33 g (0.0390 moles) calcium chloride dissolved in 5 mL water was added to the sodium salicylate solution dropwise and the mixture was left to mix at room temperature for 5 minutes. The white precipitate was filtered and washed 3 times with water, followed by acetone 3 times to remove any unreacted reagents. Finally, the white precipitate was lyophilised overnight.

2.3.4. Preparation of Calcium Hydrogel Films

For the preparation of ionically cross-linked blank alginate hydrogel films, a predetermined amount of alginate was dissolved in deionised water overnight. To the alginate solution, 5 mL of deionised water containing the appropriate amounts of calcium chloride were added, and the mixture was left to undergo gelation overnight under constant stirring. The contents of the hydrogel were poured into a petri dish and were left to dry at 60 ^{o}C in an oven or were shell frozen at -4 ^{o}C for 2 hours, and then lyophilised overnight.

2.3.5. Preparation of Hydrogel Films with Alternative Cross-Linkers

The preparation of hydrogel films cross-linked with calcium ions was done using the same method as above. Except in one case calcium chloride was replaced with copper (II) sulfate or calcium salicylate.

2.3.6. Entrapment of the Model Analgesics

The method was adapted from Perchyonok et al. (2014). The alginate films were loaded with either sodium salicylate or sodium aspirin. The films were prepared in the same manner as discussed above. Known amounts of the drug (150 mg/mL) were added to the alginate mixture before addition of calcium, stirred until homogeneous mixtures obtained, and calcium added for crosslinking.

2.4. *In Vitro* Characterisation of Hydrogel Films

2.4.1. Fourier Transform Infrared Spectroscopy (FTIR)

A select number of reactants, products, and hydrogel films were crushed into a coarse powder and FTIR spectrums were collected using potassium bromide disks. The spectra were collected for alginate, and hydrogel films B1, B4, A, and D (Islam, Yasin, Bano and Riaz, 2011).

2.4.2. Equilibrium Swelling Studies

The swelling behaviour of the hydrogel films were measured at room temperature in both water and SIF. 1.00 g of the blank hydrogel was placed in the medium and removed at certain time intervals until equilibrium swelling was obtained (after 24 hours). At each time interval, the films were dried on a tissue to remove surface liquid, and weight. The following equation was used to determine the percentage of swelling within the film:

$$\% \, Swelling = \frac{(W_w - W_d)}{W_d} \times 100 \, \%$$

where, W_d is the initial weight and W_w is the final weight of the swollen hydrogel films at the certain time intervals (Adbelhalim, 2006).

2.4.3. In Vitro Cumulative Release Studies

The *in vitro* release of the entrapped model drug was carried out by placing the dried hydrogel film in both water and SIF buffer, while being stirred gently. At certain time intervals, 1 mL of the solution was withdrawn and replaced with 1 mL of blank medium. Depending on the medium used, the method for determination of the drug amount differed. The following equation was used to determine the percentage of drug released over the time period:

$$\% \, Drug \, Release = \frac{C_d}{C_{md}} \times 100 \, \%$$

where C_d is the combined concentration of drug released into the medium at each time interval and C_{md} is the maximum concentration of drug which could be released by the hydrogel. The C_{md} value alters slightly according to amount of drug added to the hydrogel films and has been stated for each release case (Perchyonok et al., 2014).

2.4.3.1. SIF Buffer

The withdrawn drug solution was diluted with the blank medium to a dilution factor of 800 fold for sodium salicylate and 600 fold for sodium aspirin solutions. 3 mL of the diluted drug solution was measured at λ_{max} of 300 nm corresponding to the salicylate salt and result was applied to a calibration curve constructed from a series of standard solutions of sodium salicylate to determine the drug amount.

2.4.3.2. Water

The withdrawn drug solution was diluted down with the blank medium to the dilution factor of 250 fold for the sodium salicylate solutions. 1 mL of the diluted drug solution was added to 4 mL ferric chloride solution (5 w/v %). The coloured drug solution was measured at λmax of 530 nm corresponding to the salicylate salt and the result was evaluated from a calibration curve constructed from a series of standard solutions of sodium salicylate to determine the drug amount.

2.5. Methods for *In Vivo* Release in Poultry

Methods for *in vivo* experimentation were adapted from methods used by Singh (2011) Singh et al. (2010 and 2011).

2.5.1. Study Design

This study was conducted on 30 broiler chickens with an average weight of 3 Kg. The 30 chickens were divided into five separate groups, each comprising six chickens. Each chicken was restrained manually for catheterisation of the medical metatarsal vein with a 22 G catheter. All chickens were kept in the five groups under standard conditions and all were fed *ab lib*. with commercially available feed and a 24 hour supply of fresh drinking water. One out of the five groups of chickens received a subcutaneous injection of aqueous sodium salicylate solution. The other four groups received sodium salicylate hydrogel formulations (Table 2).

2.5.2. Hydrogel Preparation

All hydrogels were prepared using the same method above in 2.3.1.1; however, they were not dried after preparation. Hydrogels were diluted where appropriate for injection to be possible. In these cases, the alginate and calcium chloride content remained constant and the water and drug content were increased to maintain the drug concentration at 150 mg/mL. Hydrogels for *in vivo* injection into chickens were prepared in closed Schott bottles and all equipment was autoclaved to ensure a sterilised environment. Hydrogel C was made by Iman Kavianinia for initial *in vivo* experimentation.

2.5.3. Drug Administration

Sodium salicylate for injection was made by dissolving 150 mg of sodium salicylate in 1 mL sterile water. The injection was filtered through 0.2 um syringe filters and immediately administered at the dose rate of 150 mg/Kg. The sodium salicylate formulation in a hydrogel was also administered subcutaneously in a similar way as the aqueous solution.

2.5.4. Sample Collection

Serial blood samples of 2 mL were collected in heparinised vials at 0, 0.25, 0.5, 1, 4, 8, 16, and 24 hours from the chickens injected with the sodium salicylate standard; 0, 0.25,

1, 4, 8, 16, 24, 48, 72, 96, and 120 hours for the chickens injected with the hydrogels. The total amount of blood collected from each chicken was 16 mL for the standard group and 22 mL for the hydrogel group. The vials of blood where chilled after collection, and centrifuged at 2000 rpm for 10 minutes. The plasma was separated and flash frozen on dry ice. Finally, the plasma samples were all stored at -70 ^{o}C until analysis.

2.5.5. Sample Preparation

Liquid-liquid extraction was used to process plasma samples. 300 μL of plasma was diluted with 300 uL water and 300 uL of the diluted plasma was added to 700 μL of 10 % acetic acid in acetonitrile and vortex mixed for two minutes. The samples were centrifuged at 13000 rpm for 15 minutes and the supernatant was separated and dried under a gentle stream of compressed air at 20 degrees, unless stated otherwise. The dried samples were rehydrated with 200 μL of mobile phase and centrifuged at 13000 rpm for 10 minutes.

2.5.6. Validation Protocol

The HPLC method was validated by standard validation protocol as described by Masson (2007).

2.5.6.1. Lower limit of Quantification (LLQ) and Detection (LLD)

The LLQ and LLD were determined by measuring small concentrations of sodium salicylate (500, 400, 300, 200, 100, 50, and 10 ng/mL) diluted in water. Once LLQ and LLD were determined in solution, the same concentrations were added to drug-free plasma.

2.5.6.2. Linearity

Linearity of the method was determined by preparing multiple sodium salicylate solution at varying concentrations (500 ug/mL to 50 ng/mL). The method was repeated for sodium salicylate in drug-free plasma.

2.5.6.3. Recovery

The recovery of sodium salicylate in spiked plasma after liquid-liquid extraction was determined by comparing the area of the peaks from the spiked plasma (400, 300, and

200 ug/mL) with the calibration curve prepared above. The mean of the three samples was calculated to determine recovery rate.

2.5.6.4. Specificity

The blank plasma from each chicken used in the project was extracted and analysed using HPLC to test for the specificity.

2.5.7. Sample Analysis

The plasma samples were analysed using HPLC equipped with a PAD. The separation of sodium salicylate was achieved using a Phenomenex C18A column at 40 ^{o}C. The mobile phase consisted of water:acetonitrile:ortho-phosphoric acid (71:28:1) with a flow rate of 1 mL/min under isocratic conditions. The wavelength of the detector was set to 230 nm (Singh, 2011). The specificity of the method was evaluated by measuring the plasma from a sample taken before being introduced with the model analgesic using the same method, and subtracting the small peak from all the samples. The small sodium salicylate peak in the blank plasma is assumed to be from the feed given to the chickens. Sodium salicylate peaks eluted at around 8.5 minutes and were analysed using Chromeleon 7.2 software. The standard curve was prepared using Chromeleon and the unknown sodium salicylate concentration was calculated by linear regression using the Chromeleon software.

Table 2. Composition of Physically Cross-Linked Alginate Hydrogels

		Alginate		Calcium Chloride		Water Content	Analgesic Used	Analgesic
Type	Sample Code	g	%	g	%	mL		g
Blank Al Films	B1	1.00	5.00	1.00	5.00	20.00	-	-
	B2	1.00	5.00	0.50	2.50	20.00	-	-
	B3	1.00	5.00	0.30	1.50	20.00	-	-
	B4	1.00	5.00	0.20	1.00	20.00	-	-
	B5	1.00	5.00	0.10	0.50	20.00	-	-
Loaded Hydrogels	A	1.00	5.00	0.30	1.50	20.00	NaSA	3.00
	B	1.00	2.50	0.30	0.75	40.00	NaSA	6.00
	C	1.00	1.67	0.30	0.50	60.00	NaSA	9.00
	D	1.00	5.00	1.00	5.00	20.00	NaSA	3.00
	E	1.00	2.50	1.00	2.50	40.00	NaSA	6.00
	F	1.00	1.67	1.00	1.67	60.00	NaSA	9.00
	G	3.00	15.00	1.00	1.50	20.00	NaSA	3.00
	H	3.00	3.75	1.00	1.25	80.00	NaSA	12.00
	I	1.00	5.00	0.20	1.00	20.00	NaAp	3.00
	J	1.00	5.00	1.00	5.00	20.00	NaAp	3.00
	K	1.00	5.00	-	-	20.00	CaSA	0.30
	L	1.00	5.00	CuSO4 - 1.0	5.00	20.00	NaSA	3.00
	M	1.00	5.00	0.20	1.00	20.00	NaSA	3.00

Chapter 3: Results and Discussions

3.1. Synthesis of Calcium Salicylate

Two methods were explored in relation to the synthesis of calcium salicylate, with method one producing a yield of 13.3 % and the second method producing a yield of only 4.4 %. Given that only a small amount of the product was required to produce the hydrogels, these small yields were no problem. Furthermore, the reactants used to make the product were cheap and easy to obtain.

3.2. Synthesis of Sodium Aspirin

Many methods for the synthesis of sodium aspirin were explored, with most leading to either no or small yields. This was observed mostly when using methods that required the application of heat such as using the rotary evaporator to remove the majority of the solvent. Furthermore, the yield was also altered depending on the base used. Stronger bases such as sodium hydroxide tended to hydrolyse the aspirin to sodium salicylate, however, strong bases could be used if the pH of the reaction was maintained between 6.0-7.0.

Optimum yield results were obtained when the synthesis was completed under cold conditions, around 4 ^{o}C and lower. This accounted for the low stability of sodium aspirin at higher temperatures and the conditions required for the neutralisation of aspirin. This synthesis was easier as the mixture could be left to mix in a fridge and then lyophilised at -25 °c to obtain the product.

Overall percentage yield for this method was 85 %, with any unreacted aspirin of sodium hydrogen carbonate being filtered off before lyophilisation. In terms of observations, both reactants were white solids which were very slightly soluble in water. When mixed together, the product was clear and colourless. After lyophilisation, the final product was a white solid.

3.3. Model Analgesic Calibration Curves

The following figure illustrates the UV-Vis spectrum of sodium salicylate and sodium aspirin over a range of wavelengths from 250-350 nm.

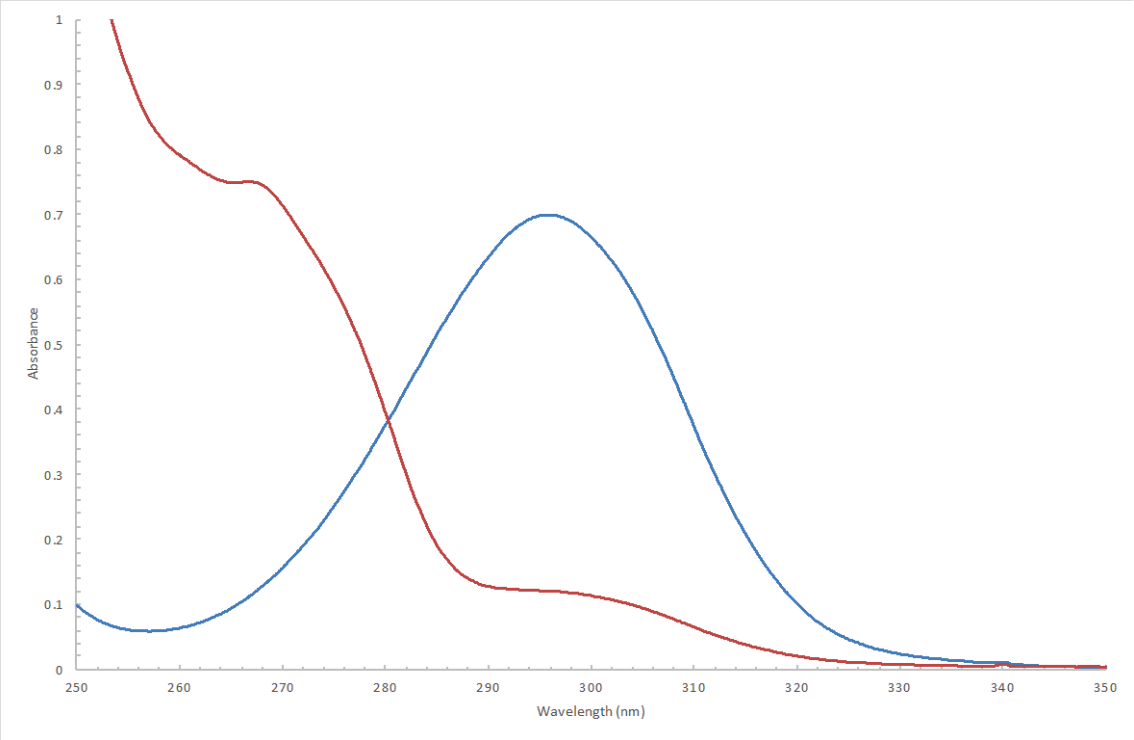

Figure 11. UV-Vis absorption spectra of sodium salicylate (blue) and sodium aspirin (red) in water over wavelength range of 250-300 nm.

Immediately, the spectra can be distinguished according to the λ_{max} each exhibit, with a λ_{max} of 300 nm and 260 nm for sodium salicylate and sodium aspirin respectively. From this data, calibration curves for both drugs were created using the λ_{max} for each and are shown below in Figures 12 and 13.

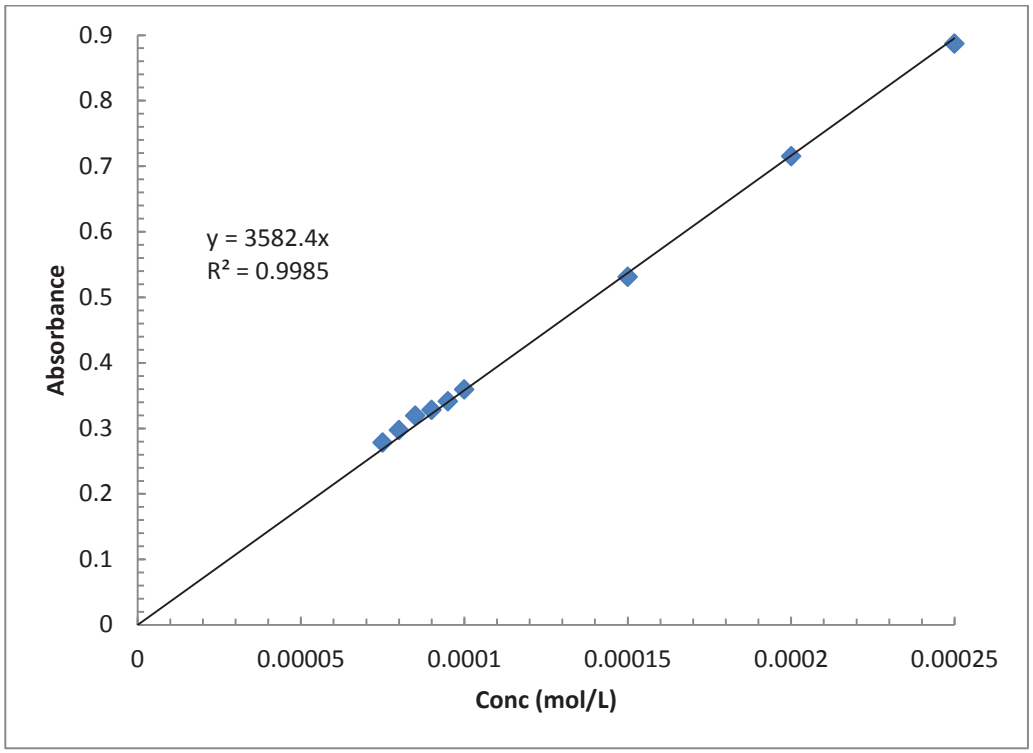

Figure 12. Calibration curve of sodium salicylate in SIF buffer using λ_{max} at 300 nm.

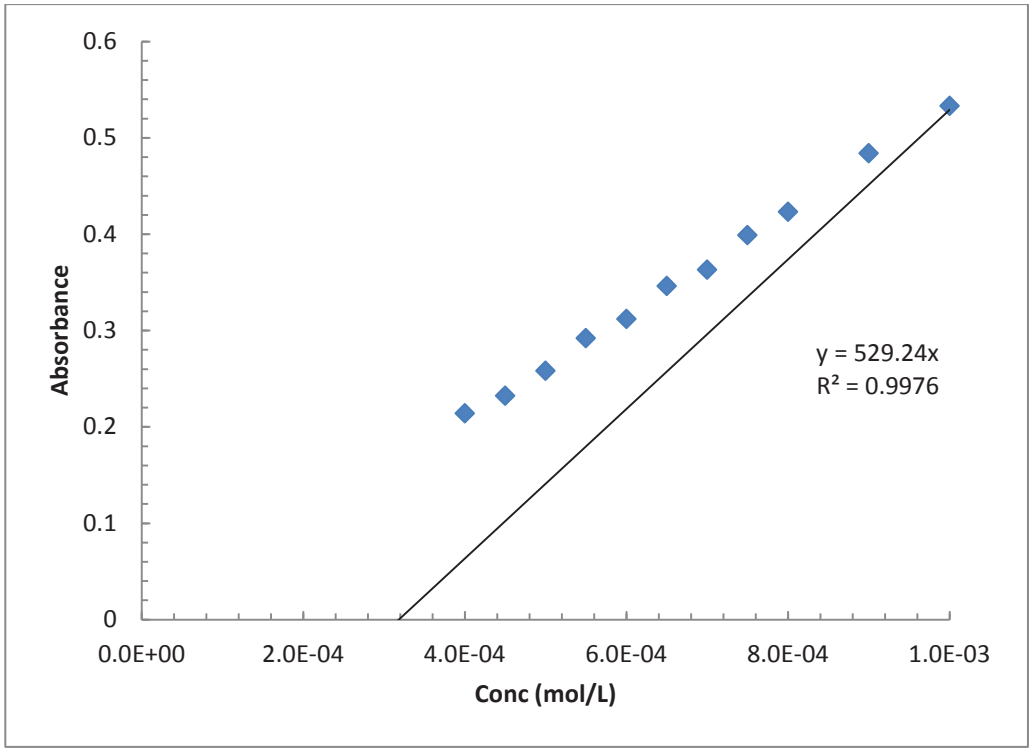

Figure 13. Calibration curve of sodium aspirin in SIF buffer using λ_{max} at 260 nm.

In both calibration curves, R^2 values are quite close to 1 indicating a strong relationship between concentration of the drug and absorbance at λ_{max}. At concentrations above 0.00025 mol/L for sodium salicylate and 0.001 mol/L for sodium aspirin, the calibration curves began to curve off and result in a less accurate line. Therefore, it is assumed that the R^2 values would begin to decrease with increasing data points.

3.4. Stability of the Model Analgesics

The stability of sodium salicylate and sodium aspirin was measured during a 48-72 hour time period. Results are shown in Table 3 and 4.

Table 3. Stability results of sodium salicylate measured in SIF buffer at 4, 21, and 37.5 ˚C using UV-Vis spectroscopy at λ_{max} of 300 nm

	4 ˚C	21 ˚C	37.5 ˚C
Time (hours)	ABS	ABS	ABS
0	0.671	0.671	0.671
24	0.678	0.678	0.682
48	0.677	0.663	0.666
72	0.677	0.667	0.681

Table 4. Stability results of sodium aspirin measured in SIF buffer at 4, 21, and 37.5 ˚C using UV-Vis spectroscopy at both λ_{max} of 300 and 260 nm.

	4 ˚C		21 ˚C		37.5 ˚C	
Time (hours)	ABS (nm=260)	ABS (nm=300)	ABS (nm=260)	ABS (nm=300)	ABS (nm=260)	ABS (nm=300)
0	0.774	0.898	0.774	0.898	0.774	0.898
24	0.78	0.996	no peak	1.686	no peak	2.923
48	0.718	1.063	no peak	2.164	no peak	3.125

Over the 72 hour time period, sodium salicylate appeared to be quite stable in the buffer at each temperature, with only minor changes to the absorbance. In contrast, sodium aspirin appears to be less stable than its counterpart at all three temperatures, with dramatic changes to the absorbance throughout the 48 hour time period. One major change is the loss of the peak at 260 nm and the increase to the 300 nm peak. This indicates the transformation of sodium aspirin to sodium salicylate, which can be accounted for by the cleaving of the acetyl group due to the slightly basic conditions of

the buffer. This was slowed down by storing the sodium aspirin at lower temperatures. However, after 48 hours, it begins to show the transformation to sodium salicylate.

3.5. Hydrogel Preparation

Preparation of hydrogels was relatively easy as the majority only required the basic components to be mixed together until gelation had occurred. The hydrogels were made with natural components which were non-toxic nor damaging to the environment, and could be described as green chemistry. Furthermore, the components were not expensive and could be purchased and prepared in large quantities to satisfy the requirements of *in-vivo* testing. Limitations to hydrogel preparation included time required to prepare each hydrogel, with the minimum of two days for most, and the model analgesics each requiring separate conditions for the hydrogel preparation and release studies due to their different stabilities and optimum environments.

3.5.1. Blank Hydrogel

Blank hydrogel refers to the hydrogels B1-B5, which were prepared in the absence of the model analgesics. These hydrogels were prepared using a fixed concentration of alginate and a decreasing concentration of calcium ions, the cross-linker. Observations made during the preparation of blank hydrogels were as expected, with many undergoing gelation immediately after the addition of the cross-linker, in most cases calcium. Figure 14 shows a photo of two blank hydrogels for comparison purposes.

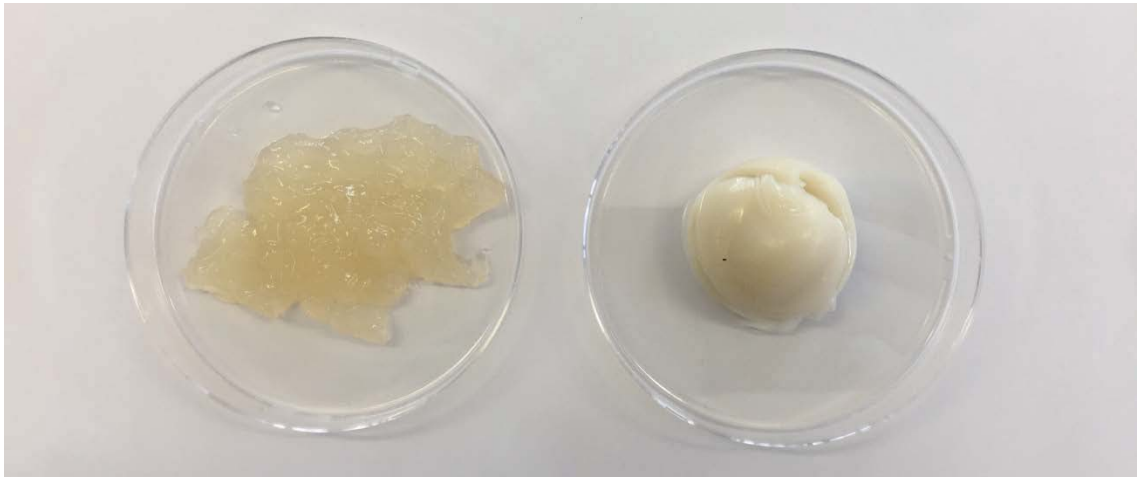

Figure 14. Images of blank hydrogels B4 (left) and B1 (right). Hydrogel compositions: both contain 1.0 g alginate and 20 mL water; B4 contains 0.2 g calcium chloride and B1 contains 1.0 g calcium chloride.

At low calcium concentrations (0.1-0.3 g per 20 mL hydrogel), hydrogels were thick, smooth, and highly viscous light brown coloured liquids which were slow moving when tilted and coated the back of a spoon. These hydrogels had a good injectable consistency for the *in vivo* experiments. Figure 15 shows an image of hydrogel B4 coating a spoon.

Figure 15. Image of hydrogel B4 and its ability to coat the back of a spoon. Hydrogel composition: 1.0 g alginate, 20 mL water, and 0.2 g calcium chloride.

At higher calcium contents (0.5-1.0 g per 20 mL hydrogel), hydrogels formed thick, solid balls of alginate/calcium with most of the water being expelled. Removal of the magnetic stirrer and drying of these “solids” required the surface area of the balls to be

increased, and this was achieved by breaking it up into smaller pieces. Unfortunately, they are not injectable when present in this "solid" form and the methods required alterations to prepare injectable blank hydrogels with higher calcium chloride content. Despite this limitation, the solid balls were easily overcome by diluting the hydrogel mixture down but keeping the amount of each component the same before the addition of the calcium.

3.5.2. Loaded Hydrogel

When the model analgesic is introduced to the alginate and calcium, the observations are not what were expected. When calcium is added to the drug/alginate mixture, instead of gelation occurring, a precipitate forms with no gelation occurring, even after the hydrogel is left to mix for a 24 hour time period. This precipitate will be addressed as the separation effect. Furthermore, when the hydrogel is left to sit at room temperature after "gelation" has occurred, the hydrogel separates into two separate layers, with the bottom layer containing the precipitate and the top layer being a cloudy, pale brown coloured liquid. These observations occurred when both model analgesics were used, but this was more predominant with sodium salicylate. All hydrogels were loaded with sufficient sodium salicylate or sodium aspirin that the final concentration of the drug in the hydrogel was 150 mg/mL. This value was pre-set by the IVABS team as the desired concentration to be injected into the poultry. This is demonstrated in Figure 16.

Figure 16. Image of a loaded hydrogel illustrating the separation effect. Hydrogel composition: 9 g sodium salicylate, 1.0 g alginate, 1.0 g calcium chloride, and 60 mL water.

Instead of gelation occurring, it appears that is a side reaction occurred, where the white solid being formed is favoured over the formation of the hydrogel. The resulting mixture is a solid suspended within a liquid phase which settles out over time if left to sit undisturbed. The term colloidal suspension can be used here instead of hydrogel given the resulting mixture and will be used for simplicity of further discussion.

These colloidal suspensions were prepared when the analgesic was added to the mix no matter the content of the calcium was. Nevertheless, differences in the colloidal suspensions were noted at different calcium contents with a more gel like state and less precipitate formed at lower calcium contents compared to the liquid like state and maximum precipitate formed at higher calcium contents. Figure 17 illustrates six separate colloidal suspensions prepared at the three standard dilutions (20, 40, and 60 mL) with two calcium chloride contents, 0.3 g and 1.0 g calcium. These two calcium chloride contents were chosen based on the blank hydrogel swelling results. Refer to sections 3.6. It can be seen that at the lower calcium content/low dilution, the colloidal suspension has formed a slightly cloudy gel like system while the higher calcium content colloidal suspensions have formed a a much cloudier liquid like state.

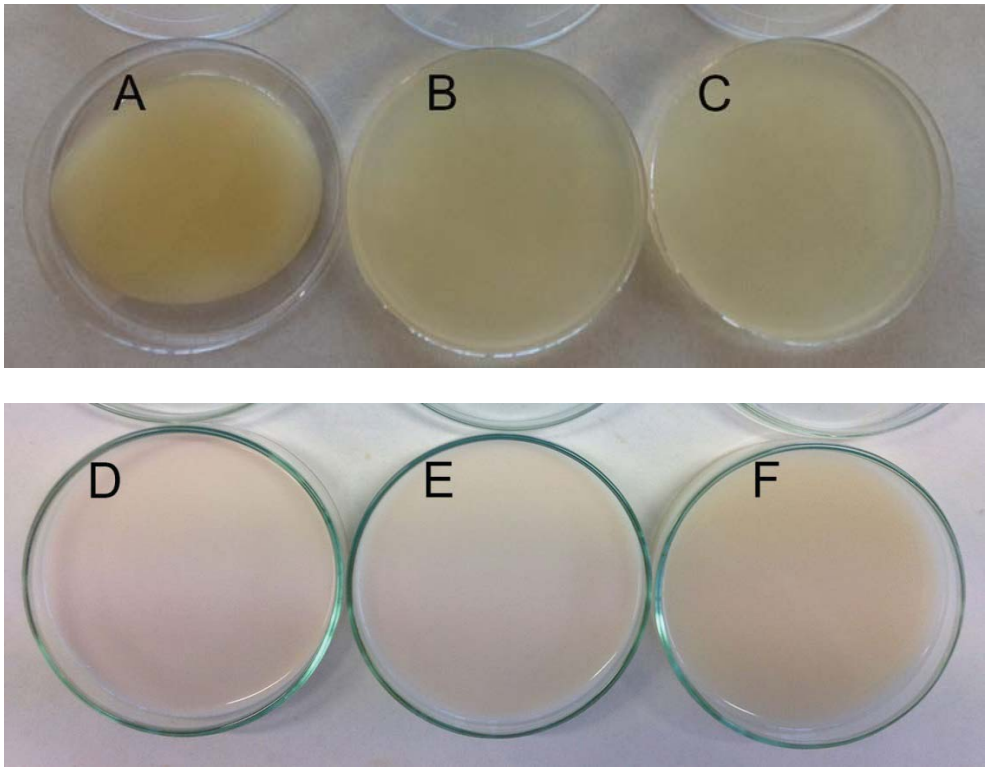

Figure 17. Images of loaded hydrogels illustrating the differences between the colloidal suspensions at different calcium contents, 0.3 g calcium chloride (upper image) and 1.0 g calcium chloride (lower image). Hydrogel composition of upper image: 1.0 g alginate and 0.3 g calcium chloride for all, 3 g sodium salicylate and 20 mL water (A), 6 g sodium salicylate and 40 mL water (B), and 9 g sodium salicylate and 60 mL water (C). Hydrogel composition of lower image: 1.0 g alginate and 1.0 g calcium chloride for all, 3 g sodium salicylate and 20 mL water (D), 6 g sodium salicylate and 40 mL water (E), and 9 g sodium salicylate and 60 mL water (F).

The only difference between the blank and the loaded hydrogels is the presence of the model analgesics. Further, the model analgesics were added as the sodium salt resulting in a slightly larger concentration of sodium ions present (1.73 %) in the hydrogel compared to the calcium ions added for crosslinking (1.44 %). The alginate is also adding to the sodium ion percentage as it is present in the sodium salt form. It was assumed that the calcium is interacting with the drug instead of the alginate, forming the insoluble calcium forms, calcium salicylate and calcium aspirin, leaving the sodium ions to continue to interact with the alginate, resulting in the colloidal suspension over the hydrogel.

Assuming a precipitate is achieved when using different divalent ions, such as copper or iron, an easy test was created to prove the divalent ion being added to cross-link the alginate was instead precipitating as a calcium salicylate salt. The divalent calcium ion was substituted by copper ions because the calcium salicylate is colourless compared to the green copper salicylate. Figure 18 illustrates a hydrogel prepared with copper sulphate and the bright green solid achieved.

Figure 18. Image illustrating the formation of copper salicylate during the preparation of a hydrogel. Hydrogel composition: 9 g sodium salicylate, 1.0 g alginate, 1.0 g copper sulfate, and 60 mL water

Unfortunately, changing the conditions in which the hydrogel is made does not change the outcome of the hydrogel. Many conditions were changed to inhibit the formation of calcium salicylate which included changing pH of the system, increasing/decreasing the temperature of the system during hydrogel preparation, pre-treating the sodium drug before adding to alginate, trying to dissolve the calcium salicylate after hydrogel formation, adding the drug after hydrogel preparation, soaking blank hydrogels in drug solutions, and using sonication to mix the hydrogel matrix during preparation. In all cases, precipitate forms and overall outcome results in colloidal system instead of the hydrogel.

One of the hydrogels loaded with sodium salicylate contained a very high concentration of alginate, hydrogel G, which resulted in a gel-like state. Once the calcium chloride

was added, cross-linking occurred immediately and the resulting hydrogel had a liquid gel-like texture with minimal cloudiness from any precipitate formed. It appears to be that the more alginate added to the mixture forces the calcium ions to cross-link with the alginate, limiting the production of the undesired side product and a more gel-like texture. Furthermore, the more dilute the hydrogel, a greater amount of precipitate formed, resulting in a less gel-like texture. Perhaps if more alginate is added to the mixture at lower dilutions, it could result in a gel-like textured hydrogel with no calcium drug product formed, however, this will result in a hydrogel which is not injectable.

From these results, it seems that the ability of calcium interacting with the model analgesic overpowers the high affinity of the calcium binding to the alginate within the system, which poses a problem in the formation of the hydrogels. Nevertheless, this phenomenon could be an advantage as injection into chickens can be altered slightly by producing an alginate/drug solution and a calcium solution, then injecting both into the chicken and allowing the hydrogel to form *in situ*. Furthermore, it still possesses the ability to create a barrier between the drug and surrounding environment, leading to a slower release of the drug from the system which is the overall outcome for the hydrogels.

3.5.3. Calcium Salicylate Cross-linked Hydrogel

Given the formation of calcium salicylate when the model analgesic is added to the alginate mixture, a hydrogel which is cross-linked with calcium salicylate is possible as an alternative to the normal hydrogel preparation (Figure 19). In this hydrogel, the calcium was able to interact with the alginate instead of the drug molecules, forming the desired gel like consistency. The major limitation to using calcium salicylate is that it is a water insoluble molecule and has to be added to the alginate in powder form. Furthermore, only a small amount can be used to prepare the hydrogel, otherwise, the drug cannot dissolve into the alginate and the resulting hydrogel contains solid masses of calcium salicylate. Unfortunately, the amount of drug required to be maintained within the system is not achievable with calcium salicylate and adding a mixture of sodium salicylate and calcium salicylate to a hydrogel system ends in the same outcome as the normal loaded hydrogel. Therefore, these hydrogels are not viable for the *in vivo* experiments.

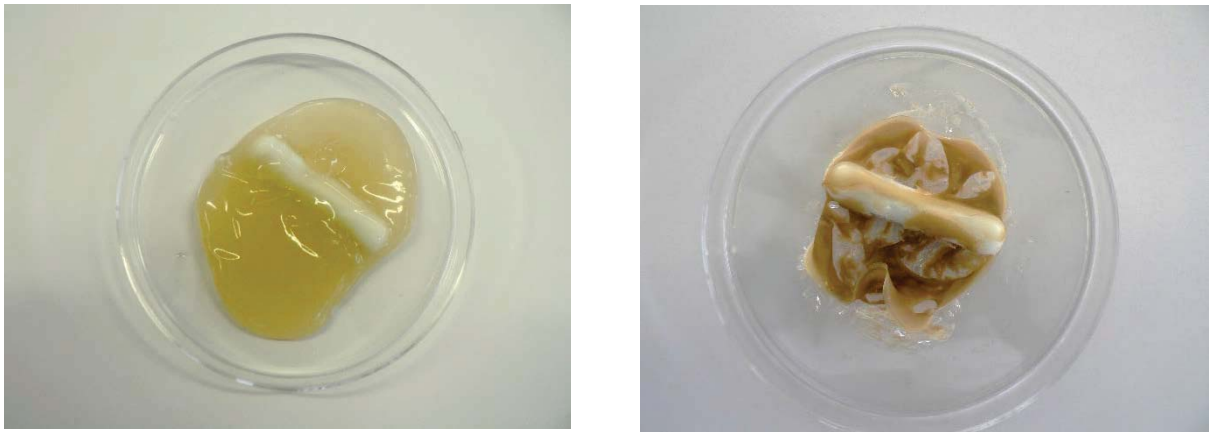

Figure 19. Images illustrating the hydrogel cross-linked with calcium salicylate, before oven drying (left) and after oven drying (right). Hydrogel composition: 1.0 g alginate, 0.3 g calcium salicylate, and 20 mL water.

3.5.4. Drying Method

The drying methods used to prepare the hydrogels were oven dried and lyophilisation. These methods speed up the process and allow the release studies and swelling studies to be done faster, compared to air drying the hydrogels. The main reason for both being used is due to the model analgesics used in this project, with sodium salicylate hydrogels being able to use both drying techniques interchangeably, and sodium aspirin only able to use lyophilisation due to its low stability under heat.

The two drying methods produced hydrogels with slightly different structural appearance. During oven drying, the heat relaxes the hydrogel system, creating more holes to form where water can escape, resulting in a flat structure with small pores and holes. Furthermore, the solid is able to sink to the bottom. In contrast, lyophilisation forces water out by applying a negative pressure to the system, creating larger pores in the system, resulting in a more porous structure with little holes. In both cases, the properties (swelling studies and drug release) remain similar as the final film are the same in composition and when introduced to the medium, the pores in the films open up allowing the medium to enter. Figure 20 illustrates a comparison between the same hydrogels dried using each method.

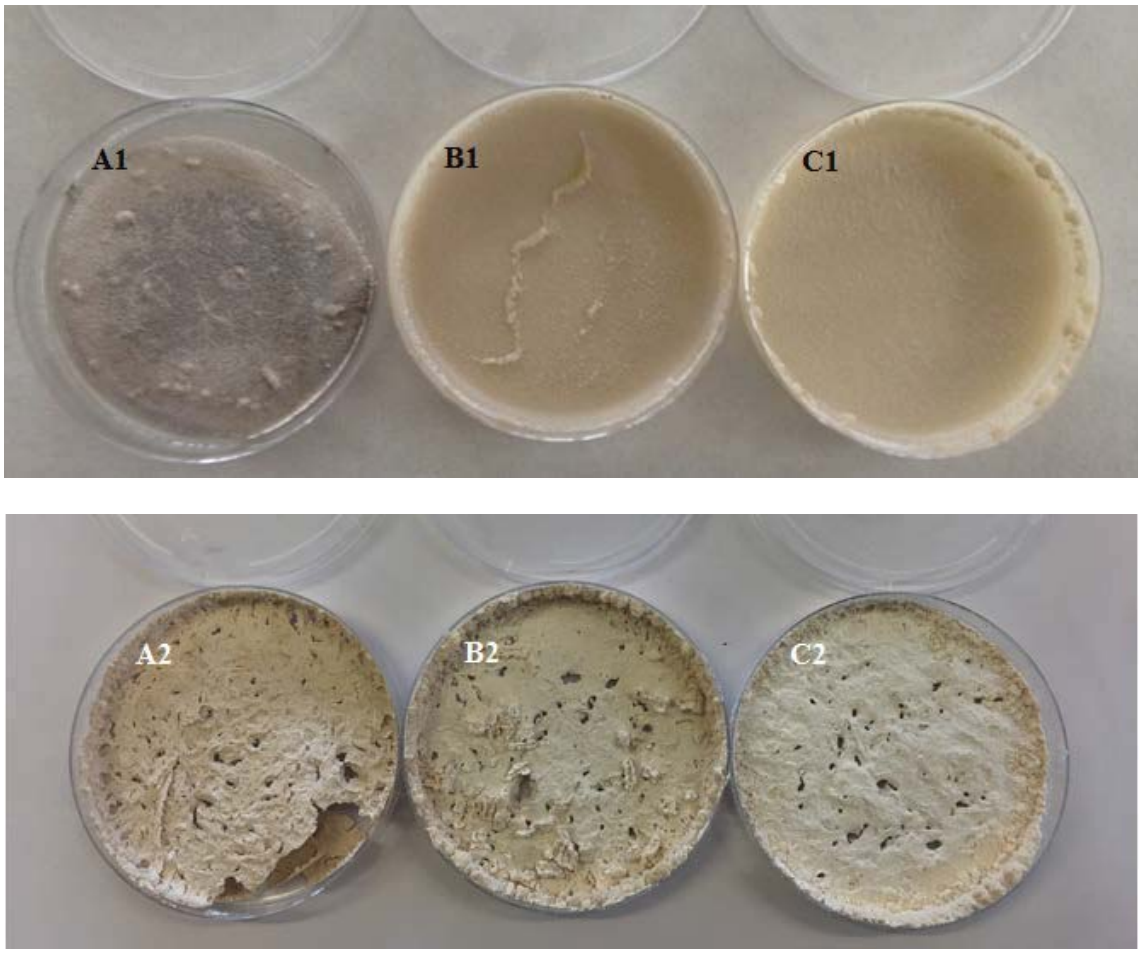

Figure 20. Image illustrating the comparison between the drying techniques used in this project, oven drying (upper image) and lyophilisation (lower image). Hydrogel composition: All hydrogels have 1.0 g alginate and 0.3 g calcium chloride, 3 g sodium salicylate and 20 mL water (A1 and A2), 6 g sodium salicylate and 40 mL water (B1 and B2), and 9 g sodium salicylate and 60 mL water (C1 and C2).

As a result, the drying methods are interchangeable with the exception of using sodium aspirin. In the case of this project, hydrogels A-F were oven dried and the rest were lyophilised.

3.6. FTIR

All IR spectra are in the appendix. Sodium alginate has many peaks of interest. The peaks at 891.8 and 1098.2 cm^{-1} indicates the presence of the polysaccharide structure. Peaks at 3448.1 and 1610.6 cm^{-1} are characteristics of the hydroxyl stretch present on the carboxylic acid groups and the intense carboxylate anion stretch respectively. Furthermore, the large amount of noise on each of the peaks indicates the presence of a

large number of each functional group. For IR spectra of B4 and B1, noise on peaks 3448.1 and 1610.6 cm^{-1} appears to decrease respectively, which is consistent with cross-linking as calcium ions are binding to the functional groups of alginate. The increasing peak at 3448.1 cm^{-1} also indicates the presence of cross-linking as OH groups are activated as calcium ions bind to the functional groups of alginate.

There are many peaks of interest in sodium salicylate. First off, the broad peaks at 1807.9 and 2547.5 cm^{-1} indicates the presence of the C=O stretch and the OH phenol stretch respectively and the peaks at 3070.9, 1582.8, and 743.8 cm^{-1} indicates the presence of phenol CH stretch, C=C bend and CH bend respectively. For hydrogels A and D, peaks at 3500 cm^{-1} have increased indicating the presence of OH stretches on carboxylic acids which in turn indicates the presence of alginate. Further, the noise on the peaks at 3500 and 1700 cm^{-1} has increased respectively indicating the lack of cross-linking as they are beginning to look similar to the naked sodium alginate. Finally, all the peaks for salicylic acid are present.

3.7. Swelling Studies for Select Hydrogel Groups

The swelling of hydrogels is an easy test which indicates the hydrogels ability to release the drug trapped within the matrix. The faster the hydrogel swells, the faster the model analgesic will be released from the system, as the drug being expelled from the matrix is replaced by water molecules. As the main goal of this project is to study drug release from the hydrogels, a select few were chosen to compare the swelling studies and illustrate the swelling capabilities of hydrogels.

3.7.1. Blank Hydrogels

The swelling profiles for the blank hydrogels cross-linked with a range of calcium chloride contents are shown in Figures 21 and 22. Note that the medium used is SIF buffer or water. All results are recorded as swelling % using equation 1. Swelling max and equilibrium results are shown in Table 5 and 6.

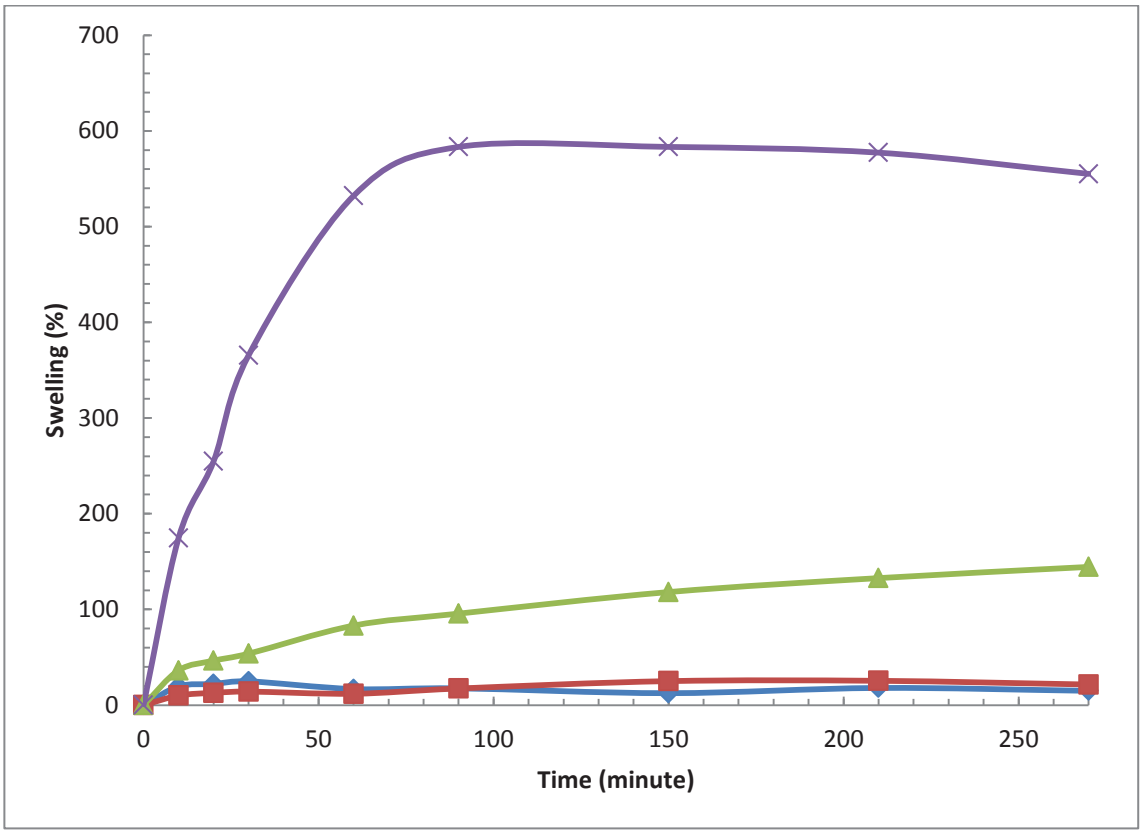

Figure 21. Swelling profiles of hydrogels: B1 (blue), B2 (red), B3 (green) and B4 (purple) cross-linked with decreasing calcium chloride content in water. Hydrogel composition: All hydrogels have 1.0 g alginate and 20 mL water, 1.0 g calcium chloride (B1), 0.5 g calcium chloride (B2), 0.3 g calcium chloride (B3), and 0.2 g calcium chloride (B4).

Table 5. Equilibrium swelling values for hydrogels B1, B2, B3, and B4 left overnight in water.

Hydrogel	Calcium Chloride added (g)	Initial weight (g)	Swollen weight (g)	Equilibrium swelling (%)
B1	1.0	0.736	0.775	2.58
B2	0.5	0.311	0.372	19.61
B3	0.3	0.590	1.604	171.86
B4	0.2	0.483	3.488	622.15

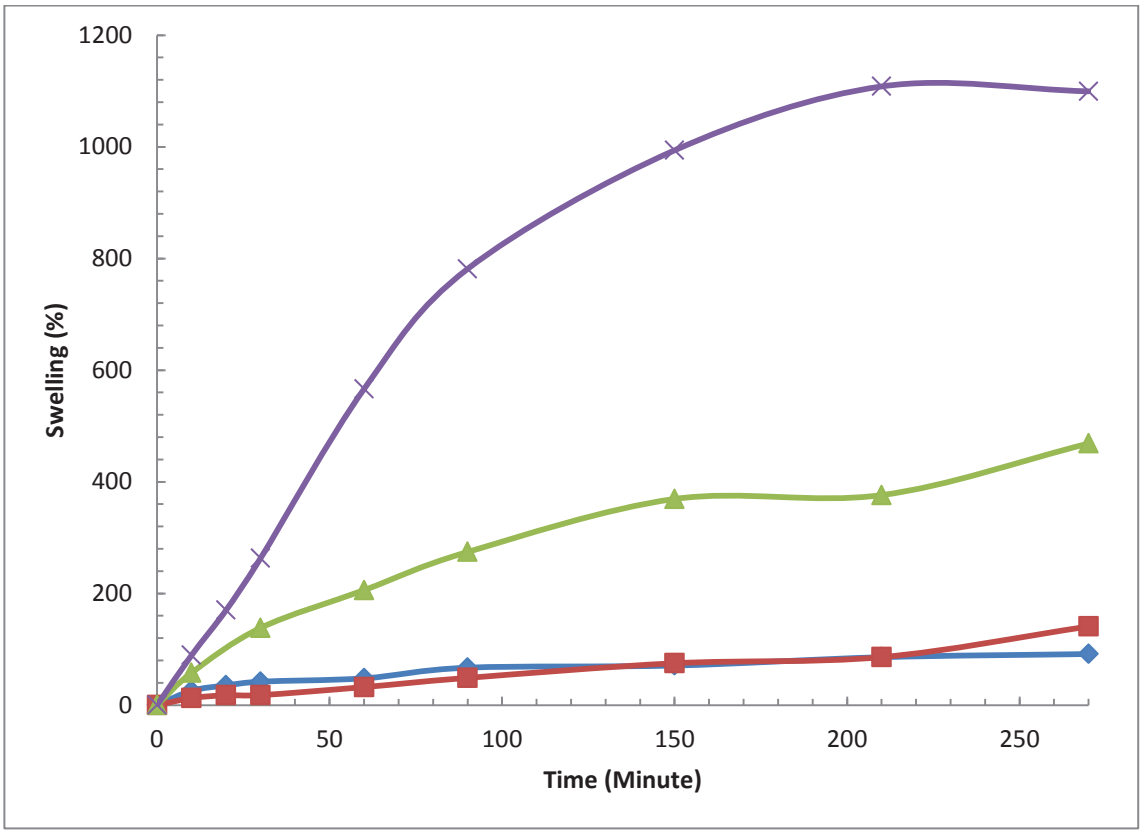

Figure 22. Swelling profiles of hydrogels: B1 (blue), B2 (red), B3 (green) and B4 (purple) cross-linked with decreasing calcium chloride content in SIF. Hydrogel composition: All hydrogels have 1.0 g alginate and 20 mL water, 1.0 g calcium chloride (B1), 0.5 g calcium chloride (B2), 0.3 g calcium chloride (B3), and 0.2 g calcium chloride (B4).

Table 6. Equilibrium swelling values for hydrogels B1, B2, B3, and B4 left overnight in SIF.

Hydrogel	Initial weight (g)	Swollen weight (g)	Equilibrium swelling (%)
B1	0.756	3.540	356.35
B2	0.492	1.864	278.86
B3	0.420	1.819	333.10
B4	0.160	N/A	N/A

As can be seen from Figures 21 and 22, the different medium does not affect the trend of the swelling profiles, only swelling % values. This was the perfect example to show that there are no differences in the trends of the profiles given the different media.

The main trend which can be observed is that the smaller the amount of calcium chloride added to the hydrogel, the more medium the water absorbs, due to the less tightly cross-linked the alginate is. As a result, there is more room for the water to exist within the hydrogel matrix, the more water the hydrogel absorbs when introduced into the medium. Further, higher crosslinking reduces porosity, limiting the free space available for water to exist. As can be seen from the figure above, hydrogels B1 and B2 absorb little water due to the higher crosslinking, while B3 and B4 absorb the most water due to the lack of crosslinking and the larger pores present within the matrix.

With calcium chloride contents below 0.2 g, the hydrogels begin to deteriorate quickly when introduced to the media. Hydrogel B5 had the lowest calcium chloride content at 0.1 g. Table 7 compares the maximum swelling of each hydrogel, and most occurred before equilibrium is achieved. In the case of B5, the swelling percentage indeed was high at the maximum swelling, however, it began to deteriorate fast after 60 minutes in both cases and eventually, it was fully dissolved in the media. This indicates that there is little cross-linking occurring due to the small amount of calcium chloride and when introduced to the medium, the uncross-linked alginate begins to dissolve back into the medium due to it being water soluble.

Table 7. Comparison of maximum swelling values for hydrogels B1, B2, B3, B4, and B5 in water and SIF.

Hydrogel	Swelling in water (%)	Swelling max in SIF (%)
B1	17.94	368.25
B2	25.40	278.86
B3	171.86	468.57
B4	622.15	1108.13
B5	716.01	654.53

From these swelling results, hydrogels B3 and B4 appear to be the most useful for the *in vivo* experiments when loaded with the analgesic, with B3 being the optimal hydrogel. Indeed, B4 absorbs the largest quantity of water, yet in the SIF medium, it appears to be structurally weaker. These results were collected for the hydrogel dissolving into the SIF medium before equilibrium is achieved. This could increase the speed of analgesic

release as the barrier between the analgesic and the medium is effectively removed, allowing a large portion of the analgesic to be released all at once.

In the case of B3, it appeared to be the most useful as it swelled to a high degree without the loss of the hydrogel matrix, which could potentially allow a sustained release of the analgesic. In addition, B1 and B2 have a higher potential for a longer sustained release of the analgesic as they have a lower degree of swelling and in theory, this could lead to the medium entering the matrix at a slower rate, thereby releasing the analgesic out slower. B1 can be useful for the *in vivo* experiments as the maximum calcium chloride content. Any hydrogels prepared with a calcium chloride content above 1.0 g are not injectable as they are large solid masses compared to the solid in liquid matrixes of B1-B5.

3.7.2. Hydrogels A, B, and C

The swelling profiles for A, B, and C are shown in Figure 23. Note that the medium is water and the swelling values are calculated in swelling g/g. The following equation is used to calculate the swelling in g/g

$$Swelling\ {}^{g}/_{g} = \frac{W_s - W_d}{W_d}$$

where W_s is the swollen weight of the hydrogel at any given time interval and W_d is the weight of the dried hydrogel before adding to the medium. Table 8 shows the equilibrium values in both swelling g/g and swelling %.

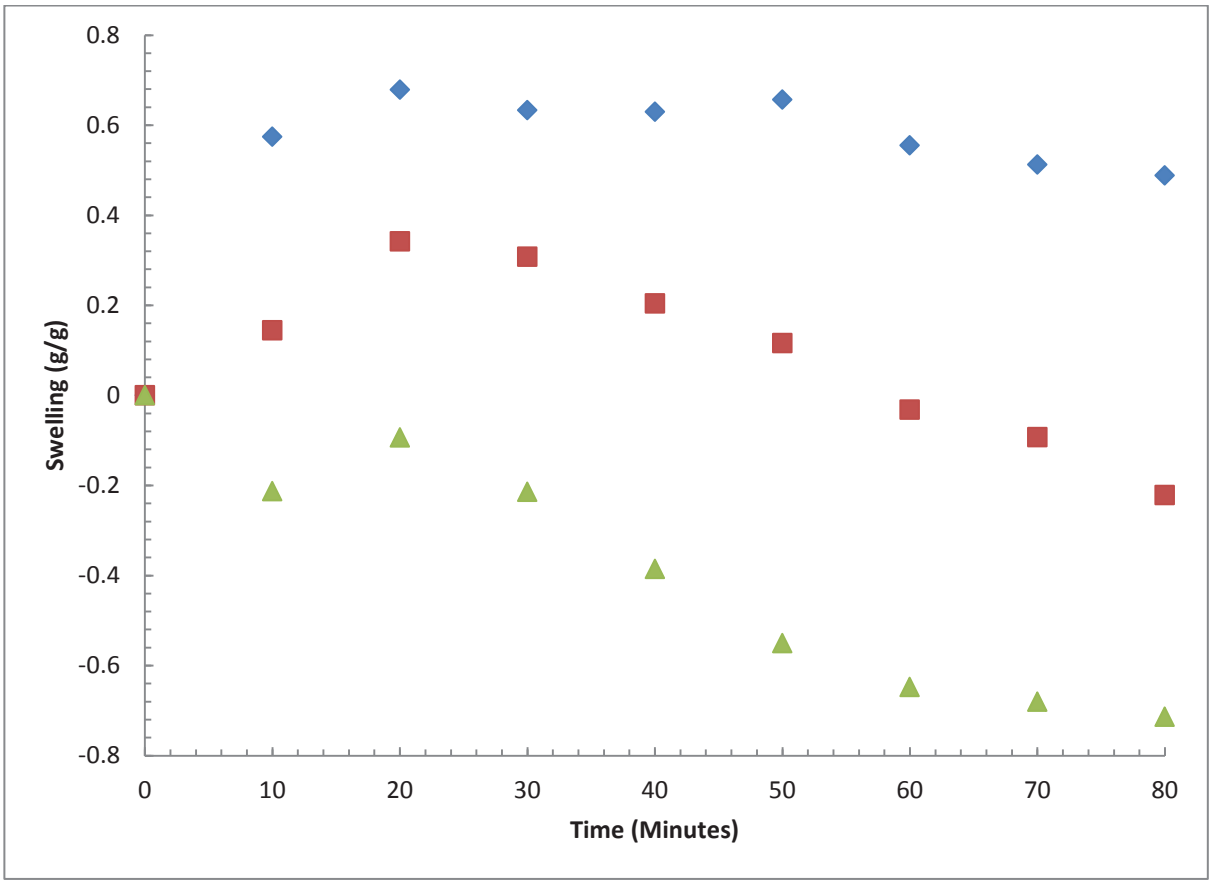

Figure 23. Swelling profiles of hydrogels: A (blue), B (red), and C (green) cross-linked with 0.3g calcium chloride in water. Hydrogel composition: The hydrogels have 1.0 g alginate and 0.3 g calcium chloride, 3 g sodium salicylate and 20 mL water (A), 6 g sodium salicylate and 40 mL water (B), and 9 g sodium salicylate and 60 mL water (C).

Table 8. Equilibrium swelling values of hydrogels A, B, and C left overnight in water.

Hydrogel	Equilibrium swelling (g/g)	Equilibrium swelling (%)
A	0.50	50
B	-0.24	-24
C	-0.71	-71

The first trend which can be seen is the less diluted the hydrogel is, the more it swells when dried down and added to the medium. Consequently, this pattern can also be explained by the gel-like state of each hydrogel, with the most gel like one absorbing the most water (Figure 23, hydrogel A) and the more liquid-like one absorbing no water (Figure 23, hydrogel C).

Given that hydrogel A is the only one that appears to be in a gel-like state and absorbing/retaining water during the swelling profile experiment, then there is an indication that some degree of cross-linking has occurred during the hydrogel preparation, producing the insoluble hydrogel product. However, given that the swelling g/g of A does not go past 1, the degree of cross-linking is smaller than expected as compared to the blank hydrogels above. The image of A in Figure 17 does support this small degree of cross-linking as a gel-like texture was achieved, yet the hydrogel is slightly cloudy, which indicates that the calcium and alginate have cross-linked, but the calcium and drug have also interacted with each other, producing the insoluble calcium salt forms of the drugs, and in turn, the cloudiness of the hydrogel. Nevertheless, this small degree of cross-linking does contribute to the insoluble hydrogel and the increased swelling profile of A.

Hydrogels B and C are a different story. Both hydrogels appear to lose weight when added to the medium, which already indicates the effect of little cross-linking between the calcium and alginate. The alginate and calcium have not formed the insoluble hydrogel product and when added to the medium, the uncross-linked alginate dissolves back into the water and releases the calcium drug from its cage, both of which contributes to the loss of weight of the hydrogels, hence contributing to the negative swelling g/g values obtained. This claim is again supported by Figure 17 where it can be seen that both B and C have a liquid texture instead of the desired gel texture, and both are extremely cloudy from the production of the insoluble calcium form of the drugs. The equilibrium swelling values from Table 8 further supports this observation with both being negative values, indicating the loss of the hydrogel matrix. It is important to note that there appears to be a higher degree of cross-linking with hydrogel B compared to C. This is accounted for by the way B absorbs and retains a higher swelling g/g above 0 g/g compared to C which immediately falls below 0 g/g when added to the medium. However, this cross-linking is small and weak and results in the g/g going below 0 after 60 minutes in the medium.

The second trend which can be observed is at the start of the swelling profiles for B and C. Both have a maximum swelling value where the hydrogel does swell but after 30 minutes, both begin to break down. This is not unusual as there are osmotic forces playing a role within the hydrogels themselves, resulting in the release of the water

molecules. The hydrogels begin to swell with water, and at a certain point, there is no more room within the network for the water and drug molecules to exist, therefore osmotic forces push both the water and the drug out as the hydrogel tries to gain equilibrium between the water entering and water exiting. This peak indicates the presence of cross-linked alginate due to its water retention, however this cross-linking is small and weak due to the breakdown of the material into the medium, hence the weight of each decreasing past the original dried weight. The same phenomenon is occurring in A, however as there is more-crosslinking and less alginate dissolving into the medium, there is more water retention, hence no obvious swelling maximum peak present as it the first point of the plot (point at 10 minutes).

A hydrogel's ability to retain equilibrium indicates the presence of cross-linked alginate. According to the equilibrium results in Table 8, the only hydrogel to retain the water over 24 hours is A, which is supported by the swelling profiles and the image from Figure 17.

3.7.3. Hydrogels D, E, and F

The swelling profiles for the D, E, and F are shown in Figure 24. The medium is water and the values are calculated in swelling g/g. Table 9 records the equilibrium values in both swelling g/g and swelling %.

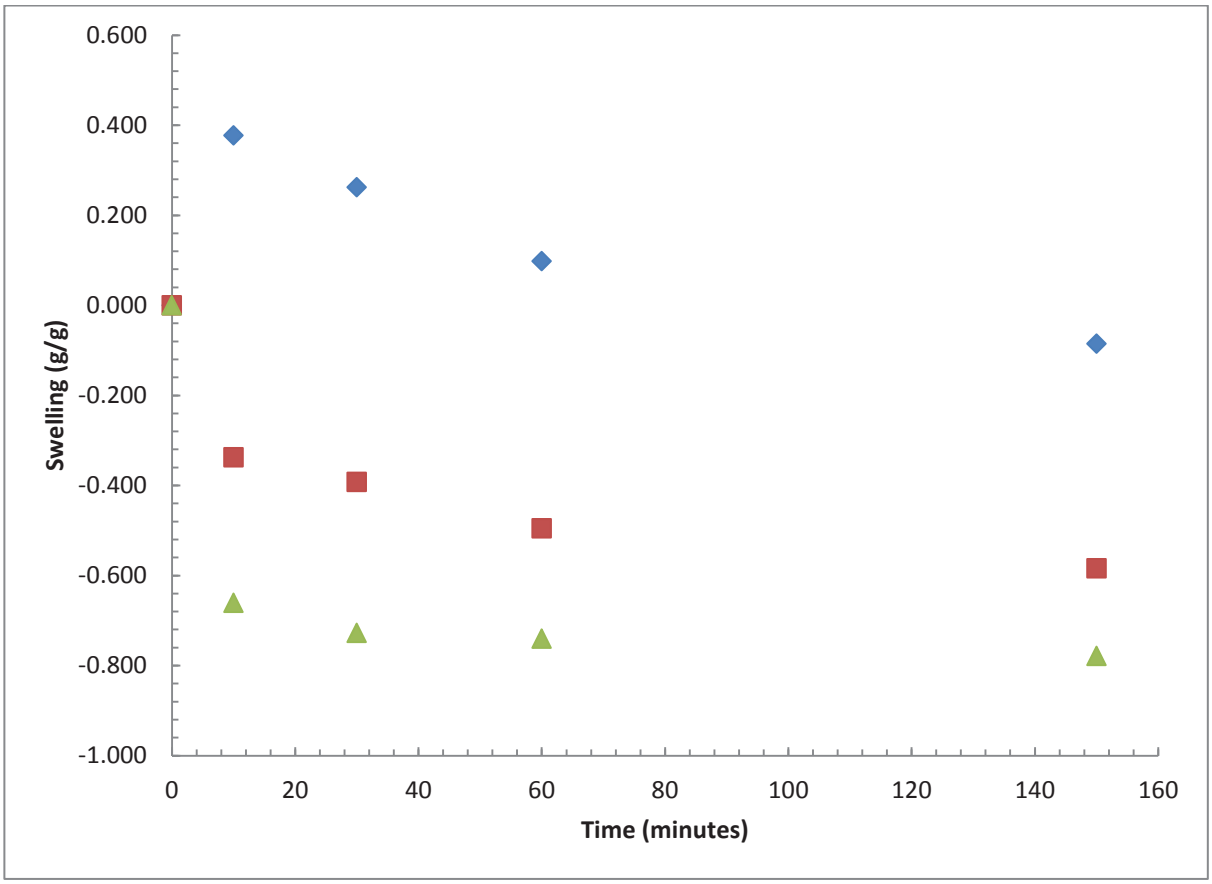

Figure 24. Swelling profiles of hydrogels D (blue), E (red), and F (green) cross-linked with 1.0 g calcium chloride in water. Hydrogel composition: All hydrogels have 1.0 g alginate and 1.0 g calcium chloride, 3 g sodium salicylate and 20 mL water (D), 6 g sodium salicylate and 40 mL water (E), and 9 g sodium salicylate and 60 mL water (F).

Table 9. Equilibrium swelling after 24 hours of hydrogels D, E, and F in water.

Hydrogel	Swelling max (g/g)	Swelling max (%)
D	-0.23	-23
E	-0.63	-63
F	-0.79	-79

Similar to the swelling profiles above (Figure 23), the hydrogel with the largest dilution retains the lesser amount of water. D has the most swelling while F has the least swelling, and the reasons behind this are the same as above.

One interesting observation made in these profiles is the swelling maximum. For D, there is one in the first 10 minutes of the profile, indicating the presence of cross-linked alginate, however, no such peak is present for E and F. In fact, the minute E and F are

introduced to the medium, they immediately begin to break up and dissolve into the water, indicating that very little cross-linked alginate is present. Furthermore, these sets of hydrogels produce a very cloudy white hydrogel which has a liquid-like texture, which further indicates the absence of cross-linked alginate. From these two points, it can be assumed that very minimal cross-linking has occurred hence explaining the swelling profiles obtained. Compared to the set above (Figure 23), where very little cross-linking had occurred to the point where swelling maximums were present, it is safe to assume no cross-linking or highly minimal cross-linking, to the point where it has no net effect on the swelling profile, has occurred in this set.

To add onto the last statement, the equilibrium values of this set are again in the negative range (Table 9). It seems the hydrogels have not retained any water during the 24-hour period and instead, some of the gel has dissolved into the water. As a result, these hydrogels have very minimal cross-linking which has no overall effect on the hydrogels properties.

3.8. Drug Release Profiles

The drug release profiles for all hydrogels were completed over a time period of up to 72 hours to determine maximum release. As the hydrogels for chicken injection were given in the wet form, the hydrogels were not washed prior to the drug release experiments to try and mimic the *in vivo* experiments – see later.

3.8.1. Hydrogels A, B, and C

The drug release profile of hydrogels A, B, and C is shown below in Figure 25. Note the medium used is water.

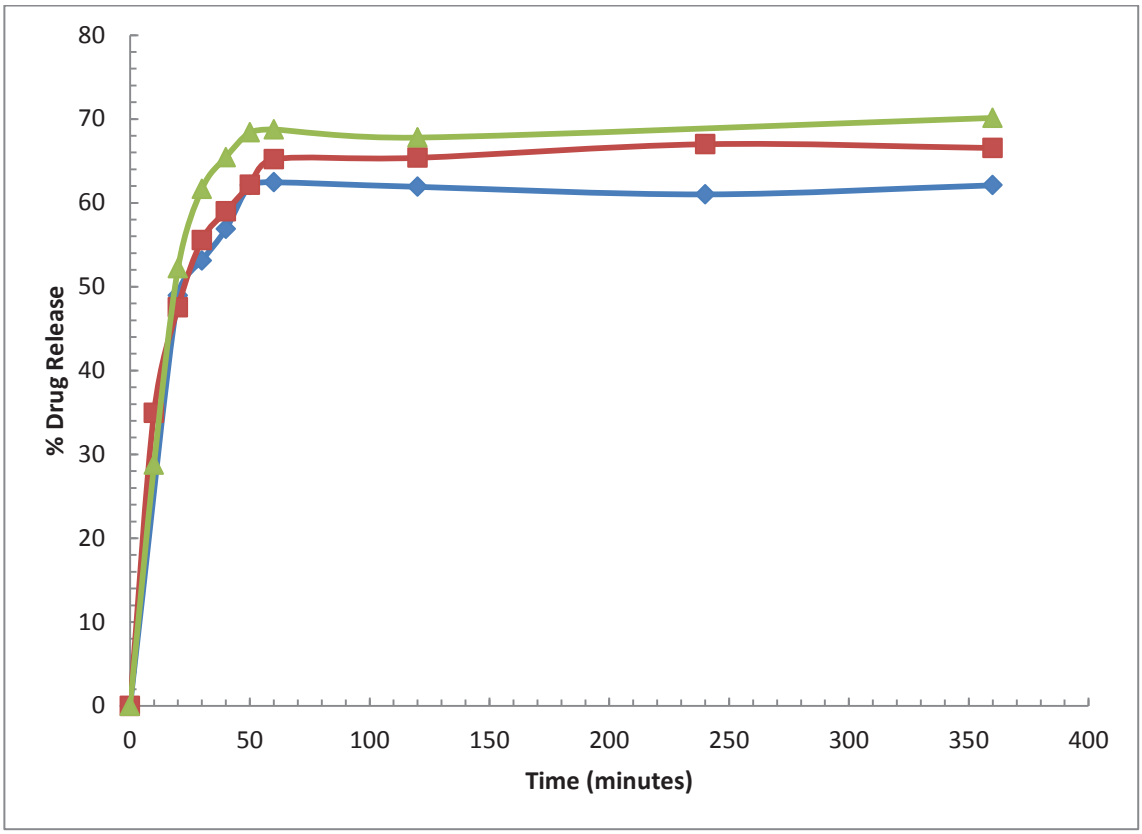

Figure 25. Drug release profiles of hydrogels: A (blue), B (red), and C (green) in water. Hydrogel compositions: All hydrogels have 1.0 g alginate and 0.3 g calcium chloride, 3 g sodium salicylate and 20 mL water (A), 6 g sodium salicylate and 40 mL water (B), and 9 g sodium salicylate and 60 mL water (C).

As can be seen from Figure 25, it appears the drug release is rapid, with the majority of the analgesic having exited the matrix after the first 50 minutes. Unfortunately, this rapid release is undesirable and would require further alterations to the hydrogel to improve release. However, it does indicate the possibilities of a slow release given that changes to the hydrogel are made. Furthermore, *in vivo* analysis of this set of hydrogels could produce differing results due to the natural buffers, barriers, proteins, and enzymes present in natural systems, meaning sustained release could be possible once the hydrogel is injected into the poultry.

3.8.2. Hydrogels D, E, and F

The drug release profile of hydrogels D, E, and F is shown below in Figure 26. Note the medium used is water.

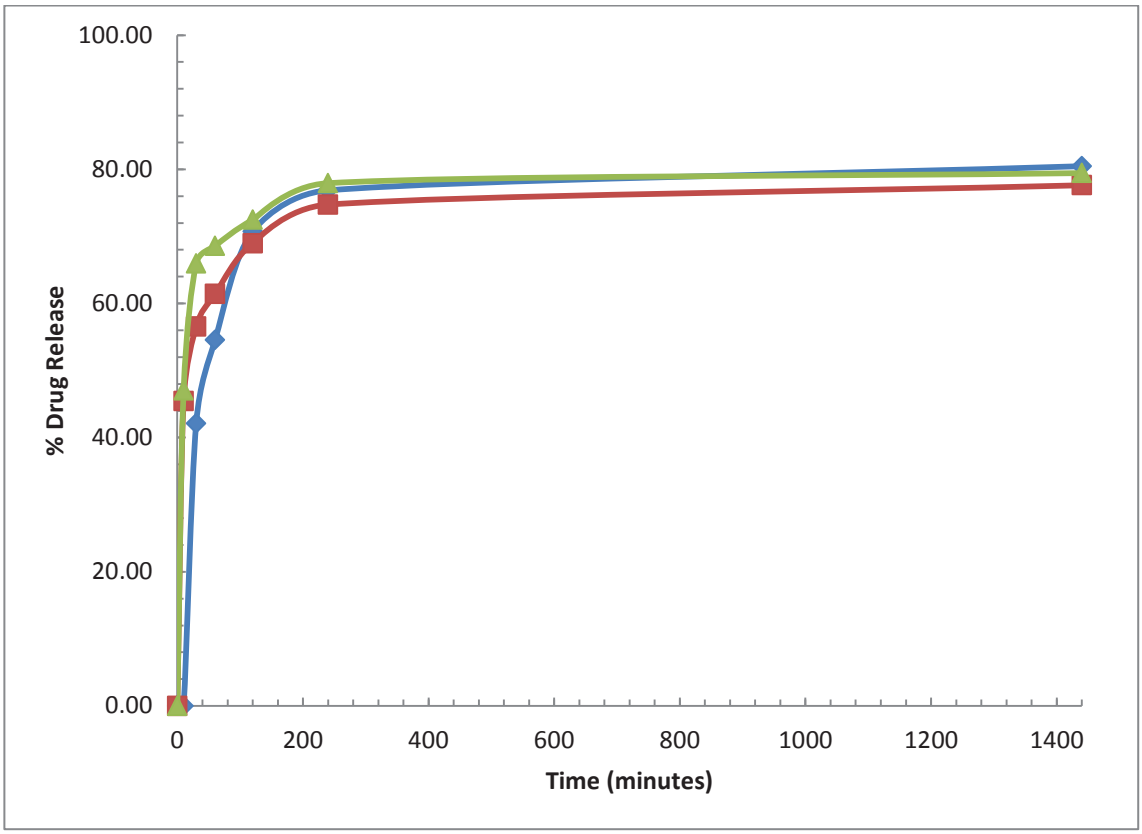

Figure 26. Drug release profiles of hydrogels D (blue), E (red), and F (green) in water. Hydrogel compositions: All hydrogels have 1.0 g alginate and 1.0 g calcium chloride, 3 g sodium salicylate and 20 mL water (D), 6 g sodium salicylate and 40 mL water (E), and 9 g sodium salicylate and 60 mL water (F).

Just by adding more of one component to the hydrogel mixture (in this case, calcium chloride), the time of release for the analgesic has increased by a factor of 4 compared to Figure 25. In this case, drug release has increased to around 3.33 hours, supporting the hydrogels ability to decrease the rate of release with altering hydrogel structures. However, again this release is not desirable as release is aimed for over 24 hours. It can be assumed that due to the longer release, *in vivo* experiments will result in the analgesic therapeutic dose remaining above the threshold for action longer then hydrogels in Figure 25.

3.8.3. Hydrogels G and H

The drug release profile of hydrogel H is shown below in Figure 27. The medium in which the measurements were taken is SIF buffer to mimic the natural pH and buffers present in the *in vivo* experiments.

For most profiles, a portion of the analgesic is released into the solution and then the analgesic concentration in the solution appears to decrease. This is not unusual due the equilibrium being created between the water and hydrogel. The drawback is that the analgesic on the surface of the hydrogel is immediately released into the medium and as the hydrogel gains equilibrium, the analgesic in solution can enter and exit with the water, hence giving the effect of the changing analgesic concentration in solution. One example is hydrogel H in Figure 27.

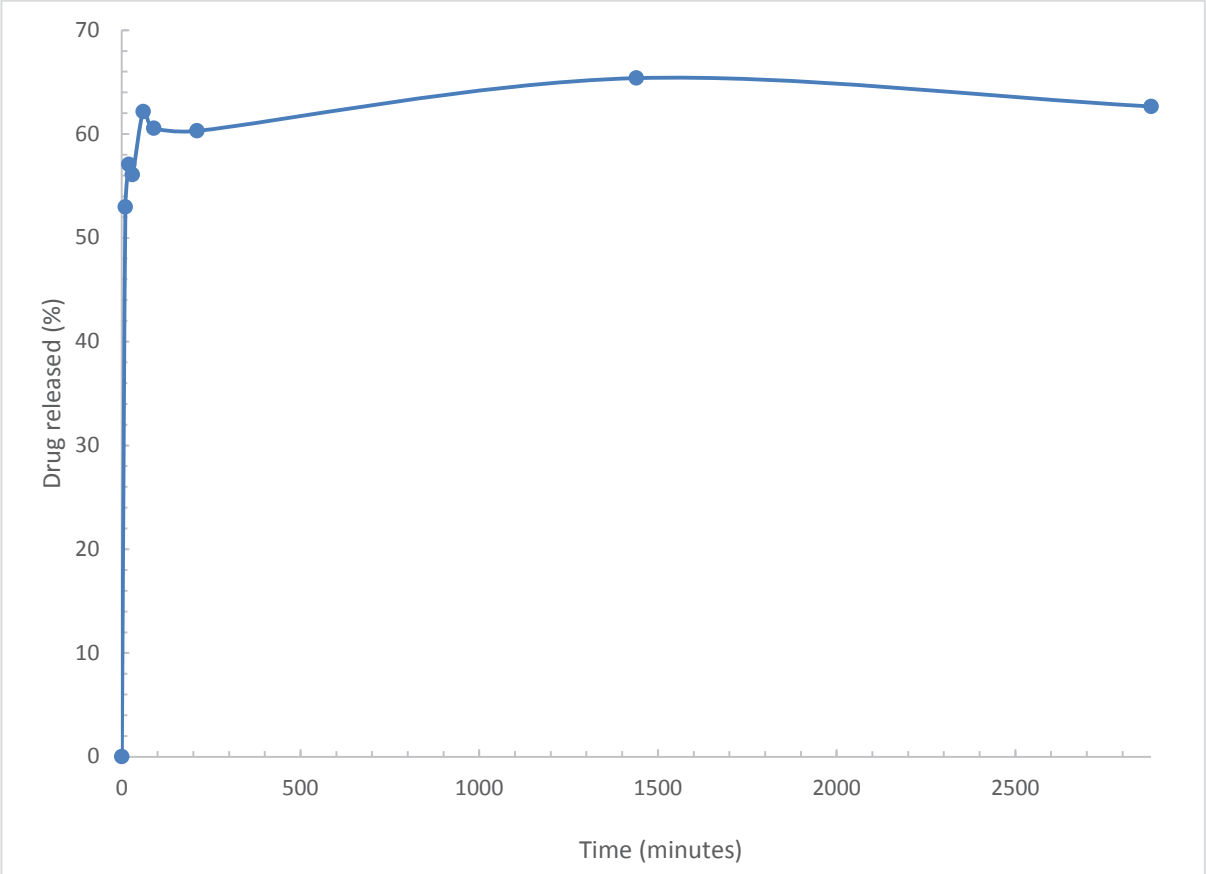

Figure 27. Drug release profile of hydrogel H in SIF buffer. Hydrogel composition: 3.0 g alginate, 1.0 g calcium chloride, 12.0 g sodium salicylate, and 80 mL water.

One point of interest is the maximum release of this gel is only around 65 %. Given that the cross-linked alginate is insoluble in water, some of the drug molecules will have bonded to this in some way, resulting in the drug release percentage not reaching 100 %. Furthermore, some of the drug will be present in the insoluble calcium form as described above. Given that the SIF is made in water, adding the hydrogel to the

medium will still result in the calcium drug being insoluble. These points account for the drug % release of 65 %.

Another point of interest is the time at which the maximum drug release point of H is at. After 3.5 hours in the medium, there is a total release of about 60 % while after 24 hours, this release increases to 65 %. This is promising as it was still releasing after the first 3.5 hours. Yet on the other hand, the release only increased by 5 % which could have been reached relatively fast after 3.5 hours, which means that the release would have only lasted for about 4 hours. Compare this to the above results for the hydrogel set D, E, and F where the release was only after the 2 hours, there is some improvement in the release and could be further slowed down when the hydrogel is introduced to the chickens due to the different enzymes, environments and layers the drug needs to go through to penetrate the blood stream.

As stated in 3.3.2 above, the more dilute hydrogel H is, the less calcium drug produced during the preparation. Figure 28 shows a comparison of the release of hydrogels G and H in SIF medium. As can be observed, G releases more drug then H does. Again, G is less dilute then H and so the cross-linked alginate is tighter and less calcium drug has formed, so there is more analgesic released from the hydrogel. One limitation to hydrogel G is that after 48 hours, analgesic release exceeds 100 %, which is not uncommon in “crude” systems. Figure 28 is given in mol/L instead of drug release percent for simplicity.

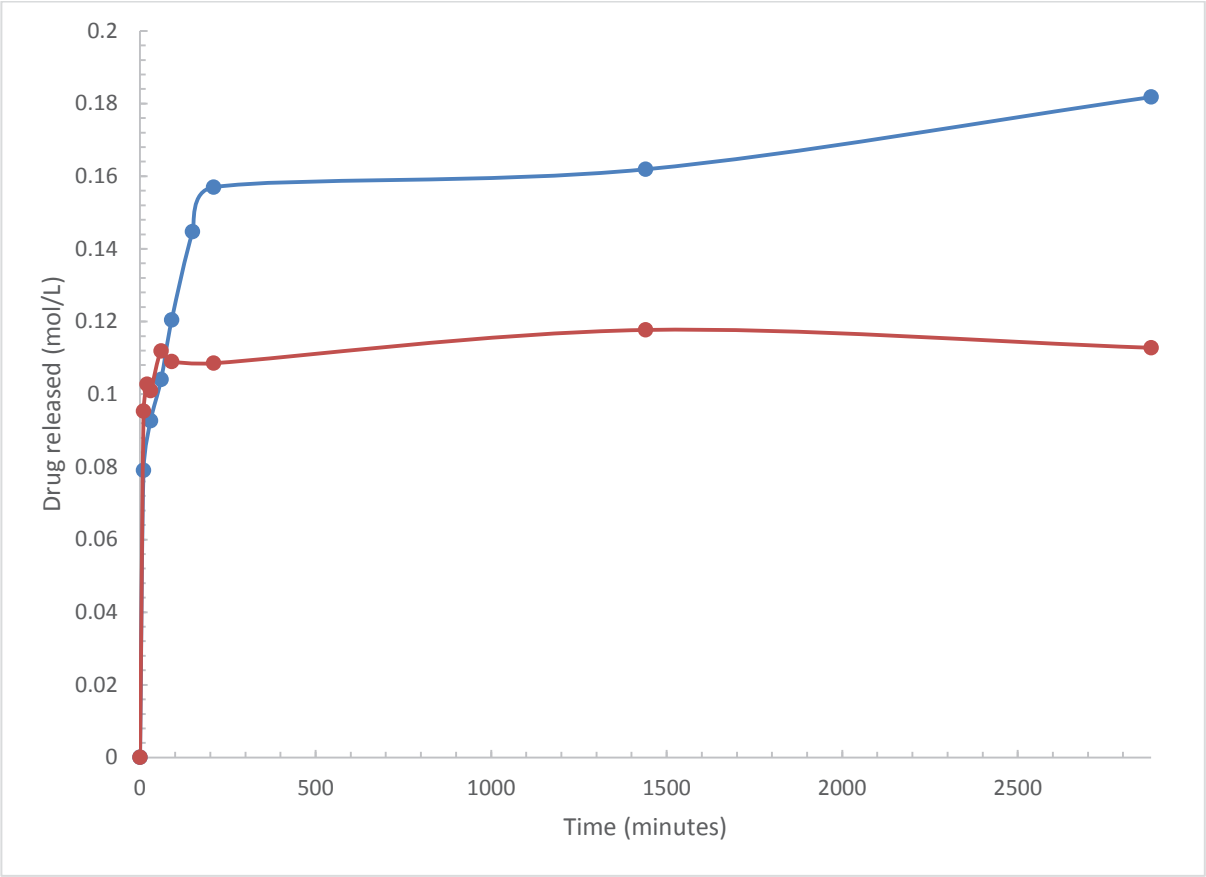

Figure 28. Comparison of drug release profiles for hydrogels G (blue) and H (red) in SIF medium. Hydrogel composition: Both have the 3.0 g alginate and 1.0 g calcium chloride, 3.0 g sodium salicylate and 20 mL water (G) and 12.0 g sodium salicylate and 80 mL water (H).

3.8.4. Hydrogels I and J

Due to the slower release of sodium salicylate within the hydrogels above, hydrogels prepared with another analgesic, sodium aspirin, were used as a comparison. The aim was to distinguish if switching the analgesic would slow down the release. All hydrogels prepared with sodium aspirin were similar in composition to their counterparts, with the exception of sodium aspirin added instead of sodium salicylate. The drug release profiles of hydrogels I and J are shown below in Figure 29. The medium in which the measurements were taken is SIF buffer to mimic the natural pH and buffers present in the *in vivo* experiments.

The first point of interest is the low drug release percentages, with I reaching a maximum of 34 % and J reaching a maximum of 22 %. Sodium aspirin was not the

easiest drug to use in these hydrogels due to its instability in solution, as described in 3.2. In combination with the SIF buffer, sodium aspirin transforms into sodium salicylate in solution at any temperature, with temperatures below 4 degrees only being stable for less than 48 hours. This made measuring the drug harder as the sodium salicylate curve had to be applied due to the majority of drug detected being in the sodium salicylate form over the sodium aspirin form, yet both drugs were present in solution. As the sodium salicylate peak overshadows the sodium aspirin peak, only sodium salicylate can be calculated, hence the small drug release percentages.

In addition, when calcium chloride is added to the hydrogel mixture, calcium aspirin is produced instead of the desired cross-linking of alginate. Calcium aspirin is also insoluble in water, therefore, the low percentage release can also be account to some extent by the presence of calcium aspirin.

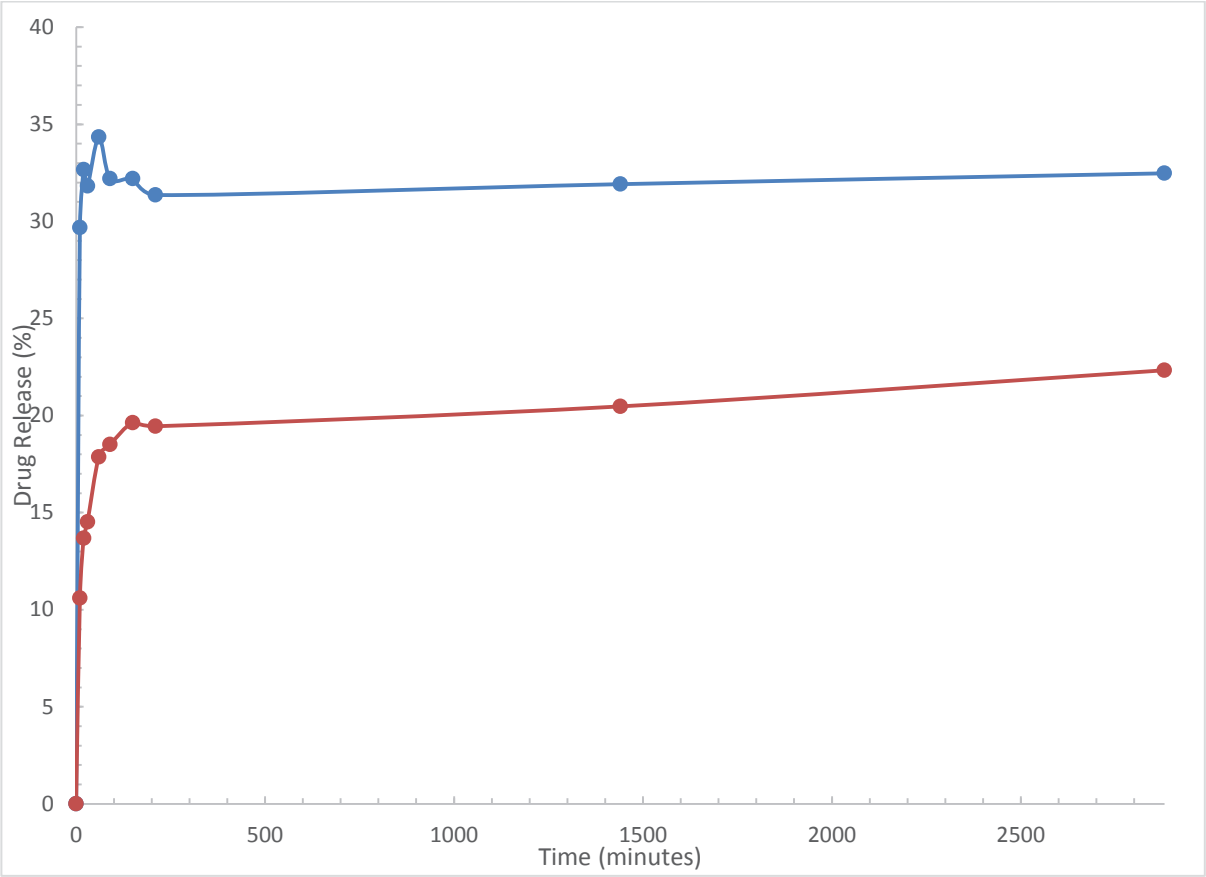

Figure 29. Drug release profiles for hydrogels I (blue) and J (red) in SIF medium. Hydrogel composition: I 1.0 g alginate, 3.0 g sodium aspirin, 0.2 g calcium chloride and 20 mL water and J 3.0 g sodium aspirin, 1.0 g calcium chloride and 20 mL water.

One trend that can be seen between these two hydrogels is that the more calcium present in the preparation, the less drug which is released during the release experiments. This can again be accounted for by the presence of calcium aspirin. As more calcium chloride is added, more calcium aspirin is produced, hence a lower release profile of the hydrogel. It is expected that a hydrogel with a calcium content in between I and J will result in a release profile larger then J and smaller then I.

For I, another maximum peak at the start of the profile can be seen, please refer to paragraph above in section 3.5.3 for explanation.

3.8.6. Hydrogel K

The drug release profile of hydrogel K is shown below in Figures 31 and 32. The two profiles are used to express the slower release of the drug compared to other profiles above. The medium in which the measurements were taken is water for simplicity.

In section 3.3.3, it was stated that hydrogels made with calcium salicylate were not viable for the *in vivo* experiments. However, the release profile is a nice comparison for the behaviour of the hydrogel. In these measurements, only a section of this hydrogel was used to measure the drug release profile, and therefore the drug release percentage is not available for this set of data, yet the important part to this profile are the trends expressed.

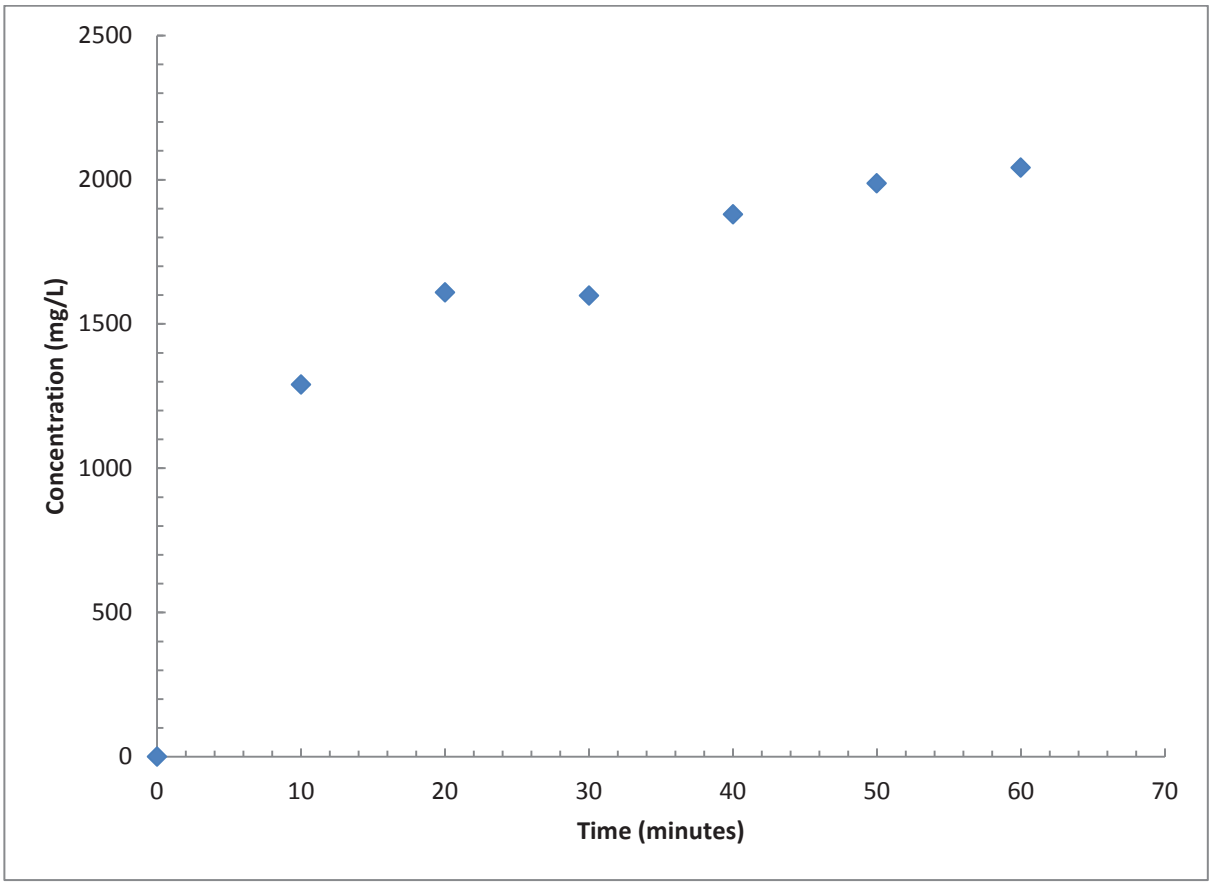

Figure 30. Drug release profile one of hydrogel K in water. Hydrogel composition: 1.0 g alginate, 0.3 g calcium salicylate, and 20 mL water.

The desired hydrogel for the *in vivo* experiments is one that slowly releases the model drug over an extended period of time. Ideally, the period would be over 24 hours

minimum. In the case of hydrogel K, the drug was released over a 4 hour period and supports the idea of the release slowing down for the more gel-like textured hydrogels. Both Figures 31 and 32 show a climbing slope over the time period of the release profile which represents the slower release of the drug as compared to the other hydrogels used. It is important to note that after 24 hours (Figure 31), the drug release of the hydrogel had increased from about 2000 mg/L to 3000 mg/L and given the slower release due to this hydrogel, it is assumed that a portion of the hydrogel was still releasing after 4 hours in the medium.

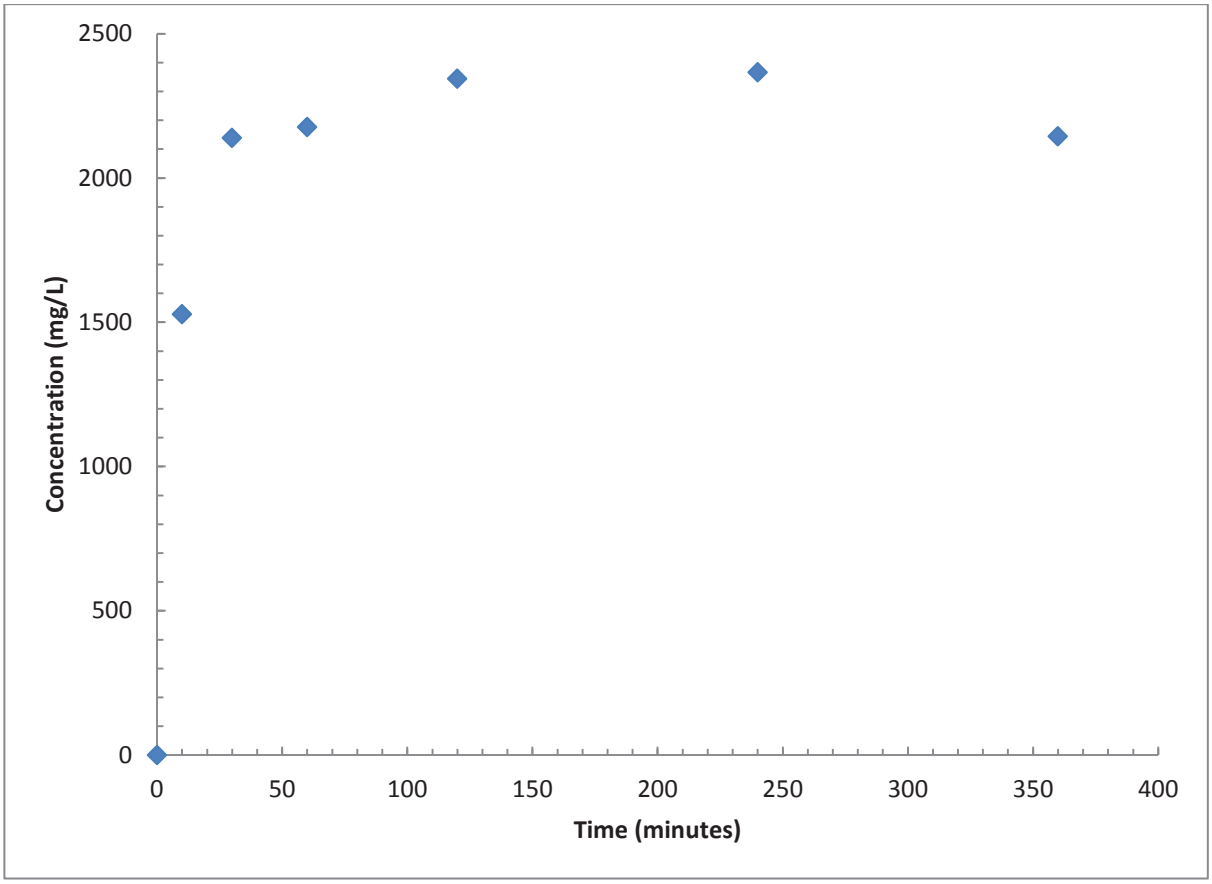

Figure 31. Drug release profile one of hydrogel K in water. Hydrogel composition: 1.0 g alginate, 0.3 g calcium salicylate, and 20 mL water.

3.9. *In Vivo* Experiments on Poultry Using Hydrogels

All poultry experiments were performed by my supervisor, Dr. Preet Singh at the Institute of Veterinary, Animal and Biomedical Sciences (IVABS). This included the ethics application, the preparation of the chickens for the experiments, caring for the chickens during testing, administration of the hydrogel, collection of the blood, the

storing of the blood before removal of plasma, and the separation of the blood plasma for analysis.

3.9.1. HPLC Analysis of Processed Plasma Samples

The technique used to analyse the processed samples was HPLC. This technique had its advantages but also limitations, mainly caused by the model analgesics chosen and the interaction between the HPLC and model analgesics. In the following sections, these advantages and limitations to the HPLC will be discussed with reference to the issues which arose during the project in terms of the model analgesics chosen.

3.9.1.1. Preparation of Samples for Analysis

There are multiple ways in the literature to prepare sodium salicylate/sodium aspirin for analysis, however, there is very little about the difficulty of using them as the model analgesics in biological samples, as both are highly sensitive to factors such as heat, pH, and solvents. Furthermore, the biological samples need to be stripped of their proteins and enzymes to prevent the HPLC column from blocking up, which the majority of the time strips the model analgesics along with it. Therefore, a method is needed that would strip the proteins from the plasma samples, while retaining the drug in the sample or a method where one could strip the proteins while transferring the model analgesics to another solvent for maximum precision.

The chosen method for the preparation of the samples was liquid-liquid extraction, as the proteins could be stripped using acetonitrile and the model analgesics being transferred to another solvent, acetic acid, where both are still soluble. This means that the solid proteins stripped from the plasma samples could be centrifuged away and the model analgesics remains dissolved in the acetic acid, hence minimal loss of drug achieved.

Another method explored was similar to liquid-liquid extraction. Instead of both acetonitrile and acetic acid added to the plasma samples, only acetonitrile was added to remove the majority of the proteins. After centrifugation and drying down to remove the solvents, the samples were re-dissolved into water for the HPLC analysis. The issue with this method was that during the removal of the proteins, the drug was also removed, resulting in low readings using HPLC. Unfortunately, there is no clear

explanation as to why this occurred as the drugs were soluble in acetonitrile and it was assumed that the drug would be in the liquid layer after centrifugation. Further, this method was used in past articles with good success rates. There is the possibility that during the removal of the proteins, the drug may have been caught between the precipitated protein particles, hence being dragged down to the solid layer after centrifugation, resulting in the low reading on the HPLC. Yet, one could assume that the small size of the drugs would prevent this phenomenon from occurring. Alternatively, the model analgesics may have transformed from their sodium salts (sodium salicylate and sodium aspirin) to their insoluble forms (salicylic acid and aspirin) with the addition of acetonitrile, resulting in the solids forming in the solid layer after centrifugation, but again, one could assume that the pH of the system is not low enough for this to occur. Nevertheless, using this method resulted in the loss of the drug during the preparation of the sample and this method was not valid.

Another method which was explored was Solid Phase Extraction (SPE) and was provided by Ms Shashwati Mathurkar. This method involved the activation of SPE columns with methanol, feeding the plasma samples through the column so the salicylic acid would stick to the column, washing the columns to remove any of the proteins, and eluding the salicylic acid for analysis. This method would be perfect for analysing plasma samples with a high lipid content such as sheep plasma, however, for chicken plasma, this method again resulted in the loss of the drug. When the plasma was sucked through the SPE column, instead of sticking to the column, it would go all the way through into the washings and the resulting eluent contained little to no drug. As a result, this method was made invalid and liquid-liquid extraction was explored instead.

One limitation to the liquid-liquid extraction method was drying down the solvent after protein removal. In the method developed by Dr. Singh, the drying method involved maintaining the sample at 20 degrees and blowing on the plasma samples with a gentle air stream until the solvent had evaporated. Given the number of samples being prepared, it was decided that initial testing would use an altered drying method which involved either freezing the plasma samples and using lyophilisation or lyophilising the "wet" plasma samples to remove the solvents after protein removal with acetonitrile. The main reasons behind this was the lack of equipment in the laboratory, the number of samples being prepared at any given time, and the amount of time required for

blowing the solvents away. This new drying technique came with its limitations including the bubbling of the solvents when samples were wet and infecting others around them, the aggressive nature of lyophilisation effecting the stability of the drugs and the time it took to lyophilise the samples. As a result, future plasma samples (i.e. the last three set of samples) were dried using the method developed by Dr. Singh for accurate results on the HPLC.

3.9.1.2. HPLC Problems

A considerable amount of time went into perfecting the HPLC analysis of the plasma samples. At every stage of the analysis, problems involving the HPLC arose and required large amounts of time to fix. Some of the more major issues are as follows:

- The pH sensitivity of the model analgesic forced the peak to constantly move between mobile phases. This was minimised by fixing the mobile phase to pH of 2.00 and comparing drug peak in plasma to standard drug peaks.
- Removal of organic solvents from HPLC to suit the reverse phase required to measure the model analgesic. This was minimised by changing to a HPLC used only for reverse phase.
- Blockage of the HPLC column due to the presence of plasma proteins and enzymes. This was minimised by attaching a guard column.
- Leakage of mobile phase from guard column when tightened using hands as per the guard column instructions. This was minimised by tightening the guard column using a vice.

3.9.2. HPLC Validation Results

The LLQ was calculated to 400 ng/mL while the LLD was 50 ng/mL. The linearity of the curve was determined between 500 ug/mL to 500 ng/mL, with the r-squared value being 0.9970. The average recovery rate of sodium salicylate in spiked plasma was 87.6 %. Retention time for sodium salicylate in solution and plasma is between 8.00 – 10.00 minutes depending on the mobile phase of the day.

3.9.3. Analysis of Chicken Plasma Samples

The analysis of the chicken plasma samples using HPLC is discussed below. Note that due to the method used to process the samples, group 2 is composed of six chickens but only four are used in the results as the other two data groups are lower in concentration due to process method, which brings into question their reliability and consistency compared to the other four. Group 1 remained consistent and reliable. As a result, the method used for the processing of the plasma samples has been refined for groups 3, 4, and 5 to ensure sodium salicylate stability and higher recovery rate. The properties of each group are shown below in table 11.

Table 10. Table 10 illustrates the 5 groups of chickens injected with the altering substances through subcutaneous injection.

Group	Number of Chickens	Injected substance
1	6	Aqueous Sodium salicylate solution (150mg/mL)
2	6	Hydrogel C
3	6	Hydrogel F
4	6	Hydrogel H
5	6	Hydrogel J

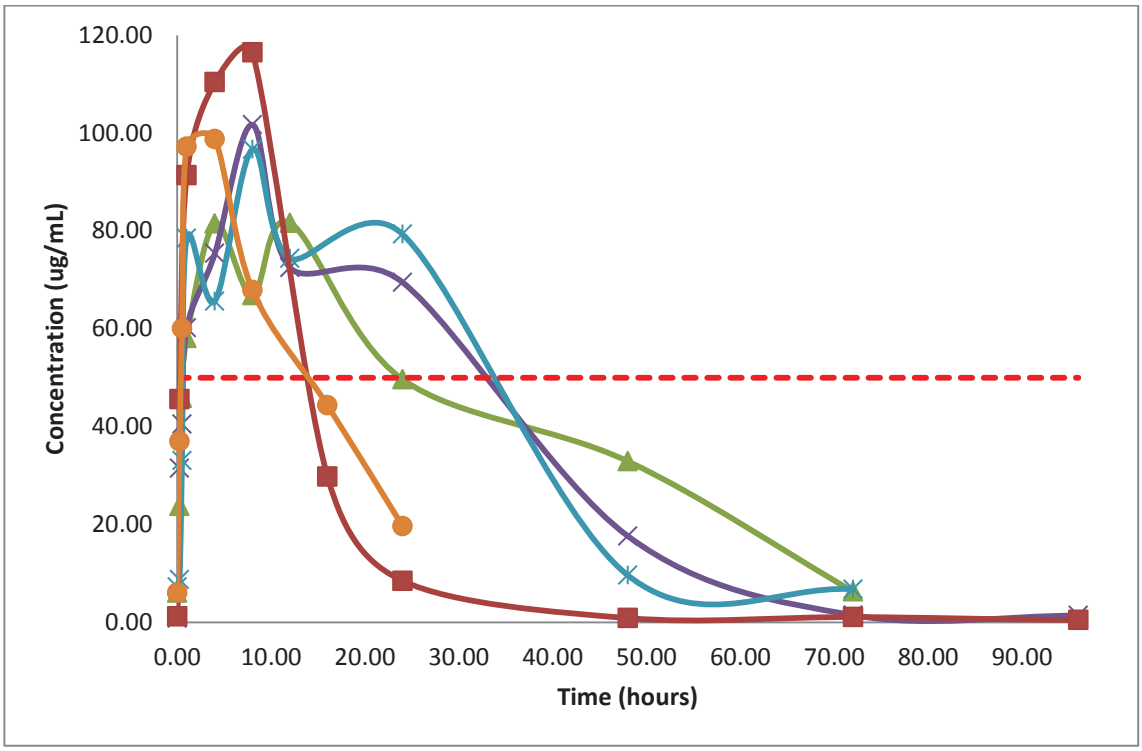

Figure 32. Concentration time curves for sodium salicylate after subcutaneous injection of group 1 (orange), group 2 (red), group 3 (blue), group 4 (purple), and group 5 (green) in broiler chickens. The data points represent the mean of the 6 chickens, with a total of 24 chickens in the whole study. The red dotted line indicates the minimum effective plasma concentration of sodium salicylate required to maintain analgesia in chickens.

The minimum effective concentration of the model analgesics in the blood plasma is 50 µg/mL (Baert & De Backer, 2002). This means the concentration of the model analgesics in the blood plasma needs to exceed 50 µg/mL to produce analgesia.

In terms of the group 1, it is not surprising that the drug exits the system rapidly. After injection, the drug must overcome the natural barriers within the body (skin, muscle, veins, etc.) to get to the blood stream. Once this is achieved, the chicken's metabolic system to processes and excretes the drug and its metabolites from the blood. Indeed, the group surpasses the effective concentration. It is not surprising that it only remains above 50 µg/mL for up to 18 hours as compared to the 36 hours achieved by the groups injected with the hydrogel formulations Y and Z. Any improvement to this will have achieved the goal of this project.

From Figure 32, the drug concentration for group 2 does exceed the 50 μg/mL mark, however, it matches the time achieved for group 1. It appears that even with the extra barrier supplied by the hydrogel structure, there is no dramatic improvement to the release of the analgesic. This indicates the degree of cross-linking present within the hydrogel structure is not at the optimum level needed to achieve the desired sustained release of the analgesic and future hydrogels with higher degrees of cross-linking would provide more beneficial results. However, the information gained from this group does provide some context to the importance of cross-linking present within the hydrogel structure and its role in the sustained release of encapsulated drugs.

Groups 3-5 all achieve a longer sustained release of the analgesic as compared to groups 1 and 2. Further, the sodium salicylate concentration of groups 3 and 5 fluctuates rapidly over the 96 hour period, resulting in the drug metabolising in sections, hence the peaks achieved in the HPLC analysis. For hydrogels, this may not be that uncommon as it is assumed that the hydrogels release the analgesic "equally" over time and when introduced to the biological system with the ability to degrade the hydrogel, the drug could be released prematurely or in large segments as the hydrogel matrix is broken down by the systems enzymes, hence the present fluctuations.

Groups 3 and 4 show similar release profiles which is not surprising given their similar compositions. In both groups, sustained release of the analgesic is achieved past the effective concentration for 36 hours, which is twice as long compared to groups 1 and 2. This massive improvement can be accounted for by the higher degree of cross-linking, hence the drug molecules diffuse more slowly out of the gel as compared to the previous groups. Further, given that both groups have the same amount of calcium ions, it is not surprising the comparable results achieved between the two groups. Nevertheless, both groups have been highly successful in achieving the desired sustained release of the encapsulated analgesic.

Group 5 has a different analgesic added to the hydrogel then the other groups, with the composition being the same as hydrogel F. It was hypothesised that altering the analgesic from sodium salicylate to sodium aspirin would limit the separation effect due to the stability of the aspirin group and possibly increase the sustained release of the analgesic from the hydrogel. This, however, was not the case. This group does exceed

the effective concentration of analgesic in the plasma for up to 25 hours. The lower concentration of analgesic could be accounted for by the presence of the sodium aspirin and sodium salicylate in the *in vitro* experiments (stability section, 3.1), or by the fast metabolism rate of the chickens. Nevertheless, sustained release was achieved up to 25 hours as compared to the 18 hours for groups 1 and 2, which further supports the idea of using hydrogels as a drug delivery system for poultry.

Chapter 4: Conclusions

It is quite clear that new drug systems need to be investigated for the delivery of drugs in birds due to their faster metabolism (Singh, 2011). Hydrogels provide a unique system of slow release of drugs over a period of time which not only could reduce the number of injections required to maintain the therapeutic dose of the drug in birds, but also improves the safety of introducing the drugs into the birds, as the high dose required is not fully introduced into the blood stream immediately. Further, hydrogels can be made in large quantities. They can be made from inexpensive, non-toxic, and biodegradable materials, and can easily be altered to accommodate different drug molecules.

The approach of this thesis was completed in two ways, the *in vitro* analysis of selected hydrogels and the *in vivo* analysis of hydrogels in poultry. The first approach included the initial structural analysis, the swelling of the hydrogels and the drug release in water and a pH mediated buffer (SIF). The swelling profiles of the blank hydrogels indicated the presence of cross-linking occurring between the alginate and calcium ions, as copious amounts of water and buffer were absorbed while maintaining the structure of the hydrogel. Further, the optimum calcium chloride amount was identified by the hydrogels ability to absorb water while retaining structure with little matrix being dissolved. The optimum calcium chloride amount was 0.3g per gram of alginate.

Loaded hydrogels produced unexpected observations and results. Firstly, when the model analgesic was introduced to the hydrogel matrix, a separation effect occurred between the alginate and the analgesic. The culprit of this effect was the slightly larger sodium ion concentration, upsetting the balance of ions present in the hydrogel mixture, resulting in a side reaction between the calcium ions and the analgesic molecules forming the insoluble calcium drug salt, with two layers formed. Next, the swelling studies indicated the lack of cross-linking between the alginate and calcium ions with the majority of the hydrogels dissolving into the media, with negative values obtained. However, this was beneficial as it indicated a method of hydrogel delivery to the consumer in the form of powders, with the consumer needing to rehydrate the powder before use. Finally, the drug release profiles indicated fast release of model analgesics, with the maximum release time being around 3.33 hours. This was undesirable as

release was aimed to be ideally over 24 hours, yet could be different once injected into the poultry due to the natural barriers and components within the system.

The *in vivo* experiments were tested on five groups of broiler chickens, each consisting of 6 chickens. Group one comprised of a 150 mg/mL aqueous sodium salicylate solution, with the other 4 groups comprising of hydrogels of slightly different compositions loaded with a standard 150 mg/mL amount of sodium salicylate or sodium aspirin (group 5). All groups were injected with the substances subcutaneously. Drug release results show that the standard aqueous solution remained in the system for up to 18 hours. Further, the results illustrate sustained release ability with three of the four hydrogel groups showing release past the 18 hours achieved by the standard aqueous solution. Furthermore, the number of injections required to keep the therapeutic dose of the analgesic past 50 µg/mL appears to have been decreased with the hydrogel group which further supports the project.

Given the unforeseen difficulties of preparing the hydrogels with sodium salicylate, the positive points of this research have indicated the possibility of using hydrogels as a drug delivery system for birds. To summarise:

- Swelling studies indicates the formation of hydrogel materials in all samples including one where separation effect is dominant
- Sustained drug release in *in vitro* experiments is present with the maximum time of over 3.33 hours being achieved
- The lowest release percentage of sodium salicylate was around 65 % and sodium aspirin around 40 %
- Sustained analgesic release in hydrogels injected in the chickens indicates release for up to 36 hours compared to the 18 hours the standard aqueous sodium salicylate solution remained within the system

4.1. Recommendations for Future

Based on the preliminary results obtained in this study, new pathways and ideas have evolved for the improvement of both the hydrogels used and the release of the model analgesic. Even though the hydrogel achieved the desired outcomes of the study, it is essential that future hydrogels are altered to improve the controlled release of the

analgesic past what has been achieved in this study. Nevertheless, this study has generated new knowledge which has not previously been documented and with further refinement, it appears this information would be highly beneficial to the veterinary community for the effect pain relief for birds. The following sections will detail future recommendations which can be made based on the results obtained from the prepared hydrogels, their swelling profiles, drug release profiles, and *in vivo* experiments.

From the initial preparations of the hydrogels, the most relevant recommendation is the replacing of the model analgesic used with one which does not contain sodium ions to reduce and remove the separating effect of the alginate mixture with the drug solution. As stated earlier, this effect was due to the side reaction between the calcium ions and the salicylate salt, forming the insoluble calcium salicylate salt. The main culprit was the presence of the sodium ions present in the solution supplied by both the sodium salicylate and the sodium alginate, which inhibited the cross-linking of alginate. Reducing the sodium ions by lowering the drug concentration or increasing the alginate concentration appeared to reduce the separation effect to an extent, with some separation occurring. Therefore, it is recommended to limit/remove the separation effect, the sodium salicylate model analgesic must be completely removed from the hydrogel preparation and replaced with something which does not contain an excess of ions. Furthermore, the new model analgesic chosen should interact with the hydrogel structure while not interfering with the cross-linking of the alginate. Ideally, the model analgesic should be in the same family as sodium salicylate (an NSAID), be more water soluble without having an excess of ions present such as the sodium ion compared to sodium salicylate and be small enough to exist within the hydrogel structure, but large enough to allow slow release from the hydrogel. Possible substitutions include meloxicam and butorphanol (Figure 33), both of which are considerably more water soluble than sodium salicylate, do not contain any ions to inhibit the cross-linking of alginate, are in the same family to sodium salicylate, and are analgesics already on the market targeted for veterinary use. The only limitation is the size, given that they are twice the size of sodium salicylate, but can be compensated for by the degree of cross-linking within the hydrogel. Other examples could include another family of analgesics such as opioids, however, special consideration to hydrogel structure may be required for effective drug release. Nevertheless, changing the model analgesic from sodium salicylate to another could have dramatic positive effects on the properties of the

hydrogel from the preparation, the swelling profile, drug release profile, and to the *in vivo* experiments and potentially result in a hydrogel with the desired outcomes required to meet the objectives of the project.

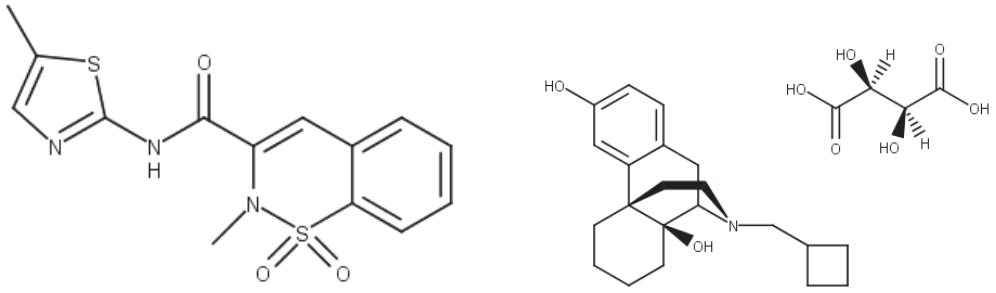

Figure 33. Molecular structures of meloxicam (left) and butorphanol tartrate (right).

Should the model analgesic be retained as the drug used for the project, the next recommendation would be to change the chemical structure of the hydrogel and potentially use a different form of cross-linking to produce the desired hydrogel. Given the limitations of sodium salicylate described above, it also has a multitude of advantages over other analgesics including its small size, cheapness, high toxicity dose, storage, stability in powder and liquid form and it is easy to obtain or synthesise. In cases where the advantages of sodium salicylate outweigh the limitations including the separating effect, a change to the chemical structure of the hydrogel would be recommended to reduce the separation effect. Unfortunately, an ionically cross-linked alginate hydrogel is not suitable due to the separation effect as shown by the results obtained and a change to either the cross-linking of the hydrogel or the polymer used in the hydrogel preparation would be required. One replacement could be a chemically cross-linked chitosan hydrogel cross-linked with a cross-linker with a low toxicity such as genipin (Figure 4) or epichlorohydrin. Chemically cross-linked hydrogels tend to be stronger than physically cross-linked hydrogels and have slower drug release profiles. Furthermore, this hydrogel completely removes one source of sodium ions supplied by the alginate and this potentially could reduce or even completely stop the separation effect. Other examples of hydrogels which could be used include synthetic polymer hydrogels, a mixture of polymers cross-linked with two or three cross-linkers, or smart hydrogels which have the ability to change their properties to different environmental factors including pH and temperature. To review, changing the chemical structure of the hydrogel would be recommended if the model analgesic continues to be sodium

salicylate. This would negate the weak physical cross-links within the hydrogel structure and replace it with stronger chemical links and in theory, this should completely minimise the separation effect as ions such as the sodium ion will not be involved in the cross-linking of the hydrogel. That aside, changing the chemical structure of hydrogel could also be beneficial for use with other analgesics such as meloxicam, due to the overwhelming evidence of slower drug release present in current literature for other types of cross-linking, taking care with the toxicity.

Another alternative method which can be recommended with the use of salicylate derivatives is to bind aspirin with chicken serum albumin and load this mixture into the hydrogel matrix. Unfortunately, the majority of the salicylate family are water insoluble molecules, which poses a problem with the use in hydrogels where the majority of the matrix is water, hence the use of sodium salicylate or sodium aspirin. Yet, there is evidence in the literature which suggests binding one of these insoluble derivatives to serum albumin and loading this mixture into the hydrogel matrix to achieve the desired slow release. For example, Hung *et al* (1997) were successful in binding aspirin and diflunisal derivatives to bovine serum albumin, with the smaller the chains, the more the drugs bound to the protein. In the case of these hydrogels, the same method which Hung *et al* used could be applied with chicken serum albumin, and loaded into the hydrogel matrix to achieve a slower release of the drug molecule, as it would have to be released from both the hydrogel and the chicken proteins.

To add to the last recommendation, another alternative could be the use of β-cyclodextrin grafted onto the hydrogel polymer, used as the cross-linker of the hydrogel or loaded into the hydrogel matrix. β-cyclodextrin is a cyclic oligosaccharide which consists of seven glucopyranose units with an α-(1,4) linkage (Martin Del Valle, 2004; Pluemsab, Fukazawa, Furuike, Nodasaka, & Sakairi, 2007; Kono, & Teshirogi, 2015). Structurally, cyclodextrins adopt a bucket like conformation with a hydrophilic surface and a hydrophobic core. Chemical structures and structural schematics are shown in Figure 34 (Martin Del Valle, 2004; Pluemsab, Fukazawa, Furuike, Nodasaka, & Sakairi, 2007). Furthermore, β-cyclodextrin does not irritate the organism when injected intramuscularly, and is non-toxic with the LD_{50} of a rat being >5000 mg/Kg orally and between 450-790 mg/Kg for intravenous injection (Martin Del Valle, 2004). The most interesting and relevant property of β-cyclodextrin is its ability to form an inclusion

complex with non-polar guest molecules such as aspirin. In these complexes, the aspirin is held within the non-polar core of the β-cyclodextrin, which increases the stability, solubility, and shelf life of aspirin (Martin Del Valle, 2004). When introduced to the hydrogel as this inclusion complex, β-cyclodextrin can help to slow down the release of the aspirin as it needs to be dissolved into water and the aspirin displaced by water molecules (Martin Del Valle, 2004). Given this ability, three options can be recommended for the use of β-cyclodextrin, with the most promising one being grafted onto the polymer used in the hydrogel. Kono and Teshirogi successfully grafted β-cyclodextrin onto chitosan and cross-linked with a water soluble carbodiimide cross-linker. They further illustrated the hydrogel's ability to slowly release aspirin from the cyclodextrin core, hence supporting the recommendation made. The other two options include cross-linking the hydrogel with β-cyclodextrin as illustrated in the work of Pluemsab *et al*, or loading the hydrogel with the inclusion complex, allowing it to exist freely within the hydrogel matrix. Again, this recommendation completely negates the separation effect due to the absence of the sodium ions from sodium salicylate and could potentially achieve a sustained release of the model analgesics.

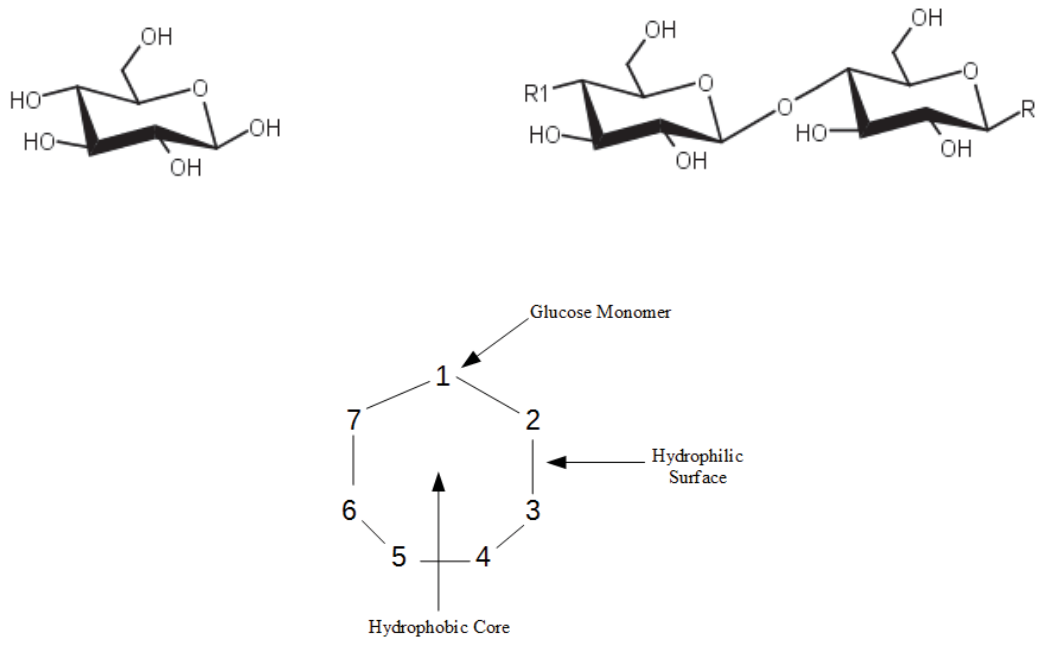

Figure 34. Chemical structures of cyclodextrin monomer (upper left) and cyclodextrin α-(1,4) linkage (upper right). Structural schematics of β-cyclodextrin (lower).

Once the hydrogel is introduced to water, either from the *in vitro* experiments or the preparation of the hydrated hydrogel, there is premature release of the analgesic from the hydrogel matrix. As a result, it is recommended that a slightly altered procedure for the preparation of the hydrogel is produced to minimise premature analgesic release. Once there is water present in the hydrogel mixture, the analgesic is released from the matrix to allow room for the water to exist. Indeed, this is the required function for the hydrogel to achieve the desired release of the analgesic, but there is one issue which comes about and that is the premature release of the analgesic before injection into the chicken. This has the potential to push the analgesic out of the matrix to the surface and a substantial portion of the analgesic would be released once injected into the chickens immediately, negating the desired effect of prolonged release. For the purpose of this project, all hydrogels which were injected into the chickens were made for injection immediately within a three day period. However, for the purpose of designing a hydrogel to be sold as an analgesic for birds, it is required that the hydrogel produced will be stable in the hydrated form. One recommendation would be to produce a hydrogel which can be injected and form *in situ*. This would compensate for the premature release of the analgesic as any analgesic to be released during the formation of the hydrogel would contribute to an initial boost to the minimal therapeutic dose. Balakrishnan and Jayakrishnan (2005) were able to produce a hydrogel comprised of periodate-oxidised sodium alginate cross-linked with proteins such as gelatin in the presence of borax. They observed that there was rapid gelation due to the higher pH of the medium along with the ability of borax to complex with the hydroxyl groups on the polysaccharides. Given their hydrogel formed *in situ*, a similar hydrogel could be created for the purpose of this project which would be injected into the chickens as separate components and can allow the gelation to occur within the chickens, hence minimising the premature release of the analgesic. Furthermore, there is the possibility for these separate components to be injected using the same syringe to minimise chicken injection.

In addition, another recommendation would be to form a hydrogel with a fast gelation rate, with the intention of supplying the hydrogel in powder form and mixing with water for a brief period of time to form the hydrogel. For example, the polymer, analgesic and cross-linker could be supplied as a mixed powder and when ready to be injected into animals, mix the powder with a pre-determined amount of water for a brief period of

time. This method would add to the stability of the hydrogel and analgesic, a cheap method to create and transport, non-toxic, biodegradable, time efficient, and easy to produce on large scale.

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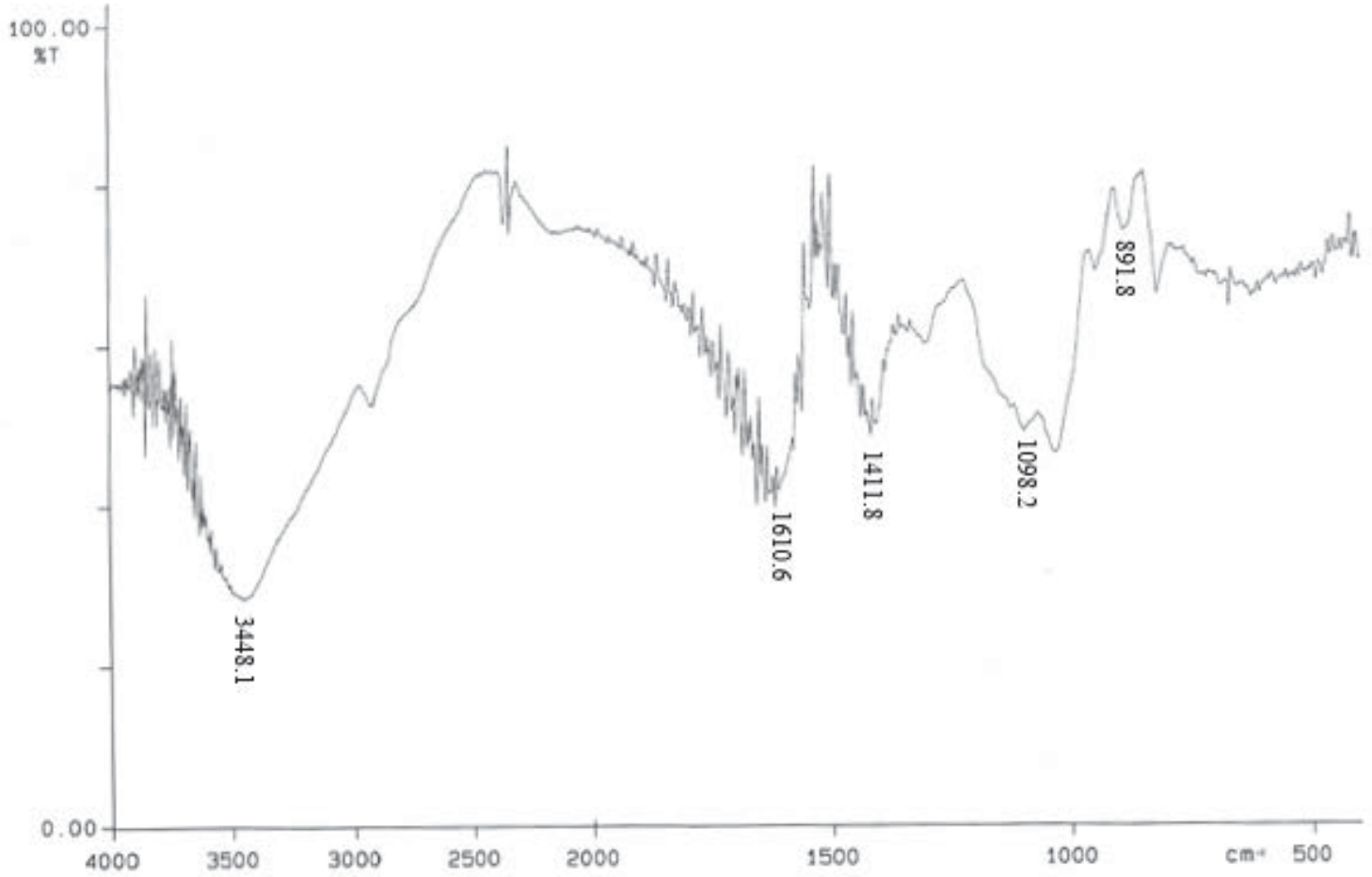
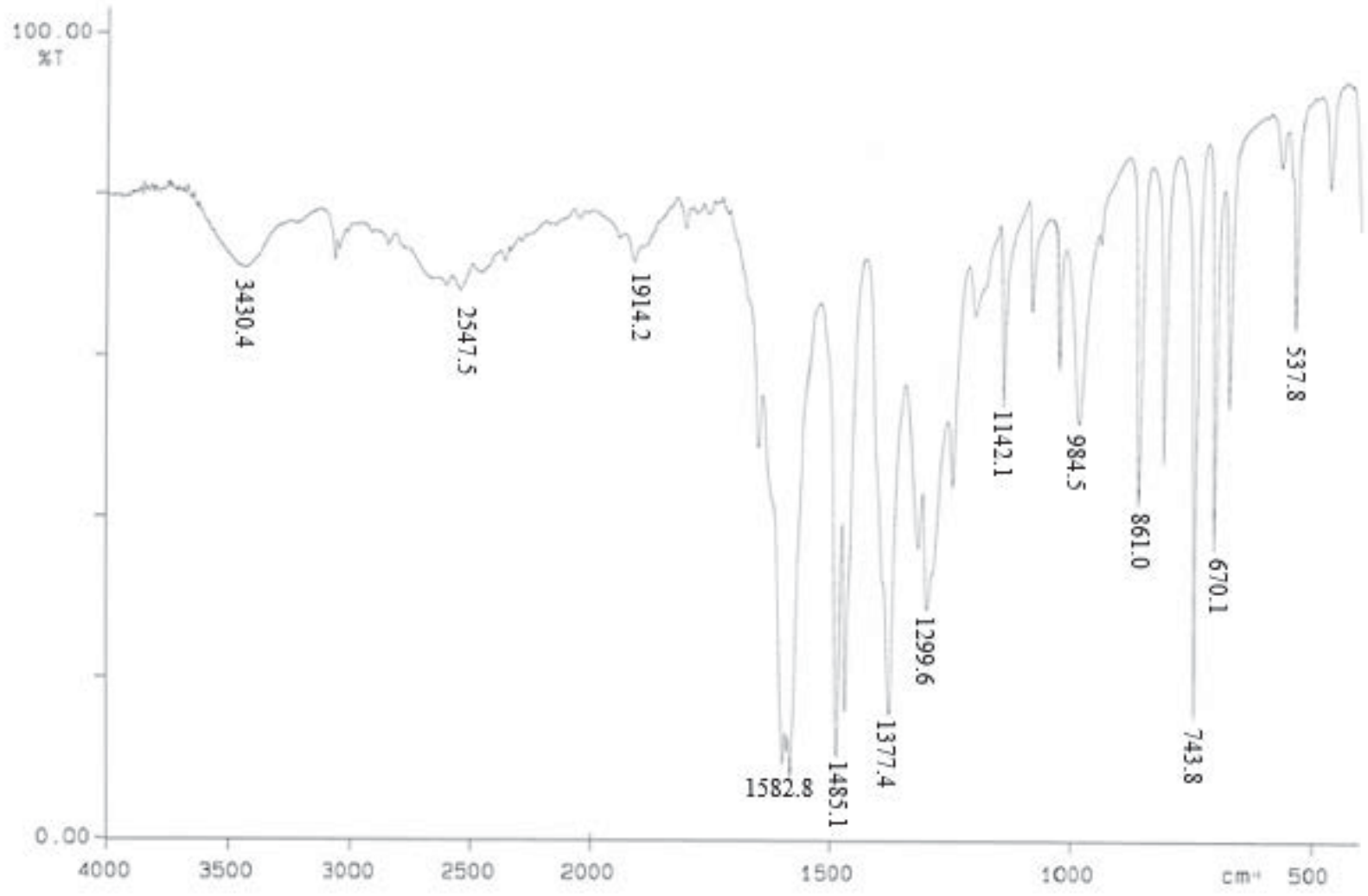
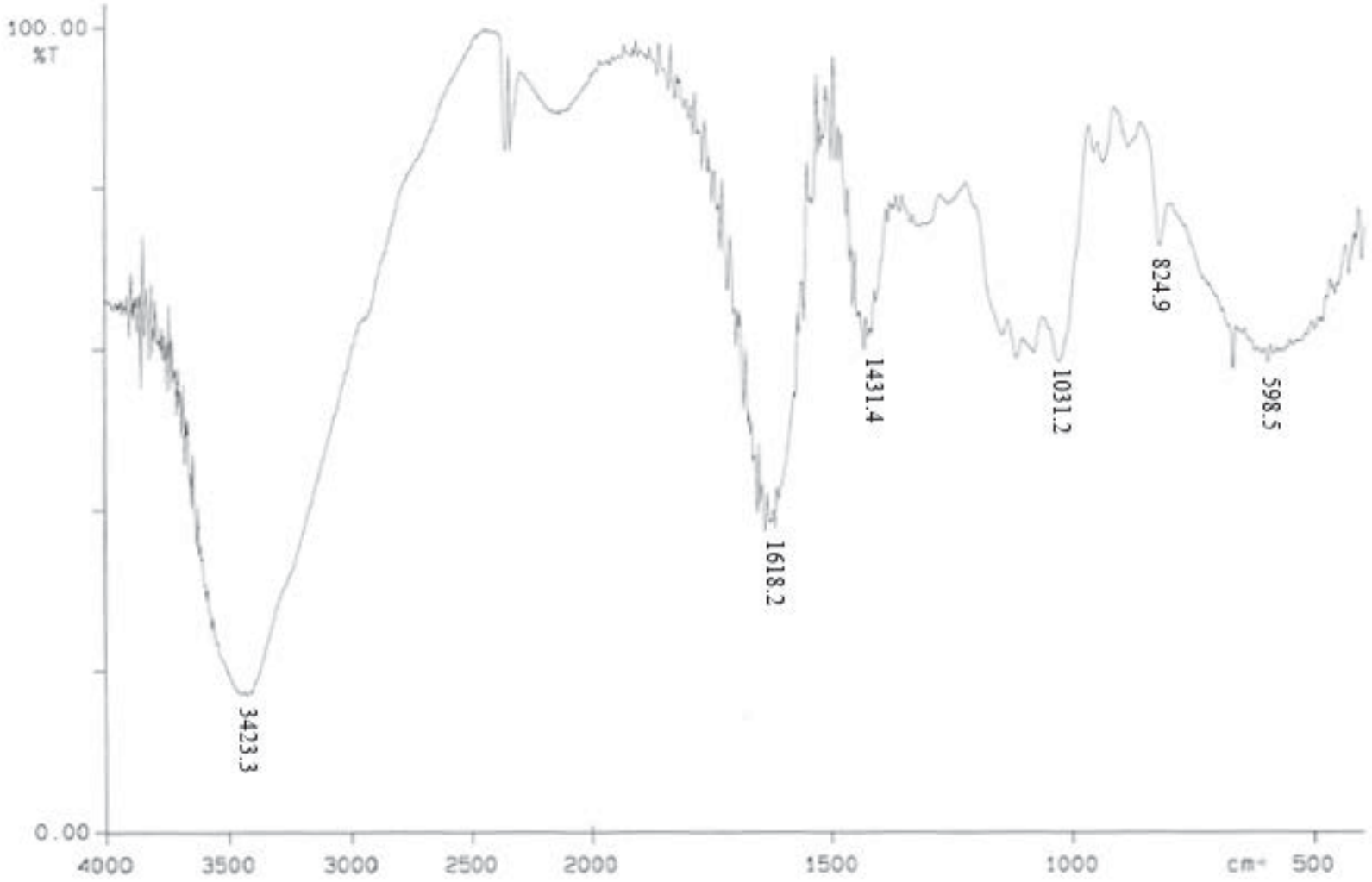
Appendix



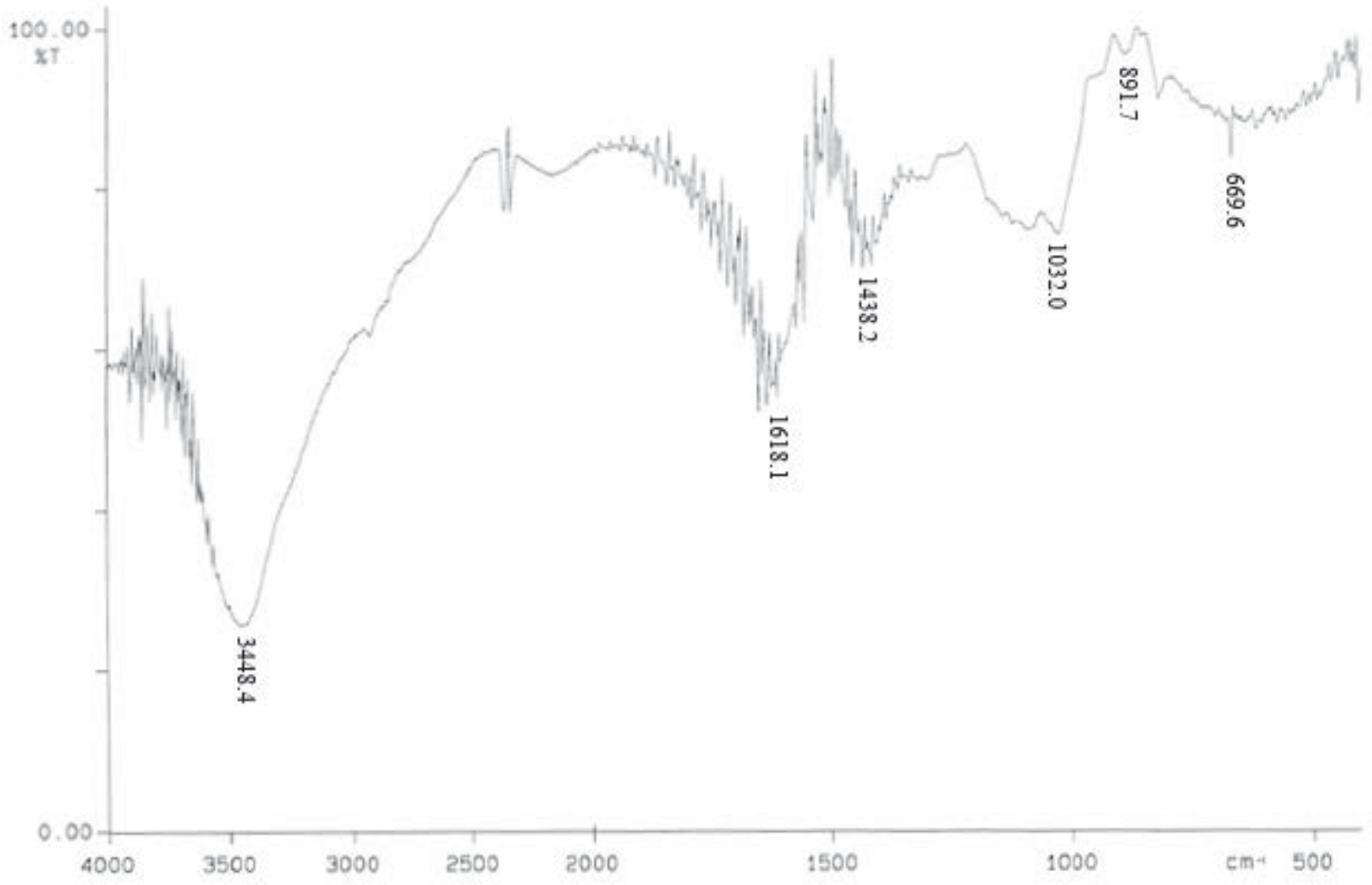
Appendix A. IR spectrum of Alginate



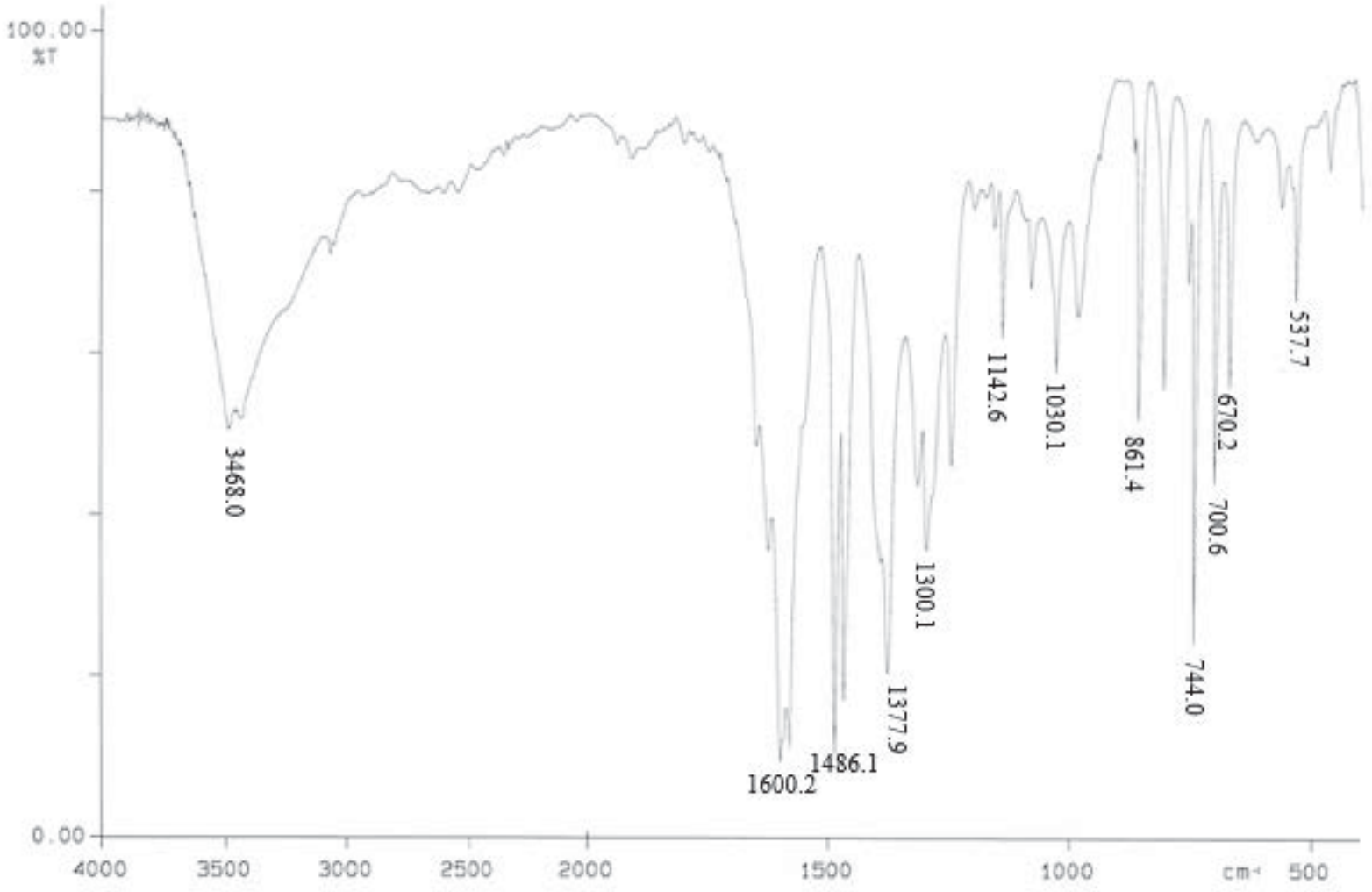
Appendix B. IR spectrum of sodium salicylate



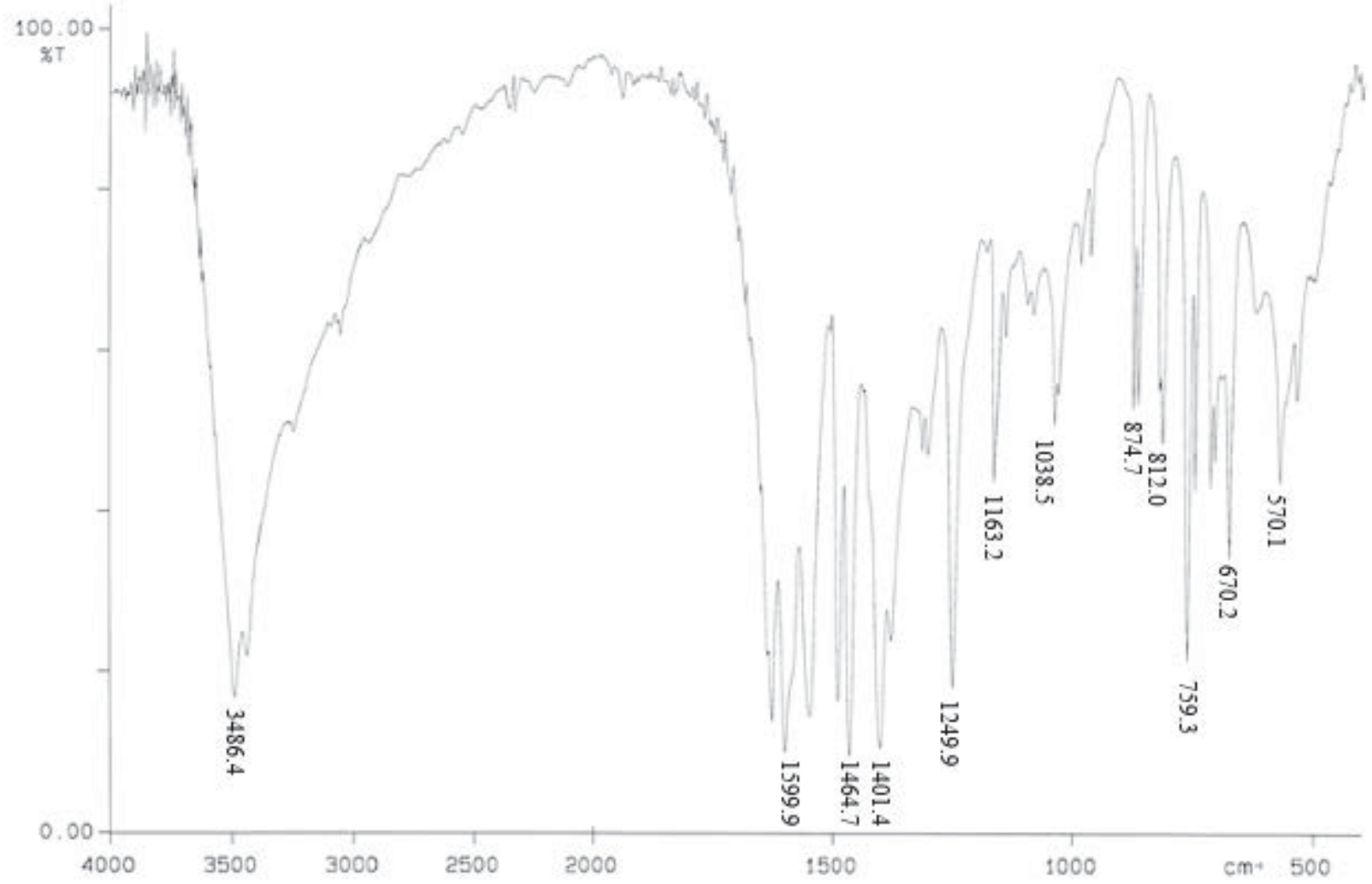
Appendix C. IR spectrum of hydrogel B1



Appendix D. IR spectrum of hydrogel B3



Appendix E. IR spectrum of hydrogel A



Appendix F. IR spectrum of hydrogel D