Synthesis Of Keratin-Based Hydrogels And Cryogels Destined For Environmental Applications

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Abstract

Bio-based polymers have been of interest to industry due to their biocompatibility, biodegradability and their relatively low toxicity. They are synthesized from renewable feedstocks such as proteins, polysaccharides and lipids. The numerous functional groups of proteins allow for a variety of chemical and physical linkages and structures. The subject of this thesis focuses on the synthesis and characterization of chemically crosslinked protein-based superabsorbent hydrogels and cryogels. The protein used in these materials was keratin extracted from chicken feather meal, which is regarded as a waste or residue product from the poultry industry. The superabsorbent hydrogels may be suitable as material for soil amendments or wastewater treatment, while the cryogels may be used in oil spill clean-up and remediation.

As part of this thesis, acrylic acid grafted hydrolyzed keratin hydrogels were synthesized and characterized. The effects of the crosslinker concentration, protein concentration, and acrylic acid concentration on the swelling of these hydrogels were investigated. The swelling tests showed that these hydrogels had a much higher swelling capacity (mean = 501 g/g) than previously reported keratin hydrogels (50-63 g/g). They were shown to have different swelling capacities in different media. This was demonstrated by testing the swelling degree in solutions with varying salinity and pH.

Keratin is an insoluble fibrous protein that has many hydrophobic amino groups. As a second study, keratin was modified with oleic acid to increase its oil holding capacity.

The modified keratin and unmodified keratin, were used to synthesize cryogels. The oil holding tests showed that the oleic acid modification had a positive effect on the oil holding capacity of the cryogels. The results also indicated that these materials had an oil holding capacity (10.76 g/g) superior to that of the industry standard material, polypropylene (6 g/g), or other oleic acid modified materials made from cellulose (6.3 g/g).

Résumé

Les polymères bio-sourcés sont reconnus en raison de leur biocompatibilité, biodégradabilité ou de leur toxicité peu élevée. Les protéines, polysaccharides et lipides sont les principaux matériaux biologiques utilisés pour leur synthèse. Les nombreux groupes fonctionnels des protéines permettent une variété de liaisons chimiques permettant des structures variées. Ainsi, le sujet de ce mémoire porte sur la synthèse et la caractérisation des hydrogels et cryogels à base de protéines modifiées chimiquement. La protéine utilisée fut la kératine extraite des plumes de poulet, qui sont généralement considérées comme déchets. Les hydrogels super-absorbants peuvent convenir comme matériau pour les amendements du sol ou traitement des eaux usées, tandis que les cryogels peuvent être utilisés dans le nettoyage déversement d'huile de nettoyage et la remédiation des sols contaminés.

Dans le cadre de ce projet, la synthèse et la caractérisation d'hydrogels à base de d'hydrolysats de kératine et d'acide acrylique furent réalisées. Les effets de la concentration en agent de réticulation, la concentration en protéine et la concentration en acide acrylique sur le gonflement de ces hydrogels furent étudiés. Les essais ont démontré que le gonflement de ces hydrogels avaient une bonne capacité de gonflement (501 g / g) dans l'eau distillée. Ces hydrogels ont également été testés dans des solutions ayant des degrés de salinité différente ainsi que différent pH.

La kératine est une protéine fibreuse insoluble qui présente de nombreux groupes d'acides

aminés hydrophobes. Afin d'augmenter la capacité d'absorption d'hydrocarbure, de l'acide oléique fut greffé à la kératine. . La kératine modifiée, ainsi que la kératine non modifiée, furent utilisées pour synthétiser des cryogels ayant une capacité de rétention d'huile pétrochimique élevée. La modification de la protéine avec l'acide oléique a eu un effet positif sur la capacité d'absorption des cryogels (10,76 g d'huile/g cryogel). Ce résultat est supérieur au polypropylène (6 g/g) qui est considéré comme standard pour cette application.

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CONTRIBUTION OF AUTHORS

This thesis is submitted in the form of original papers submitted to peer-reviewed journals. The candidate, Bryan Wattie, was responsible for designing and conducting the experiments, handling the data analysis, as well as the preparation of the manuscripts and the thesis. Marie-Josée Dumont, supervisor of the candidate and Assistant Professor in the Department of Bioresource Engineering of McGill University, and Dr. Mark Lefsrud, co-supervisor of the candidate, Associate Professor in the Department of Bioresource Engineering of McGill University, contributed to all aspects of this research work. They provided assistance, including planning and directing the research, editing and reviewing the thesis and the manuscripts before submission for publication. The details of the manuscripts that have been submitted for publications are provided below:

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CHAPTER 1

INTRODUCTION

General Introduction

Approximately 5 million tons of feathers are produced yearly as a byproduct of the meat and egg industries. Feathers are sometimes used a cheap animal feed, however their nutritional value and ease of digestion are quite poor [1]. Much of the feather byproduct is disposed of in landfills [2]. However, feathers are roughly 90% proteins [3], and proteins have gathered considerable interest as a feedstock for the development of biomaterial [2, 4-10]. This represents an opportunity for the valorization of this by-product, and the reduction in wastes from the poultry industry.

Proteins can be classified based on their molecular structure as globular or fibrous protein. Both have a primary structure that is defined as the linear sequence of amino acids forming the polypeptide chain, and a secondary structure of either coiled alpha helices or pleated beta-sheets. For fibrous proteins, such as collagen and keratin, the secondary structure dominates the formation of the protein. For globular proteins, such as albumin, a weak tertiary and quaternary structure can be formed primarily through hydrogen bonding. The main differences between these two groups of proteins are that globular proteins are water-soluble, have weak bonding, and three-dimensional structures whereas fibrous proteins are

water insoluble, have strong intermolecular bonds, and form a fibrous or filamentous structure. [11]

Keratin is a fibrous protein found in feathers, horns, nails, and hair of many animals. Due to the fibrous structure of keratin the secondary structure dominates its formation. The protein found in the outer feather structure is mainly a beta-sheet conformation with disulfide bridges while the harder quill is predominantly alpha helix [12]. Disulfide bonds crosslink the beta sheet conformation giving it a rigid structure, and rendering it insoluble in water[13]. Thus, feather keratins serve their purpose as being, stiff water insoluble fibers, and light enough for flight.

Utilizing feather keratins as feedstock for polymer synthesis is challenging due to insolubility in water and the presence of cross-linked disulfide bridges. To solubilize keratin, a number of techniques exist including alkaline/acid hydrolysis [14], steam explosion [15], microwave radiation [16], reduction of disulfide bridges by 2-mercaptoethanol [17, 18] or sodium sulfide [19], and ionic liquids [20].

Proteins and their derivatives - keratin included- can be used to produce several different types of products for a variety of industries. Proteins have been used to synthesize composites [3], superabsorbent hydrogels [4], and films [19]. Proteins can also be hydrolyzed [15-16] and be used as feed or fertilizer amendment.

Study Objectives

Chicken feathers are an underutilized protein feedstock for biomaterials production. Their amino acid profile, mainly the high cysteine content, represents an obstacle to processing. The potential for keratin-based materials was therefore investigated. The primary objective of this thesis was to synthesize and characterize novel keratin-based hydrogels and aerogels. The specific objectives of this research are stated as follows:

I. To review existing keratin processing and solubilization techniques, and their utilization for the production of various keratin based biomaterials such as films, sponges, hydrogels, and fibers.

II. To synthesize superabsorbent hydrogels by redox initiated graft copolymerization of acrylic acid/sodium acrylate onto protein chains, crosslinked by N,N'-methylene bisacrylamide. A partial factorial design was used to optimize the equilibrium swelling ratio by using the initiator, monomer, and crosslinker contents as the three factors with 3 levels.

III. To modify the protein using a graft reaction with oleic acid and to synthesize keratin cryogels by freeze-drying using varying concentrations of crosslinker and protein, resulting in a lightweight material with high oil-holding capacity.

CHAPTER 2

A Review of Keratin Processing Techniques, Conditions, and Solvents for Environmental Biomaterials Applications.

2.1 Introduction

Keratin is a protein found in feathers, wool, hair, nails and hooves, and is known as a naturally biocompatible and biodegradable material. In the United States, approximately 5 million tons of feathers are produced annually, and are sometimes used as cheap animal feed; however their nutritional value and ease of digestion are quite poor [1]. More often waste feathers are disposed of in landfills [2]. Feathers are currently considered a waste stream but have great commercial opportunities as the market for biobased materials expands. The use of undervalued feathers from the poultry and egg production industries could reduce pressure on landfills. Materials made from keratin share in its native qualities of being biocompatible and biodegradable. The earliest research on keratin began in the 1930's focusing on keratin formation and applications for the textiles, biomedical, and cosmetic industries [21-24], with many reviews published on the topic [25-27].

A wide variety of techniques have been employed to solubilize and extract keratin from feather, wool, and other sources. Conventionally most techniques involve solvents and reducing agents to extract and solubilize keratin, whereas other techniques may be regarded

as “green” as they do not use harsh chemicals that need to be treated as an wastewater effluent, or employ solvents such as ionic liquid that can be reused, or the active agent is a non-toxic enzyme. The purpose of this review is to compare different processing techniques and summarize the environmental applications of keratin-based materials.

2.1.2 Properties of Keratin

Proteins are composed of amino acid building blocks that are linked together to form a protein. Variation in the amino acid sequences gives each protein a unique function, bioactivity, and structure. The amino acid profile of keratin was determined by Arai [28]. Keratin most notably has a high percentage of the amino acid cysteine in its composition. For example, plant proteins such as canola and soy isolates contain 0.63% and 1.26% cysteine [29], collagen has only trace amounts of cysteine [30], whereas feather keratin and wool keratin have up to 7% and 17% cysteine, respectively [28, 31]. The high cysteine content results in the formation of a high number of crosslinks between the sulfhydryl groups of the amino acids on the protein backbone, called disulfide bonds or bridges. These crosslinks render keratin insoluble in water and consequently, performing reactions in aqueous media involve disruption of the disulfide bonds.

Keratin’s native material properties are an important consideration in the production of a new material and its process conditions. Feather keratins are reported to have a molecular weight of 10 kDa [32]. To preserve the native keratin structure, it is important to never exceed the thermal degradation temperature (T_d) of 201^{o}C at any point in the process. The isoelectric point (pI) of proteins must be considered when controlling the pH of the aqueous

solution during processing. At a pH near the pI, proteins will arrange closer together producing smaller fiber diameters [33]. If the process pH reaches the pI or higher, then the protein will precipitate from the solution [34]. Cysteine can impart strength to any materials they are added to due to the presence of the numerous disulfide bonds. Cysteine can be highly reactive and is a suitable reaction site for modifications of the protein such as carboxymethylation [35]. The extent of carboxymethylation is determined by the total cysteine content [17]. Hydroxyl groups can form radicals, as protons can readily be abstracted from the protein chain by chemical initiators such as potassium persulfate [36]. The molecular weight will have an influence on the mechanical properties and biodegradation times of the materials produced [37]. The molecular weight can be increased through polymerization or grafting, or decreased by peptide bond scission.

Table 2.1 Physical Properties of Keratins

Physical Properties	**Measured Value**	**Additional comments**	**References**
E (Young's Modulus)	2.5 GPa	Native feather.	[38]
Degradation Temperature T_d	201 °C	_____	[29]
Cysteine content	7%	Forms disulfide bonds	[39]
Hydroxyl containing groups	22%	Serine, threonine, tyrosine.	[32]
Molecular weight	10-14 kDa	~90-100 amino acids in sequence.	[32]
Density	1.15g/cm^3	Compacted	[40]
Fracture stress (σ_f)	226 N/m^2	_____	[40]
Isoelectric point (pI)	pH 4.5-5.5	_____	[29]

2.1.3 Conventional Processing Techniques

Proteins can be converted into films, coating or thermoplastics through wet or dry processing methods. Briefly, dry processing (or thermomechanical processing) involves blending of the reagents, such as plasticizers, compatibilizers, and cross-linkers, with

proteins without the use of solvent, and then relies on the thermomechanical energy of the process to activate the reaction and form the material. The reagents may be reducing agents such as sodium bisulfite to act on the disulfide bridges, or plasticizers such as glycerol to increase flexibility of the materials, or compatibilizers to blend what may otherwise be immiscible ingredients. Keratin has been investigated for its suitability as extruded materials such as films and fibers [41-43], and for compression molding [44-46].

Conversely during wet processing, the proteins are dissolved in solution by mixing with the appropriate solvent and reagents. Wet processing allows for more varied modification of the protein, through methods such as graft copolymerization [47-49], or functional group modification [50, 51]. The remainder of this paper will focus on processes to solubilize feather keratins, but will include studies on wool keratins to understand the full body of literature.

2.1.3.1 Acid or Alkaline Hydrolysis

The simplest of these solubilization techniques involves the utilization of a strong acid (e.g. hydrochloric acid) or a strong base (e.g. sodium hydroxide) to hydrolyze the protein primary chain [22, 32, 52, 53]. The lower molecular weight protein will have less hydrophobic interactions, and be more soluble in aqueous solutions. The reaction is performed at a moderate temperature, 80-120 °C, for a period of time ranging from 15 minutes to 24 hours [54]. These conditions high and low pH conditions catalyze the H^+ or OH^- to undergo acyl nucleophilic substitution [55]. The insoluble fraction is removed by filtration or centrifugation, and the liquid is neutralized, frozen, and lyophilized to yield

the hydrolyzate. Without additives, hydrochloric acid hydrolysis damages the tryptophan groups of the protein, and makes cysteine groups unstable, making them unquantifiable for sequencing [56] or modification [50].

Conventional acid hydrolysis processes often rely on reflux heating, which when used for several hours result in a large cost at an industrial scale. Jou [54] reported that microwave heating could be used to shorten the residence time from 12-24 hours to 30 minutes using hog hair as the source of keratins. They hypothesized that the alpha helix conformation of the keratin would produce a high dielectric constant, making it prone to destabilization under microwave heating. The effect of microwave heating on the solubility and the destabilization of keratins secondary structure has been investigated in water by later research. Nevertheless the reduction in heating and residence time from microwave technology represents a significant cost saving.

The residence time (12-24 hours) of reflux heated acid hydrolysis prevents it from being a viable industrial process, whereas alkaline hydrolysis can be as short as 15 minutes to yield significant soluble fractions of keratin [57]. Alkaline hydrolysis has been shown to induce racemization of isomers [58], which affects interactions with other biological systems [29]. Degradation of the proteins through peptide bond scission and deamidation of asparagine and glutamine groups may occur at pH 10.0 or higher [17]. It has been reported that high pH in the 12-14 range can disrupt hydrogen bonding and cleave cysteine bonds [59, 60]. In either type of hydrolysis, the resultant products are free amino acids and peptides of smaller molecular weights than the initial product. These shorter chains are no longer

crosslinked to the same high degree, and are therefore soluble in water. However, these shorter chains from either acid or alkaline hydrolysis (hydrolyzate), will no longer share the same mechanical properties as the original native protein structure. Alkaline hydrolysis is requires a large amount of reagents needed, up to 30% w/w [61]. A further disadvantage of these techniques is the required neutralization step that produces impurities in the form of salts. However, due to the simplicity and availability of the reagents, these techniques are still widely used in research.

2.1.3.2 Oxidative Hydrolysis

Peroxy acids, such as performic acid, and peracetic acid can be used for acidic hydrolysis of proteins to induce an oxidation reaction [62]. This technique has become an industry standard since being introduced in 1949 from Sanger's work on insulin [63]. The thiol group of cysteine is a nucleophile and oxidizes readily to the disulfide derivative cystine which crosslinks the proteins and limits the protein solubility. Hydrolysis with strong acids limits the reactions to the scission of ester and peptide bonds, and does not break the disulfide bonds. In the presence of peroxy acids, the peptide bonds are broken to a lesser degree, and the disulfide bridges undergo oxidation. The complete oxidation of cysteine occurs in 30 minutes, however treatment time is extended to 1-6 hours to increase solubilization. The disulfide bridges are broken from and modified to $NHCH(CH_2SO_3)CO$- (Figure 2.1) yielding water soluble keratose [14]. Similar to strong acid hydrolysis, tryptophan residues are not preserved during this process [64]. Blackburn reported that keratin fibers swelled noticeably in performic acid, and swelled but more slowly in

peracetic acid and hydrogen peroxide, yet remained mostly undissolved [62]. The yield of soluble proteins from the peroxy acid hydrolysis method, is quite low, in the range of 7-40% of the initial mass, with longer treatment times of 6 hours giving the highest yield [62].

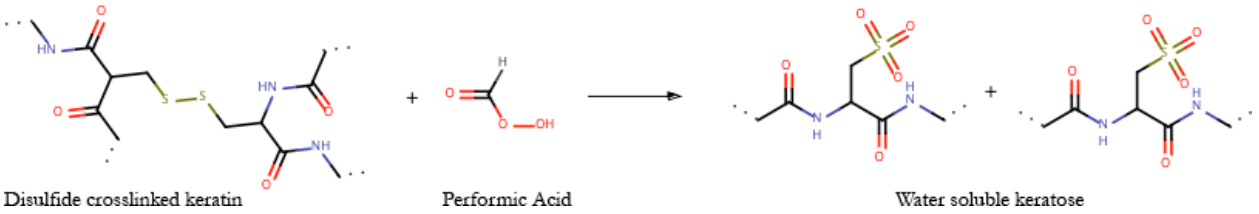


Figure 2.1 Oxidation of Keratin by Performic Acid [65]

2.1.3.3 Sulfitolysis

Sulfitolysis is an approach used to cleave disulfide bonds, using a redox mechanism with sulfite anions, which is depicted in Figure 2.2[65]. Each disulfide bond is broken into one sulfite derivative and one thiol group (-SH). In the presence of an oxidizing agent, the SH groups will oxidize to form new disulfide bridges. Solid-state copper catalysts have been reported to be the strongest oxidizing agents, because of the specificity to the thiol groups in cysteine [66]. Sulfitolysis process continues until the sulfite is consumed, or all of the cystine groups are converted. The advantage of this reaction is that the disulfide bonds are broken without hydrolysis of the protein chains. Furthermore, it introduces negative charges on the cysteine groups, which promotes solubility. The reaction normally occurs at 65°C for 0.5-2 hours [46, 67, 68]. Some protocols employ urea to disrupt the secondary structure and hydrogen bonding [69]. Sodium dodecyl sulfate may also be employed as an

anionic surfactant to stabilize the proteins in solution and prevent aggregation [67]. The main drawback of the sulfitolysis procedure is the extensive dialysis that is required after extraction. Dialysis can take up to 3 days, and represents a costly and time consuming processing step. Furthermore, the yield is limited to 30% soluble protein as compared to the initial mass of the untreated keratin. To obtain an unmodified keratin, the sulfo-keratin derivative can be reduced with 2-mercaptoethanol to yield free SH groups [70].

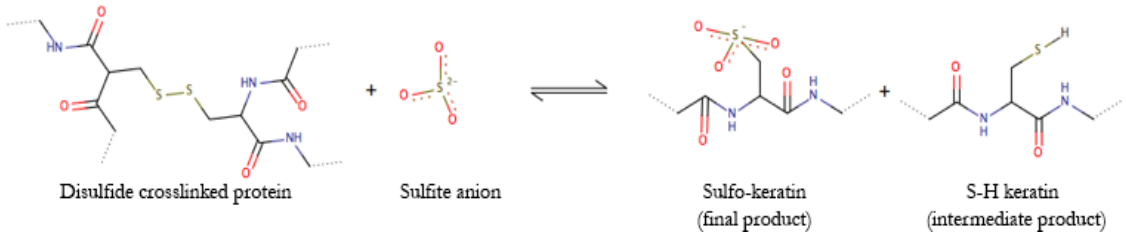


Figure 2.2 Oxidation of Disulfide Bridge by Sulfite Anion [65].

2.1.3.4 Reduction with 2-mercaptoethanol

The most common technique used for keratin extraction in recent studies involves a mild reducing condition with 2-mercaptoethanol as a reducing agent, sodium dodecyl sulfate as a stabilizing surfactant, and urea to disrupt hydrogen bonds [17, 18, 46, 71-77]. Yields of 75% with a short reaction time of only 30 minutes have been reported [17]. The remaining 25% of insoluble proteins are either ε-N-(γ-glutamyl)lysyl cross-linked or trapped in cellular envelopes [78]. In this reaction, nucleophilic substitution of cystine with 2-mercaptoethanol thiolate anion occurs. Due to the slow reaction rate, a 10 fold excess of thiol to cysteine is used. However, upon removal of 2-mercaptoethanol and urea during dialysis, the protein aggregates and the cysteine reoxidizes to cystine to form an opaque

white gel. Schrooyen reported on a method to create a stable dispersion through partial modification of cysteine with reagents such as iodoacetic acid or iodoacetamide to improve water solubility; while leaving some cysteine to recross-link to impart the mechanical strength of the films [17]. They reported that iodoacetic acid treatments (carboxymethylation) gave the best improvements to solubility while forming less aggregates [17]. The described carboxymethylation technique still requires the time-consuming step of dialysis; however the yield of 75% was much larger when compared to the yield of sulfitolysis, which only approaches 30% under optimal conditions. Schrooyen included sodium dodecyl sulfate as an anionic surfactant to help stabilize the solution for a longer shelf life [79].

Yin [80] reported on a modified pretreatment to increase the yield of the Schrooyen method [17]. Schrooyen degreased the chicken feather by Soxhlet extraction with petroleum ether for 12 hours [79]. Yin et al. used an ethanol reflux for 2 hours to remove fats, and included an HCl treatment of 1 mL of 12M HCl per gram of feather material for 2 hours prior to reduction with 2-mercaptoethanol [80]. Yin reported yields of up to 93% with the improved method [80]. Molecular weight was reported to be 20 kDa, which suggests that the process induces dimerization as feather keratins are reported to have a molecular weight of 10 kDa [32].

2.1.3.5 Sodium sulfide

The reductive extraction technique with 2-mercaptoethanol optimized by Schrooyen [17, 79] serves as an excellent benchmark in terms of yield and preservation of the proteins' primary chain; however, it has also received criticism that its high cost is unviable on an industrial scale [19]. Poole [19] instead suggested that the use of sodium sulfide, first proposed by Jones and Meecham in 1943 [81], be revisited. Sodium sulfide is a strong alkaline salt that is commonly used in the pulp and paper industry as part of the Kraft process [82]. Poole [19] found that it was a more effective reaction pathway, using only 3.5 fold excess of thiol, while the 2-mercaptoethanol scheme used a 10 fold excess [19]. It was found that increasing the concentration of sodium sulfide beyond 10 g L^{-1}, did not result in any significant increase in yield. At a pH >10, peptide bond scission may be expected, and the pH of the reaction with sodium sulfide was 14. However, it was found that for reaction times of 1-6 hours, little damage occurred to the protein backbone with 70% being above the 10 kDa weight fraction [19]. Subsequent research by Tsuda et al. found that alkaline hydrolysis with 10g/L NaOH at 120°C for 10 minutes produced smaller molecular weights of 767 Da [83]. Therefore the high alkalinity of sodium sulfide does not greatly affect the molecular weight of the proteins. The higher molecular weight contributes to improved tensile properties of the film. Moreover, the solution could be used without dialysis. The sodium sulfide process shows promise despite its main shortcoming being yield, which was limited to 55% when the physical properties of their product, films, were optimized.

2.1.4 Green Processing Techniques

The aforementioned techniques may be regarded as more conventional chemical processing techniques, yet it is of increasing interest to find techniques that use fewer chemicals, or less energy, and ideally create fewer wastes. The following three processes for solubilizing keratin may be regarded as greener techniques: 1) hydrothermal treatment, 2) enzymatic hydrolysis and 3) the use of ionic liquids. These techniques are described in the following paragraphs.

2.1.4.1 Hydrothermal Treatment

Superheated water has been shown to disrupt both the primary and secondary structure of keratin as Yin et al. showed nearly complete dissolution of feather keratin after 120 minute treatment times at a temperature of 220 °C and approximately 22 bar of pressure [84]. The superheated water simultaneously acts on all three bonding sites of the protein, the hydrogen bonding of the secondary beta sheet, the disulfide bridge, and the peptide bonds in the primary chain. This investigation of feather keratins built on previous works using superheated water for the dissolution of wool [85, 86]. Esteban reported the extraction of amino acids from hog hair using subcritical water, they reported yields of 32.5% total amino acids based on the initial mass of hog hair [87]. It was found that these extended treatments at high temperature and pressure while they use only water as a solvent-effectively hydrolyzed the protein chain into small oligopeptides and free amino acids [85, 86], but to a limit. Increasing the temperature over 100°C and residence time longer than 60 minutes have resulted in amino acid degradation [87]. The short chain length of the

resulting product may limit its applications in biomaterial development. Interestingly, Yin reported that these small oligopeptide displayed self-assembling dendritic patterns depending on the drying conditions and concentration [84]. The opportunities to use natural fibers in cosmetics, feed additives, and textiles are numerous and thus other research [73, 88-91] has investigated hydrothermal treatments of wool and feather to produce small particles for these applications. However, yield or recovery of soluble protein was not reported and materials such as films have not been investigated [86, 92, 93].

Zoccola et al. investigated the use of superheated water on wool keratins pairing with microwave heating [16]. Their research showed that the optimal conditions for protein extraction were at a temperature of 150 °C and a residence time of 60 minutes. Zoccola et al. had a recovered yield of 74% for keratin greater than 3kDa [16]. While the molecular weight of keratin is usually in the 10-14 kDa range, proteins of 3 kDa demonstrate that this process only cleaves a small number of bonds along the primary chain. Zocolla [16] and Jou [94] used microwave heating on wool and hog hairs to extract keratin and Chen [95] extended it to poultry feathers. Chen used the same parameters for residence time (30-60 minutes) and temperature (150-200 °C) and reported similar yields near 71% of soluble oligopeptides and amino acids based on the starting mass of feathers [95].

These hydrothermal treatments include steps to cool the heating vessel, or release steam through a valve [85]. High-density steam explosion employs a catapulting mechanism allowing for a much faster release of steam than traditional valve release. This innovation

decreases the release time from 10's of seconds to 0.0875 seconds [96]. Zhang et al. focused mainly on the effect on enzyme digestibility of the protein using the high-density steam explosion [93]. Wei argued that the main effect of the treatment is attributable to the shearing forces of exploding steam that has seeped deep within the feathers, and found that they could improve the solubility of the feathers in distilled water from 0% to 12.49% for 90% of the protein mass [15], the remaining 10% was insoluble in water. Using X-ray diffraction, Wei [15] determined that steam explosion decreased the crystallinity and denatured the beta-sheet structures of the keratin for easier downstream processing. This process is quick and incorporates three phases: the steam penetration phase (3−5 s), the boiling phase (1−3 min), and the explosion or deflation phase (within 0.00875 s) [93]. The short time of the explosion phase was measured using pressure sensors, with deflation time being defined as the time where pressure reached ambient conditions. With the development of 5 m^3 reaction vessels, this technique may be a cost effective treatment for the utilization of feather both as a feed and as a biopolymer feedstock but still will have a high energy demand [15].

2.1.4.2 Enzymatic Hydrolysis

Enzymes are a special class of proteins, which are biologically active, and often act on specific substrates. The group of enzymes that act on keratin are called keratinases, and they are both sulfitolytic and proteolytic [1]. These enzymatic treatments are often combined with a pretreatment step [97, 98]. Mokrejs et al. used a two stage process with alkaline hydrolysis using potassium hydroxide [97] while Eslahi et al. used sodium sulfite

to reduce cysteine bonds and sodium dodecyl sulfate as a stabilizing surfactant [98]. Eslahi et al. reported a yield of keratin hydrolyzate of 21.6%. These studies do not focus on material development but rather suggest that hydrolyzate be used as feed additives or nitrogenous fertilizer. Enzymatic hydrolysis can be cost prohibitive at the industrial scale due to the cost of producing the enzyme, and the difficulty involved in reusing the enzyme.

2.1.4.3 Ionic Liquids

Ionic liquids (IL) are salts with melting points below 100 °C. They have unique properties as solvents, known for their ability to dissolve many biological macromolecules such as cellulose [99], and silk fibroin [100]. IL have versatile operating properties; they are typically non-volatile, non-flammable, chemically and thermally stable, and reusable. Several studies have investigated a variety of ionic liquids and their suitability as solvents for keratins from both wool [65, 101-105] and feather [20, 106-108]. Sun used 1-butyl-3-methylimidazolium chloride ([BMIM]Cl), a common IL, and found that the treatment caused distinct changes in the keratin secondary structure [106]. The crystallinity and the alpha-helix and beta-sheet conformations were reduced, while random and beta-turn conformations increased. The hydrophilicity of the regenerated keratin was increased, as well as its capacity to adsorb Cr(IV) ions. The authors did not report a yield or losses of recovered material compared to initial mass, however a solution of 23% keratin was obtained after 48 hours of stirring.

Idris investigated a series of ionic liquids, including [choline][thioglycolate], examining cysteine cleavage during dissolution [108]. They found the thioglycolate anion did not increase the total yield or the solubility, but did impact the initial rates of dissolution, suggesting that cysteine bond cleavage occurred at a higher rate, but was not a limit on solubility. Idris [108] used the same IL [BMIM]Cl as Sun [106], but limited dissolution time to 10 hours, while increasing the temperature to 130°C. Idris [108] surpassed the solubility point of 23% achieved by Sun's process [106], reaching up to 45% solubility (mass of feather/mass of ionic liquid) [108]. Furthermore, they achieved a recovered material yield of 51% [108]. Interestingly, Idris [108] did not find the same differences in the secondary structure of the keratin as Sun [106], rather they found that only [choline][thioglycolate] altered the structure. It was shown that [Choline][thioglycolate] produced keratins solely in their monomeric form in the 10-14 kDA range while the other ILs showed evidence of dimer (20-28 kDA) and trimer forms (30-42 kDa) [108].

[BMIM]Cl is a water-soluble or hydrophilic IL, and the recovery of the dissolved material relies on rinsing with many other solvents. To address the challenge of recovering the ionic liquid, Wang et al. investigated the use of a hydrophobic IL 1-hydroxyethyl-3-methylimidazoliumbis(trifluoromethanesulfonyl)amide ([HOEMIm][NTf2]), with sodium bisulfite as a reagent to reduce the cysteine bonds [107]. They found a yield of 21.7% based on an initial mass, with optimal conditions of 4 hours at 80 °C [107]. The yield was limited to 7.86% without sodium bisulfite. Wang et al. argued that the advantage of a hydrophobic solvent is the immiscibility with water, and that the keratin could easily be separated using water, while 95% of the IL could be recovered [107]. The yield was relatively low, however

the results from gel permeation chromatography showed a nearly monodisperse molecular weight distribution. Moreover, the authors used dialysis tubing with a cut-off of 3500-5000 Da, and so smaller peptides may have been lost.

Idris [20] later investigated aprotic ionic liquid dimethylammonium formate ([DMEA][HCOO])[20]. The main advantage of this IL is that it may be recovered through distillation at 122 °C and 0.5−0.6 mbar [20]. Near complete recovery of the IL was achieved with 99.6% of the initial mass of the feather being recovered [20]. The solubility of keratin was substantially lower reaching only 15% w/w [20]. The authors suggested that this may have been due to a lower temperature of 100 °C processing temperature and a shorter time of 7 hours, used to avoid undesired volatilization of the IL [20]. The yield of the recovered material was 63% based on the initial mass [20].

2.1.5 Summary of techniques

Keratin from various agri-food waste streams has significant potential as a feedstock for biomaterial development due to its strength, biodegradability, and biocompatibility. However, it is a challenging material to modify and process, which can be mainly attributed to high cysteine crosslinks and the hydrogen bonding of the beta-sheet structure. The techniques used in the body of scientific literature are varied, and are summarized in Table 2. Of the treatments reviewed, treatment with sodium sulfite is the most industrially applicable; the compound is well understood from the Kraft process, is inexpensive, and yields a satisfactory recovery of undamaged material. Sodium sulfite is often added in

solutions due to its sulfitolytic action which reduce cysteine bonds. The extraction with 2-mercaptoethanol, urea and sodium dodecyl sulfate serves as an excellent benchmark, and the technique is suitable for applications able to support a higher cost such as biomedical applications. Ionic liquids show substantial promise for the development of biomaterials from many feedstocks, but our understanding of them is in its nascent stage. When designing a process utilizing keratin, one must ensure that they have a cost effective choice of inputs, both chemical and energetic, and that their recovered product has the desired properties.

Table 2.2. Processing Techniques and Yields of Feather Keratin.

Process	**Process time**[b]	**Process Temperature**	**Active Agent**	**Yield**[c]	**Reference**
Acid Hydrolysis	12-24 hours	90-110 °C	Hydrochloric acid Sulfuric acid	50%	[14] [94]
Acid Hydrolysis with Microwave	50 minutes	90-110 °C	900 W/100 W cycling microwave power	50%	[94]
Alkaline Hydrolysis	15 minutes	90°C	Sodium Hydroxide	90%	[57, 83, 109]
Alkaline-Enzymatic hydrolysis	4,8 hours	55 °C, 70°C	Sodium Borate Buffer, 0.3% Potassium Hydroxide	22%, 91%	[98], [97]

Oxidative Hydrolysis	30-45 minutes	40-80°C	Peroxy Acids (performic & peracetic acid)	7-40%	[62]
Reductive Extraction	30-45 minutes	40°C	2-mercaptoethanol[a]	75%	[17, 18, 79]
Sulfitolysis	2 hours	65°C	Sodium sulfite	30%	[52]
Metal Sulfides	1-6 hours	35°C	Sodium sulfide[a]	65%	[19]
Superheated Water	60-120 minutes	120-180°C	Water	90-98%	[84]
Steam Explosion	60-120 minutes	120 C	Water		[85]
High Density Steam Explosion	3 minutes	120°C	Water	90%	[15]
Microwave assisted Superheated Water	30-120 minutes	150-180°C	Water	20-80%	[16]
Ionic Liquids	4-48 hours	80-130°C	[BMIM]Cl [Choline] [thioglycolate] ([HOEMIm] [NTf2]) ([DMEA] [HCOO])	20-60%	[20, 106, 107]

a: with urea and sodium dodecyl sulfate; b: without dialysis time ; c: Soluble material/Initial mass

2.1.6 Summary of selected application

The purpose of the following section is to briefly summarize applications of keratin for use in soil fertilizer and water treatment as they relate to their processing techniques. A thorough review of other industrial applications of keratin was recently published in 2015 by Reddy including but not limited to films, fibers, and composites [110].

2.1.6.1 Feed and Fertilizer

Two essential considerations in feed and fertilizer compositions are protein and amino acid content [111]. The high protein content of feathers could make them a viable feed ingredient however, their structure and insolubility render them relatively inaccessible to digestive enzymes [112]. The use of acid or alkaline hydrolysis would require high-energy inputs in the form of heat. The hydrolyzate would require a neutral pH, and neutralization would produce salts that are undesirable and difficult to remove. More viable options are hydrothermal treatment and enzymatic hydrolysis[113]. Steam treatment has short treatment times, but requires a large energy input.

Feathers may be directly applied to fields as fertilizer. From a practical perspective this approach is difficult as lightweight feathers do not spread well with conventional machinery, and may not stay on the soil [114]. Free amino acids and oligopeptides have been shown to have a beneficial effect on the growth of plants as biostimulants, promoting plant growth, nutrient uptake, stress tolerance, and yield [29]. Two studies have

investigated the effect of enzymatic hydrolyzates on plants [115, 116]. Cao used a dilute foliar application and only remarked on the morphological improvements on Chinese cabbage [115]; while Gurav reported improvement in harvest times, antioxidant content, and yields in banana crops testing both root and stem dosages [116]. These studies demonstrate the potential of feather hydrolyzates as fertilizer for crops, and require further study for additional crops. For minimally added value products like feed and for fertilizer purposes, a low cost method is desirable, and so enzymatic treatments are often preferred, while hydrothermal treatments may be cost-effective.

2.1.6.2 Adsorbents

In recent years, the body of research concerning native feathers and derived materials as adsorbents has grown considerably. A comprehensive review was done by Ghosh et al., summarizing the adsorption capabilities of keratin materials such as membranes, fibers, powders, and meshes on different contaminant metal ions such as zinc, lead, and chromium [117]. Ghosh et al. did not address keratin sorption capabilities beyond contaminant metals [74, 118, 119] such as Cr(IV) and Pb(II), but they have been used on various dyes [120-122] such as Azo Red and Blue 80 from the textile industry, which must be removed from wastewater during treatment [120, 122, 123]. The material form used varies from unmodified keratin powders [124] and fibers [120] to composite filters and membranes [125].

2.1.6.3 Sponges and Hydrogels

Hydrogels and spongy materials have an important role to play in the biomedical fields for controlled drug release and delivery matrices, as well as tissue engineering. Many different keratin hydrogel studies have been conducted investigating their effects on cell growth and regeneration [77, 126-129], as well as drug loading [76, 130]. Very few papers with applications of keratin hydrogels outside the field of biomedicine such as superabsorbent hydrogels or controlled release agrochemicals have been reported. One salt and pH responsive hydrogel from keratin was synthesized by ethylenediaminetetraacetic dianhydride grafting onto keratin, and moderate swelling of 64 g/g was reported [131]. There has been one report of a keratin colloidal solution used as an adsorbent [74]. Keratin sponges may be used for oil spill cleanup. Zhou et al produced a sponge capable of holding 30 g/g of oil [132], which is a significant improvement from native keratin fibers which hold 3-5 g/g [133], and even compared to industry standard polypropylene materials which hold 8-10 g/g [134]. This increase in oil absorption capacity is likely due to increased porosity and surface area of the sponge as compared to native fibers. Further modification strategies such as acetylation [45, 135] or fatty acid grafting [134, 136] to increase oil absorption capacity should be investigated.

2.1.7 Conclusion

This review has investigated a range of techniques for processing keratin. In its native or unmodified structure, keratin is a low value protein feedstock. It is a challenging protein feedstock for processing due to its solubility properties, but has shown promise in several

materials. For example, keratin-based hydrogels and adsorbents can find application in controlled-release of agrochemicals, superabsorbent, wastewater and oil treatments. The process for purifying and modifying keratin must be chosen with careful consideration of cost, molecular weight, and material properties, depending on the target application. Keratin based materials or composite will have improved biocompatibility, and biodegradability for environmental applications.

Connecting Statement

The literature review (Chapter 2) presented various published techniques for the extraction of feather keratin, and the potential applications of keratin as a bio-based material. In Chapter 3, acid hydrolysis is used to produce keratin hydrolysate which is then utilized as a feedstock for the synthesis of hydrogels. The resulting hydrogels were tested for their swelling capacity in different media and swelling under load.

Chapter 3 has been accepted for publication in Waste and Biomass Valorization.

Chapter 3

Synthesis and properties of feather keratin-based superabsorbent hydrogels

3.1 Abstract

The present work reports significant improvement in the performance of keratin based hydrogels. These hydrogels were synthesized by solution based graft copolymerization of acrylic acid monomers on the hydrolyzed keratin proteins' backbones in the presence of a crosslinker (N,N0-methylenebis (acrylamide)) and initiators (sodium bisulfite and potassium persulfate). The grafting was confirmed by means of Fourier transform infrared spectroscopy. The contributions of the crosslinker, initiator and neutralization degree to the hydrogels were investigated through differential scanning calorimetry, thermogravimetric analysis, swelling test, and scanning electron microscopy. The macromolecules exhibited extraordinary water absorbency capacity in distilled water. The highest equilibrium swelling of hydrogel in distilled water reached 501 g/g of hydrogel in 48 h. The swelling properties of the optimized hydrogel were also studied at various pH and saline concentrations.

3.2. Introduction

Superabsorbent hydrogels are 3-dimensional hydrophilic cross-linked polymer networks. The prominent applications of biocompatible and biodegradable hydrogels materials are in the biomedical industry [27]. The concept of controlled release is not limited to drug delivery in medicine, but is also applicable in agriculture. Hydrogels may be impregnated with fertilizers [137] or other agro-chemicals [138], and then applied to the soil where release is controlled, preventing run-off pollution and erosion [139]. The superabsorbent properties of hydrogels are beneficial in soils with high rates of water infiltration or where water is limited [140].

Superabsorbent polymers are most commonly made from acrylic monomers such as acrylamide, acrylic acid, and sodium acrylate. Acrylic acid has been copolymerized with biopolymers such as proteins and polysaccharide to improve the biocompatibility and the biodegradability [141]. Polysaccharides such as starch, chitosan, alginate, and cellulose have been grafted with acrylic acid to synthesize hydrogels [142]. Collagen [36, 143], cottonseed [144], canola [145], and keratin [137, 146] proteins-acrylic acid copolymers have been reported. Proteins have many reactive groups along their primary chain that are suitable for modification, grafting, and crosslinking [147].

Chicken feathers are a waste product of the poultry and egg industry, and it is estimated that 2 million tons are produced annually in the United States [148], and more than 75 thousand tons in Canada [149]. Over 90% of the feather's composition is keratin, a

structural protein. Keratin has been investigated as a starting material in the synthesis of films [109], fibres [69], fertilizers [114, 116], composites [53, 147], adsorbents [117, 120, 132] and hydrogels [77, 131].

Keratin has a high percentage of the amino acid cysteine in its composition. For example, plant proteins such as canola and soy isolates contain 0.63 and 1.26% cysteine [26], whereas feather keratin and wool keratin have up to 7 and 17% cysteine, respectively [27, 28]. The high cysteine content results in the formation of a high number of crosslinks between the sulfhydryl groups of the amino acids on the protein backbone, called disulfide bonds or bridges. These crosslinks render keratin insoluble in water and consequently, performing reactions in aqueous media involve disruption of the disulfide bonds.

Keratin' s native material properties are an important consideration in the production of a new material and its process conditions. Feather keratins are reported to have a molecular weight of 10 kDa [29]. To preserve the native keratin structure, it is important to never exceed the ther-mal degradation temperature (Td) of 201 °C at any point in the process. The isoelectric point (pI) of proteins must be considered when controlling the pH of the aqueous solution during processing. At a pH near the pI, proteins will arrange closer together producing smaller fiber diameters [30]. If the process pH reaches the pI or higher, then the protein will precipitate from the solution [31]. Cysteine can impart strength to any materials they are added to due to the presence of the numerous disulfide bonds. Cysteine can be highly reactive and is a suitable reaction site for mod-ifications of the protein such as carboxymethylation [32]. The extent of carboxymethylation is determined by the total

cysteine content [33]. Hydroxyl groups can form radicals, as protons can readily be abstracted from the protein chain by chemical initiators such as potassium persulfate [8]. The molecular weight will have an influence on the mechanical properties and biodegradation times of the materials produced [34]. The molecular weight can be increased through polymerization or grafting, or decreased by peptide bond scission

This study presents the synthesis and characterization of hydrogels from chicken feather and acrylic acid by graft copolymerization using an L9 orthogonal array as the experimental design [150]. There have been two reports of keratin materials produced by graft copolymerization with acrylic acid. First, Yang developed a composite hydrogel material for slow release fertilizer [137] where water-soluble keratin was achieved through a two-step process of alkaline hydrolysis, and sulfitolysis. The authors did not attempt to optimize their formulations, and low water absorbency in the range of 40-50g/g was achieved. Similarly, Li et al. reported the application of keratin-g-acrylic acid as a sizing agent for textile applications [146]. The objective of this work was to develop superabsorbent hydrogels with a high swelling ratio, using feather protein derivatives as one of the main constituents. The hydrogel properties were studied by Fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), and scanning electron microscopy (SEM).. The optimized hydrogel formulation reported in this study reached a higher swelling equilibrium than previously reported results for keratin-based hydrogels.

3.3. Materials and Methods

3.3.1 Materials

Feather meal with a protein content of 96% was provided by Rothsay (Guelph, Ontario). Acrylic acid (AA), sodium bisulfite (SBS), potassium persulfate (KPS), N,N-methylenebis (acrylamide) (NMBA), aprotinin from bovine lung (6.5 kDa), and cytochrome c (12.4 kDa) as molecular weight standards, 2-mercaptoethanol (2-ME), Coomassie Brilliant Blue R-250, N,N,N',N'-tetramethyl ethylene diamine (TEMED) from Sigma Chemical Co. (St. Louis, MO, USA), and anhydrous ethanol were of analytical grades and purchased from Sigma Aldrich (St. Louis, MO, USA). Laemmli sample buffer was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Sodium chloride methanol sodium dodecyl sulfate (SDS), and hydrochloric acid (HCL) (ACS reagent grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sodium hydroxide was purchased from EMD (Damstadt, Germany). Acetic acid glacial was purchased from Fisher Scientific (Nepean, Ontario, Canada).

Feather meal was partially hydrolyzed using 1M HCL. Briefly, 10 g of feather meal was added to 100 mL of 1M HCL and kept at 95 °C for 24 hours. The solution was filtered and lyophilized. The yield of hydrolyzed feather keratin (HFK) was 5.8 g.

3.3.2 Methods

3.3.2.1 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli to determine the changes in the molecular weight of the feathers before and after hydrolysis. Samples (1 mg) were dissolved in 100 µL of 2 × Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) containing 150 mM of 2-β-mercaptoethanol and heated at 80 °C for 10 min to ensure complete dissolution. The samples (10 µL) and molecular weight standards (10 µL) were loaded into the individual lanes in the freshly prepared 17% acrylamide gel. After 1 hour of electrophoresis at 120 V, the gel was stained with Coomasie brilliant blue staining solution for 30 min and rinsed in 10% glacial acetic acid. Gels were allowed to dry under ambient conditions and the images of the bands in the gel were collected. Aprotinin from bovine lung (6.5 kDa), and cytochrome c (12.4 kDa) were run as additional standards.

3.3.2.2 Preparation of Hydrogels

In a typical experiment, hydrolyzed feather keratin-poly acrylic acid (HFK-PAA) hydrogels were prepared as follows: 0.03 g of NMBA in 5 ml degassed distilled water was added to 6 g of partially neutralized (70 mol%) AA. Hydrolyzed feather keratin (1.5 g) was dissolved in 25 mL of distilled degassed water at 70 °C in a thermostat water bath with agitation using a magnetic stirrer for 5 min. Thereafter, initiators (0.5 g KPS and 0.25 g SBS) were added to the protein solution. After stirring for 10 min, the protein solution was

mixed with the prepared AA and NMBA solution. The mixture was incubated in a water bath at 70 °C for 60 min for completion of the reaction. The preceding manipulations were all conducted under a nitrogen atmosphere. The resulting gel was immersed in an excess of non-solvent ethanol (200 ml) to dewater it. After 3 h, the ethanol was decanted. The gel was cut into small pieces and re-immersed with 100 mL of fresh ethanol for 24 h. The gel was filtered and freeze-dried at -50 °C for 24 h, the dried gel pieces were treated with liquid nitrogen before being ground into a powder. The powdered hydrogel was stored away from moisture, heat and light. To study the effects of the crosslinker, initiators, and monomers, hydrogels with different compositions were synthesized according to a partial factorial L9 array as described in Table 3.1.

Table 3.1 L9 Orthogonal Array

Sample	**HFK**		**KPS/SBS**		**AA**		**NMBA**	
	m (g)	m (%)	m (g)	,m(%)	m (g)	m(%)	m (g)	m.(%)
HFK-AA-1	1.5	38.41	0.25/0.125	9.60	2	51.22	0.03	0.77
HFK-AA-2	1.5	25.27	0.25/0.125	6.32	4	67.40	0.06	1.01
HFK-AA-3	1.5	18.81	0.25/0.125	4.70	6	75.24	0.1	1.25
HFK-AA-4	1.5	32.02	0.75/0.375	24.01	2	42.69	0.06	1.28
HFK-AA-5	1.5	22.30	0.75/0.375	16.73	4	59.48	0.1	1.49
HFK-AA-6	1.5	17.33	0.75/0.375	13.00	6	69.32	0.03	0.35
HFK-AA-7	1.5	34.48	0.5/0.25	17.24	2	45.98	0.1	2.30
HFK-AA-8	1.5	23.89	0.5/0.25	11.94	4	63.69	0.03	0.48
HFK-AA-9	1.5	18.05	0.5/0.25	9.03	6	72.20	0.06	0.72

L9 orthogonal array used as the experimental design for the formulation of hydrogels; HFK – hydroylyzed feather keratin; KPS – potassium persulfate; SBS – sodium bisulfite; AA- acrylic acid; NMBA - N,N'-Methylenebisacrylamide.

3.3.2.3 Infrared Analysis (FT-IR)

The FT-IR spectra of the HFK, hydrogels and additives were conducted in triplicate on a Nicolet iS5 FT-IR spectrometer (Thermo, Madison, WI, USA). The spectra were recorded at 32 scans and 4 cm^{-1} resolution in the 4000– 400 cm^{-1} range. The spectra were analyzed using the OMNIC software package (version 8.2, Thermo Nicolet Corp).

3.3.2.4 Differential Scanning Calorimetry (DSC)

10 mg of HFK and hydrogels were compressed in hermetic aluminum pans and scanned in duplicate using a DSC (Q100, TA Instruments, Inc., New Castle, DE, USA) under a stream of nitrogen (50 mL/min). Samples were heated from 0 °C to 350 °C at a rate of 10 °C/min.

3.3.2.5 Measurement of gel content

The gel content values were determined by taking 0.03 g of dried sample and dispersed in double distilled water to swell for 48 h. After filtration, the extracted gel was frozen at -80 °C, freeze-dried for 24 hours at -50 °C, and then reweighed. The gel content (gel %) was calculated by Equation 1.

Equation 1. Gel Content

$$Gel\ \% = \left(\frac{M}{m}\right) * 100\%$$

where, M and m stand for final and initial mass of the sample, respectively.

3.3.2.6 Scanning Electron Microscopy

A thin layer of feather meal, feather hydrolysate, and the hydrogels were deposited on a double-sided adhesive carbon tape mounted on an aluminum specimen holder, and any unattached particles were removed. Samples were examined at a voltage of 5 kV using a scanning electron microscope (model S-3000N, Hitachi, Tokyo, Japan).

3.3.2.7 Swelling Measurements

The swelling capacity of the hydrogels was determined by using approximately 30 mg of dried hydrogels, placed in nylon mesh tea bags with a screen size of 100 microns. The bags were immersed in 500 mL of distilled water. The bags were removed from the water, and blotted lightly on paper towel to remove surface water. The mass of the sample was measured every 1-hour for 6 hours and then at 12, 24, and 48 hours. The swelling capacity of the gels was determined by Equation 2. Each hydrogel sample was tested using three replicates.

Equation 2. Swelling Capacity

$$\text{Swelling capacity (g/g)} = \frac{Wet\ mass - mass\ of\ bag - mass\ of\ dried\ sanple}{mass\ of\ dried\ sample}$$

3.3.2.8 Absorbency under load (AUL)

Absorbency under a load was determined by the following procedure. Approximately 30 mg of hydrogel was place into a nylon mesh bag, and the bag was sealed. The bag was placed in a plastic cylinder with a 2.50 cm diameter, with an 800-mesh nylon filter across the bottom. A disc of diameter, 2.49 cm, was placed on the hydrogel sample, and a mass either of 100 g or 200 g, was placed on top of the disc to apply the desired pressure. The entire assembly was then immersed in either water or 0.9% NaCl for 1 hour. AUL was calculated using Equation 2.

3.3.2.9 Swelling in different media

The swelling property of the hydrogel samples was measured using different concentrations of NaCl by using the same method as the Swelling Measurements (2.2.7) but varying the NaCl concentration in the solution, with each sample measured in duplicate. The swelling property of the hydrogel samples was studied in various pH solutions. Individual solutions with a pH of 1, 3, 5, 7, 9, and 11 were prepared by using HCl (10 M) and NaOH (1 M) solutions. The pH value of each solution was adjusted using a Symphony SB70P pH meter (VWR, Wayne, NJ, US).

3.4. Results and Discussion

3.4.1 SDS-PAGE

Figure 3.1 reports the changes in the molecular weights (kDa) of the feather proteins before and after hydrolysis. Lane 1-3 contains proteins of known molecular weight as a standard. Lane 4 contains the feathers hydrolyzed with 1 M hydrochloric acid solution and had lost most of the high molecular weight proteins seen in the unhydrolyzed feathers in lane 5. Lane 5 contains the unhydrolyzed feather keratins and has a molecular weight in the 10-14 kDa range. Feathers treated with 1 M hydrochloric acid solution did not show any major bands (lane 4) indicating severe hydrolysis; similar results for keratin have been found using alkaline hydrolysis [83, 109]. The decrease in molecular weight due to hydrolysis makes the protein more hydrophilic and soluble in water. To demonstrate the increase in solubility, the authors prepared 15% (w/w) solution the hydrolysate, which readily dissolved, whereas the native feather meal did not dissolve at all in water.

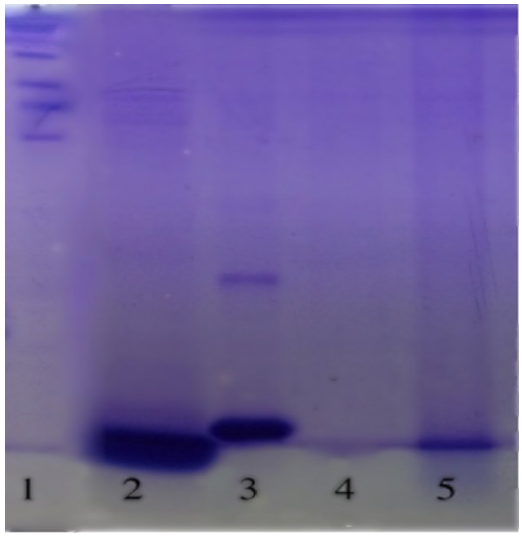


Figure 3.1. SDS-PAGE of Keratin and Hydrolyzed Keratin

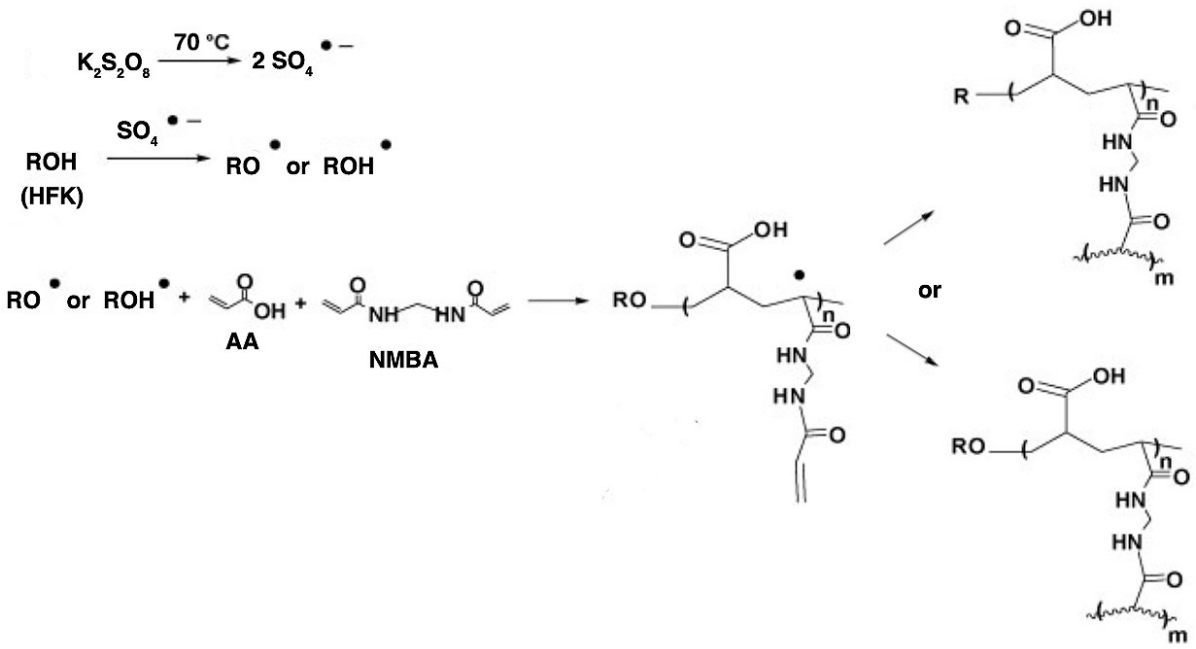
Lane 1 molecular weight markers 25-100 kDa; Lane 2 Apotinin molecular weight 6 kDa; Lane 3 Cytochrome C molecular weight 12 kDa; Lane 4 hydrolyzed keratin; Lane 5 Keratin extracted by Na_2S (~10 kDa)

3.4.2 Synthesis and Mechanism

The hydrolysis step was used to solubilize the proteins. The hydrolysis process disrupts the secondary structure of the proteins making reactive groups more accessible for graft copolymerization [53, 117]. The resulting shorter oligopeptides are water-soluble. Feather meal has been investigated as a potential source of nitrogenous fertilizers. However, it was found that feathers do not biodegrade at an appreciable rate to release the nitrogen due to a lack of proteolytic enzymes in soil [114]. Feather hydrolysate was shown to have better availability for plant uptake and showed beneficial effects for plant growth and yield [116]. The alkaline hydrolysis process is known to induce racemization of amino acids [58] which is not beneficial for plant growth [29].

The synthesis mechanism (Figure 3.2) is a redox-couple initiated free radical copolymerization with acrylic acid, N,N'methylene bisacrylamide, and protein hydrolysates. This mechanism has been reported for the synthesis of hydrogels using different proteins [144, 145]. It should be noted that for initiator values lower than 0.25 g KPS/ 0.125 g SBS, no gel was formed, but a tacky white product precipitated upon addition of ethanol. This indicates insufficient crosslinking of the graft copolymer. Process parameters of reaction temperature (70 °C), and degree of neutralization (70%) were chosen based on previously reported optimum values [5, 145, 150, 151]. The persulfate initiator decomposes with heating (> 60 °C) and produces sulfate anion radicals that abstract hydrogen atoms from the hydroxyl groups of the keratin hydrolysate chains forming macroradicals. These macroradicals initiate the polymerization of acrylic acid forming the graft copolymer [144, 145, 152].

Figure 3.2. Schematic of Acrylic Acid Hydrogel Prepared With HFK (HFK-AA).



3.4.3 Fourier Transform Infrared Spectroscopy

Evidence of grafting was obtained by analysis of the FTIR spectra of HFK, HFK-AA, and a hydrogel synthesized without HFK. The broad absorption band region from 3200 cm^{-1} to 2800 cm^{-1} for HFK can be attributed to the O–H, N–H, and C–H stretching. The shoulder in the curve at 2600 cm^{-1} is attributable to S–H stretching of the cysteine groups, whereas S–S bonds are not visible [153]. The absorption bands at 1665 cm^{-1}, 1560 cm^{-1} and, 1210 cm^{-1}, visible in the HFK spectra are attributed to the amide I, amide II, and amide III bonds [146]. The broad peak in the HFK spectra at 2961 cm^{-1} represents the characteristic IR bands of aliphatic hydrocarbons of methylene asymmetric C-H stretching and symmetric C-H stretching [154]. The absorption bands in HFK-AA hydrogel in Figure 3.2 at 1712, 1580 and 1403 cm^{-1} are ascribed to -COOH stretching, COO^- asymmetric stretching and

COO^- symmetric stretching, respectively. The result confirms that part of the acrylic acid was partially neutralized by NaOH solution. The FTIR spectra of the hydrogel do not show a characteristic band in the 1665 cm^{-1} region, while the band absorbance at 1550 cm^{-1} increased significantly. This indicated a significant decrease in amide I stretching. The increase in the C-N stretching at 1240 cm^{-1} and 1180 cm^{-1} suggests that the carboxylic group of feather hydrolysate had been replaced with amide bonds linked to the acrylic acid monomers. The comparison of these spectra suggests that acrylic acid was grafted onto the primary chain of HFK.

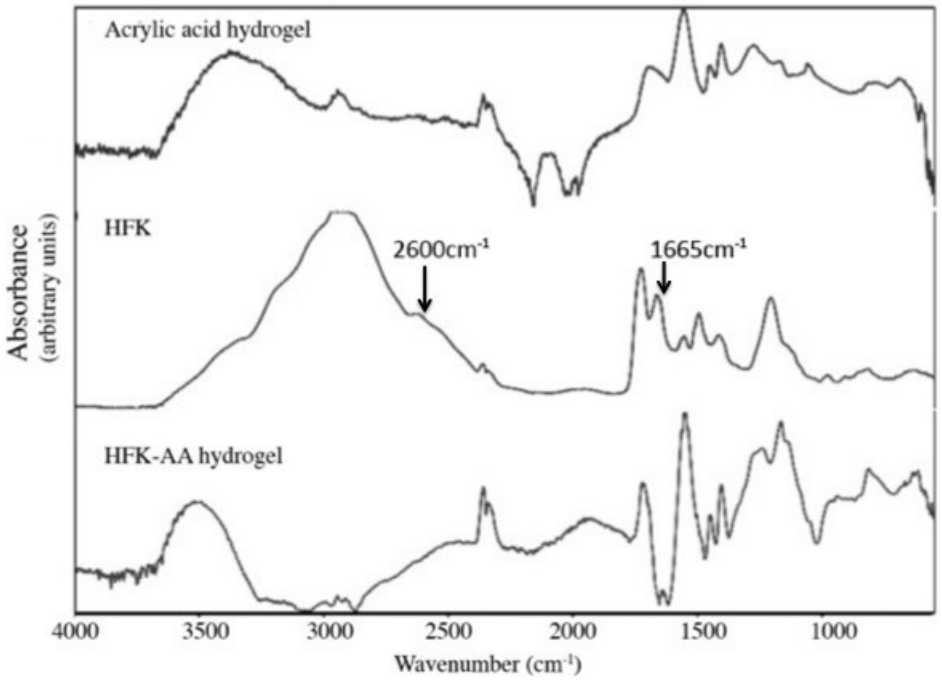


Figure 3.3. FTIR Spectra of Hydrolyzed Feather Keratin (HFK), an Acrylic Acid Hydrogel Prepared Without HFK and an Acrylic Acid Hydrogel Prepared With HFK (HFK-AA).

3.4.4 Thermal Properties

The DSC thermograms show evidence of improved thermal stability of the hydrogel as compared to the hydrolysate. Furthermore, indication of a glass transition temperature was present in the hydrogels near 50 °C, increasing with higher crosslinker content. The hydrolysate shows several distinct denaturation peaks after 100 °C, representing different polypeptide fractions. Only two peaks are present in the hydrogel samples (near 109 °C and 190 °C). The glass transition temperature of poly(acrylic acid) is reported to be in the range of 105-120 °C [155]. Residual polymer chains that are not crosslinked may act as plasticizers, lowering the T_g. Sample 9 had a higher gel content, 70% compared to 48-50% in other samples, meaning less residual chains as plasticizer and showed no T_g in the DSC thermogram. Crosslinked polymers may not exhibit a glass transition temperature, due to immobilization of the polymer chains at crosslinking junctions [156]. The improved thermal stability of the material is additional evidence of graft copolymerization.

Table 3.2 Thermal Properties of Keratin Based Hydrogels

Sample	NMBA (%wt)	Gel Content (%)	T_g(°C)	First Denaturation Temperature (°C)	Second Denaturation Temperature (°C)
HFK-AA-3	1.25	50.81	48	109	185
HFK-AA-5	1.49	40.93	51	108	180
HFK-AA-7	2.30	48.15	52	108	190
HFK-AA-9	0.72	71.04	---	120	195

Increasing NMBA concentration. increases the T_g, Two denaturation peaks are clearly in all sample around 108-120 °C and 180-195(°C).

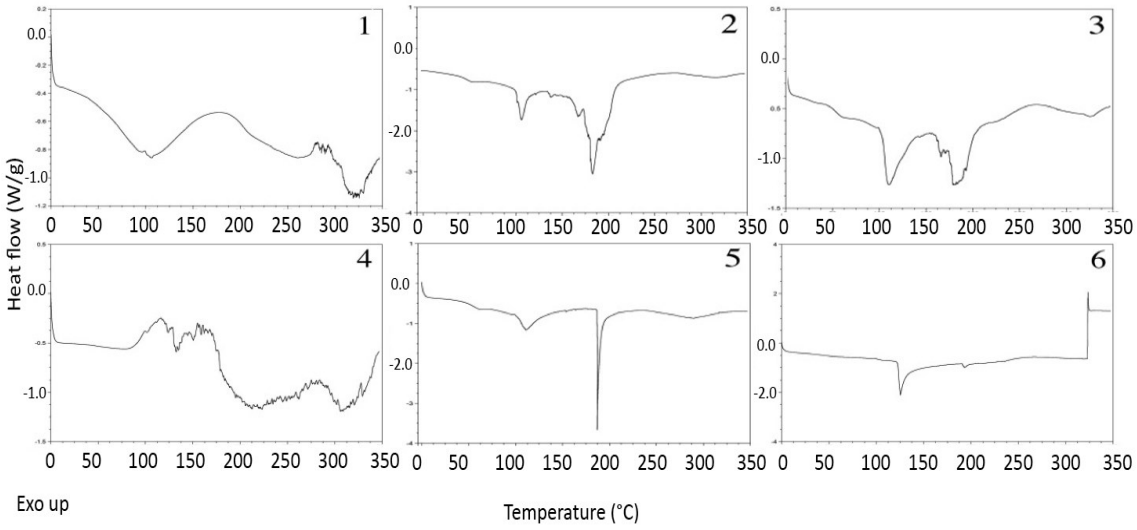


Figure 3.3. DSC Thermograms of feathermeal and HFK-AA Hydrogels

1: Feathermeal; 2: HFK-AA-3; 3: HFK-AA-5; 4: HFK; 5:HFK-AA-7; 6: HFK-AA-9; Increasing NMBA concentration. increases the T_g, Two denaturation peaks are clearly in all sample around 108-120 °C and 180-195(°C).

3.4.5 Microstructure

The surface morphology of the feather meal, feather hydrolysate, and HFK-AA hydrogel were observed by SEM (Figure 3.4). The feather meal shows the typical pattern of the filamentous fibres and the thin striations on the surface [120]. The fibrous structure of the keratin after hydrolysis became smooth and the ordered fibrous structure was no longer observable. The surface morphology of the hydrogel showed a porous structure. For porous hydrogels, absorption occurs mainly by capillary action rather than conventional diffusion which occurs across a concentration gradient [157].

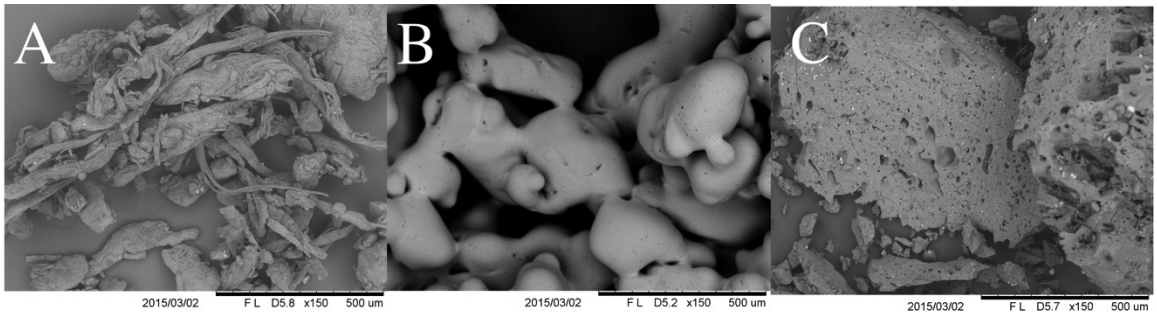


Figure 3.4. SEM Images of Feathermeal, HFK, and HFK-AA Hydrogel

Feathermeal (A), feather hydrolysate (B), HFK-AA hydrogel (C) at 150X.

3.4.6 Swelling properties

3.4.6.1 Swelling in distilled water

Swelling of superabsorbent hydrogels relies on the anion-anion electrostatic repulsion force. The experimental formulations were selected based on publications that reported the highest swelling values for biobased acrylic acid hydrogel, being 920 g/g [36], 1410 g/g [141], 3310 g/g [150]. The keratin-based hydrogels of this work did not have swelling ratios as large as the other biobased acrylic acid hydrogels. However, the swelling ratios of this work are much larger (330-502 g/g) compared to the results from other keratin hydrogel publications of 48 g/g [137], and 72g/g [131]. Not all formulations selected as part of the experimental array had sufficient dimensional stability, and dissolved during the swelling test. This is due to low crosslinking density, or insufficient initiation of the crosslinking reaction. The highest swelling ratio in distilled water occurred at 48 hours for the hydrogel synthesized with 34.48% HFK, 17.24 %KPS/SBS, 2.30% NMBA, and 45.98% AA.

Table 3.3 Equilibrium Swelling in Distilled Water

Sample	1	2	3	4	5	6	7	8	9
Equilibrium Swelling (g/g)	*D*	*D*	487.17±2.71	*D*	430.54±11.01	*D*	501.58±6.26	*D*	335.47±15.46

D: dissolved in water; no dimensional stability.

3.4.6.3 Effect of load on swelling

Absorbency under load (AUL) is a common industrial metric for superabsorbent hydrogels [141, 151]. AUL values change proportionally with the mechanical strength of the swollen gel, and can be considered a measure of the gel strength of the superabsorbent materials. Increasing the load from 2 kPa to 4 kPa reduced the swelling capacity of the gel from a maximum of 49 g/g to 20 g/g (Figure 3.7).

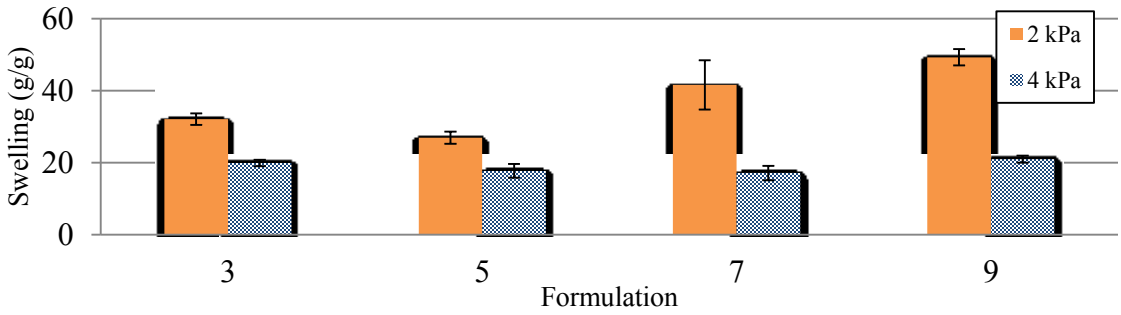


Figure 3.5. Absorbency Under Load in 0.9% NaCl (AUL)

3.4.6.4 Effect of salinity on swelling

The water absorbency of hydrogels decreased significantly in saline solutions using sample formulation 3 as it showed a very high swelling ratio of 487 g/g. As the NaCl concentration increased, the water absorbency decreased. This effect is attributed to the reduced osmotic pressure difference between the superabsorbent hydrogel and the external salt solution with increasing ionic strength [5, 143]. The sodium ions created electrostatic screening of the anionic groups of the hydrogel, preventing the anion-anion electrostatic repulsion force, and resulted in the contraction of the hydrogel network and reduced swelling.

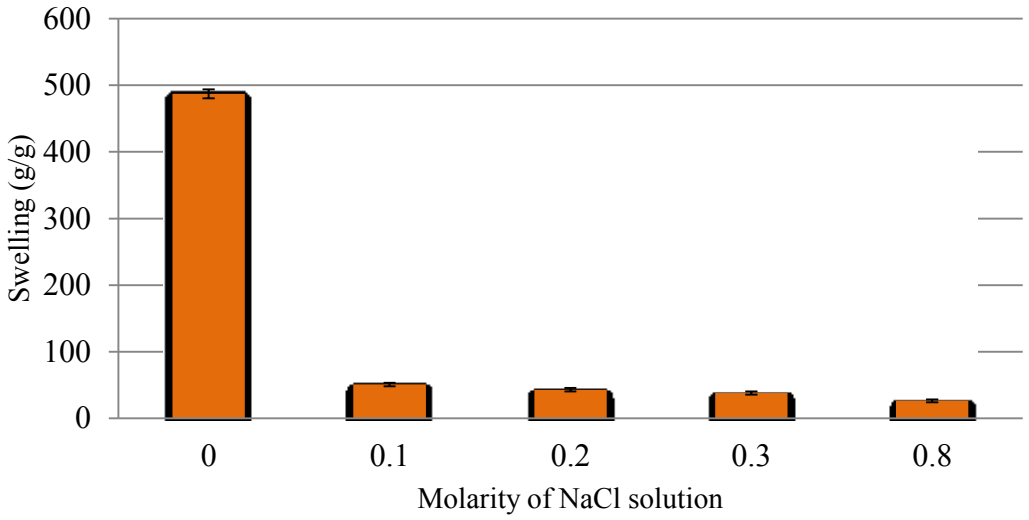


Figure 3.6. Swelling in Various Saline Solutions for Sample Formulation 3

3.4.6.5 Effect of pH on swelling

The swelling capacity of the hydrogels was studied at various pHs, ranging from 1.0 to 13.0. The swelling capacity of the hydrogels was sensitive to the ionic strength of the swelling medium; therefore no buffer solution was used. According to Figure 3.7, swelling increased from pH 1 to pH 9. An increase of pH, gradually ionizes of the carboxylate groups of the hydrogels. As the carboxylate groups were ionized, anion-anion electrostatic repulsion occurs, and therefore swelling occurs. At a pH>9, swelling decreased due to charge screening from an increased concentration of sodium cations. Maximum swelling (524 g/g) was achieved at pH 9 after 48 hours. Typically the literature reports the highest swelling in distilled water. This difference may be explained by partial hydrolysis of the

hydrogel network. As the hydrogel remained in pH 9 for a longer period of time, some of the linkages of the network may have been hydrolyzed, allowing the hydrogel to further expand. This same action on the network explains the mass loss between the 24 and 48 hour measurements, as chains are hydrolyzed. The response of the gel to changing pH was also investigated. These materials swelled and shrank continuously depending on the pH (Figure 8), this response to changing environmental factors defines them as smart materials.

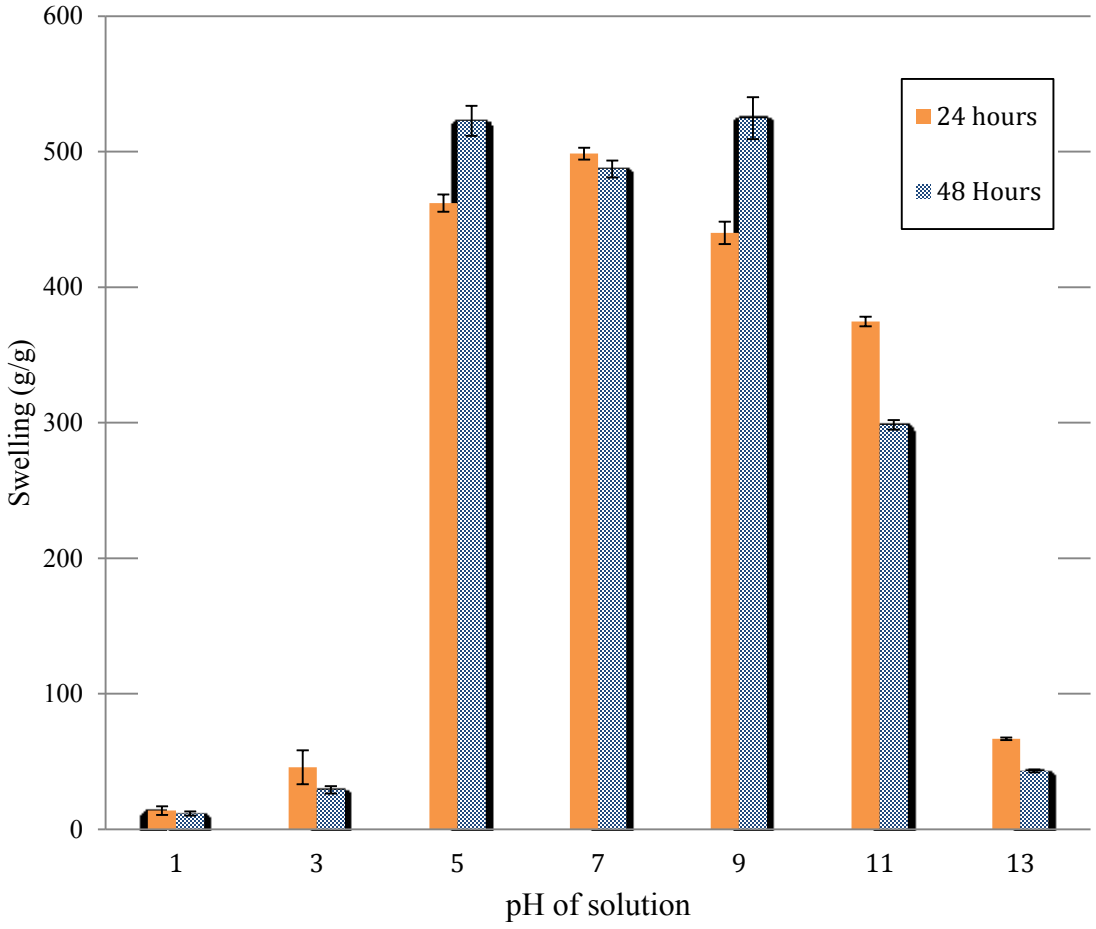


Figure 3.7. Swelling in Water With Various Ph for Sample Formulation 3

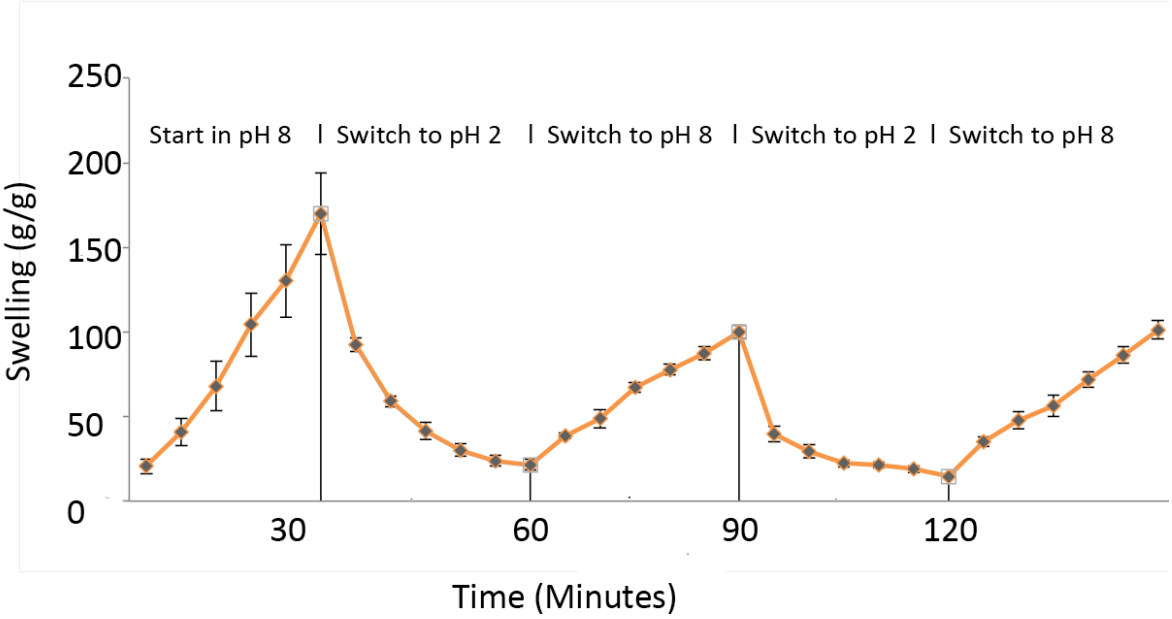


Figure 3.8. pH Switching Behavior for Sample Formulation 3

3.4. Conclusion

Superabsorbent hydrogels were synthesized by free radical graft copolymerization of hydrolyzed feather protein, partially neutralized acrylic acid, and crosslinker N,N' methylenebisacrylamide. The optimum formulation for swelling capacity was the hydrogel synthesized with 34.48% HFK, 17.24 %KPS/SBS, 2.30% NMBA, and 45.98% AA. The highest swelling of 501 g/g was achieved in distilled water. The hydrogels were sensitive to the pH and salinity of the swelling media. This material may be a suitable smart material for land remediation or agriculture.

Connecting Statement

Chapter 3 showed the potential for feather hydrolysate to be used as superabsorbent hydrogels. The ability of the protein backbone to undergo graft reactions with acrylic acid suggested that the protein may be modified by other graft reactions as well. Chapter 4 presents the graft modification of keratin protein with oleic acid to improve the oil holding capacity of the feather. The effect of oleic acid modification, protein concentration, and crosslinker concentration on the oil holding capacity of the cryogels has been studied.

Chapter 4 has been submitted to the Journal of Polymers and the Environment.

Chapter 4

Synthesis and properties of oleic acid modified feather keratin-based motor oil sorbing cryogels with high oil holding capacity

4.1 Abstract

Oleic acid was used to modify keratin extracted from chicken feathers by free radical initiated graft copolymerization. Thereafter, the modified keratin was used for the synthesis of cryogels. The influence of oleic acid modification, the crosslinker content, and the protein concentration on the properties of the cryogels were investigated by Fourier transform infrared spectroscopy, scanning electron microscopy, SDS-PAGE, and through oil holding capacity tests. To enhance the sorption properties, the cryogels were crosslinked with glutaraldehyde. Varying protein concentration from 1.27% to 5.09% and glutaraldehyde concentration from 0% to 10% produced cryogels with oil holding capacity ranging from 4.56 g/g to 10.76 g/g. The highest results exceeded the sorption capacity of previously published oleic acid modified woodchips (6.3 g/g) as well as polypropylene (6 g/g), which is the standard material used in industry.

4.2. Introduction

Natural and synthetic sorbents are used for site remediation of oil spills [158].

Polypropylene is one of the most common materials used in sorbents, predominantly because of its hydrophobic and oleophilic properties [159]. Apart from the hydrophobicity and oleophilicity character of the materials, properties required for good oil sorbents include high oil uptake capacity, high rate of uptake, and biodegradability [160].

Chicken feathers are a residue product from the poultry and egg industry. It is estimated that 2 million tons are produced annually in the United States [148], and more than 75 thousand tons are produced in Canada [149]. Over 90% of the feather's composition is keratin, a structural protein found in feathers, hairs, nails, and hooves [148]. Keratin has been investigated as a feedstock material in the synthesis of biomedical films [109], fibers [69], composites [53, 147], heavy metal adsorbents [117, 120, 132], and oil sorbents [132, 133, 161]. Chicken feathers have several features desirable for oil sorbents such as surface toughness, flexibility, high fiber length to diameter ratio, and hydrophobicity [133].

Keratin sorbents have been reported both as native feather [133, 161], and synthesized sponges [132]. This work presents cryogels synthesized by using a fatty acid grafting technique for protein modification, which has previously only been reported on cellulosic feedstocks [134, 136]. The cryogel was crosslinked to increase the oil holding capacity, and freeze-dried to produce a stable oil-sorbing cryogel. Furthermore, the previously reported unmodified keratin sponge was only tested for the sorption of soy oil and paraffin oil [132]. This work presents the physical properties of newly synthesized keratin cryogels and investigates the oil holding capacity with respect to motor oil, a known contaminant of concern [162].

4.3. Materials and Methods

4.3.1 Materials

Feather meal with a protein content of 96% was provided by Rothsay Canada (Guelph, Ontario). Sodium sulfide, hydrochloric acid, 2,2-azobis(2-methylpropionitrile) (AIBN), oleic acid, glutaraldehyde, N,N-methylenebis (acrylamide) (NMBA), aprotinin from bovine lung (6.5 kDa), cytochrome c (12.4 kDa), 2-mercaptoethanol, Coomassie Brilliant Blue, R-250, N,N,N',N'-tetramethyl ethylene diamine, and acetic acid were of analytical grades and purchased from Sigma Aldrich (St. Louis, MO, USA). A molecular weight marker kit containing a lyophilized mixture of the six following proteins: carbonic anhydrase, bovine 29 kda, albumin, egg 45 kda, albumin, bovine 66 kda, phosphorylase b, rabbit 97 kda, β-galactosidase, e. coli 116 kda, myosin, rabbit muscle 200 kda was purchased from Sigma Aldrich (St. Louis, MO, USA). The lyophilized proteins were of analytical grade. Laemmli sample buffer was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Motor oil (Castrol SL-10W-30) was obtained from Petro-Canada (Calgary, AB, Canada).

4.3.2.1 Oleic acid modification of feather meal

Oleic acid modified feather meal was obtained by free radical initiated graft copolymerization. 5 g of feather meal was mixed with 1 g of oleic acid in 100 mL of n-hexane with 0.01 g of AIBN initiator in a 500 mL conical glass flask placed in a hot water bath. The 6 hours reaction was performed at 65 °C under reflux and stirring. The modified feather meal was collected by filtration and the solid filtrate was washed with hexane to remove unreacted reagents. The modified feather meal was then dried in an oven at 50 °C until constant weight.

4.3.2.2 Infrared Analysis (FT-IR)

The FT-IR analysis of the feather meal and oleic acid modified feather meal were conducted in triplicates on a Nicolet iS5 FT-IR spectrometer (Thermo, Madison, WI, USA). The spectra were recorded at 32 scans and 4 cm^{-1} resolution through the 4000–400 cm^{-1} range. The spectra were analyzed using the OMNIC software (version 8.2, Thermo Nicolet Corp).

4.3.2.3 Preparation of keratin solution

Keratin was solubilized as outlined in a previously published method [19]. Briefly, 5g of feather meal were dissolved in 50 mL of aqueous solution containing 10 g/L of Na_2S for 1.5 hours at 70 °C in a temperature controlled hot water bath under gentle stirring. After

reaction, the solution was neutralized with 1 M HCl to a pH of 7.5 under well-ventilated conditions. Lowering the pH of these solutions with acids such as HCl must be done under appropriate ventilation as it may release hydrogen sulfide gas.

4.3.2.4 SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli [163] to determine the molecular weight of the feather keratin obtained after sodium sulfide extraction. Samples (1 mg) were dissolved in 100 µL of 2 × Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol, 0.01% bromophenol blue) containing 150 mM of 2-β-mercaptoethanol and heated at 80 °C for 10 min to ensure complete dissolution. The samples (10 µL) and molecular weight standards (10 µL) (Sigma Aldrich St. Louis, MO, USA) were loaded into the individual lanes in the freshly prepared 17% acrylamide gel. Aprotinin from bovine lung (6.5 kDa) and cytochrome c (12.4 kDa) were run as additional standards to ensure to cover the full spectrum of the expected molecular weight for the keratin proteins. After 1 hour of electrophoresis at 120 V, the gel was stained with Coomasie brilliant blue staining solution for 30 min and rinsed in 10% glacial acetic acid. Gels were dried under ambient conditions and the images of the bands in the gel were collected using an 8.0 megapixel digital camera.

4.3.2.5 Estimation of the protein concentration

The protein concentration was estimated after freezing to -40°C and freeze-drying 10 mL of the keratin solution prepared following the method described in Section 2.2.3 before the addition of HCl. After freeze-drying, the resulting powder was a mixture of keratin and Na_2S. Since the concentration of Na_2S was known, the protein content was estimated by Equation 3.

Equation 3. Estimation of protein concentration (Pc)

$$\mathrm{Pc} = \frac{mass\ of\ freeze\ dried\ material\ (Mfm) - mass\ of\ Na_2S\ (Mn)}{mass\ of\ water\ (Mw)}$$

Where the estimated protein concentration (Pc) (m/m) = (Mfm−Mn)/ Mw, where Mfm is the mass of the matter after freeze drying (g), Mn is the mass of the Na_2S (g), and Mw is the mass of the water (approximately 10 mg) obtained by measuring the mass of the sample before and after freeze-drying.

4.3.2.6 Preparation of keratin cryogels sponges

10-20 mL of keratin solution of varying concentrations was poured into a 50 mL plastic test tube (Fisher Scientific, Waltham, MA, USA) and stored at -80 °C for at least 2 days. After freezing, the samples were freeze-dried in a vacuum freeze-dryer (7670520, Labconco Co., Kansas City, KA, USA) at −45 °C and 12 Pa for 36 h. To vary the protein

concentration, 0-30 mL of distilled water was added to the protein solution before freezing. To synthesize crosslinked keratin cryogels, various dosages (0-10%) of glutaraldehyde crosslinker was added prior to freezing and freeze-drying the keratin solution. 20 mL of keratin solution of known concentration (2.55%) was poured into a 50 mL plastic test-tube. Varying amounts (0, 0.2, and 1.0 mL) of 50% glutaraldehyde was added, and the tubes were left at room temperature for 6 hours to allow the crosslinking reaction to occur. After 6 hours, the tubes were frozen at -80 °C for at least 2 days. After freezing, the samples were freeze-dried to produce the keratin cryogels. The various formulations for the preparation of keratin cryogels are found in Table 4.1.

4.3.2.7 Scanning Electron Microscopy (SEM)

A thin layer of feather meal and cryogels were deposited on separate double-sided adhesive carbon tapes mounted on an aluminum specimen holder. Samples were examined at a voltage of 5 kV using a scanning electron microscope (model S-3000N, Hitachi, Tokyo, Japan)

Table 4.1. Experimental Design Used for Preparation of Keratin Cryogels.

Sample #	Feedstock material	Dilution with distilled water	Glutaraldehyde
1	Feather meal	0	0
2	Feather meal	1:1	0
3	Feather meal	1:1	0.2 mL
4	Oleic acid modified feather meal	0	0
5	Oleic acid modified feather meal	1:1	0
6	Oleic acid modified feather meal	1:1	0.2 mL
7	Feather meal	1:2	0
8	Feather meal	1:3	0
9	Feather meal	1:1	1 mL

4.3.2.8 Measurement of oil holding capacity

The oil holding capacity of the cryogels was determined as follows: prior to insertion of the dried sample, an empty nylon mesh tea bag with screen size of 100 microns was immersed in excess motor oil for 15 minutes. The bag was removed from the oil, and allowed to hang for 2 minutes. Thereafter the bags were blotted lightly on paper towel to remove any remaining free surface liquid, and placed on a digital balance to determine the mass of the wet bag (Mb). Subsequently, approximately 30 mg of dried cryogel (Mo) was placed in a pre-wetted bag. It was then immersed in excess motor oil for 15 minutes. The

bags were removed from the oil, and allowed to hang for 2 minutes. 2 minutes allowed sufficient time for dripping to stop. Thereafter the bags were blotted lightly on paper towel to remove any remaining free surface liquid, and placed on a digital balance to determine the final mass (Mf). Each cryogel samples were tested using three replicates. The standard deviation was calculated and recorded. The oil holding capacity of the cryogels was determined by Equation 4.22.

Equation 4.2. Oil holding capacity

$$\text{Oil holding capacity } \left(\frac{\text{g}}{\text{g}}\right) = \left(\frac{Wet\ mass\ (Mf) - mass\ of\ dried\ sample\ (Mo) - mass\ of\ wet\ bag\ \ (Mb)}{mass\ of\ dried\ sample\ (Mo)}\right)$$

Where Mf is the mass of the sorbent after oil exposure (g), Mo is the initial mass of the sorbent (g), and Mb is the mass of the bag pre-wetted in the motor oil (g).

4.4. Results and Discussion

4.4.1 Oleic acid modification of chicken fibers.

Keratin has an amino acid sequence up to 20% hydroxyl containing groups, serine and threonine, which can be modified [28]. Therefore oleic acid was grafted to the hydroxyl groups of the primary protein chain. Oleic acid modification has been performed on sawdust [134] and other cellulosic fibers [136] to improve the oleophilicity of the materials. The grafting mechanism was achieved by free-radical initiation with AIBN at 65 °C using

n-hexane as the solvent. The solubility of oleic acid is 720 g per 100 g of n-hexane at 10 °C [164].

It can be observed from the FTIR spectrum (Figure 4.1) of oleic acid-modified feather that the intensity of the –OH stretching band at 1685 cm^{-1} present in native feather meal has considerably decreased due to the esterification with oleic acid. Two new bands appeared at 1735 and 1235 cm^{-1}, representing the C=O and C–O stretching bands respectively, providing evidence that oleic acid has been esterified with the –OH group of the feather to form protein–OCOC moiety [22]. However, considerable hydroxyl character remains in the 3600 cm^{-1} to 3330 cm^{-1} region. This results suggests that while esterification has occurred, process variables for the grafting reaction such as the choice of initiator, initiator concentration, substrate pretreatment, could be investigated and optimized.

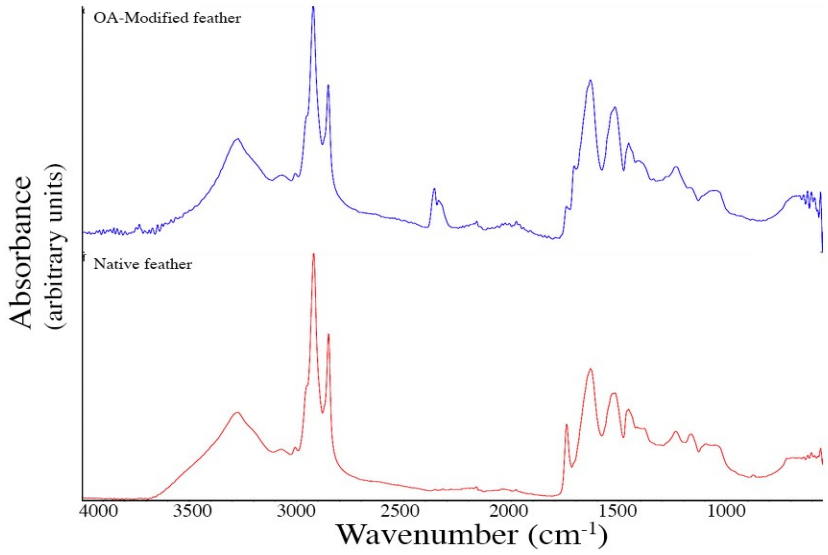


Figure 4.1. FTIR Spectra of Modified and Native Feather

Modified feather fiber with new peaks around 1734 and 1245 cm^{-1} evidencing grafting reaction and new ester bonds.

4.4.2 Solubilization of feathers using Na_2S

Keratin proteins were dissolved using Na_2S. The molecular weight of the dissolved proteins was investigated using SDS-PAGE. Figure 2 shows the molecular weights (kDa) of the feather proteins obtained by solubilization with Na_2S. Lane 4 contained the feather keratins and had a molecular weight in the 10-14 kDa range. Lane 1-3 contained proteins of known molecular weight as standards. Lane 1 was prepared from a molecular weight marker kit containing a lyophilized mixture of the six following proteins: carbonic anhydrase, bovine 29 kDa, albumin, egg 45 kDa, albumin, bovine 66 kDa, phosphorylase b, rabbit 97 kDa, β-galactosidase, e. coli 116 kDa, myosin, and rabbit muscle 200 kDa. Lane 2 contained aprotinin, which has a molecular weight 6 kDa. Lane 3 contained cytochrome, which has molecular weight of 12 kDa. Solubilizing feather meal using Na_2S [19] is less costly than other schemes such as using 2-mercaptoethanol [17, 18] and more efficient than using sulfites for sulfitolysis [32].

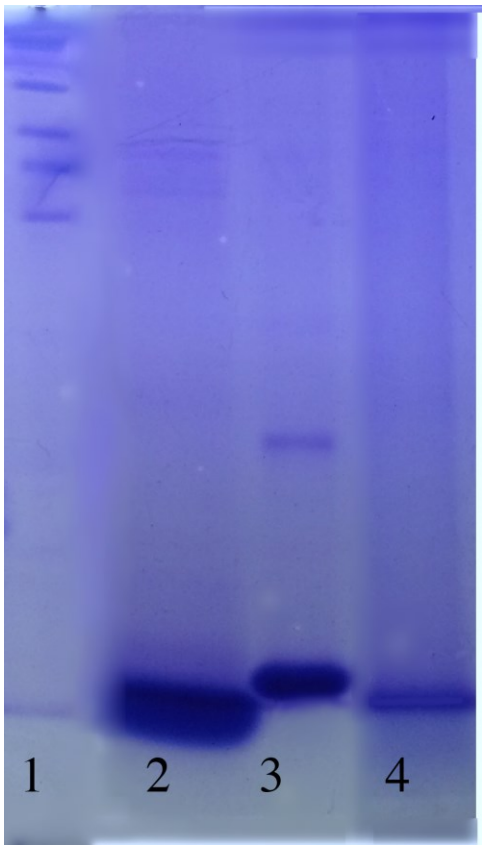


Figure 4.2. SDS-PAGE of Keratin Solubilized by Na_2S

Lane 1 molecular weight markers 25-100 kDa; Lane 2 Aprotinin molecular weight 6 kDa; Lane 3 Cytochrome C molecular weight 12 kDa; Lane 4 Keratin extracted by Na_2S (~10 kDa)

4.4.3 Synthesis of keratin cryogels

The freeze dried keratin solutions produced stable keratin cryogels (Figure 4.3). When keratin is dissolved by Na_2S, the cysteine S-S disulfide bond is cleaved [19] and after freeze-drying, the disulfide bridges reform upon oxidation [132]. This stabilizes the cryogels, creating a porous solid structure as shown in Figure 3. The synthesis of keratin cryogels through freeze-drying has been previously reported [72, 127, 132].

Figure 4.3. Keratin Cryogel

4.4.5 Oil holding capacity

The highest oil holding capacity of the cryogels was 10.8±0.5 g oil/g sorbent, and was superior to polypropylene (6 g/g) [160]. The oil holding capacity of the cryogel sponges, as well as unmodified feather meal and oleic acid modified feather meal are presented in Table 2. The porosity created during the synthesis of the cryogels contributed to a large increase in oil holding capacity ranging from 4.56 g/g to 10.76 g/g as compared to 0.12 g/g for the unmodified feather. Furthermore the oleic acid modification was found to be an effective strategy to increase the oil holding capacity of chicken feathers.

Table 4.2 Oil Holding Capacity of Keratin Cryogel Sponges and Feather Meal

Sample	Feather	Modified Feather	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9
Oil holding capacity (g/g)	0.12 ± 0.06	0.77 ± 0.13	4.58 ± 0.21	8.14 ± 0.30	9.07 ± 0.19	4.68 ± 0.39	9.78 ± 0.49	9.28 ± 0.36	10.76 ± 0.53	10.22 ± 0.85	7.71 ± 0.33

4.4.5.1 Effect of glutaraldehyde concentration on oil holding capacity of keratin cryogels

Glutaraldehyde is a commonly used biodegradable crosslinker for proteins [166, 167]. The use of glutaraldehyde in the synthesis of the sponges had two effects. First, glutaraldehyde crosslinked with the hydrophilic groups along the protein backbone [166], further exposing hydrophobic groups and therefore increasing the oleophilicity of the material. Secondly, it enhanced the formation of fibrous structures within the protein matrix as evidenced in the SEM images shown in Figure 4.4. The formation of these structures increased the porosity and the specific surface area of the material. However, increasing the crosslinking density can restrict the swelling ability of the keratin sponges. From Table 3, the oil holding capacity increased from 4.58 g/g to 9.07 g/g with the addition of 1% crosslinker however, as the glutaraldehyde concentration increased to 10%, the oil holding capacity decreased to 7.71 g/g.

Table 4.3. Effect of Glutaraldehyde Concentration on Oil Holding Capacity of Keratin Cryogels

Glutaraldehyde concentration	0%	1%	10%
Oil holding capacity (g/g)	4.58±0.21	9.07±0.19	7.71±0.33

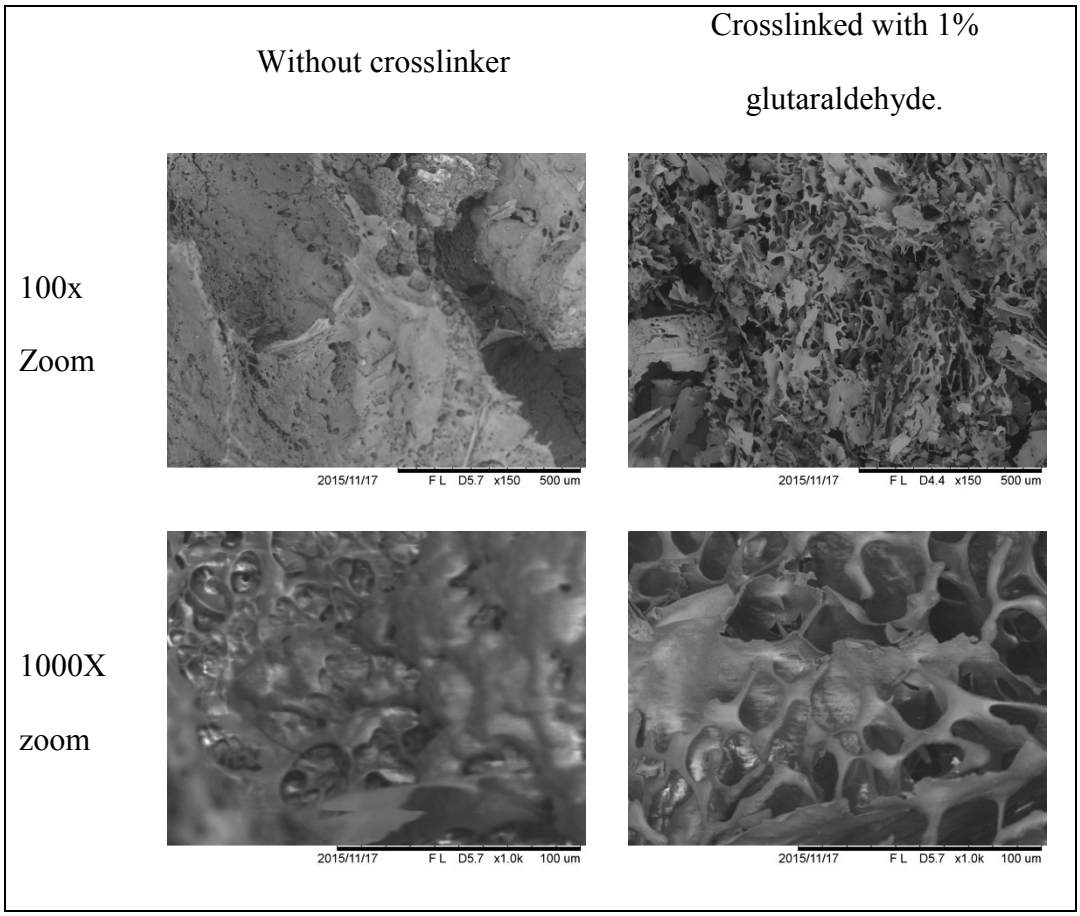


Figure 4.4. SEM Images of Crosslinked and Un-crosslinked Cryogels

4.4.5.2 Effect of protein concentration on oil holding capacity of keratin cryogels

The concentration of the protein solution had a significant effect on the oil holding capacity of the sponges. Increasing the protein concentration of the solution increased the density of the cryogels and decreased their pore size [168], decreasing the oil holding capacity (Table 4.4). The protein concentration was estimated to be 5.09% (Equation 2) representing a protein extraction yield of 50.9%, which is similar to previously reported

yields [19]. Below a 1.69% protein concentration, the oil holding capacity began to decrease, as the structure became too weak and the porous structure collapsed.

Table 4.4. Effect of Protein Concentration on Oil Holding Capacity of Keratin Cryogels

Dilution factor (protein solution: distilled water):	0	1:1	1:2	1:3
Oil holding capacity (g/g)	4.58±0.21	8.14±0.30	10.76±0.53	10.22±0.85

4.4.5.3 Effect of oleic acid modification

The effects of oleic acid modification were confirmed by statistical analysis using two-way ANOVA, resulting in a p-value of 0.0059, indicating that oleic acid modification had a significant effect on the oil holding capacity of feathermeal and keratin cryogels. Figure 4.5 presents the effect of oleic acid modification on oil holding capacity on four treatment groups. Treatment group 1 is the modified and unmodified feather. Treatment group 2 is the keratin cryogel prepared with modified feather, and unmodified feather, 5.09% protein solution, no crosslinker. Treatment group 3 is the keratin cryogel sponge prepared with modified feathers, and unmodified feathers, 2.545% protein solution, no crosslinker. Treatment group 4 is the keratin cryogel sponge synthesized with modified feathers and unmodified feathers, 2.545% protein solution, and 0.2 mL crosslinker solution. While the difference was statistically significant in all treatment groups, oleic acid modification had

the largest total effect on the oil holding capacity on sponges (treatment group 3), increasing the oil holding capacity from 8.14 g/g to 9.75 g/g. The oil holding capacity of modified feather meal was 0.77 g/g, representing a 700% increase in oil as compared to unmodified feather meal, which only held 0.11 g/g.

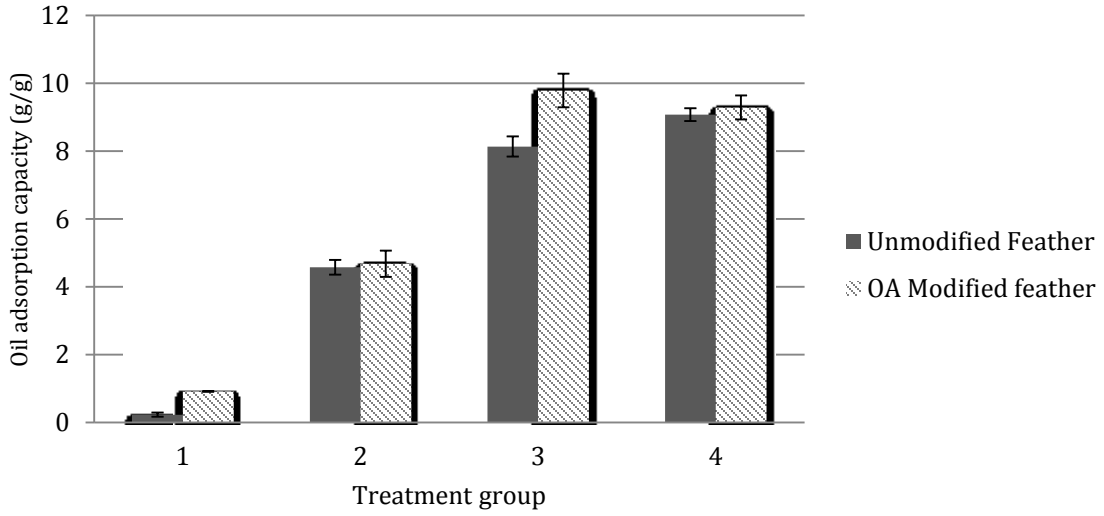


Figure 4.5. Effect of Oleic Acid Modification on Oil Holding Capacity of Keratin Cryogels

4.5. Conclusion

Oil sorbing cryogels were produced from chicken feather meal. A novel fatty acid modification for proteins that increased the oleophilicity and oil holding capacity of keratin-based materials was presented. The modification improved the oil holding capacity of all treatment groups. The keratin cryogels synthesized with a 1.27% protein

concentration and no additional crosslinker produced the cryogel with an oil holding capacity of 10.72 g motor oil/ g of sorbent, a 150% improvement over existing materials. This particular treatment group with the highest oil capacity was made from unmodified feather meal. This demonstrates the significant effect of protein concentration when preparing cryogels. This material may be suitable as a sorbent for oil spills or as an oleophilic fertilizer for remediation of contaminated soils.

CHAPTER 5

GENERAL CONCLUSION AND RECOMMENDATIONS

5.1 General conclusion

The objectives of this thesis were to 1. Synthesize superabsorbent keratin based hydrogels, and 2. To modify the protein using a graft reaction with oleic acid and to synthesize keratin cryogels by freeze-drying using varying concentrations of crosslinker and protein. Detailed conclusions related to both objectives are listed as follows:

1. Superabsorbent hydrogels were synthesized with keratin hydrolysate by redox initiated graft copolymerization of the hydrolysate with acrylic acid and sodium acrylate. The hydrogels were crosslinked using N,N'-methylene bisacrylamide. A high degree of swelling was achieved in distilled water (501 g/g). These hydrogels exhibited a different swelling response in media with different pH and salinity.
2. Keratin was successfully modified with oleic acid to increase the oil holding capacity. Oil-sorbing cryogels with high oil holding capacity were synthesized. The cryogels had oil holding capacities of 10.76 g/g which outperforms the industry standard of polypropylene at 6 g/g.

5.2 Recommendations and future work

The main weakness of the keratin hydrogels for environmental applications was the use of N,N'-methylene bisacrylamide as crosslinker. This crosslinker is toxic, and may prohibit the use of these materials in long-term or sensitive applications. The selection of a less toxic crosslinker can be of interest for future studies.

The main weakness of the keratin cryogels as oil sorbing sponges would be the economy of scale for the production of the material. Freeze-drying is costly process at an industrial scale for mass production of materials. The production of materials from modified feather keratin using different methods such as extrusion, co-extrusion with polypropylene, or electrospinning may be of interest for future studies.

These two different crosslinked keratin matrices may also be suitable for wastewater treatment containing heavy metals or other organic contaminants.

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