# Thermal, Sonochemical and Mechanochemical Generation of Glycated Gelatins: Effect on their Stability and Functional Properties

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# ABSTRACT

The reaction between the carbonyl groups of reducing sugars and ε-amino groups of proteins is a promising approach for the modification of functional properties of proteins. Glycation is usually carried out using thermal energy. In this study gelatin was glycated with saccharides (at a molar ratio of 1:16 protein to sugar) using ultrasonic and mechanochemical energy to evaluate their effectiveness in controlled production of glycated and stable conjugates and to further evaluate their ability to improve the functional properties of glycated gelatin relative to thermally induced process. Fluorescamine and dinitrosalicylic acid tests were used to evaluate the extent of glycation. The results have indicated that the rates of ultrasonic and mechanochemical glycations were faster than the thermal glycation. Glycoconjugates formed in 15 minutes of sonication (20 kHz) and 30 minutes of mechanochemical milling (30 Hz) showed 35.42% and 36.01% of glycation efficiency respectively. Thermal glycation at 55$^{o}$C required 24 hours of incubation to obtain 34.54% of glycation. Unlike mechanochemically and thermally produced glycoconjugates, sonochemical glycation mainly generated Schiff base conjugates which upon storage at room temperature hydrolyzed with concomitant release of the attached sugar moiety. Overall, the resulting glycoconjugates from thermal, sonochemical and mechanochemical milling resulted in improved functional properties compared to the untreated gelatin. The mechanochemically formed glycoconjugates in particular exhibited improvements in solubility from pH 3 through pH 11, emulsion stability, thermal stability and at the same time producing stronger films with higher tensile strengths and elongation break compared to native gelatin. Foaming stability was greatly enhanced by ultrasonic and mechanochemical glycation. Mechanochemical glycation was found to be the most suitable method combining shorter reaction times and stability of the products formed with improvements in functional properties. These improvements in functional properties are mainly attributed to the changes in charge distribution, solvation and conformational modifications. The introduction of the sugar moieties causes an increase in net negative charge thus increasing electrostatic repulsion and hydrophilicity of the protein, resulting in an overall increase in functional properties.

# RÉSUME

La réaction entre les groupes carbonyles de réduction des sucres et des groupes amino de protéines est une méthode prometteuse pour la modification des propriétés fonctionnelles des protéines. La glycation est habituellement effectuée à l'aide d'énergie thermique. Dans cette étude, la gélatine a été glyquée avec des saccharides (à un rapport molaire de protéine à sucre de 1:16) en utilisant une énergie ultrasonique et mécanochimique pour évaluer leur efficacité dans la production contrôlée des conjugués glyqués et leur stabilité, ainsi mieux évaluer leur capacité à améliorer les propriétés fonctionnelles de la gélatine glyquée par rapport au procédé induit thermiquement. Des tests de fluorescamine et d'acide dinitrosalicylique ont été utilisés pour évaluer l'étendue de la réaction entre la gélatine et le sucre. Les résultats ont indiqué que les taux de glycations ultrasoniques et mécanochimiques étaient plus rapides que la glycation thermique. Les glycoconjugués formés en 15 minutes de traitement ultrasonique (20 kHz) et 30 minutes de traitement mécanochimique (30 Hz) ont montré respectivement 35,42% et 36,01% d'efficacité de glycation. La glycation thermique à 55°C a nécessité 24 heures d'incubation pour obtenir 34,54% de glycation. Contrairement aux glycoconjugués produits mécanochimiquement et thermiquement, la glycation sonochimique a généré principalement des conjugués de base de Schiff qui, lors du stockage à la température ambiante, s'hydrolysent avec une libération concomitante de la fraction de sucre attachée. Dans l'ensemble, les glycoconjugués résultants d'un traitement thermique, sonochimique et mécanochimique ont donné des propriétés fonctionnelles améliorées par rapport à la gélatine non traitée. Les glycoconjugués formés mécanochimiquement présentent en particulier des améliorations de solubilité de pH 3 à pH 11, la stabilité à l'émulsion, la stabilité thermique et en même temps la production de films plus forts avec des résistances à la traction et une rupture d'allongement supérieures à celles de la gélatine d'origine. La stabilité de la mousse a été grandement améliorée par la glycation ultrasonique et mécanochimique. Nous avons trouvé que la glycation mécanochimique était la méthode la plus appropriée combinant des temps de réaction plus courts et la stabilité des produits formés avec des améliorations dans les propriétés fonctionnelles. Ces améliorations des propriétés fonctionnelles sont principalement attribuées aux changements dans la répartition des charges, la solvatation et les modifications conformationnelles. L'introduction des fractions de sucre provoque une augmentation de la charge négative nette augmentant ainsi la répulsion électrostatique et l'hydrophilie de la protéine, ce qui entraîne une augmentation globale des propriétés fonctionnelles.

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# CHAPTER 1:

## 1. General Introduction

### 1.1 Rationale of the Proposed Research

Proteins are essential components of the human diet, as they provide nutritive value through the supply of amino acids. In the food industry, proteins are valued not only for their exceptional nutritional profile, but also for their ability to interact as functional ingredients in a given food system. The functional properties exhibited by a protein helps determine its potential applications in a food matrix, and also contributes to the worthiness of the protein in the industry. As the changes in the functional properties of proteins have a direct influence on the overall quality, sensory attributes and acceptability of the food system, there exists an increased demand for proteins with multiple functional properties.

With this growing demand, there exists a need to explore innovative research methods directed towards enhancing functional properties of selected food proteins. This will further ease the use of the proteins as functional ingredients for a wider range of food applications. To date, numerous methods have been developed and studied precisely regarding the generation of modified food proteins with improved or novel functional properties. Aside from intrinsic and extrinsic factors, which may affect the protein's functional properties, genetic, chemical and physical methods are the most studied methods for the generation of modified proteins with improved functional properties. Although, many studies have been attempted to develop modified proteins, only a few have been successful in the actual improvement of their functional properties. Recent attention has been geared towards the conjugation of proteins with carbohydrates, mainly sugars, through the Maillard reaction. This is due to its promising approach for both the modification and enhancement of the functional properties of food proteins. The conjugation reaction induces changes in the charge, solvation and conformation of the conjugated protein, which thereby alters the existing functional properties of the protein (Khan et al., 1999).

The Maillard reaction is a spontaneous reaction occurring naturally between the carbonyl group of the reducing sugar and the ε-amino group of the protein molecule (Liu, Ru, & Ding, 2012) The reaction consists of three stages; early, intermediate and advanced. The early stage favors the

formation of the protein-carbohydrate conjugates. In this stage, the free amino group of the protein reacts with the carbonyl group of the reducing sugar to form a Schiff base. This intermediate is unstable and undergoes a rearrangement reaction to form stable Amadori products. The later advanced stages of the Maillard reaction will result in the degradation of the Amadori products into dicarbonyl intermediates. These compounds will further undergo various pathways to produce undesirable end products. It is therefore important to limit the Maillard reaction to its early stages. Parameters including the incubation time, temperature, pH, water activity ($a_w$) and the protein/sugar ratio, need to be controlled to limit propagation to advanced stages. Although many studies have been conducted to improve the functionality of the protein via the Maillard reaction, many focus primarily on the improvement of hydration and surface properties. Most studies have been directed towards thermal generation of glycated proteins. To our knowledge the glycation of food proteins via methods other than thermal reaction has been little investigated. Specifically, the effect of mechanochemically or sonochemically induced glycation on the functional properties of the modified proteins have not previously been investigated.

## 1.2 Objectives of the Research

Due to its desirable functional properties such as emulsifying, foaming, and gelling ability, gelatin has been widely used in food, pharmaceutical and cosmetic industries. For this reason, gelatin was selected as a model protein for this study.

To date many researchers have attempted to glycated proteins through thermal incubation, and have been successful in proving the grafting reaction and its subsequent effects on the functional properties of the conjugates. However the protein/saccharide conjugation can take several days or weeks when classical heating is used (Mu et al., 2010). Therefore, it is necessary to investigate novel technologies to conduct the glycation reaction in a more time efficient manner. The aim of the study is to explore different sources of energy aside thermal incubation, and their effect on the glycation reaction, rate and stability. Sonochemistry and mechanochemistry are emerging technologies presently used in the food industry for numerous applications. The use of the energy generated from sound waves or repeated mechanical collision respectively is hypothesized to give rise to different types of glycation, degree of glycation, glycation sites, and glycoforms. The significant technological advances in sonochemistry and mechanochemistry, render these

processes suitable for glycation reactions comparative to thermal incubation as these non-thermal methods are environmentally friendly, hold shorter processing time, have higher product yield, and present lower operating and maintenance costs (Mu et al., 2010; Perusko et al., 2015). It is of great interest to study the resulting conjugates produced by the thermal, sonochemical and mechanochemical methods and broaden the applications of proteins as functional ingredients.

The specific objectives of the study are as follows:

1. Explore the differences of thermal, sonochemical and mechanochemical glycation reactions and their effect on the stability of the resulting glycoconjugates.
2. Explore the effect of the different means of glycation on the functional properties of the resulting glycoconjugates
3. Comparative study on the effect of thermal glycation of gelatin with glucose and ribose.

# CHAPTER 2:

## 2. Literature Review

### 2.1 Introduction

In the past decade considerable amount of information has been gathered regarding protein functionality and their uses as functional ingredients. Regardless of their source whether plant or animal each protein is unique and is composed of a specific amino acid sequence which imparts unique physical and functional properties. Factors such as amino acid content, molecular weight distribution, net charge and isoelectric point contribute to the protein's functionality. Recent attention has been directed towards the use of proteins from industrial waste as food ingredients. However, these byproducts and industrial waste proteins lack the requisite functional properties expected from the industry. To compete with the existing functional ingredients, the proteins must contain functional characteristic, aesthetic and organoleptic appeal, and maintain their cost advantage (Kinsella & Melachouris, 1976). With such demands increasing over the years, there is an ongoing research for methods of modification for the improvement of the functional properties of proteins that are promising and cost effective.

The functional properties of proteins play a major role in a given food system. The modification of these properties is common to obtain enhanced and improved functionalities, thus allowing a broader use of the protein for a more versatile array of applications. Proteins hold the ability to add functional properties such as emulsification, solubility, texture, foaming and gelation amongst other properties to a food matrix. Although intrinsic factors such as molecular composition and structure and extrinsic factors such as pH and temperature have a significant impact on the properties exhibited by the proteins, there exist additional methods that have a potential impact on them as well (Oliver, Melton, & Stanley, 2006). The most extensively studied methods include chemical and enzymatic procedures such as acetylation, alkylation, esterification, amidation, and deamination. Studies have proven the improvement of functional and physico-chemical properties by these means (Li et al., 2015). However as these methods use potentially toxic chemicals that are often prohibited for use in industrial applications, recent studies have turned towards alternate means to improve the functional properties of proteins (Chevalier et al., 2001). Amongst these new methods, non-enzymatic glycation, otherwise known as the Maillard reaction is a method of great

interest to the food industry for enhancing both functional and structural properties of proteins. In fact, glycation is more desirable as it does not require any extraneous chemical, and is rather a naturally occurring reaction between sugars and proteins. In addition, sugars are safe and stable compounds that are highly abundant and very diverse, hence allowing it to not only have different effects on the improvement of functional properties but also render's protein glycation a more cost effective method.

For the purpose of this study, gelatin was used as a model protein, as it is a commonly utilized protein in the industry. Gelatin is an exceptional protein known for holding a wide range of applications and serving multiple functions in a given food system. In fact, gelatin is a protein that is superior to commercial hydrocolloids commonly used in the food industry. However, its applications are not limited to one field; they are also predominant in the nutraceutical and pharmaceutical industries. Although many correlate gelatin with gummy bears and other gel-like candies, gelatin is known for more than just its gelling ability. It is an excellent emulsifying agent, foaming agent, protective colloid, and thickening agent amongst other functionalities (Schrieber & Gareis, 2007a). Despite its advantageous properties, gelatin presents some weakness. Some of its disadvantages include its low heat stability, high temperature solubility, low gelling temperature, and slow gelation process (Schrieber & Gareis, 2007a). For this reason, in order for gelatin to be a well-rounded protein, some aspects of its functionalities must be improved. The improvement of the lacking properties will render gelatin a food ingredient with perhaps novel properties and applications. For such, protein modification techniques must be applied to optimize gelatins' functional properties.

## 2.2 Protein Glycation

Protein glycation is a form of the Maillard reaction that takes place between the aldehyde group of the open chain form of a reducing sugar and the amino groups of the protein molecule (Liu et al., 2012). It is accelerated by heat and does not require the addition of any chemicals. The mechanism for protein glycation consists of the simple attachment of the sugar residue to the protein via the Maillard reaction. Subsequently, the change in charge, solvation and conformation of the protein results in changes induced to the functional properties. The primary reactive amino group in the protein is the ε-amino group of the lysine residues in the proteins. In addition to such, the imidazole

group of histidine, the indole group of tryptophan and the guanidino group of arginine are also reactive, but to a lesser extent when compared to lysine (Oliver et al., 2006).

## 2.3 Maillard Reaction

The Maillard reaction was first discovered by Louis Camille Maillard in the early 20$^{th}$ century. His research focused mainly on the effect of the conjugation of a sugar and amino acid to induce changes in color, flavor and aroma (Fayle & Gerrard, 2002). He extensively studied the reaction and was not only the first to do so, but was also the first to describe the production of increased color formation as a result of the condensation reaction between glucose and lysine. The same was found to be true between sugars and proteins a few decades later. The first scheme representing the reaction mechanism was first introduced by Hodge in 1953. The reaction is of particular interest to food scientist and flavor chemists, as the protein/sugar conjugation imparts changes in texture, sensory properties, nutritional profile, and toxicological aspects (Nursten, 2005). The changes in textural attributes include the enhancement of the protein solubility, stability, gelling ability, water-holding capacity and emulsifying capacity, thus causing changes in the product development. The sensory attributes include the development of aroma compounds, color, as well as the generation of volatile and non-volatile flavor. Negative implications such as a reduced nutritional profile due to loss of essential amino acids and formation of toxic or mutagenic compounds with prolonged reaction are highly possible as well (Nursten, 2005).

### 2.3.1 Chemistry of the Maillard Reaction

Although the Maillard reaction was initially described in the early 1900s, it is still studied today due to its complex reaction pathways, intermediates and products. The reaction is comprised of three stages; initial, intermediate and final. As outlined by Hodge (1953) the early stage of the Maillard reaction is the most understood. In the initial stage of the reaction, there are actually two main reactions taking place as shown in Figure 2.1. This stage corresponds to the reversible formation of the glycosylamine and the Amadori rearrangement. Primarily, a carbonyl-amine reaction will take place between the carbonyl of the reducing sugars and the amino group of the protein (Oliver et al., 2006). The reaction is optimum at weakly-acidic conditions. Subsequently, a sugar-amine condensation is observed to form a Schiff base with the release of water. This

unstable intermediate is then cyclized into an N-substituted glycosylamine. Later, Amadori rearrangement or Heyns rearrangement, will result in the partial isomerization to form more stable compound. Via the Amadori arrangement, the glycosylamine is converted to 1-amino-1-deoxy-2-ketose, while via the Heyns rearrangement, the ketosamine is converted to 2-amino-2-deoxy-1-aldose. At this point, depending on pH, further reactions of the Schiff base and Amadori/Heyns compounds will take place (Oliver et al., 2006).

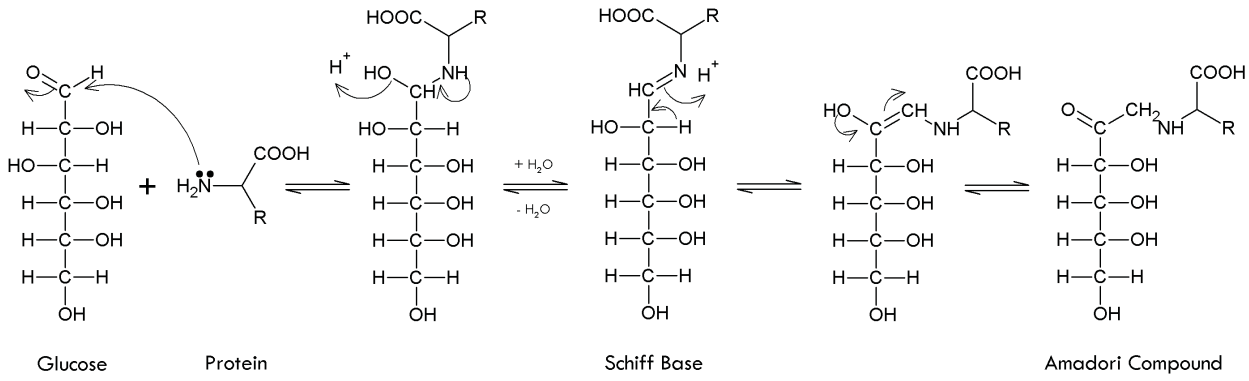


Figure 2.1. Early stage Maillard reaction: condensation reaction of open-chain form glucose with free amino group of a protein

The later occurring intermediate and final stages are less characterized compared to the early stage. The intermediate stage involves the degradation of the Amadori or Heyns product, which thereby undergo transformations under the form of sugar dehydration, sugar fragmentation, amino acid degradation, oxidation, acid hydrolysis, and enolization (Friedman, 1996; Ledl & Schleicher, 1990). Sugar dehydration produces furfurals and/or reductones based on the acidic or alkaline environment. Sugar fragmentation creates a variety of dicarbonyl compounds. At the end of this stage, a slight color production is observed, however most of the color production is observed during the final stage.

During the final stage two main observations are noted. Firstly, through the overall reaction, the Amadori compound is degraded to α-dicarbonyl compounds such as deoxyosones and other flavor and aroma compounds. At this stage a possible further reaction is the Strecker degradation of the α-dicarbonyl compounds which will give rise to aminoketones, aldehydes and acrylamide. During

the final stages, polymerization can occur to yield the formation of melanoidins and heterocyclic nitrogen containing compounds (Mottram, 2007). These further reactions however are known to have more negative outcomes such as the reduction of protein digestibility and formation of mutagenic compounds. The overall Maillard reaction is shown in Figure 2.2.

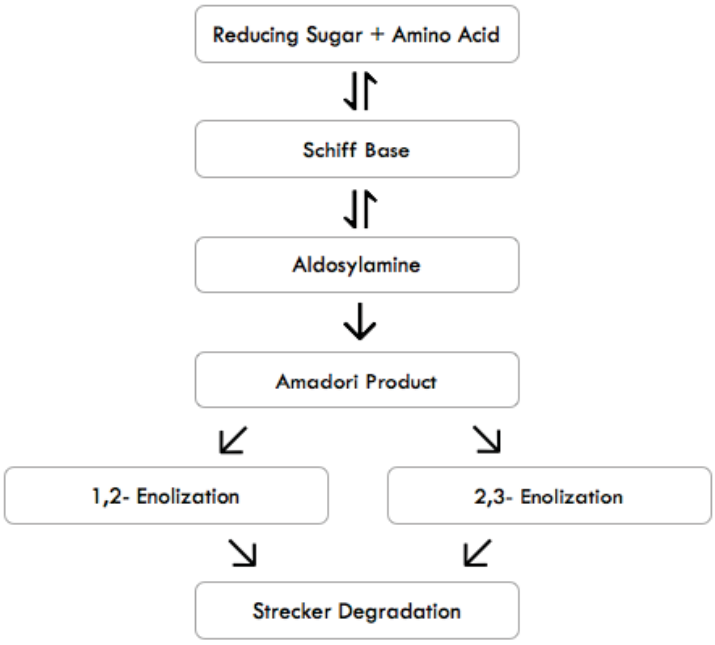


Figure 2.2. Simplified overview of the overall Maillard Reaction

## 2.4 Methods of inducing Glycation

For decades, the protein glycation reactions have been studied by the aid of thermal energy, this involves the presence of heat to initiate the reaction between the reducing sugar and protein. However as this method is known to require greater incubation times, new techniques of glycation requiring shorter reaction periods would be beneficial to the industry. In addition, the new techniques may give rise to novel glycoconjugates with novel properties. Each method may differ with respect to glycation sites, extent of glycation possible and rate of glycation which will be important respect to the conjugate formed, its functional properties, and its potential applications.

### 2.4.1 Ultrasonically Induced Glycation

As thermal processing provides numerous advantages in the processing of food, such as inactivation of enzymes and reduction in microbial content, it has been the most common method used in the food industry (Soria & Villamiel, 2010). However, with the search for cost effective methods for the production of high quality food, numerous technological advances have been seen over the recent years. Amongst these advances, sonication is a rapidly growing field of research. The use of high frequency ultrasounds has previously been seen for analytical purposes such as quality assurance and process control. However the application of low frequency high energy ultrasound in the food industry is limited and emerging rather slowly (Jambrak et al., 2008). Although the application of ultrasound is common for the acceleration of decomposition reactions, polymerization reactions and degradation of polymers (Floros & Liang, 1994), it is believed that sonochemistry has a great potential in other potential food applications including the conjugation reaction of sugars and proteins.

#### 2.4.1.1 Principle of Sonochemistry

The relevance of sonication in chemistry springs from the fact that ultrasound generates a form of energy that allows chemical reactivity to occur in such a way that is different from the commonly seen heat, light or pressure. The principle of the ultrasound relies on the production of a series of mechanical waves at frequencies above 16kHz (Soria & Villamiel, 2010). The effect is observed through compression and rarefaction waves. In a liquid system, the ultrasound effect is related to the cavitation phenomenon, where the effects of ultrasound are produced through the cavitation bubbles (Mu et al., 2010). These bubbles are generated during the refraction cycle and collapsed in the compression cycle. In the refraction cycle, cavitation bubbles are formed from the gas nuclei that exist within the fluid and are distributed throughout the liquid. These bubbles grow over the period of the cycle, until a critical size is reached. At this critical size, the bubbles becomes unstable and collapse in the compression cycle (Soria & Villamiel, 2010). The phenomenon is depicted in Figure 2.3. The collapse of the bubbles results in the accumulation of energy in “hot spots”, hence referred to as the Hot-Spot theory (Mason & Lorimer, 2002). This theory refers to the generation of extreme temperatures and high pressures as a result of the violent and rapid collapse of the cavitation bubbles.

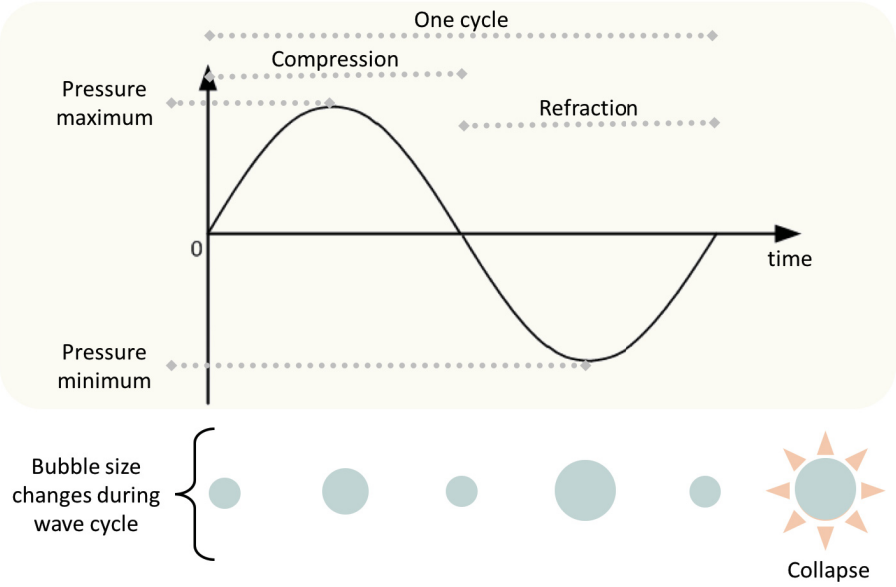


Figure 2.3. Principle of ultrasonic cavitation, adapted from Soria and Villamiel (2010)

Although the significant effect of ultrasound on the rate of various processes in the food industry has greatly been studied, considerable interest is also due to its effects on food processing and preservation. Ultrasound is known to be a cost effective alternative while providing high product yield, hold short processing times, and improve taste, texture, flavor and color (Freitas et al., 2006; Mu et al., 2010). Recent interest has however been focused on the acceleration effect of the ultrasound treatment on the Maillard reaction and the possible effects on the functional properties of the generated glycoconjugate. As this is relatively a new application for ultrasound and the food industry, there exists very minimal studies conducted on the effect of sonication induced glycation. It is suggested that the local translational motion induced through cavitation allows reactive groups to be brought closer in proximity, thus resulting in a an accelerated yet steady Maillard reaction (Li, Xue, et al., 2014). Furthermore, several studies have shown that high ultrasonic treatment will result in the disruption of the tertiary and quaternary structure of the protein, thus giving rise to increased exposure of free amino groups. Therefore is expected to facilitate the conjugation reactions (Li, Xue, et al., 2014; Mu et al., 2010).

### 2.4.1.2 Sonochemistry and Improvement of Functional Properties

The physical, mechanical and chemical effects induced by acoustic cavitation is thought to be the principle reason for the modification of protein through ultrasound treatment. Many studies have shown that the ultrasonic treatment of proteins, can induces changes in viscosity, solubility, texture and emulsifying properties (Soria & Villamiel, 2010). It has extensively been experimented in the milk industry where it has been found that high-intensity ultrasound processing of milk can result in a size reduction of fat globules up to 81.5%, improves yield of cheese due to increased binding locations, improve viscosity and rheological properties of yoghurt, amongst other findings (Bermúdez-Aguirre, Mawson, & Barbosa-Cánovas, 2008; Soria & Villamiel, 2010; Vercet et al., 2002; Villamiel & de Jong, 2000). Only a limited number of studies however exist on the effect of sonication on glycation and the properties of the glycoconjugates formed. The existing literature show that sonication can be a promising method for changing the conformation of protein and thereby infer changes in its functional properties. Most studies show mainly positive results for the use of sonication to conjugate proteins and saccharides.

A study by Li, Huang, et al. (2014), showed that the ultrasonic treatment was able to accelerate the conjugation reaction between peanut protein isolate (PPI) and dextran or gum Arabic in a significantly shorter period of time. The solubility of the resulting conjugates was enhanced between pH 3-9, while the emulsifying activity and stability was also remarkably improved for both polysaccharide conjugates. The study also noted that there exist a structural difference between the thermally glycated and ultrasonically glycated conjugates. The ultrasonically treated samples contained fewer α-helix and greater β-structures, while the thermal conjugates showed the opposite results. Similarly, Mu et al. (2010), demonstrated that the use of ultrasound is effective in accelerating the conjugation reaction between soy protein isolates and gum acacia. 60 minutes of sonication was found to give equivalent degree of glycation in comparison to 48h of thermally induced glycation. The study further showed improvement in emulsifying activity, emulsifying stability, and surface hydrophobicity in soy protein isolate/gum acacia conjugates. Jambrak et al. (2008) found that the foam capacities and foam stabilities of whey protein isolates were improved after ultrasound treatment at low (20 kHz) and high (40 kHz) intensities. The enhanced foaming ability is expected as a result of the homogenization effect of ultrasound, where the ultrasound

allowed the dispersion of the protein and fat molecules more evenly thus resulting in the improvement of the foaming property. It was also observed that the change in protein conformation and structure was altered in such a way that it allowed the hydrophilic parts of the amino acid to open towards water, enhancing the solubility. Chen et al. (2016) found that the conjugation of PPI with maltodextrin through sonication enhanced solubility and surface hydrophobicity of the conjugates. Near the isoelectric point of the PPI, limited improvement in solubility was observed, however at the other pH ranges, ultrasonically treated PPI with maltodextrin showed enhanced protein solubility. In terms of emulsifying properties, it was noted that the native and thermally glycated PPI formed unstable emulsions and caused flocculation and coalescence during homogenization, while the ultrasonically glycated conjugates not only showed a decrease in droplet size but showed remarkably improved emulsifying properties at even at a small degree of glycation.

Contrary to the glycation studies above mentions, a study by O'Sullivan et al. (2016), studied the effect of sonication on plant and animal proteins in the absence of sugars, amongst which bovine gelatin was a protein studied showed that sonication did not induce significantly enhanced effects. The study did show that the ultrasonic treatment reduced the size of the proteins studied without altering the size of the primary structure of the protein, where the reduction of size was attributed to the pressure denaturation of the protein molecule resulting from the homogenization effect. Moreover, with the change in structure resulting from the ultrasound treatment, the hydrophobicity of the protein was also found to increase. The reduced particle size and increased hydrophobicity is expected to cause faster adsorption of the protein at the oil-water interface of emulsion, however the study showed that the rate of protein adsorption remained unchanged between the treated and untreated samples.

The existing studies on the effect of ultrasonically induced glycation on the functional properties of the protein/saccharide conjugates focus mainly on the effect on hydration and surface properties such as solubility and emulsifying properties. A better understanding of the mechanism of action of the ultrasonically induced glycation reaction, in addition to the effect of the intensity and frequency will contribute to comprehend its effects on the functional properties of the resulting

conjugates. Similarly, the effect of sonication with respect to the protein denaturation either by hydrolysis or aggregation is highly important to establish its effects on the functional properties.

### 2.4.2 Mechanochemically induced Glycation

The use of mechanochemistry was first reported by Lea (1892) to allow mechanical activation of chemical reactions. However, in the recent years, its use for promoting reactions between solids have been emerging and intensively studied. Mechanochemistry relies on the generation of mechanical energy where intramolecular bonds are broken by either grinding, milling, shearing, kneading, stirring, pulling or cutting (Kaupp, 2009). This method is thought to be a promising method for activation of chemical reactions as it is advantageous, effective and does not require solvents. The lack of solvent enhances the reaction between the two reactive groups and also contributes to a cost effective advantage.

#### 2.4.2.1 Principle of Mechanochemistry

Mechanochemistry, unlike regular chemical reactions which use heat, light and pressure, utilizes force to cause the reacting molecules to deform and further carry out other reactions. The basis of mechanochemistry relies primarily on shear and extensional deformations. Similar to other reactions, mechanochemical milling will cause physical, chemical and biochemical changes. The main form of energy produced is mechanical energy which is based on three key factors; friction, impact and collision (Stolle et al., 2011). Mechanochemistry is known to cause mechanical activation of solids, mechanical alloying and reactive milling of solids. This process thereby causes particle refinement, thus increases surface area and surface energy. This energy in turn allows mechanochemical activation. Hence alterations in the chemical composition and structure will occur as a result of chemical reactivity induced by the milling process (Stolle et al., 2011).

The milling or grinding reactions for chemical transformations has been studied during the evolution of chemistry. However in the early stages, mechanochemistry was promoted via hand grinding and provided a limited reaction as a result of low grinding strength and varying grinding speed (Stolle, 2014). It was not a convenient or effective mean for chemical transformations to occur. With the generation of high speed mixers/millers and ball assisted milling, attention has

spurred towards mechanochemistry in recent years. The mechanochemical milling is a more reliable and reproducible method as compared to the hand grinding or manual grinding reactions. The chemical activation of a reaction using a ball mill will depend on factors such as milling time, frequency, size of the milling ball amongst other factors, however will still produce sophisticated results in comparison to the hand grinding (Wang, 2013). Generally, mechanochemistry is used for the refinement of particles, de-agglomeration and for cracking of bacteria (Stolle et al., 2011). Its uses are being extensively investigated in numerous fields, however, to a lesser extent in the food industry.

#### 2.4.2.2 Conjugation Reaction using Mechanochemistry

The field of mechanochemistry has been mainly studied for the breakdown of polymers to synthesize smaller chain polymers. However, with increasing interest on the branch of mechanochemistry and mechanochemical activation, the conjugation of solid state reactants has increasingly been studied. Mechanochemical milling has been studied to understand solvent-free organic reactions, particularly addressing the formation of carbon-carbon, carbon-nitrogen, carbon-oxygen, carbon-sulfur, or carbon-halide (Cl, Br) bonds (Stolle, 2014). A cascade of condensation, oxidation, or reduction reactions using the mechanochemical milling process have also been investigated.

Although the mechanochemical reaction has not been studied for its effectiveness in protein glycation reactions, some studies have previously been conducted on its effect on C-N bond forming reactions; the primary reaction between a carbonyl and amino group. Kaupp, Schmeyers, and Boy (2001) studied the condensation reaction between 4-nitroaniline and 4-hydroxybenzaldehyde. The study demonstrated the formation of imines through the ball milling reaction in less than 30 minutes. Likewise Mokhtari et al. (2009), used kneading ball milling to react 2,4-dinitrophenylhydrazaine with aldehydes to give rise to hydrazones. The reaction was made possible at a reaction temperature ranging between 25-70°C for 10-20 min. The formation of Schiff bases with solid aromatic amines and aldehydes was studied by Cinčić, Brekalo, and Kaitner (2012). The study explored the use of liquid assisted mechanochemical reactions, where they concluded that liquid assisted grinding reaction can increase the potential to create Schiff

bases. Mechanochemical reaction between 5-aminosalicylic acid and vanillin, σ-vanillin or 2-hydroxy-1-naphthaldehyde for 5-30 minutes, with minute amount of ethanol gave yields of Schiff bases. Although Amadori compounds are more stable in conjugation reactions, perhaps the studies focused mainly on the condensation reaction between the sugar and protein only and did not further explore the stability of the conjugates formed.

Although the above mentioned studies and reactions are not direct evidence of protein glycation reactions, literature shows that chemical reaction is possible through mechanochemical activation. With most literature focusing on the use of mechanochemistry to initiate reactions, there exists a lack of studies on the effect of mechanochemistry on the reacted species or conjugates formed. There is however evidence that mechanochemistry improves solubility as a result of particle size reduction and increase in surface area. Loh, Samanta, and Sia Heng (2015) studied the effect of mechanochemistry on poorly soluble drugs, and demonstrated that as a result of the milling process, drug dissolution and solubility is greatly enhanced. The alteration in particle size, surface area and shape of the drug, in addition to the structural changes through mechanical energy and amorphization of the drug molecule, improvements in dissolution, solubility and bioavailability of the drug was made possible. The effect of mechanochemistry on other functional or physico-chemical properties is not found in literature. However, as mechanochemistry, like thermal incubation or sonication, results in changes in structure as a result of the conjugation reaction, it is believed that this method can also be used to impart improved functional properties in glycated protein. In addition, the conjugation of a hydrophilic sugar moiety, which result in change in charge and solvation properties contributing to changes in functional properties respectively.

## 2.5 Model Protein: Gelatin

Gelatin is a protein that has gained increased prominence in the recent years as it serves multiple functions and holds a wide range of applications. Although one may associate gelatin with the food industry, the application of gelatin is seen in numerous other industries as well. In fact, the first use of gelatin dates back to over 8000 years ago where is was used as an adhesive. Today its applications have grown to expand over a multitude of industries and specific product groups rendering it a very popular protein. The global demand for gelatin has increased tremendously as

gelatin is a protein in abundance, relatively low in cost and holds exceptional functional properties (Gómez-Guillén et al., 2009). The demand has increased from 120 000 tons in 1976 (Ofori, 1999) to 326 000 tons in 2009 (Haug & Draget, 2011), and is expected to increase further to 426 kilo ton by 2020 (Grand View Research., 2014). Such can be justified by its use as a functional ingredient in the food industry, as a processing aid in the pharmaceutical industry, as a wound dressing in the biomedical field, amongst other applications (Karim & Bhat, 2008). Generally, gelatin is extracted from porcine and bovine sources, however with increasing demand for vegetarian, halal and kosher options, piscine sources are of high interest in the recent years. With growing interest for this protein and the search for alternate sources of gelatin, the global demand is only expected to increase.

### 2.5.1 Gelatin Structure & Composition

Gelatin is a protein obtained by partial hydrolysis of collagen, the principle component of the skin, bones and hides of specific animal bodies. The conversion of collagen to gelatin involves the transformation of the highly organized collagen fibers to a depolymerized system (Poppe, 1997). The production of gelatin involves the pretreatment of the raw material with either dilute acid or alkali, producing either Type A or Type B gelatin respectively. The main differences between the two is depicted in Table 2.1. In terms of food applications of Type A and Type B gelatin, the notable difference is in the viscosity and isoelectric point. Type B gelatin is more viscous compared to Type A gelatin (Poppe, 1997). Due to the alkaline treatment of Type B gelatin, the process deaminates asparagine and glutamine into aspartic and glutamic acid respectively. This explains the differences in the isoelectric point of Type B gelatin compared to that of Type A (Schrieber & Gareis, 2007b).

Table 2.1. Characteristics of gelatin Type A and type B

| | **Gelatin Type A** | **Gelatin Type B** |
|---|---|---|
| Raw material | Pigskin & bone | Bovine hide & bone |
| Pretreatment | acidic | alkaline |
| Isoelectric point | 7-9.4 | 4.5-5.3 |

Gelatin's structure and composition is very similar to that of its parent molecule; collagen. Collagen molecules are characterized by a repeating amino acid sequence where glycine is present as every third amino acid in the chain (Haug & Draget, 2011). It consists of three α chains which follows a repeating Gly-X-Y amino acid sequence, where X and Y are mostly proline and hydroxyproline. Due to this nature, collagen takes the form of a right handed triple helical structure stabilized by inter and intramolecular hydrogen bonds (Djabourov, Lechaire, & Gaill, 1992; Wagermaier & Fratzl, 2016). While the glycine residues face the center of the structure, the X and Y residues are oriented in such way that they will be exposed to solvents (Duconseille et al., 2015). The general structure of gelatin is depicted in Figure 2.4. The triple helix is roughly 300nm in length and has a molecular weight of around 300 000 Da. During the gelatin manufacturing process, the partial cleavage of the protein cross links result in the breakdown of the collagen structure, causing the loss of the native collagen structure to form gelatin (Schrieber & Gareis, 2007a). Gelatin is a mixture of high molecular weight polypeptides and unlike its parent molecule, it consists of three dominating fragments; α, β, and γ. The α-chains are known as the building blocks of the β and γ chains as they consist of either two or three covalently bound α-chains, respectively (Haug & Draget, 2011). Each chain has varying molecular weights. Hence gelatin is considered a polydiverse protein with a molecular weight distribution ranging from 10,000 to 1,000,000 Da (Babel et al., 2000).

Similar to its parent molecule, gelatin contains 18 different amino acids, where the predominant amino acids are glycine, proline and hydroxyproline at 24%, 17% and 10% respectively. The high content of these amino acids can be explained by comparing the amino acid sequence of gelatin to that of the collagen molecule. Considerably different from collagen molecule, gelatin contains alanine at high levels (14%). The other amino acids are present at levels below 10%, such as glutamic acid (7%), aspartic acid (6%), lysine (4%), serine (3%), arginine (3%) and leucine (3%) (Keenan, 2012). The remaining amino acids, which include serine, arginine, leucine, valine, phenylalanine, threonine, isoleucine, hydroxylysine, histidine, methionine and tyrosine, account for 9%. The least abundant amino acid accounting for 0.2% is tyrosine (Keenan, 2012). As notable tryptophan and cysteine, two essential amino acids are not present in gelatin. Likewise, the acidic

amino acids, glutamic acid and aspartic acid, are only found in collagen, in gelatin they are found in the form of glutamine and asparagine respectively (Keenan, 2012).

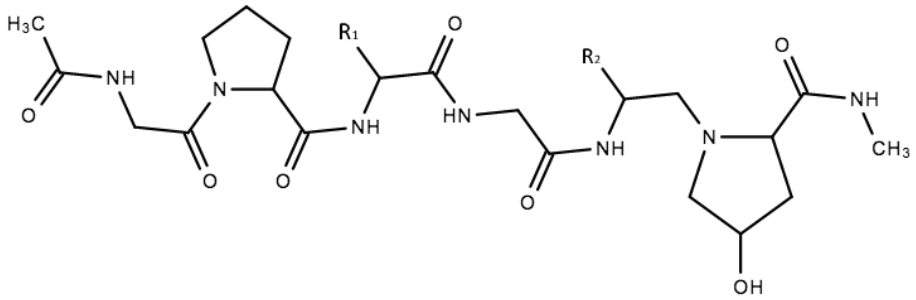


Figure 2.4. Chemical configuration of gelatin

## 2.6 Effect of Glycation on Functional Properties

Although proteins are widely used to add functional properties into a food system, there exist substantial evidence that suggests the functional properties of proteins can be further improved by derivatization of the protein. Glycation which results from the attachment of reducing sugars to proteins through the Maillard reaction affects the functional properties as a result of change in charge, solvation and conformation (Darewicz & Dziuba, 2001; Kato, Minaki, & Kobayashi, 1993). The expected mechanism of reaction between gelatin and glucose is shown in Figure 2.5. The yield of the Maillard products are highly influenced by temperature, pH, and relative humidity amongst other intrinsic properties. Although numerous studies have focused on the modification and changes in functionality of Maillard glycoconjugates, the studies showed both beneficial and detrimental outcomes (Oliver et al., 2006). The most important properties affected by glycation includes hydration and surface properties such as solubility, emulsifying properties, and foaming ability.

Protein glycation limited to the early stages of the Maillard reaction showed an increase in protein solubility (Katayama, Shima, & Saeki, 2002; Sato et al., 2000), increase in emulsifying activity (Darewicz & Dziuba, 2001; Kato et al., 1992), improved foaming ability (Dickinson & Izgi, 1996), and increased heat stability (Chevalier et al., 2001; Hattori et al., 1997). Studies focusing on the effect of advanced stages of Maillard reaction also showed beneficial effects with the formation of

antioxidant (Chevalier et al., 2001), anticarcinogenic and antimutagenic properties (Hosono, 1997). On the contrary, some studies on the conjugation of proteins and reducing sugars reported formation of mutagenic compounds and decreased nutritional value of the protein. As the sugars react with the free amino group of lysine, the blockage of the lysine group and other essential amino acids results in the decreases in nutritional value. In addition to the decreased nutritional value, studies have shown decreased digestibility (Culver & Swaisgood, 1989), development of off flavors (Walker, 1972), extensive browning (Guerra-Hernandez et al., 2002), and reduced solubility (Kato et al., 1986). Although beneficial and detrimental outcomes were reported by the studies, the common conclusion derived from the studies is the potential use of glycation as a means for protein modification to yield or manipulate protein functional properties.

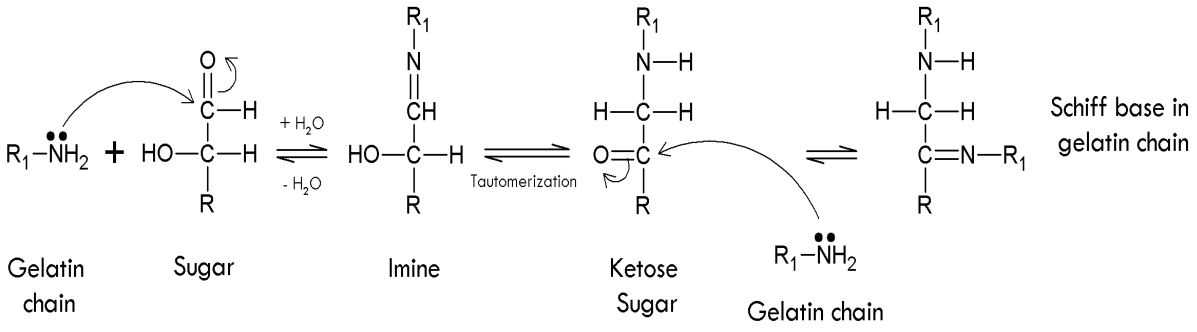


Figure 2.5. Representative scheme of gelatin glycation with a sugar; adapted from Duconseille et al. (2015)

### 2.6.1 Improvement in Functional Properties of Glycoconjugates

#### 2.6.1.1 Protein Solubility

The effect of glycation on protein solubility remains debatable. Studies conducted so far indicate that the conjugation of sugars with proteins result in a decrease in protein solubility (Kato et al., 1986; Lea & Hannan, 1949). In contrast to these studies, more recent studies have shown that following glycation, a significant improvement can be observed in protein solubility. Oliver et al. (2006), suggest that the protein solubility will be enhanced given that the extent of glycation is limited. The decreased solubility at higher glycation degree can be explained by the favored formation of protein cross links at advanced stages of the Maillard reaction. The protein cross links

will enforce substantial changes in the structure of the protein thus causing changes in the functional properties. In addition, with prolonged heating, there is higher possibility of thermal denaturation which can give rise to insoluble protein. It is however debated that the improved solubility cannot be limited to the degree of glycation, as the possibility of disulfide bonding, and cross linking needs to be taken into account (Oliver et al., 2006).

On the contrary, the increase in solubility at limited degree of conjugation can be partially due to the hydrating ability of sugars. It is evident that the conjugation of a sugar to a protein will induce several structural and physicochemical changes. The binding of the sugar to the protein will cause a change in charge, where an increase in net negative charge will be observed resulting in a shift to a rather acidic isoelectric point (Chevalier et al., 2001; Wang & Ismail, 2012). The overall increased electrostatic repulsion, hydrophilicity and steric hindrance will reduce the occurrence of protein-protein interactions and disulfide linkages. The change in net charge will allow the protein to resist denaturation and allow enhanced solubility and stability properties. (Wang & Ismail, 2012).

Seo et al. (2013) studied the effect of lysozyme glycation with galactose, galactan and galactooligosaccharides on several functional properties including protein solubility. The study concluded that lysozyme conjugated with galactan showed unfavorable effects on solubility at acidic pH, perhaps due to its linearity which formed aggregates through intermolecular hydrogen bonding. The lysozyme/galactose and lysozyme/galactan conjugates showed improved solubility in comparison to the native lysozyme at pH close to the isoelectric point. The increased solubility was attributed to the decreased hydrophobicity of the protein and increased hydrophilicity due to the conjugation of the saccharides. Niu et al. (2011) studied the glycation of wheat germ protein (WGP) with glucose, lactose, xylose, dextran and maltodextrin. Conjugation of WGP with dextran showed the greatest improvement in solubility from 52.16% to 84.84%, followed by lactose and glucose. They concluded that the greater the carbohydrate length the greater the improvement in solubility. Contradicting such, Li et al. (2009) found that the conjugation of rice protein with glucose and lactose showed higher solubility than with dextran and maltodextrin as a result of higher degree of glycation. Hence it can be concluded that numerous factors includind

carbohydrate length and degree of glycation play significant roles on the solvation properties of the protein/saccharide conjugate.

#### 2.6.1.2 Viscosity

Viscosity is a fundamental parameter that characterizes a fluid's resistance to flow (Schmitt et al., 1998). Viscosity, or gelling ability depends mainly on the molecular weight, shape, and size of the biopolymers. Generally, polysaccharide dispersions display greater viscosity in comparison to protein dispersions, hence following glycation, the glycoconjugate is expected to exhibit an increase in viscosity as a larger complex is formed. In other words, the greater the molecular weight of the glycoconjugate, the greater the increase in the viscosity or viscoelastic property. An increase in viscosity therefore improves gelling ability as well. An example of such can be observed in a study conducted by Paraman et al. (2007). The study observed the increase in viscosity of rice protein when glycated with glucose and xanthan gum. The rice protein glycated with xanthan gum had a viscosity of 31.6 mPas, whereas that glycated with glucose had a viscosity of 12.4 mPas. The higher viscosity expressed by the xanthan gum glycated residue can be explained by the complex size of the xanthan gum compared to glucose. It is also suggested that the increase in surface hydrophilicity and partial unfolding of the quaternary structure can give rise to the increased viscosity (Baniel et al., 1992; Liu et al., 2012).

#### 2.6.1.3 Emulsifying Properties

One of the most important structural functions of proteins is its ability to stabilize emulsions. The emulsifying ability is expressed by a molecule, when it is capable of stabilizing an emulsion and is validated in terms of emulsifying activity, capacity and stability (Ahmed, 2013). Both proteins and carbohydrates have significant roles in the stabilization of an oil in water emulsion. Generally, proteins can adsorb at the oil water interface in order to form a coherent viscoelastic layer, while polysaccharides can confer the colloidal stability by their thickening and gelling behaviors (Kato et al., 1993). Hence when the two polymers work together it is expected to see enhanced results. The protein-saccharide conjugate surface will be much more active than the surface of the individual polymers, hence the conjugates can saturate the surface layer at a much lower concentration and provide greater stabilization. The balance of hydrophobic and hydrophilic character of the resulting conjugate plays an important role in the alteration of emulsifying

properties. The hydrophobic residues of the protein will anchor to the oil droplet while the saccharide will attract water molecules around the oil droplet (Khan et al., 1999). This will speed the formation of a thick layer around the stabilized emulsion, hence preventing or ceasing coalescence (Magdassi, 1996). With an increased number of sugar residues attached, it is expected to observe an increase in steric stabilization, this having an effect on emulsifying properties (Oliver et al., 2006). In comparison to shorter chain carbohydrates, longer chained polysaccharides have a higher ability in stabilizing emulsions as they have a better hydration capacity (Seo et al., 2013).

Amongst the numerous studies present on the effect of conjugation of proteins and sugar on their functional proteins, Kato was amongst the first to report the effect on the emulsifying properties of the resultant conjugate. Kato's studies revealed that the heating of sugars with the protein had little or no effect on the emulsifying properties of the conjugate. The conjugation of glucose with α-casein (Kato et al., 1992), protamine (Matsudomi et al., 1994) and ovalbumin (Kato et al., 1990) showed reduction in surface properties. Likewise, a study by Groubet et al (1999), showed that the emulsification properties remained unchanged upon glycation of casein with various sugars; glucose, arabinose, lactose, ribose and galactose. In rather recent studies, it was found that the emulsifying activity of conjugates can be greatly enhanced. Moreno, López-Fandiño, and Olano (2002) showed that smaller chain carbohydrates can show an improvement in emulsifying properties given a high degree of glycation. This justification was also observed in Kato's work, where the study showed that the conjugation of lysozyme with galactomannan increased emulsifying properties, however the effect was greater in double conjugated lysozyme (Kato, 2000). The recent studies have shown that conjugation with small carbohydrates are an effective method for improving functional properties of food proteins. Factors such as amount of unreacted lysine, reaction time, structure of reacting saccharide or sugar and protein: sugar ratio contribute to the overall effect of increasing or decreasing emulsifying effect (Oliver et al., 2006).

#### 2.6.1.4 Heat Stability

It has been proven by many studies that the conjugation of proteins and sugars can result in a more heat stable form of the native protein (Aoki and others 2001; Kato and others 1995; Jiménez-Castaño and others 2007; Liu and Zhong 2013; Liu and Zhong 2012; Wang and Ismail 2012). Chevalier et al. (2001), demonstrated that the conjugation of $\beta$-lactoglobulin with either galactose,

glucose, lactose and rhamnose led to increased heat stability. Kato (2002) justified that the increase in heat stability is partially due to the fact that the carbohydrate conjugated to the protein stabilizes the protein in such a way that it protects its aggregation and inhibits protein-protein interaction. Therefore, the conjugation allows the protein to reverse its denaturation, thereby increasing heat stability. It is also proven that the increased hydrophobicity (Wang & Ismail, 2012) and increased denaturation temperature (Liu & Zhong, 2012) play a significant role in the alteration of heat stability. Studies suggest that there exists a positive correlation between the molecular weight of the sugar being conjugated and the heat stability of the glycoconjugate. Shu et al. (1996) showed that lysozyme glycated with galactomannan was far more heat stable than when glycated with xyloglucan, which have a molecular weight of 24000 and 1400kDa respectively. Likewise, a study by Liu and Zhong (2012) showed that whey protein isolates conjugated with lactose and maltodextrin were more heat stable than those glycated with glucose.

Based on literature, it is observed that molecular weight of the sugars were not the only factors imparting a greater increase in heat stability. In fact, increased electrostatic repulsion as a result of change in charge, increased denaturation temperature (Liu & Zhong, 2012), and increased surface hydrophobicity (Liu & Zhong, 2012; Wang & Ismail, 2012) also contribute to the improvement of heat stability in glycated conjugates.

## 2.7 Analytical Methods for Monitoring Maillard Reaction

Depending on the protein molecule, its composition and structure, different analytical methods can be applied to characterize the conjugate resulting from the reaction. In addition, the method of analysis can help further investigate the progress of the Maillard reaction, the percent of lysine blocked, available free sugars as well as other aspects. Methods such as mass spectrometry (MS), Fourier Transform Infrared Spectroscopy (FTIR), and Tryptophan fluorescence are amongst the common methods for structural analysis of the conjugates. On the other hand, the monitoring of glycation can be conducted with sugar tests, florescamine assay, furosine assay and other tests.

### 2.7.1 Mass Spectrometry

Mass spectrometry is one of the most common techniques used to characterize protein glycation reactions. The use of soft ionization methods such as matrix assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI), have been greatly favored due to their ability to generate intact molecular ions under atmospheric conditions. In addition, the Time of Flight ESI-MS, helps generate high resolution results due to its high sensitivity, thus aiding in accurate interpretations in distinguishing between glycated and non-glycated proteins. MS is one of the most advantageous methods for the analysis of protein/saccharide conjugates as the technique will provide accurate relative molecular mass measurements, and allows for a quantitative estimation of the extent of glycation (Yeboah et al., 2000). With ESI-MS or TOF/ESI-MS, the change in molecular weight during glycation can be seen where the mass will increase by 162 units for every glucose molecule attached as an example. Although the molecular weight of glucose is 180 units ($C_6H_{12}$), the sugar will undergo dehydration upon conjugation thus causing a change in the molecular weight by 18 units (loss of water; $H_2O$). The method is rather more direct; however, it requires the protein to be a single molecular weight protein (pure protein). Complex proteins render the monitoring of protein glycation difficult by ESI-MS, resulting in mass overlays, thus rendering the method not suitable for those complex proteins (Yeboah et al., 2000).

### 2.7.2 Combination of Fluorescamine and Sugar Test to estimate Extent of Glycation

#### 2.7.2.1 Fluorescamine Assay

The quantification of total protein content is generally common in numerous scientific studies. There exist numerous absorbance-based colorimetric methods for the quantification of protein where the fundamental basis relies mainly on the reduction of copper in the presence of a chromogenic reagent (Held, 2006). Generally, the furosine assay is used widely for assaying glycated proteins. This method involves hydrolyzing the glycated protein in 6N HCl for 18h. The hydrolysates are then analyzed by high performance liquid chromatography (HPLC) to detect the presence of furosine, a product resulting from the cyclization of the lysine amadori product. (Yaylayan, Huyghues-Despointes, & Polydorides, 1992). The amount of furosine is indicative of the glycation extent. In addition to periodate oxidation methods where the amount of formaldehyde released correlated to the extent of glycation, the thiobarbituric acid is another commonly used

method for assaying glycated proteins. Similarly sugar tests have also been used. Although these methods have been long used, it is noted that these methods are not highly accurate as they can be subject to interference caused by the agents being used. To correct these issues, dye-binding protein assays were generated, such as the Bradford method. However, this method can also lose its reliability with the formation of aggregates overtime. The formation of the aggregates can result in the reduction in signal (Held, 2006).

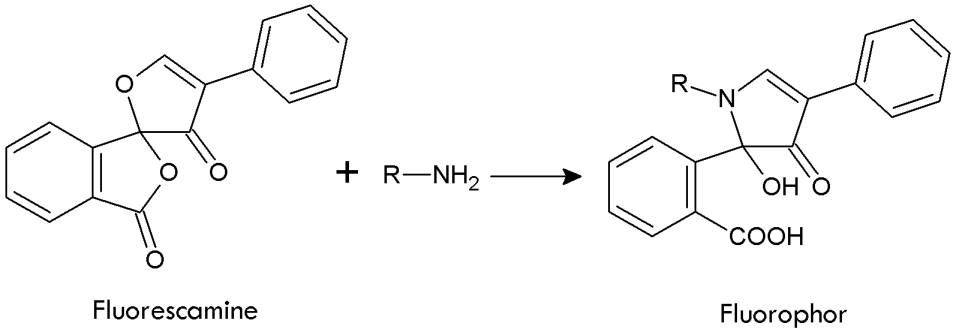


Figure 2.6. Reaction of fluorescamine (non fluorescent) with free amino group to form fluorophor (fluoroscent)

Fluorescamine assay is yet another method used for the estimation of degree of glycation. It is a selective reaction, where fluorescamine reacts with the primary amino groups of proteins or amino acids. When the fluorescamine reacts with the N terminal group, it produces products with enhanced intrinsic fluorescence. The reaction is depicted in Figure 2.6. The resultant fluorescence is an indication of the number of free amino groups present in the protein (Yaylayan et al., 1992). Thus when the native protein is reacted with the fluorescamine reagent, it should produce a higher fluorescence in comparison to the glycated protein which has sugar molecules attached at its N-terminal or $\varepsilon$-amino group of the protein (Figure 2.7). The fluorescence of the glycated and non-glycated protein can be converted into the number of glycated sites in the protein of interest. In the advanced stages of the Maillard reaction, products with fluorescence are also formed. However, these products will not interfere with the assay as the excitation and wavelength of these fluorophores are different from that of the fluorescamine fluorophores (Yaylayan et al., 1992). The advanced Maillard reaction products fluoresce at an excitation and emission wavelength of 340nm and 410nm respectively, while the fluorophors resulting from the attachment of the fluorescamine

to the protein fluoresces at am excitation wavelength of 390nm and emission wavelength of 475 nm (Yaylayan et al., 1992).

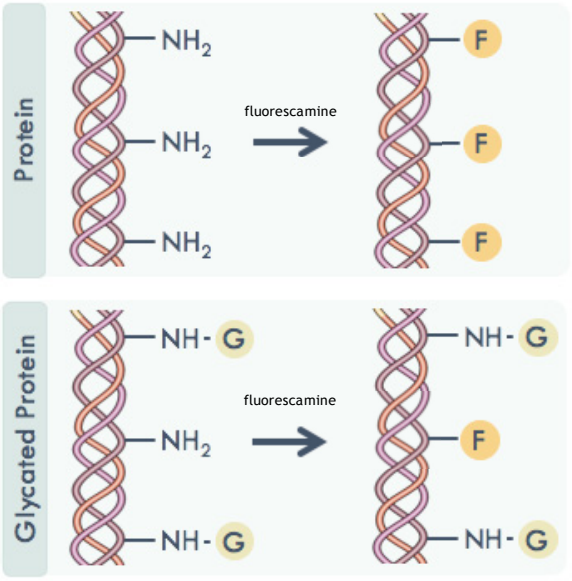


Figure 2.7. Reaction process of fluorescamine with glycated and non-glycated proteins (G = reducing sugar, F = fluorophor); adapted from Yaylayan et al. (1992)

### 2.7.2.2 Dinitrosalicylic acid (DNS) method

Spectrophometric total sugar determination methods are commonly used as they are highly sensitive in comparison to other colorimetric methods. Amongst the spectrophometric methods, includes the Dinitrosalicylic acid (DNS) method. This method has been used by many studies for estimating the amount of reducing sugar content present in numerous samples (Breuil & Saddler, 1985; Negrulescu et al., 2012). Under alkaline conditions, DNS will react with the free carbonyl group of the reducing sugar forming 3-amino-5-nitrosalicylic acid as shown in Figure 2.8. This aromatic compound has a maximum absorption at 540 nm. The resulting absorbance is directly proportional to the amount of reducing sugar present in a given sample (Başkan et al., 2016). The DNS assay solution requires: DNS, sodium potassium tartrate, phenol, sodium bisulfate and sodium hydroxide. Sumner et al., the original developers of the DNS assay, explain the purpose of each reactant for the DNS assay. The sodium potassium tartrate, also referred to as Rochelle salt, is used to prevent the desolation of oxygen by the reagent. The phenol helps increase the

production of color, while the sodium bisulfate stabilizes the color obtained in the presence of the phenol (Miller, 1959).

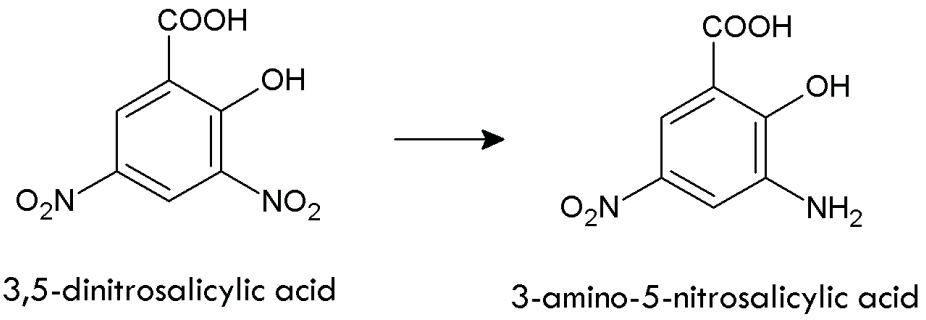


Figure 2.8. Reaction of 3,5-dinitrosalicylic acid in the presence of reducing sugars to form 3-amino-5-nitrosalicylic acid

The combination of the fluorescamine and DNS assay is expected to give a better understanding in relation to the glycated amino sites and remaining sugar content by the respective methods. In addition, based on the amount of glycated sites occupied by the reducing sugar, an indirect approach as to the amount of sugar reacted and amount of unreacted amount of sugar can be determined based on the information provided by the fluorescamine assay. Using the information obtained by both methods, a more vivid understanding can be obtained pertaining to the relative extent of glycation.

## 2.8 Conclusion

Amongst the existing methods for modification of functional properties of proteins, glycation through the Maillard reaction is an acceptable approach compared with other chemical modifications. Protein glycation holds a great potential for not only the modification of properties but also for the creation of value added functional ingredients. Although it is documented in numerous studies that glycation through the Maillard reaction can improve the functional properties of food proteins, many of these studies focus on thermally induced glycation. Studying the effect of the reaction conditions and different approaches to induce protein glycation such as the use of sonication or mechanochemistry, and evaluating their respective effects on the functional properties of the conjugates in comparison to thermal glycation can potentially provide a more successful alternative in the food industry as well as increased applications. Protein

glycation through the Maillard reaction holds great potential, however there is still more research required in order to have a better understanding on reaction conditions, relationship between the reactants, as well as other properties. With greater understanding, glycoconjugates can be introduced as novel functional ingredients.

# CHAPTER 3:

## 3. Material & Methods

### 3.1 Materials

Porcine gelatin Type A (40,000-50,000 Da), D-glucose, D-ribose, fluorescamine, potassium borate tetrahydrate, 3,5-dinitrosalicylic acid (DNS), potassium sodium tartrate tetrahydrate, potassium disulfite, phenol, sodium hydroxide, hydrochloric acid, phosphate, sodium phosphate, sodium dodecyl sulfate (SDS), and sorbitol were obtained from Sigma Aldrich Chemical Co. (St. Louis, Missouri, United States). Acetone Optima (spectroscopy grade) and hydrochloric acid were purchased from Fisher Chemicals Co. (Hampton, New Hampshire, United States)

### 3.2 Preparation of Glycated Proteins

#### 3.2.1 Thermally generated samples

Gelatin (0.1g) and saccharide (0.0075g) were dissolved in distilled water (2 mL) at a molar ratio of 1:16 (approximately 1:2 molar ratio of available lysine residues to sugar carbonyl). To ensure homogeneity of the mixture, the saccharide was first added to the distilled water and allowed to solubilize prior to the addition of gelatin. The sample was then vortexed, and incubated for 24, 48 and 72 hours at 55°C in a Digital Shaking Dry bath (Thermo Scientific, Ohio, USA). The samples (2 mL) were contained in a closed 4mL vials (15 x 44mm) throughout the experimental procedure to ensure that oxidation was not a contributing factor. Samples were then freeze dried (Labconco Freeze Dryer, Missouri, USA) for 48 hours and then stored at -20°C, until further use (testing functional properties).

#### 3.2.2 Sonochemically generated samples

Similar sample preparation was carried out as the thermally treated samples. After mixing of the gelatin (0.750g) and the saccharide (0.05625g) in distilled water (15mL) to obtain a 5.4% w/v of protein/sugar solution (gelatin/glucose ratio of 1:16), the samples were exposed to ultrasonic treatment. The gelatin samples were sonicated with a frequency of 20 KHz and amplitude of 100% with a High Intensity Microprocessor Controlled Ultrasonic Processor (Autotune Series, 750 Watt) for 5, 10 and 15 minutes. The 12 mm diameter probe was used to sonicate 15 mL samples held in a 30 mL flat bottom beaker. The ultrasound probe was immersed at a depth of 1.5 cm into

the sample. The ultrasonic treatment was carried out without cooling, allowing for the sample temperature to rise from 25°C to 65°C; an uncontrolled temperature sonication was carried out. Samples were freeze dried (Labconco, Missouri, USA) for 48 hours and then stored at -20°C, until further use (testing functional properties).

### 3.2.3 Mechanochemically generated samples

Gelatin and glucose in a 1:16 molar ratio were mixed together using a pestle and motar (0.1g gelatin and 0.0075g of glucose). After sufficient crushing to ensure homogeneity, the dry sample mix underwent mechanochemical milling for 10, 20, and 30 mins, at a frequency of 30Hz, using a Mixer Mill MM400 (Restech, Haan, Germany). The milling process was aided with the addition of a small metallic ball (5mm in diameter). Following the preparation of the conjugates, the samples were collected from the walls of the compartment used for mechanochemical milling, to which distilled water (2mL) was added to obtain the same protein concentration as the thermal and sonochemical methods (when measuring fluorescence and determining residual sugar content). As the mechanochemically prepared conjugates lack solvent, the samples did not require to be freeze dried. Excess samples prepared were stored in air tight 4mL vials (15 x 44mm). However fresh samples were prepared when functional properties were to be tested.

## 3.3 Determination of the Extent of Glycation of Gelatin with Selected Saccharides

### 3.3.1 Measurement of Free Available Amino Groups

A modification of the fluorescamine assay (Yaylayan et al., 1992) was used to quantify the proportion of reacted ε-amino groups in the glycated samples. 100µL of the native gelatin solution (0.1g gelatin in 2 mL distilled water) and thermally, ultrasonically and mechanochemically glycated gelatin solutions (0.1g gelatin, 0.0075g glucose and 2 mL distilled water) were diluted (50x) and stirred continuously on a magnetic stirrer. A 200µL aliquot of the diluted solution was transferred into a 10 mL vial containing 4mL of 0.2M potassium borate buffer, pH 8.5 and was vigorously vortexed. An aliquot (1mL) of the fluorescamine reagent (15mg/100 mL acetone) was rapidly added. A potassium borate buffer containing no protein was used as the blank, where the blank was run routinely. The fluorescence was measured using a Kontron SFM 25 spectrofluorometer (Kontron Instruments, Zurich, Switzerland), at an excitation wavelength of 390

nm and emission wavelength of 475 nm. The results are expressed as the percent glycation (fluorescence after treatment over fluorescence of native protein) as per Perusko et al. (2015).

### 3.3.2 Measurement of Residual Sugar

#### 3.3.2.1 Preparation of Reagent

The DNS reagent was prepared by combining 1% (w/v) DNS solution, 40% (w/v) sodium potassium tartrate solution, 0.2% (w/v) phenol solution and 0.5% (w/v) potassium disulfite solution prepared in 1.5% (w/v) NaOH solution at a 1:1:1:1 (v/v/v/v) ratio

#### 3.3.2.2 Use of dinitrosalicylic acid (DNS) assay for the Determination of Reducing Sugar Content

To a test tube, 1mL of native gelatin solution (0.1g gelatin in 2mL distilled water) or thermally, sonochemcially or mechanochemically glycated gelatin solution (gelatin (0.1g), glucose (0.0075g), distilled water (2 mL)), DNS reagent (2 mL) and distilled water (1 mL) was added and vortexed for 30 seconds. The samples were then incubated in boiling water for 5 minutes, and then cooled in an ice bath for 5 minutes. After cooling, the solution was transferred to a cuvette and the absorbance of the samples were recorded at 540 nm against a reagent blank. The DNS reagent was used as a blank. An Evolution 300 UV Visible Spectrophotometer (Thermo Electron Corporation, Cambridge, United Kingdom) was used in conjunction with the VisionPro software to obtain the absorbance values.

Glucose was used as the chemical standard for this assay. Glucose was dissolved in distilled water to obtain a concentration of 5 mg/mL and then diluted to obtain concentrations between 1 mg/mL – 5mg/mL. The standard solution (1 mL), DNS reagent (2mL) and distilled water (1 mL) were mixed together and vortexed. The samples were heated and then cooled as indicated above. The absorbance of the standard solutions were carried out using a spectrophotometer to obtain the calibration curve. The analysis was carried out in triplicates at a wavelength of 540nm. The DNS reagent was used as a blank. The linear region of the curve was used to obtain a linear regression equation.

## 3.4 Determination of Functional Properties of the Glycoconjugates

For the analysis of the functional properties, freeze dried samples of the thermal and sonochemical conjugates and freshly prepared mechanochemical conjugates were used.

### 3.4.1 Protein Solubility

Protein solubility at various pH values were determined by the method of Betschart (1974) with slight modifications. Protein solutions (1%, w/v) were prepared using distilled water (0.02g conjugate in 2mL distilled water). The pH of the solution was adjusted to 3, 5, 7, 9 or 11 with 0.1M HCl or 0.1M NaOH and vortxed for 1 min at room temperature. The dispersion was then centrifuged with a Baxter Canlab Biofuge 13m (Heraeus instruments, Mont-Royal, Canada) at 3000 rpm for 30 minutes. The supernatant of the centrifuged samples was recovered, and the amount of protein in the supernatant was determined by the fluorescamine assay method (see section 3.3.1). Protein solubility was calculated as the percent ratio of protein in the supernatant to the total protein present in the sample as shown in the equation below. The total protein present for each form of glycated gelatin was obtained by determining the fluorescence exhibited by the completely solubilized protein sample at 40°C. The solubility profile was obtained by plotting the protein solubility as a function of pH.

$$solubility\ (\%) = \frac{Protein\ content\ in\ supernatant}{Total\ protein\ content\ in\ sample} \times 100$$

### 3.4.2 Thermal Stability

Thermal stability of native and glycated gelatin was determined by the method of Shu et al. (1996) with several modifications. The turbidity of 10mg/ml protein solution in 0.1M sodium phosphate, pH 7, was measured between 50 to 85°C, at every 5°C interval. The solutions were heated in a Digital Shaking Drybath (Thermo Scientific, Ohio, United States), and the temperature was increased at a rate of 1°C every 2 minutes. Once the desired temperature is reached, the turbidity of the sample was measured spectrophotometrically at a wavelength of 500nm. The thermal stability was expressed as the percentage of relative turbidity obtained in comparison to the sample with the highest turbidity.

### 3.4.3 Emulsifying Properties

The emulsifying properties (emulsifying activity (EAI) and emulsifying stability index (ESI)) were measured by the method of Pearce and Kinsella (1978). Pure coconut oil (1.5 mL) and 4.5 mL of 0.1% (w/v) protein solution prepared in 0.01 M phosphate buffer, pH 7, were homogenized together at room temperature for 1 min, using a Vortex Genie 2 (Fisher, Scientific Industries Inc., Bohemia, New York), at a vortex level of 6. Fifty microliter portions of the emulsions were pipetted from the bottom of the test tube at 0 and 10 min after homogenization. These portions were diluted with 5 mL of 0.1% sodium dodecyl sulfate (SDS) solution. The absorbance of each diluted emulsion was determined at 500 nm with a Evolution 300 UV Visible Spectrophometer (Thermo Electron Corporation, Cambridge, United Kingdom). The absorbance measured immediately and at 10 min after emulsion formation were used to calculate the EAI and ESI. Each sample was prepared in triplicate for the EAI and ESI measurement.

The EAI was expressed as:

$$EAI\ \left(m^2/g\right) = \frac{2T \times A_0 \times DF}{C \times \varphi \times \theta \times 10000}$$

Where T = 2.303; DF = dilution factor =100; $A_0$ = absorbance measured immediately after emulsion formation (t=0 min); C = weight of protein/ unit volume (g/mL) of the aqueous phase before emulsion formation; $\varphi$ = wave path length = 1 cm; $\theta$ = oil volume fraction of the emulsion = 0.25

And the ESI was expressed as:

$$ESI\ (min) = A_0 \times \frac{\Delta t}{\Delta A}$$

Where $\Delta t$ is the time interval = 10 min and $\Delta A$ is the decrease in absorbance between 0 and 10 min = $A_0 - A_{10}$

### 3.4.4 Foaming Properties

The foaming properties (foaming capacity (FC) and foaming stability (FS)) of the native and glycated protein were determined by the method of Lin et al. (1974) with modifications. The 5 mL of 1.0% (w/v) protein solutions were prepared in 0.05 M phosphate buffer, pH 7.4. Complete solubility of the protein was ensured by heating the mixture in a Digital Shaking Drybath Thermo Scientific for 2 minutes at 40°C. The samples were then homogenized using a Vortex Genie 2, for 2 minutes. The volume of foam generated immediately after homogenization and after 10 minutes and 24 hours of storage at room temperature were recorded, and used to calculate the foaming capacity and foaming stability.

The foaming capacity was expressed as the maximum volume of foam produced immediately after homogenization.

The foaming stability was expressed as:

$$FS\ (hour) = V_0 \times \frac{\Delta t}{\Delta V}$$

Where $V_0$ is the volume of foam at 0 min; $\Delta t$ is the time interval = 24 hours and $\Delta V$ is the decrease in the volume of foam between 0 and 24 hours = $V_0 - V_{24}$

### 3.4.5 Film Forming Properties

#### 3.4.5.1 Film Preparation

Gelatin and glycated gelatin based films were prepared according to the formulation shown in Table 3.1. Gelatin or glycated gelatin were dissolved in 25 mL of distilled water, and heated at 80°C for 15 min as per Etxabide et al. (2015). The mixture was covered and continuously stirred using a magnetic stirrer in order to obtain a good homogenous blend. Following such, sorbitol was added to the solution at a 30% w/w of gelatin or glycated gelatin and was continued to be heated at 80°C with continuous stirring for another 15 min. The film forming solution (20 mL) was poured into a Petri dish and allowed to dry for 48 h at room temperature. The films were then conditioned in a bio-chamber at 25°C and 50% relative humidity for 48 h prior to conducting tests.

Table 3.1 Formulation of gelatin based film

| | Native or glycated gelatin | Sorbitol | Water |
|---|---|---|---|
| **Formulation 1** | 0.25g | 0.075g | 25mL |

### 3.4.5.2 Film Thickness

The film thickness was measured with an electronic digital micrometer (CO 030025, Marathon Management Co, Richmondhill, Ontario) with a sensitivity to the nearest 0.001 mm. The thickness of the film was calculated as the average of the readings obtained at 5 random positions of the film.

### 3.4.5.3 Mechanical Properties

Tensile strength, elongation at break and the Young Modulus of the gelatin and glycated gelatin based films were measured with an Instron Universal Testing Machine (model 4500, Instron Corporation, Canton, MA), operated by the ASTM standard 638-10 method. The preconditioned films were cut into dog bone shape with the measurements shown in Figure 3.1, and clamped between the grips of the machine with an initial grip distance set at 30mm. The clamped samples were deformed under a tensile loading using a 50kg load cell and a crosshead speed of 50mm/min until deformation of sample. The maximum load and the extension at break values obtained were used to calculate the tensile strength (TS) and elongation at break (EB) using the equations mentioned below. Each film sample was tested in duplicates.

Tensile strength was expressed as:

$$TS\ \left({}^{N}/_{mm^2}\right) = \frac{Maximum\ load\ (N)}{Cross\ sectional\ area\ of\ film}$$

where cross sectional area corresponds to the thickness of the film multiplied by the width of the film.

Elongation at break was expressed as:

$$EB\ (\%) = \left(\frac{displacement\ at\ maximum\ load}{original\ gage\ length}\right) \times 100$$

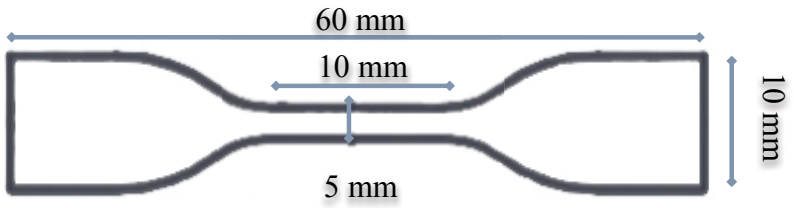


Figure 3.1. Dimension of the dogbone shape die cut used for film analysis

# CHAPTER 4:

## 4. Results & Discussion

Typically, the use of Electrospray Ionization Time of Flight Mass Spectrometry (ESI/TOF-MS) is the best approach to evaluate the degree of glycation. However, gelatin is a composite mixture of various protein fractions with a molecular weight ranging from 40,000-50,000 Da and due to its non-uniform molecular weight distribution the ESI-MS generates a bell curve shaped spectrum as opposed to sharp peaks at specific masses. This renders the monitoring of gelatin/saccharide conjugates by ESI/TOF-MS very difficult. The glycation of the saccharide molecules will not be evident in a bell curve shaped spectrum containing overlapping clusters of multiple charges peaks. As this method was not suitable for confirming gelatin/saccharide conjugation, the conjugation reaction and sugar reactivity were assessed based on monitoring the reduction in available free amino groups through fluorescamine assay, and verifying sugar consumption through the DNS assay.

The fluorescamine assay was used to monitor the changes in the free amino groups of the protein molecule. As the protein structure will continuously undergo changes despite attaining a specific degree of glycation after a given reaction time, a direct assumption cannot be made between the change in amino groups and degree of glycation. More specifically, the results of the fluorescamine assay should be interpreted with caution when proteins are subjected to environments which cause hydrolysis, modification of secondary structure, aggregation and cross-linking. The changes in fluorescence between the untreated protein and treated protein therefore depicts an overall net effect of protein hydrolysis, aggregation and crosslinking. In the presence of sugar, the fluorescence will include the effect of protein glycation as well. Such changes can either overestimate the measured fluorescence such as hydrolysis or underestimate in the case of aggregation and cross-linking involving lysine. However, with the use of appropriate control experiments these drawbacks can be minimized. Figure 4.1 illustrates the respective effect of the above mentioned reactions on the measured fluorescence value.

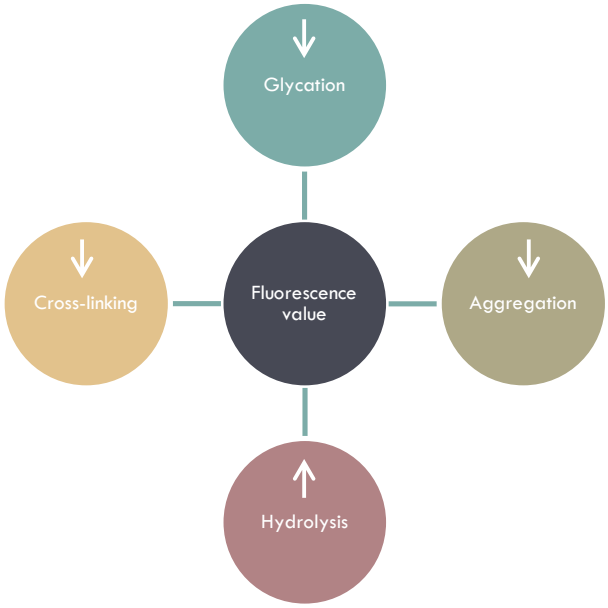


Figure 4.1. The effect of different reactions on the measured fluorescence (↓ decrease; ↑increase)

## 4.1 Thermal Glycation of Gelatin with Ribose and Glucose

Thermal glycation was conducted with a pentose (ribose) and hexose (glucose) sugar to elucidate in general, the effect of carbohydrate type on the conjugation reaction between gelatin, since the glycation of gelatin was not reported in the literature. Figure 4.2 demonstrates the change in the fluorescence measured bi-hourly when gelatin and ribose were subjected to thermal treatment at 55°C for an 8 hour period. The figure shows a fluctuation in the measured fluorescence values, where an oscillating pattern is observed with respect to the reaction time. It is evident that the protein structure will be affected by heat, where the structure will unravel from its coil or helical form and expose a greater number of hidden amino groups, thus resulting in a net increase in fluorescence value. However, in the presence of sugar, the conjugation reaction between the gelatin and ribose should demonstrate a decrease in fluorescence as the free available amino groups will be conjugated with the reducing saccharide. However, this is based on the assumption that the glycation reaction is the major reaction taking place alongside the hydrolysis, aggregation and cross-linking reactions. The unexpected pattern depicted in changes in fluorescence as a function of reaction time seen in Figure 4.2 is a clear observation of the competition between protein glycation and protein denaturation reactions.

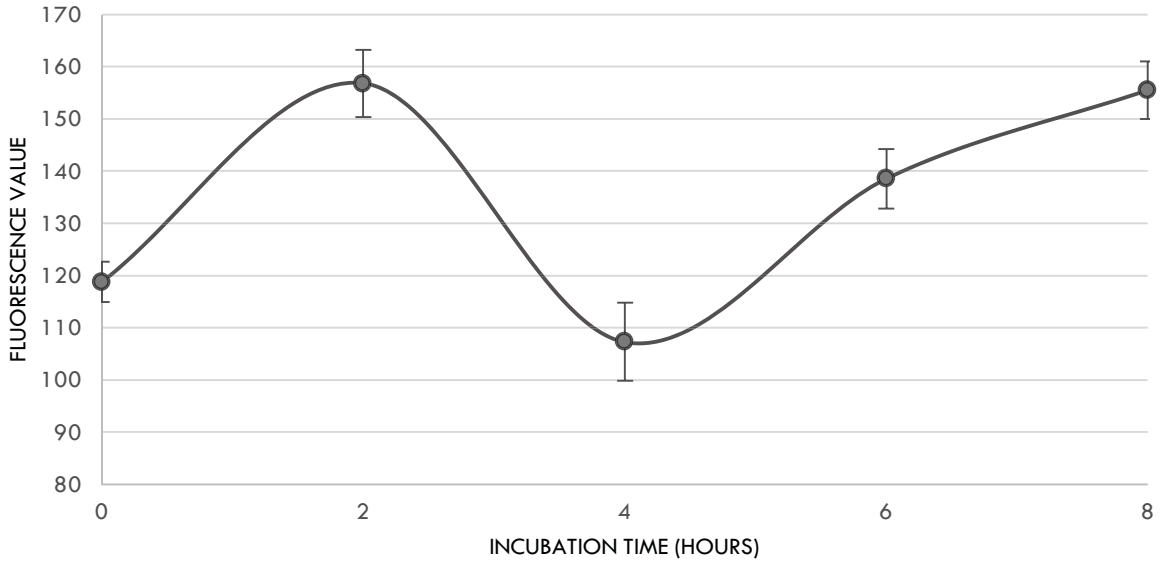


Figure 4.2. Bi-hourly monitoring of the changes in fluorescence as a function of time for the incubation mixtures of ribose and gelatin at 55°C

Ribose is known to be a highly reactive sugar due to its structural properties. The high content of acyclic forms (Burton & McWeeny, 1963; Eskin & Shahidi, 2012; Laroque et al., 2008; Northrop & Connor, 2008) and its high rate of mutarotation contributes to its reactivity (Eskin & Shahidi, 2012). Due to its reactivity ribose was expected to undergo glycation at a fast rate, however, according to Figure 4.2 an increase in fluorescence is observed in the initial two hours of thermal heating, as opposed to a decrease in fluorescence as a result of ribose reacting with the free lysyl groups of gelatin. Although hypothesized that this observation was mainly due to the changes in protein structure resulting from denaturation causing greater exposure of lysyl groups relative to the rate of protein glycation, it is however expected that with the high reactivity of ribose, glycation should be the predominant reaction over denaturation especially in such short reaction times. It is likely that despite the high reactivity of ribose, during the initial 2 hours of thermal reaction, the ribose attaches to the lysyl group of the protein as a Schiff base, but quickly releases itself as a result of structural changes, aggregation or instability of the protein. The figure however shows that after 2 hours of incubation, the resulting protein undergoes glycation again followed by continuous denaturation and hydrolysis. Hence the bi-hourly measurement of the fluorescence shows the denaturation of the protein in the first 2 hours as opposed to the increase in overall

glycation. After 4 hours of incubation with ribose at 55°C, gelatin undergoes irreversible denaturation as confirmed by the observation of increased florescence as measured by flourescamine assay (see Figure 4.2). To understand this phenomena, the glycation reaction between gelatin and ribose was repeated under the same reaction conditions but was monitored at shorter intervals (30 minute) for 2 hours. The reaction progress is depicted in Figure 4.3 as percent glycation over reaction time. The monitoring at shorter intervals revealed the predicted glycation and its reversal in the first 90 minutes of the thermal incubation. The maximum glycation was attained within 60 minutes of the reaction followed by hydrolysis of the Schiff base. This indicates that monitoring the glycation reaction with reactive sugars such as ribose at longer intervals may be misleading. Following the maximum degree of glycation after 60 minutes, the reaction shows a decrease in glycation in agreement with results observed when the reaction was monitored bi-hourly (Figure 4.2). The decrease in extent of glycation can be due to the increased hydrolysis of the protein giving rise to the exposure of more amino groups. With increasing number of available amino groups, the proportion of glycated sample will decrease. Therefore this renders the assumption or calculation of the degree of glycation inaccurate, or an overestimation. In addition, the reduction in extent of glycation can be a result of instability of the glycated protein with reactive ribose moieties which may cause further degradation of sugar.

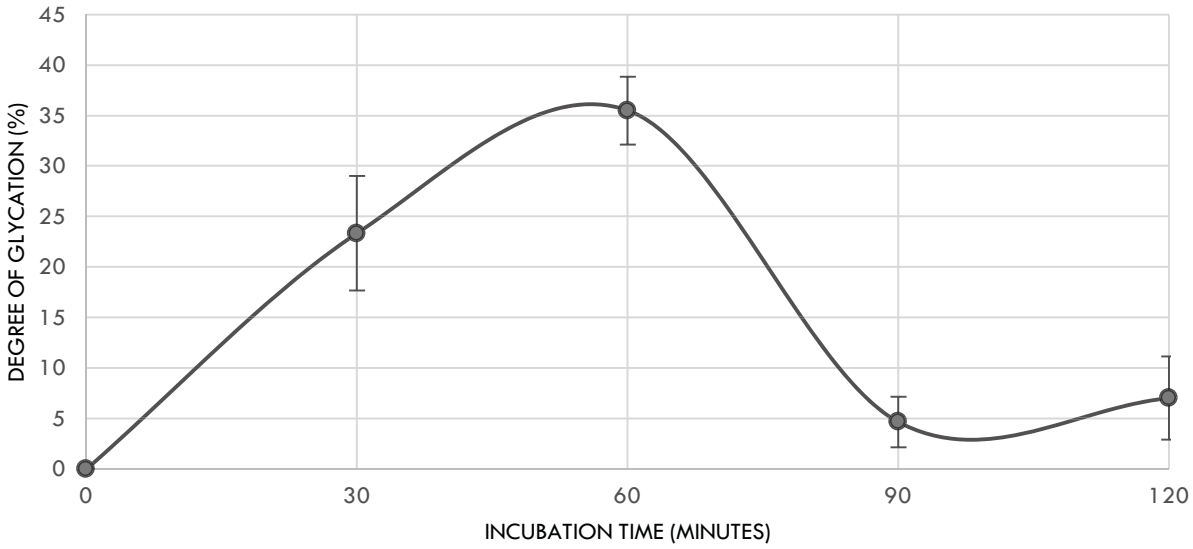


Figure 4.3. Time-dependent degree of glycation of gelatin with ribose as measured by the fluorescamine assay at 30 minute intervals. Data are reported as means±standard deviation of six replicates.

In addition to monitoring the free amino groups through the fluorescamine assay, the residual sugar content was also measured through the DNS assay. As per study conducted by Başkan et al. (2016) the linear regression equations generated for the reducing sugars, D-glucose, D-fructose, D-galactose, D-maltose and D-lactose monohydrate showed no significant differences with respect to the slope of the linear equation. Therefore, the standard curve obtained with glucose was used to quantify the residual sugar content in the gelatin/ribose mixtures (Figure 4.5). As ribose holds greater reducing ability than glucose, slight variation is expected when using the linear regression equation of the glucose curve. Table 4.1 shows a residual ribose content of 93.43% at time zero which is approximately 4-5% lower than that measured in gelatin/glucose mixtures. Table 4.1 shows the increase or decrease in residual sugar content with respect to the reaction time. As seen the residual sugar content fluctuates and shows a decrease over the first 3 hours of the reaction. This reduction in the initial sugar content suggests the progressive glycation reaction over the reaction time. This is consistent with the observations seen in Figure 4.3, confirming the glycation reaction. However in Figure 4.2, an increase in fluorescence was observed in the initial 2 hour period. This increase in fluorescence measured by the flurescamine assay and decrease in residual

sugar measured by the DNS assay at 2 hours, show that the glycation reaction did occur over the observed period, however at the 2 hour mark, the influence of heat imposed structural changes including hydrolysis of the protein thus causing greater exposure of amino groups compared to the rate of glycation. Furthermore, Table 4.1 shows a slight increase in residual sugar content measured from the DNS assay after 4 hours of thermal incubation. The experimental results therefore suggest that after 4 hours of heat treatment at 55°C, ribose decomposes and produce reactive sugars (ex; glycoaldehyde, pyruvaldehyde etc) which exhibit a positive result when tested with DNS.

Table 4.1. Residual sugar content as a function of glycation reaction with ribose, determined by DNS assay

| Time of Analysis (h) | Residual Sugar Content[a] (%) |
|---|---|
| 0 | 93.43±2.75 |
| 1 | 75.10±3.56 |
| 2 | 69.79±1.26 |
| 3 | 66.53±4.61 |
| 4 | 75.38±1.59 |

[a]Experimental results are means of triplicate measurements

Unlike the glycation with ribose, gelatin exhibits higher stability when incubated with glucose at 55°C as shown in Figure 4.4. According to this figure gelatin remains stable and undergoes progressive glycation over 48 hours of incubation time after which it slowly undergoes denaturation and reversal of glycation as evidenced by the release of free sugar after 48 hours (see Figure 4.4). The progressive glycation is assumed based on the continuous decrease in fluorescence value that was obtained over the studied reaction times. The decrease in fluorescence is however a net representation of various side reactions occurring as previously mentioned. Therefore, the results suggest that the glycation reaction overwhelms the hydrolysis of the protein induced by heat. A maximum glycation of 48.63% was obtained at 48 hours of incubation at 55°C with glucose, while only 35.48% was attained through the glycation reaction with ribose. The decrease in degree of glycation after 48 hours of incubation with glucose or after 60 minutes with ribose can be attributed to the presence of degradation reactions. As per Yeboah et al. (2000) and ter Haar, Schols, and Gruppen (2011) the extent of degradation reactions are highly dependent on

two factors; a) the incubation time and b) the hydrophilicity of the saccharide of interest. With the presence of the degradation reactions, the release of amino groups is likely (Oliyai et al., 1994), therefore explaining the increase in available amino groups (increase in fluorescence) and thus resulting in a decrease in degree of glycation. Although results are shown up to 72 hours of incubation for gelatin/glucose mixtures, from Figure 4.4, it is evident that after 48 hours of incubation the reaction mixture is problematic as sugar degradation is influencing the DNS test. At 72 hours, while degree of glycation is seen to decrease, the residual sugar content increases. The presence of sugar degradation and possibility of aggregation and cross-linking with prolonged exposure to heat, interferes with our ability to generate accurate conclusions with respect to the extent of glycation.

Although difficult to establish the optimum glycation condition for thermal glycation of gelatin with glucose or ribose, glycation with ribose showed a faster initial rate of glycation than glucose. After 60 minutes of incubation at 55°C, 35.47% glycation was attained, while 24 hours was needed to reach a similar degree of glycation with glucose. The rate of the reaction is related to the sugars reactivity. As reported by Laroque et al. (2008) and Northrop and Connor (2008), pentose sugars are highly reactive and have greater molecular mobility, of which ribose is the most reactive pentose sugar. In addition, in relation to glucose, ribose is 100 times more reactive in glycation reactions. A study by ter Haar et al. (2011) showed that carbohydrate chain length have an effect on the rate of glycation. They reported that increased carbohydrate length results in a decrease in rate of glycation. The reactivity of ribose in addition to its higher molecular mobility (Guan et al., 2006) in comparison to glucose may contribute to the conjugation reaction of gelatin with ribose occurring at a faster reaction rate.

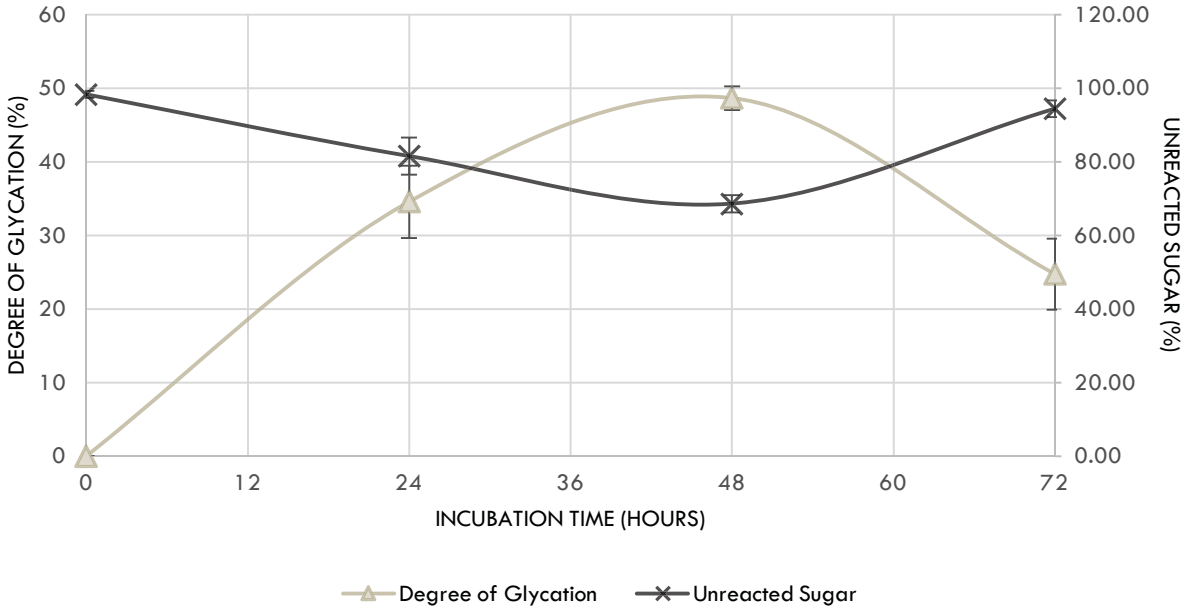


Figure 4.4. Degree of glycation of gelatin with glucose as a function of time at 55°C and the corresponding amount of unreacted glucose. Data are reported as means±standard deviation of nine replicates.

## 4.2 Comparison of the Stability of Gelatin under Thermal, Sonochemical and Mechanochemical Treatments

After studying the effect of glucose and ribose and their ability to undergo thermal glycation of gelatin, it was observed that the glycation reaction with ribose occurred quickly and produced unpredictable results relative to fluorescence measurements. Therefore, the behaviour of the highly reactive sugar when exposed to new techniques of glycation such as ultrasound or mechanochemical milling will be more complicated and difficult to control. Hence to study the effect of the different glycation methods, glucose will be used as the saccharide of choice as it displayed more reproducible and consistent results relative to ribose. The use of glucose will therefore facilitate the interpretation of the role of the new techniques on glycation.

Prior to conducting the glycation reactions, the native protein was subjected to various control treatments in the absence of glucose by the different methods; thermal treatment of 55°C for 0, 24, 48, and 72 hours, ultrasonic treatment at 20Hz for 0, 5, 10, and 15 minutes, and mechanochemical treatment at a frequency of 30Hz for 0, 10, 20, and 30 minutes. The relative changes in available

amino groups quantified by the fluorescamine method are depicted in Table 4.2. The table depicts the effect of the different reaction treatment on the structure of the protein and in particular the changes in the available amino groups. The change in fluorescence value is indicative of the increase or decrease in exposed amino groups as a result protein denaturation or hydrolysis. The table shows that each method imposed different effect on the protein with respect to treatment time.

Table 4.2. Differences in fluorescence values relative to time zero over the course of thermal, ultrasonic and mechanochemical treatments of native gelatin in the absence of sugar

| **Thermal Treatment** | | **Ultrasonic Treatment** | | **Mechanochemical Treatment** | |
|---|---|---|---|---|---|
| Treatment time (hour) | Change in Fluorescence | Treatment time (min) | Change in Fluorescence | Treatment time (min) | Change in Fluorescence |
| 0 | - | 0 | - | 0 | - |
| 24 | +5.4 | 5 | -19.9 | 10 | +13.1 |
| 48 | +44.1 | 10 | -57.8 | 20 | +22.5 |
| 72 | +23.4 | 15 | +12.2 | 30 | +31.4 |

+ indicates an increase in fluorescence value from the untreated sample (percent error of < 20%)
- indicates a decrease in fluorescence value from the untreated sample (percent error of < 25%)

Generally the presence of different amino acid constituents, covalent bonds and hydrogen bonds help fold the protein into its shape and maintain the chemical structure of the protein (Zayas, 1997). With the application of heat, proteins undergo both physical and chemical changes which will disrupt the hydrogen bonds and affect the helical structure of the protein. The protein will therefore unravel and randomly organize itself into random chains stabilized by cross linkages (Pomeranz, 2012). This unfolding process of the protein chain will increase the exposure of the hidden amino acids (van-der-Heijden, 1999), where an increase in fluorescence is therefore expected. Hence, as depicted in Table 4.2 the increase observed with both thermal incubation and mechanochemical activation is in agreement with literature.

The prolonged exposure of gelatin to heat at 55°C caused an increase in fluorescence as a function of treatment time, where the increase in fluorescence correlates to the increase in available amino groups present in the protein molecule. The overall fluorescence values measured depicts the net effect of protein hydrolysis, aggregation or cross-linking as previously shown in Figure 4.1. The

increase in fluorescence between time zero and 48 hours, is due to the change in chemical structure of the protein. It is evident that the hydrolysis reaction is overwhelming the aggregation and cross-linking reactions within the initial 48 hours. The 72 hours of thermal treatment resulted in greater aggregation of the protein molecule and was manifested by the drop in fluorescence between 48 and 72 hours, where a further decrease may indicate cross-linking involving lysine. This decrease in fluorescence can also be caused by prolonged heating, which causes thermal decomposition of the constituent amino acids. A report by van-der-Heijden (1999) indicated that lysine and cysteine are the most vulnerable amino acids, and they release both hydrogen sulfide and ammonia upon decomposition and increase the likely formation of isopeptide bonds through the cross-linking between the amino group of lysine and the carboxyl group of aspartic acid, glutamic acid or degraded cysteine residues (van-der-Heijden, 1999). This suggests that with 72 hours of prolonged heating, the ε-amino group of the lysine residues crosslinked with adjacent amino groups showing a reduction in the available amino groups. Therefore, the drop in fluorescence demonstrates the increased occurrence of aggregation and cross-linking reactions and to a lesser extent protein hydrolysis.

The mechanochemical treatment of gelatin in the absence of solvent showed an increase in fluorescence over the reaction time studied. An increase of 31.4 was observed with 30 minutes of reaction. This dry treatment, induces particle size refinement where hydrolysis can increase the number of available amino groups. In addition to the effect of heat on the gelatin structure, increase in amino groups demonstrated by the mechanochemical treatment can be mainly attributed to its milling effect. The repeated collisions between the protein and stainless steel ball will result in the breakdown of the gelatin molecule into smaller fragments. As a function of treatment time, it is expected that the α-chain of the gelatin molecule will uncoil or breakdown with the disruption of hydrogen bonds (Stolle, 2014), whereby it will expose more ε-amino group of lysine. As a result of the heat produced and the mechanochemical effect, the mechanochemical method causes greater denaturation of the protein. It is evident that more protein hydrolysis through particle refinement is observed than protein aggregation or formation of cross-links as indicated by the net increase in fluorescence.

Contrary to the thermal and mechanochemical treatments, the opposite effect on the fluorescence can be seen in the ultrasonically treated samples. The fluorescence values decrease within the first 10 minutes of the reaction, and then increase after 15 minutes of ultrasonic treatment. This shows that the sonication treatment imposes more reversible changes in the protein structure, where the folding or unfolding of the protein is not stabilized by hydrogen bonds as rapidly as the thermal or mechanochemical means. In addition, the high sheer forces generated by the cavitation phenomenon which underlies the sonication treatment will cause the unfolding of the protein and increase aggregation when exposed to ultrasonic treatment (Mason & Peters, 2002). The physical shearing will disrupt the fold of the protein structure, but will leave the secondary structure intact and unaffected, which will enhance both intermolecular interactions and aggregation (Carrion-Vazquez et al., 2000). Studies (Stathopulos et al., 2004; Zisu et al., 2011) have shown that upon ultrasonic treatment for even less than 2 minutes, many proteins including BSA, lysozyme and myoglobin can undergo formation of aggregates in a similar fashion to amyloid peptides. These studies have shown that the formation of these aggregates can accelerate the formation of additional aggregated when exposed to heating (Stathopulos et al., 2004; Zisu et al., 2011). These observations can perhaps illustrate the reason as to why a decrease in fluorescence is observed in the initial reaction time of 5 and 10 minutes. The overall net decrease in fluorescence can be attributed to the enhanced formation of aggregates, where this effect is more pronounced with increased sonication time as seen from 5 to 10 minutes of treatment as shown in Table 4.2. On the contrary, with greater exposure time (15 min) to ultrasound treatment, an increased disruption of hydrogen bonds and non-covalent forces including hydrophobic and electrostatic interactions is also observed (O'Sullivan et al., 2016) that can explain the increase in fluorescence after 15 min. This increased effect of the cavitation phenomenon can alter the protein structure despite the aggregation, implying the greater competition between hydrolysis and aggregation reactions

## 4.3 Thermal, Sonochemical and Mechanochemical Glycation of Gelatin with Glucose

During glycation, the lysyl group of the protein is substituted with a reducing sugars. This phenomena is regarded as a definitive loss of the lysyl groups (Cayot & Tainturier, 1997). Therefore the monitoring of the percent available or unavailable amino groups, can be an indicator of protein glycation (Ajandouz et al., 2001; Brands & van Boekel, 2002; Cayot & Tainturier, 1997;

Chevalier et al., 2001; Oliver et al., 2006; Yaylayan et al., 1992). Likewise, as the reaction progresses with the consumption of sugar, verifying the amount of remaining sugar as a function of reaction time will aid in assessing the reactivity of the sugar and validate the occurrence of glycation (Ajandouz et al., 2001; Brands & van Boekel, 2002; Kwak & Lim, 2004). To evaluate the differences of thermal, sonochemical and mechanochemical glycation, gelatin/glucose model system was investigated with respect to their ability to induce similar degree of glycation in a shorter period of time. The degree of glycation based on the reduction in available amino groups and the percent remaining sugar is presented in Table 4.3 for each method of glycation studied; thermal, sonication, and mechanochemical milling.

### 4.3.1 Changes in Free Amino Group Content

According to Table 4.3, the number of free amino groups continuously decreased during the initial reaction time, suggesting the progressive conjugation of the saccharide to the amino groups of lysine in combination with hydrolysis under all glycation methods tested. The glycation reaction occurred fastest between the initial glycation reaction times studied and slowly progressed thereafter to reach an optimum degree of glycation. Kato et al. (1993) had reported that this behavior is expected in the earlier stage of the reaction as there exist greater amount of accessible lysyl groups capable of interacting with saccharides thus facilitating the carbonyl-amino interaction. Kato also states that with progressing reaction time, the reaction is expected to reach a saturation point due to the increasing occurrence of inter and intramolecular crosslinking which result in a decrease in available amino groups and limits glycation. This was observed with the thermal glycation method.

The thermal glycation reaction showed an increase in degree of glycation over the initial reaction time, thus corresponding to a decrease in fluorescence values. As previously mentioned above, the decrease in fluorescence value in the presence of sugar can be attributed to 3 possible reasons; either protein glycation, aggregation or cross-linking. Over the initial 48 hours of thermal incubation, the net fluorescence depicts a combination of hydrolysis, protein glycation, aggregation and cross-linking. The new decrease in fluorescence however implies that the rate of glycation, aggregation or cross-linking is more predominant than the hydrolysis reaction.

Furthermore, Table 4.3 shows that the thermally induced glycation reaction had a tendency to exhibit a decrease in degree of glycation (increase in fluorescence value) subsequent to reaching an optimum glycation point after 48 hours, thus suggesting in the loss of the glycation ability of the saccharide and protein beyond that point. The degree of glycation dropped from 48.63% at 48 hours of thermal incubation to 24.73% by 72 hours. This phenomena can be attributed to the possible degradation, structural changes, protein polymerization or crosslinking induced upon prolonged reaction conditions (Chevalier et al., 2002; Jiménez-Castaño et al., 2005; Oliyai et al., 1994). After 48 hours of thermal treatment, the degree of glycation decreases thus causing an increase in fluorescence. The increase in fluorescence depicts an overall increased protein denaturation reaction causing structural modifications in addition to aggregation and cross-linking as a result of prolonged reaction times. Depending on the protein's environment, the protein will undergo changes with respect to the folding and unfolding of the protein. The process by which the misfolding of the protein occurs is known as protein aggregation. Protein cross-linking on the other hand is regarded as the formation of the covalent bonds between the polypeptide chains of the protein molecule. Given that cross-linking is an inevitable reaction in a protein/saccharide reaction, many authors has attempted to characterize these cross-links and have found lysine dimer, methylglyoxal lysine dimer, methylglyoxal lysine arginine dimer, glyoxal lysine dimer, and glyoxal lysine arginine dimer, amongst others to be some of the common cross-links formed during the glycation reaction (Biemel et al., 2001; Nagaraj, Shipanova, & Faust, 1996). The authors further state that dicarbonyls such as methylglyoxal or glyoxal formed during the Maillard reaction and degradation (glycoxidation or autoxidation) of the sugar used, react with the available lysine and arginine residues favouring the polymerization of the protein. The increase in fluorescence suggests the occurrence of the combined effect of protein aggregation, cross-linking, and hydrolysis, and to a lesser extent the protein glycation.

Contrary to the observations seen with the thermal treatment, both ultrasonic and mechanochemically induced glycation showed an increasing glycation ability over the reaction times studied. The enhanced glycation reaction seen by the ultrasonic method can result from the increase in cavitational intensity and cavitational activity observed in the ultrasonic reactions. This phenomenon will generate greater cavitational active volume, where the collapse temperature and pressure will increase simultaneously causing enhanced cavitational effects (Sutkar & Gogate,

2009). This will impart structural changes which may expose greater amino groups thus favoring protein glycation and hydrolysis. Similarly the mechanochemical milling of the gelatin and glucose showed increasing glycation ability with reaction time as shown in Table 4.3. The maximum glycation point was not determined, however with 30 minutes of ball-assisted milling, a degree of glycation of 36.09% was attained. As mentioned previously, the changes in the protein structure induced by the milling effect such as hydrolysis will result in greater exposure of hidden amino residue thus allowing greater extent of glycation to take place. Many studies pertaining to protein/saccharide conjugation are in agreement with the glycation pattern observed with gelatin and glucose conjugation as seen by the ultrasonic and mechanochemical methods. In addition those studies have shown that the degree of glycation, extent of degradation and stability of the saccharide conjugated is highly dependent on the incubation time (Achouri et al., 2005; Niu et al., 2011; Seo et al., 2013; ter Haar et al., 2011; Zhang et al., 2015).

Table 4.3. Comparison of the degree of thermal, sonochemical and mechanochemical glycation and the corresponding remaining unreacted glucose as % of initial sugar content

| **Method of Glycation** | **Incubation Time** | **Degree of Glycation (%)** | **Residual Sugar Content[a] (%)** | |
|---|---|---|---|---|
| | | | **Fluorescamine assay** | **DNS assay** |
| Thermal[b] | 0 h | 0 | 100 | 98.28±0.91 |
| | 24h | 34.54±4.91 | 81.80±2.32 | 81.47±5.04 |
| | 48h | 48.63±1.61 | 76.00±2.63 | 68.54±2.37 |
| | 72h | 24.73±4.83 | 87.14±3.26 | 94.37±2.21 |
| Sonochemical[c] | 0 min | 0 | 100 | 98.60±1.11 |
| | 5 min | 15.36±1.71 | 91.70±0.84 | 83.80±5.88 |
| | 10 min | 27.91±3.44 | 84.93±1.69 | 81.94±4.46 |
| | 15 min | 35.42±2.35 | 80.87±2.84 | 74.31±3.63 |
| Mechanochemical[c] | 0 min | 0 | 100 | 98.39±1.38 |
| | 10 min | 19.64±1.45 | 90.01±1.99 | 88.41±3.24 |
| | 20 min | 28.12±0.99 | 86.34±1.31 | 72.91±0.99 |
| | 30 min | 36.01±2.68 | 84.72±1.56 | 69.21±2.68 |

[a]Residual sugar content determined based on initial sugar content
[b]Experimental results are mean±standard deviation of 9 replicates
[c]Experimental results are mean±standard deviation of 6 replicates

### 4.3.2 Monitoring Changes in Residual Glucose Content

In addition to the degree of glycation, Table 4.3 depicts the percent unreacted glucose quantified by the fluorescamine assay and DNS assay respectively for the thermally, ultrasonically and mechanochemically glycated gelatin with respect to the treatment time. While the fluorescamine assay is used to quantify the degree of glycation based on the reduction in available amino groups, the DNS assay is a more direct method for assessing the residual sugar content. The DNS assay is specific to reducing sugars, as the reaction is based on the conversion of dinitrosalicylic acid in the presence of reducing sugars to 3-amino-5-nitrosalicylic acid. The indirect fluorescamine method relies on the degree of glycation quantified by the fluorescamine assay, from which the moles of lysine substituted with reducing sugar can be calculated. As the moles of lysine conjugated with sugar is equivalent to the moles of glucose consumed, using the moles of glucose initially reacted and the moles of sugar consumed, the expected unreacted sugar content can be determined. Comparison of the percent remaining sugar based on the indirect fluorescamine method and direct DNS assay method will provide an understanding of the use of these methods for monitoring the relative extent of glycation. The residual sugar content is expected to decrease with increasing glycation, as the reducing sugars will react with the available amino groups. Since DNS assay is not specific to the structure of glucose and can produce positive results from interaction with any reactive sugar fragments resulting from glucose degradation, the results obtained at longer thermal incubation times should be viewed as an overestimation.

The calculation of the remaining sugar content based on the DNS assay was carried out by generating a standard curve for the saccharide of interest; glucose. Figure 4.5 shows the standard curve obtained for glucose at 5 different concentrations using the DNS assay. The standard curve was linear over a concentration ranging between 0.001g/ml and 0.003g/ml as shown below. The equation of the line was used to determine the concentration of glucose present in the reaction mixture, from which the residual sugar content was calculated.

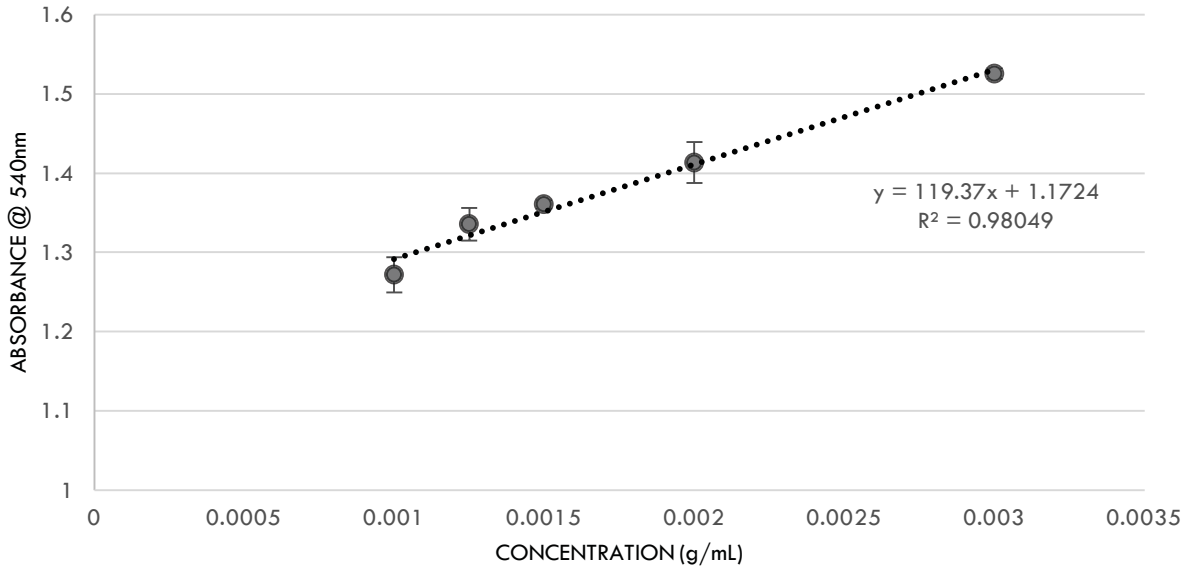


Figure 4.5. Standard curve of glucose using DNS assay

A progressively noticeable decrease in sugar content is observed for the thermally, ultrasonically and mechanochemically induced glycation methods by both the fluorescamine and DNS assays. The remaining sugar content corresponds significantly with the increase or decrease in degree of glycation, as shown in Figure 4.4, Figure 4.6, and Figure 4.7 for the respective methods. These figures show the increase in degree of glycation with the respective changes in residual sugar content. As mentioned in the previous section, the thermal incubation showed an increase in glycation over the initial 48 hours, followed by a decrease at 72 hours. The remaining sugar content depicted in Table 4.3 corresponds well to the changes in glycation extent. As the degree of glycation increases, the residual sugar content decreases. Similarly, as degree of glycation decreases with protein denaturation, the unreacted sugar content increases. This either suggests release of the attached glucose moieties from the lysyl group therefore explaining the decrease in glycation and increase in sugar as a result of increased protein denaturation occurring over prolonged heating or the prolonged heating resulting in the generation of aldehyde groups through denaturation of the protein and degradation of the sugar moiety. The prolonged exposure of the sugar in aqueous environment can result in the formation of reactive sugar components causing an unbalance in the DNS test. The results therefore show an over estimation of the remaining residual

glucose content. The continuous decrease in residual glucose content shown for the mechanochemical and sonochemical reactions depicts the progressive glycation reaction occurring between gelatin and glucose. Despite the presence of hydrolysis and other side reactions, the decrease in sugar content is evidence of the occurrence of protein glycation.

Between the two methods used to quantify the remaining sugar content, there exist slight variation as noted in Table 4.3. The fluorescamine based method shows higher unreacted sugar content than quantified by the DNS method. There exists a variation of up to 18.5% between the two methods. The variation is however within a 10% range for the thermal and sonochemical glycation reactions while mechanochemical glycation reaction showed a discrepancy relative to the upper-hand of the variation range of 18.5%. This greater difference is expected between the two methods as the DNS assay is representative of the actual amount of reducing sugar present in the reaction mixture, whereas the fluorescamine method indirectly determines the amount of unreacted sugar from the degree of glycation. This method does not take into consideration the possible degradation of sugar, or other possible reaction that may occur causing the loss of sugar molecules. Hence justifying the greater sugar content revealed by the fluorescamine method. The higher percent variation seen for the mechanochemical glycation can be attributed to the greater degree of degradation through the breakdown of molecules caused by the milling process.

Despite having similar degree of glycation after 24 hours of thermal reaction, 15 minutes of sonochemical treatment and 30 minutes of mechanochemical milling, the residual glucose content varied with 81.47%, 74.31%, and 69.21% respectively. The differences in residual sugar content can be attributed to two possible explanations; a) the calculated degree of glycation by the change in fluorescence values determined by the fluorescamine accounted for greater aggregation and cross-linking as opposed to protein glycation, thus resulting in reduced or blocked amino groups or b) the lower residual sugar content is a result of sugar degradation.

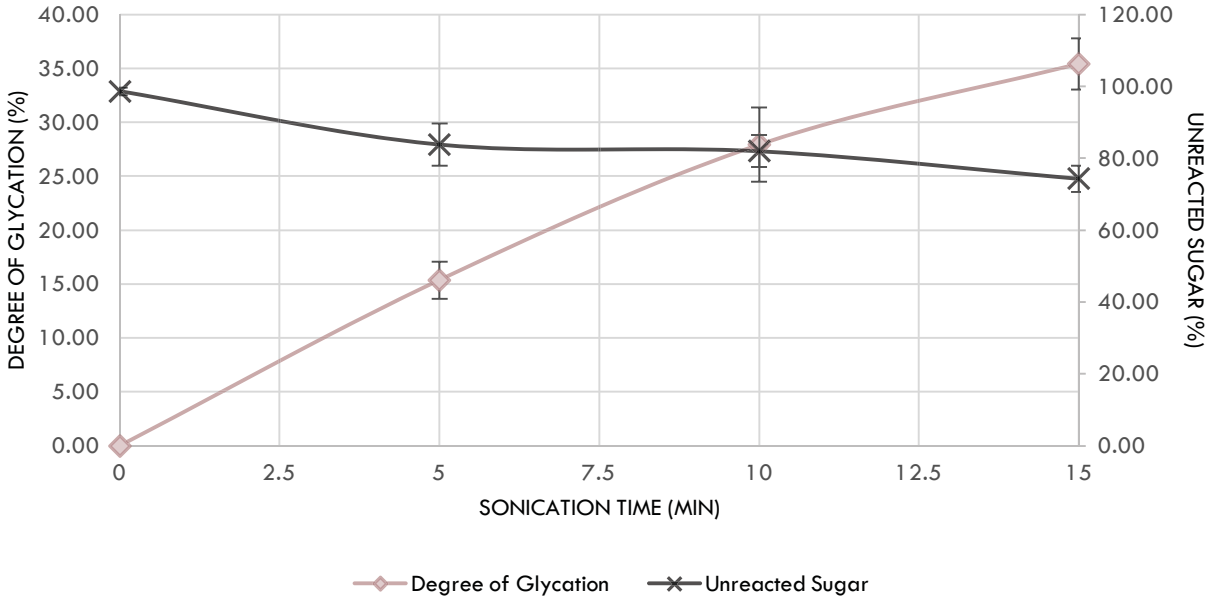


Figure 4.6. Percent of sonochemical glycation of gelatin using glucose and corresponding amount of unreacted glucose calculated using DNS assay

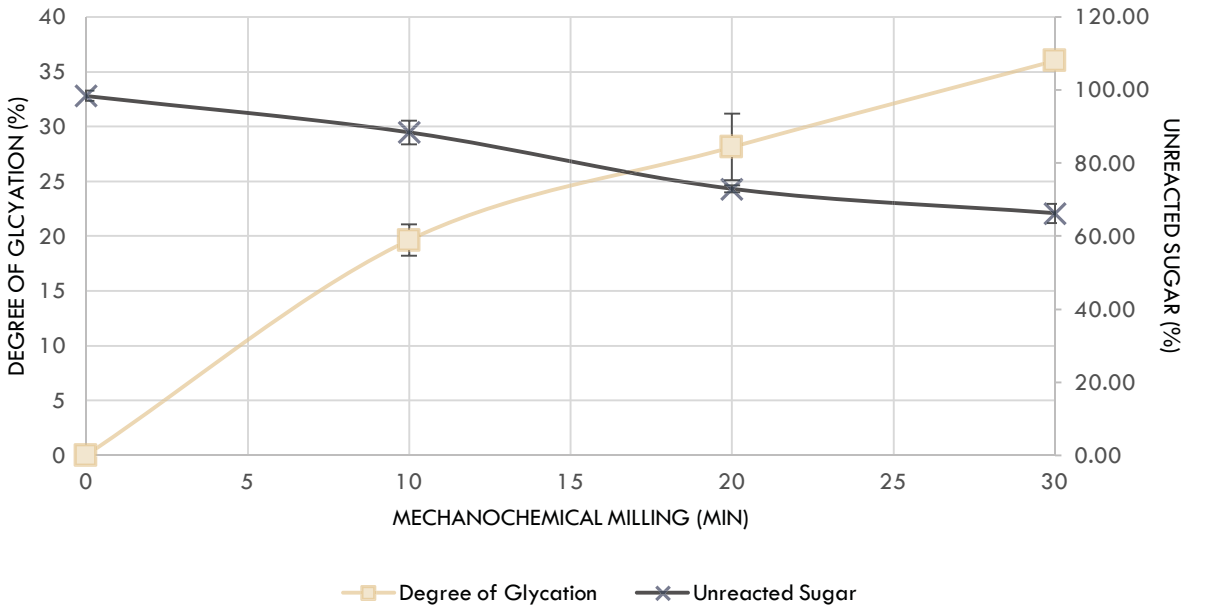


Figure 4.7. Percent of ball assisted-mechanochemical glycation of gelatin using glucose and the corresponding amount of unreacted glucose calculated using DNS assay

### 4.3.3 Overall Comparison of the Thermal, Sonochemical and Mechanochemical Glycation Methods

All the three methods investigated were found to be promising with respect to the ability to conjugate gelatin with glucose. As seen in the earlier sections, sonication and mechanochemical milling demonstrated faster glycation times; while it required 24 hours of traditional heating to obtain 34.54% glycation, 30 minutes by mechanochemical milling and 15 minutes of ultrasonic treatment was sufficient to obtain comparable degree of glycation of 36.09% and 35.42% respectively. The higher reactivity of the gelatin and glucose by the ultrasound method can be attributed to the generation of large number of cavitation bubbles which cause protein unfolding and hydrolysis of the peptide bonds upon collapse at high temperatures and pressure (Zhang, Chi, & Li, 2014). This enables greater collision between the protein and sugar hence facilitating the conjugation reaction at a much faster rate. Similarly, the short reaction time of mechanochemical glycation can be due to its dry-state reaction condition. Compared to the samples reacted under wet conditions (thermal and ultrasonic), the mechanochemically induced glycation under dry conditions was seen to be one of the effective methods of glycation. Despite the lack of studies on the use of mechanochemistry for the conjugation reaction of proteins and saccharides through the Maillard reaction, the study shows evidence that the conjugation reaction is highly possible with this method. The mechanochemical milling process is based on the rapid collision effect between the molecules and the mechanical ball, which increases friction to generate energy and heat. The resulting heat is approximately 55°C, rendering the reaction comparable to the thermal heating process. Although the reaction temperature is similar, the observations from Table 4.3 with respect to the increase in glycation and sugar loss brought upon the conclusion that mechanochemically induced glycation occurs at a faster rate than classical heating methods. Despite the higher degree of glycation reached by the thermal method, Figure 4.7 shows that the glycation curve can further increase with mechanochemical treatment time. In addition to the energy and heat generated by the mechanochemical treatment, the collision effect of two materials when subjected to mechanochemical milling poses stress upon the reactants initiating decomposition reactions. This leads to particle refinement, increase in surface area and surface energy which enable mechanical activation to take place between the reactants (Stolle et al., 2011). In addition it results in the

formation of nucleation sites that allow further reactions to occur, including physical transformations and chemical reactions (Qiu et al., 2005).

## 4.4 Stability of the Conjugates Produced by Different Methods

For the comparison of the resulting glycoconjugates formed by the different methods, the conjugates generated after 24 hours of thermal treatment, 15 minutes of sonochemical reaction and 30 minutes of mechanochemical milling were used since under these treatments comparable degree of glycation (~ 35% glycation) were achieved. In order to compare the stability of the glycated conjugates generated, the sugar content of the solutions was monitored during 24 hour of storage at room temperature (RT) through the DNS assay. Table 4.4 depicts the changes in residual sugar content observed in the samples prepared by different methods of glycation. During storage, it is expected that the conjugates show either a constant sugar content as a result of stable adduct formation or a change in sugar content indicating reversibility of the adducts.

Table 4.4. Stability of the thermal, sonochemical and mechanochemical glycoconjugates estimated by the change in % sugar content during storage as measured by DNS method

| Time of Sugar Analysis | % Residual Sugar[a] | | |
|---|---|---|---|
| | Thermal[b] | Sonochemical[b] | Mechanochemical[b,c] |
| Immediate | 81.47±5.04 | 74.31±3.63 | 69.21±2.68 |
| 1h later | 80.70±2.96 | 72.39±3.55 | 66.35±3.16 |
| 24h later | 83.45±2.37 | 86.92±4.34 | 67.67±2.26 |

[a]Residual sugar content determined based on initial sugar content
[b]Experimental results are average of triplicates±standard deviation
[c]Mechanochemical samples were reconstituted in 2 mL of water before storage

The monitoring of the residual sugar content of the thermally and mechanochemically treated samples showed slight variations in sugar content between the start of the storage and at the end. These variations however were within an experimental error of ~ ±3%. On the other hand, the values recorded for the sonochemical glycation showed statistically significant increase in the residual sugar content at the end of the storage period indicating a higher percentage of reversible Schiff base formation during glycation reaction. To better understand the effect of sonochemistry and its stability after 5, 10, 15 minutes of treatments was further analyzed (Table 4.5)

Table 4.5. Stability at room temperature storage of the sonochemically generated glycoconjugates estimated by the change in % sugar content, measured by DNS method

| Time of Sugar Analysis | % Residual Sugar[a] | | |
|---|---|---|---|
| | 5 min sonicated conjugate[b] | 10 min sonicated conjugate[b] | 15 min sonicated conjugate[b] |
| Immediate | 83.80±5.88 | 81.94±4.46 | 74.31±3.63 |
| 1h later | 84.50±0.79 | 82.54±1.18 | 72.39±3.55 |
| 24h later | 83.41±5.92 | 79.82±6.91 | 86.92±4.34 |

[a]Residual sugar content determined based on initial sugar content
[b]Experimental results are average of triplicates±standard deviation

From the previous sections it was clear that the sonochemical glycation occurs at a much faster rate relative to the traditional heating and mechanochemical milling based on the flourescamine assay. According to Table 4.5, sonochemically generated glycoconjugates after 5 and 10 min treatments show relatively good stability after 24 h of storage at room temperature based on the changes in the sugar content. In contrast to the 5 and 10 minute treated samples, the 15 minute samples showed a statistically significant increase in the unreacted sugar content when the sample was allowed to stand at room temperature (RT) for 24 hours. This suggests that with increased sonochemical treatment time, the ratio of Schiff base to Amadori is higher in the glycated protein. Although the immediate reaction favors the attachment of sugar as a result of the conformational changes induced by the cavitation phenomenon, higher proportion of Schiff base were formed especially in the last 5 min of sonication. From literature it is known that the formation of the chemically reversible Schiff base can revert back to its original products through hydrolysis (Yaylayan et al., 1992). Thus it is likely that with increased sonochemical treatment time, the attached sugar molecules to the lysine begin to detach as a result of the sonication effect. It is also possible that the structure of the protein in addition to the neighboring amino acids to the glycation site favor the catalysis of the hydrolysis reaction resulting in the release of the attached glucose. This phenomenon may explain the increase in unreacted sugar content observed in the 15 minute treated samples stored at RT.

Furthermore, in section 4.2 and the Table 4.2 the effect of sonication on the available amino groups of gelatin in the absence of sugar was shown. It was observed that 5 or 10 minute of sonochemical treatment caused a decrease in fluorescence value corresponding to the greater aggregation of the

protein than hydrolysis. However, with 15 minutes of treatment, the fluorescence decreased suggesting greater exposure of amino groups. This information may explain the differences seen in the stability of the 5, 10 and 15 minute treated conjugates. The greater denaturation and uncoiling of protein structure and the disruption of the hydrogen and covalent bonds may induce these unfavorable results with longer treatment times. Although the cavitation phenomenon overall favored the rapid glycation reaction, perhaps the reaction conditions were not highly favorable for the formation of stable conjugates. With increasing ultrasonic treatment, greater Schiff base are formed, where the reaction conditions do not allow the stabilization of the conjugate structure with hydrogen bond quick enough to prohibit the release of the sugar. Therefore, since more Schiff base and less Amadori compounds are formed, the samples treated for 15 minutes showed greater reversibility, when compared to the 5 and 10 minute conjugates.

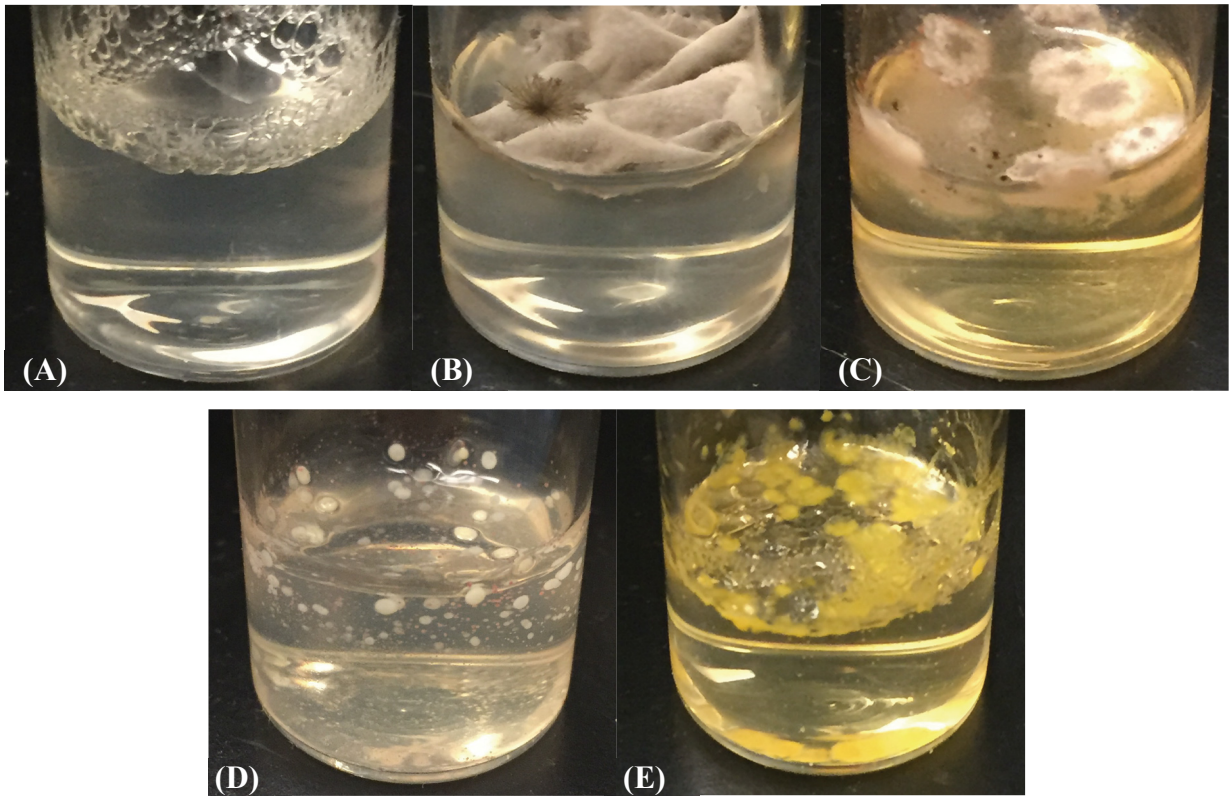


Figure 4.8. Growth of microorganisms in the 15 min ultrasonically treated samples incubated in closed vials at room temperature for up to 3 months; stored gelatin in the absence of glucose (A), stored ultrasonically treated gelatin/glucose samples (B, C, D, E)

With the release of sugar, and notably high amount of unreacted sugar, the sonicated mixture is a possible growth environment for microorganisms. Over the course of storage of the ultrasonically treated samples, at RT, growth of microorganism was observed particularly in the 15 min treated samples. Figure 4.8 depicts the growth of the microorganisms in the various ultrasonically treated samples. Figure 4.8A depicts the gelatin solution stored at RT with the absence of glucose for several months. Amongst the stored samples of thermally, ultrasonically and mechanochemically glycated conjugates in solution form, the ultrasonically treated samples showed growth of organism within a week of storage at RT. The thermal and mechanochemical treated samples showed microbial growth only after prolonged storage of the sample (> 1 month). As shown in Figure 4.8, interestingly, it was noted that amongst the ultrasonically treated samples stored at RT, different types of species were grown as depicted by B, C, D and E. The preparation of the sample at different times showed differences in terms of microbial growth, as represented by Figure 4.8 B, C, D and E. As the environment of the samples remains the same, the growth of different species was an interesting additional factor. Although the free sugar residues left after the various glycation methods can support robust bacterial growth, it is hypothesized that one of the factors promoting bacterial growth in the sonochemically treated samples, is the release of sugar as seen in Table 4.5. This perhaps enhanced the favorable environment for microbial growth, in comparison with the other methods which contained lower residual glucose content with storage at RT.

## 4.5 Functional Properties of Glycated Samples

### 4.5.1 Protein Solubility

Solubility is an important physicochemical property of proteins as it has a direct influence on other properties such as the gelling, film forming, and emulsifying ability (Sikorski, 2001). The solubility of the native and glycated gelatin samples were measured as a function of pH. It is notable that the native gelatin (control with no glycation), had greater solubility at acidic pH value of 3 in comparison to all glycated conjugates. It was least soluble closest to its isoelectric point, between pH 7 and 9 with 62.66 and 66.30% solubility respectively. The solubility of proteins generally decreases at pH values near its isoelectric point (pI); the pH at which the protein has a neutral charge. At pI, the zwitterionic form prevails, thereby allowing attractive forces to predominate. The neutral charge allows a reduction in the electrostatic repulsions, and allows

hydrophobic interactions to occur thus resulting in insolubility (Damodaran, 1997; Zayas, 1997). At pH values below or above the isoelectric point, the protein solubility is expected to increase (Whitaker, 1993; Zayas, 1997). As expected, the solubility of the native gelatin was greater at pH values above and below pH 7-9 (Figure 4.9).

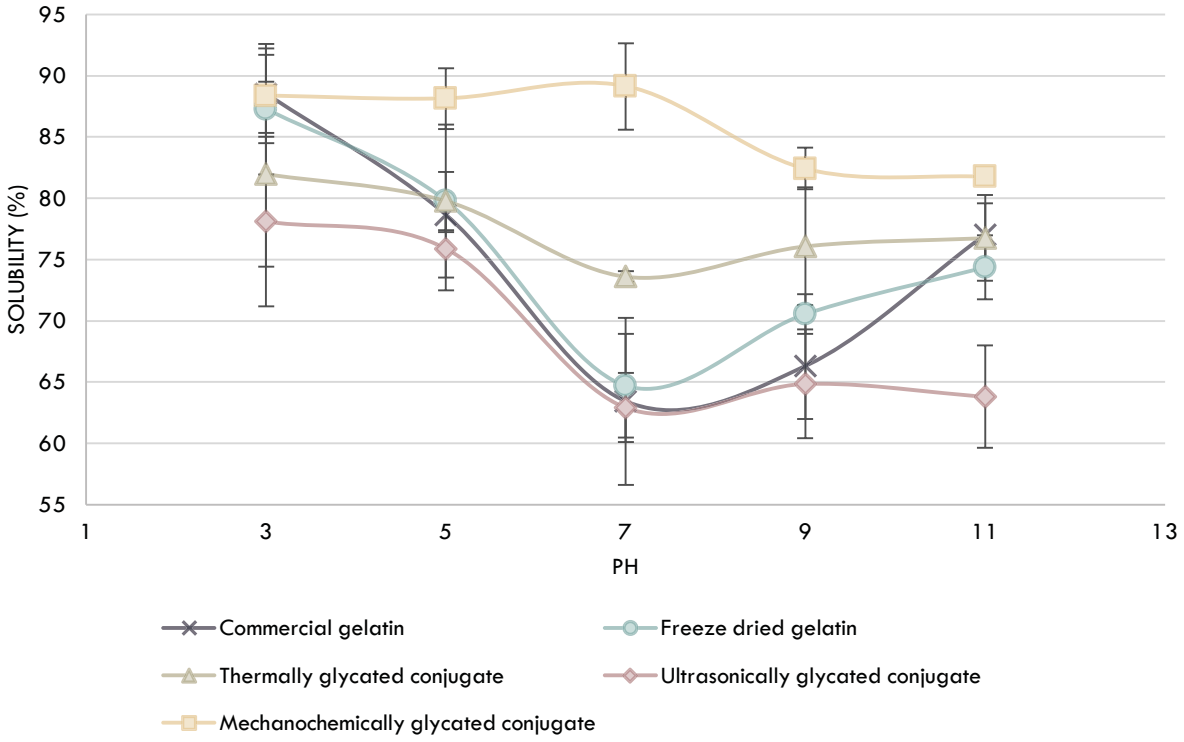


Figure 4.9. Solubility of native gelatin and gelatin/glucose conjugates as a function of pH; native gelatin (×), freeze dried gelatin (o), thermally glycated conjugate (Δ), ultrasonically glycated conjugate (◊), and mechanochemically glycated conjugate (□)

Despite the pI of gelatin being between pH 7-9 it is noted that the glycated gelatins with the exception of samples generated by sonication, did not show a similar behavior to native gelatin where solubility decreases near that pH range; instead a significant improvement in solubility at the native gelatins' isoelectric point was observed. As shown in Figure 4.9**,** substitution of the lysyl groups with reducing sugars by thermal and mechanochemical milling have increased solubility at pH range of 7-9 from 62.66-66.30% to 70.55-78.12 and 83.23-80.75% respectively. This can be due to several contributing factors such as the increased net negative charge on the protein resulting

from the conjugation of sugar, increase in hydrophilicity of the protein, decrease in exposed hydrophobicity of protein, and/or increased electrostatic repulsion between the proteins (Nakamura, Kobayashi, & Kato, 1994; Wang & Ismail, 2012).

At all pH values studied, the solubility of mechanochemically generated sample was remarkably enhanced in comparison to the thermal and ultrasonically induced glycated samples. In addition to the enhanced hydrophilic properties imparted by the conjugated saccharide moiety, mechanochemistry itself could have been an additional factor. Rojas-Oviedo et al. (2012) demonstrated an apparent improvement in solubility of indomethacin by 3 fold with the aid of mechanochemistry in the presence of lactose to increase hydrophilicity of the compound. The enhanced solubility by mechanochemistry can also be attributed to the reduction of particle size of compounds due to the breaking of cross links and hydrogen bonds due to the ball-assisted milling process (Stolle et al., 2011). The milling process can cause alterations related to reduction in particle size, change in surface area and shape of the particles (Loh et al., 2015). With decreasing particle sizes, the surface area of the particle increases, allowing greater solvent contact, solvation and interaction to occur with water molecules therefore exhibiting greater solubility. Furthermore, in previous sections it was observed that the mechanochemical treatment induces greater hydrolysis of the protein with increasing reaction time (see Table 4.2). This hydrolysis effect will favor protein solubility as well.

Although the conjugates generated by mechanochemical milling were superior in solubility, the thermally glycated conjugates showed enhancement of solubility at pH 7, 9, and 11 as well. On the other hand, ultrasonically generated conjugates showed reduced solubility at all given pH ranges in comparison with the native gelatin. The loss in solubility can be attributed to the extensive cross-linking reaction (Achouri et al., 2005) that may occur between gelatin and glucose, forming larger covalent complexes inhibiting the solubility of the conjugate. It is also highly possible that cross-links occurred between the reactive intermediates of the glucose (dicarbonyl derivatives), or the Amadori or Heyns rearrangement products resulting from the conjugation reaction (Saeki & Inoue, 1997). Damodaran (1997) states that the solubility of the protein can also be affected by the effect of pH and ionic strength on the electrostatic forces. However, monitoring the changes in pH during sonication, revealed that the pH was unaffected by the sonication

reaction. From Table 4.2 however, it was seen that the ultrasonic effect causes increased aggregation in the protein even at shorter reaction times (O'Sullivan et al., 2016). Despite the increased hydrolysis effect seen with 15 minutes of sonication, the 5 and 10 minute samples showed the overwhelming effect of aggregation relative to hydrolysis. Although 15 minutes of sonication favored hydrolysis reactions, aggregation was also seen in the net fluorescence measurements. As known from literature, aggregation will decrease protein solubility. This suggests that the decrease in protein solubility of the ultrasonically treated samples is largely due to the formation of aggregates. In addition, it was previously confirmed in section 4.4, that sonochemically generated conjugates showed high instability as a result of the formation of higher percentage of Schiff base over Amadori compounds. Thus resulting in the release of the attached sugar therefore suggesting that the degree of glycation calculated was an overestimation. Hence it is possible that the lower degree of glycation of these samples could have effected negatively on their solubility characteristics, suggesting the existence of a correlation between the extent of glycation and the functional properties imparted on the conjugate.

To ensure that the freeze drying process to which the ultrasonically glycated samples were subjected to, did not interfere with its solvation properties, native gelatin was solubilized in water at the same concentration (5% w/w gelatin solutions) and freeze dried. It is observed through Figure 4.9 that the native and freeze dried gelatin showed similar pattern in solubility over the studied pH range. Confirming that freeze drying had minimal effect on the solubility of the protein.

The solubility of modified gelatin has not been previously studied; hence the results cannot be compared to existing literature. Partially glycated whey protein hydrolysates showed enhanced solubility at pI under heated and non-heated conditions. The solubility was improved from 46.3% in native whey protein hydrolysates to 91.0% when partially glycated. Many authors studying the effect of whey protein glycation were in agreement with these results (Akhtar and Dickinson 2007; Zhu et al 2010; Wang and Ismail 2012). Likewise a study by Seo et al. (2013) showed that protein solubility was greater at the pI of lyzozyme when glycated with galactose and galactan. Solubility was increased from 49.6% to 79.3 and 82.5% respectively. The existing studies give great insight on the enhancement of protein solubility through glycation.

### 4.5.2 Effect of Sonochemical Glycation on Solubility Properties

The effect of the extent of protein glycation on solubility was assessed using the ultrasonically treated samples. Figure 4.10, shows the effect of ultrasound treatment of gelatin and glucose for 5, 10 and 15 minutes on the solubility in comparison with the commercial gelatin (untreated). As depicted in the figure, it is observed that the samples sonicated for 5 and 10 minutes show enhanced solubility relative to the commercial gelatin, while samples sonicated for 15 minutes showed reduced solubility. At the isoelectric point of pI of 7 and 9, where the commercial gelatin showed a solubility of 62.66% and 66.30%, the 5 minute sonicated samples showed 76.27% and 72.44% solubility, while the 10 minute sonicated samples showed 84.79%and 83.91% solubility at the respective isoelectric points. In fact, a remarkable increase in solubility can be noticed at all pH values tested for the samples ultrasonically treated for 5 and 10 minutes when compared to native gelatin. This shows that with increasing degree of glycation, there exists enhancement in the respective functional properties. With increased glycation, the hydrophilicity of the protein increases thus improving the affinity between the protein and water molecules. In addition, the enhanced solubility observed in the ultrasonically generated conjugates can be due to the cavitation phenomenon. The production of cavitation bubbles which collapse with increased temperature will results in the disruption of peptide bonds causing the unfolding of the protein structure. This in turn will expose the hydrophilic amino acid residues buried within the inner site of the protein structure, thus also contributing to the enhanced solubility (Morel et al., 2000).

On the contrary, the ultrasonically treated sample for 15 minutes showed unfavorable effects on the solubility of the protein. From Table 4.5 it is understood that 5 and 10 minute of ultrasonic treatment produced stable conjugates comparative to conjugates treated for 15 minutes. This instability of the conjugate may have played an effect on the solubility parameter, thus explaining the enhanced solubility observed by the conjugates with 5 and 10 minutes of treatment. Perhaps also possible that the extensive reaction caused protein aggregation which may have led to the formation of insoluble polymerization products hence bring upon a loss of solubility (Sorgentini, Wagner, & Anon, 1995).

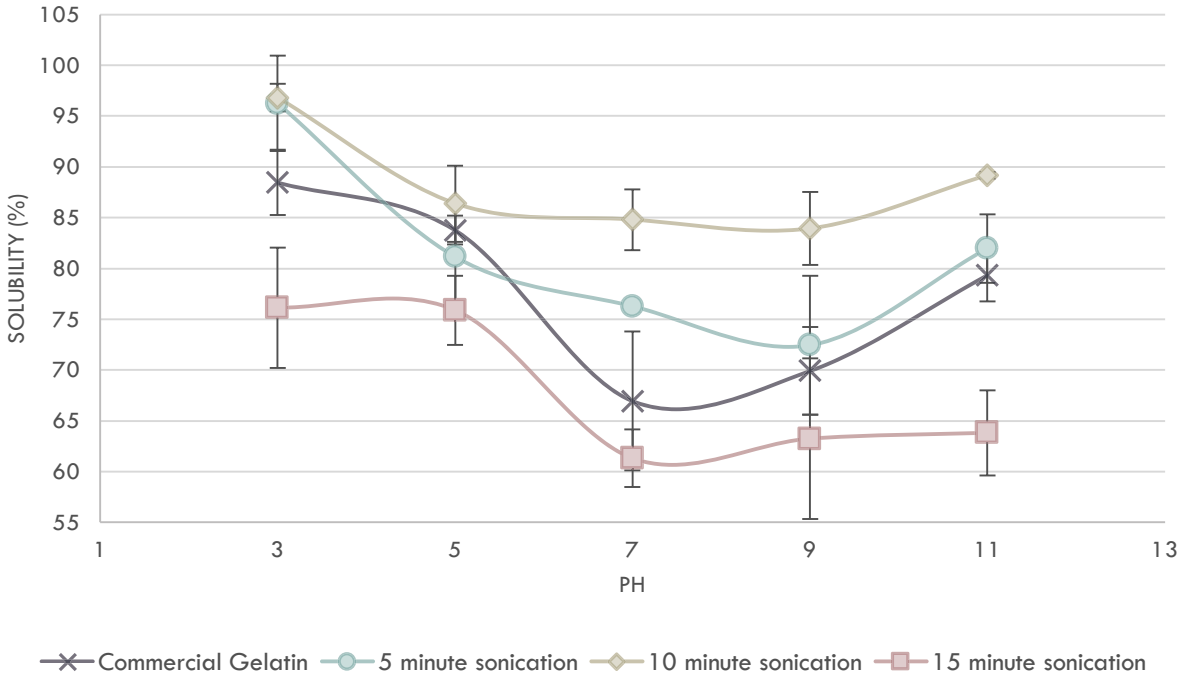


Figure 4.10. Effect of sonication time on percent solubility; commercial gelatin (×), ultrasonically derived conjugate after 5 minutes (o), 10 minutes (◊) and 15 minutes (□) of sonication

### 4.5.3 Thermal Stability

The thermal stability of gelatin and gelatin conjugates was measured over a temperature range of 50-85°C (without pH adjustment), where the turbidity of the mixture was monitored at 500 nm. A lower turbidity refers to a greater heat stability. Figure 4.11 showed the thermal stability of the conjugates formed through the different glycation methods in comparison to native gelatin. It can be seen that the native gelatin showcased a high thermal stability in comparison to the glycated gelatin conjugates. As can be seen in the figure, the thermally and ultrasonically glycated gelatin/glucose conjugates showed higher turbidity than the mechanochemically glycated conjugates. Between 50 - 75°C, the ultrasonically treated (91.8 - 95.6%) and thermally treated conjugates (92.1 - 95.1%) have a higher turbidity compared to the native gelatin (88.9 - 92.3%). Although the relative turbidity is lower in the native gelatin, the difference is small. The results in general indicate the low thermal stability of the gelatin/glucose conjugates produced by thermal and ultrasonic means. It is possible that conformational changes induced as a result of the sugar

conjugation, resulted in a reduction in α-helical content. This will cause increased protein aggregation indicative of the high percentages of turbidity (Shu et al., 1996; Takahashi et al., 2000).

Contrary to the ultrasonically and thermally glycated samples, the mechanochemically glycated samples showed lower turbidity between 50-80°C. Its thermal stability was more comparable to that of the unmodified gelatin, which remained relatively constant at the temperatures measured. The lower turbidity and higher stability can be attributed to the conjugation effect of glucose to the gelatin molecule. From literature it is found that the addition of the saccharide to a protein allows protein stabilization in such a way that it protects the protein from aggregation and protein-protein interactions (Kato, 2002). The lesser formation of aggregates therefore show lesser turbidity compared to the other studied methods. From above sections it was also noted that the prolonged heating and sonochemical treatment favored greater formation of aggregates, thus explaining the enhanced thermal properties seen in the mechanochemical conjugates. Furthermore, although the mechanochemical conjugates showed lower turbidity than native gelatin at lower temperatures, at higher temperatures the conjugate showed higher turbidity indicating low thermal stability.

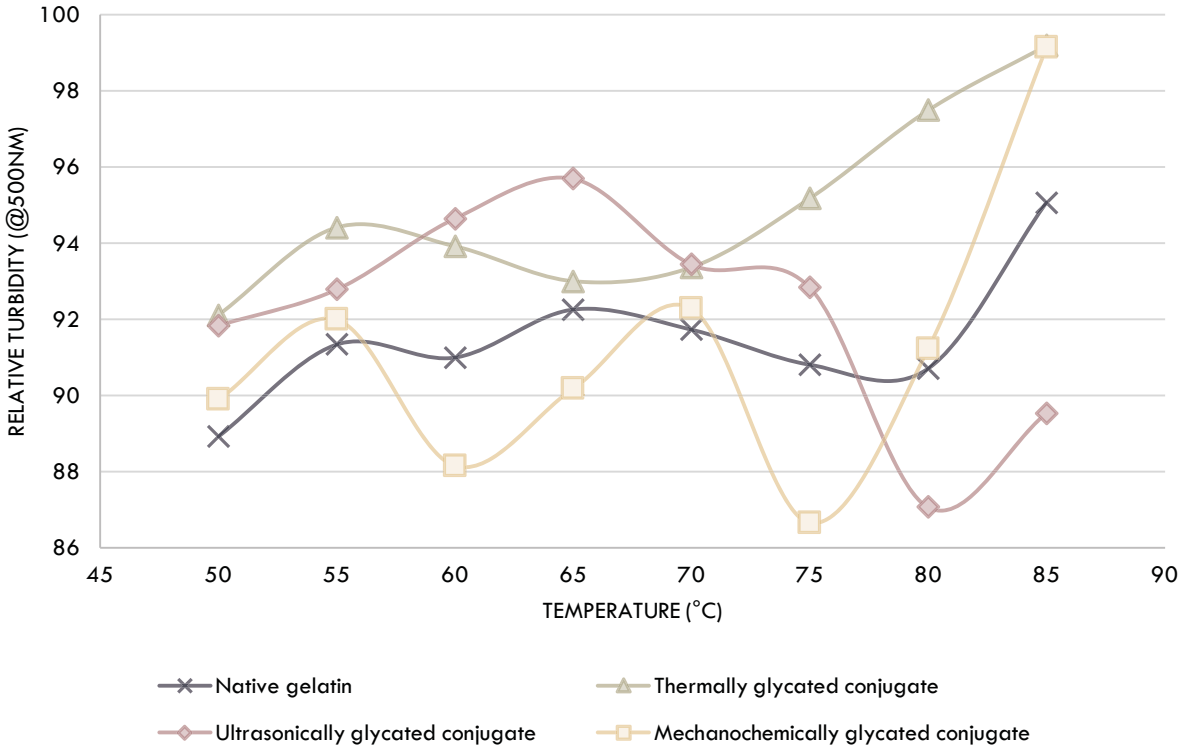


Figure 4.11. Effect of gelatin glycation on its thermal stability. Experimental results are average of triplicates. Error bars removed for clarity (Percent error < 6%)

In contrast to the thermally (97.48 - 100%) and mechanochemically (91.23 - 99.13%) glycated conjugates, the ultrasonically glycated samples showed a smaller increase (87.1 - 89.5%) in turbidity at higher temperatures (between 80 - 85°C). The greater turbidity of the glycated sample at higher temperatures can be due to the sugar moieties causing limited aggregation and reduced protein-protein interaction at high temperatures by reducing the hydrophobicity of the protein (Shu et al., 1996).

### 4.5.4 Emulsifying Properties

The emulsifying activity index (EAI) and the emulsion stability index (ESI) of the native and glycated gelatin conjugates are presented in Table 4.6. The EAI which is expressed as the area of oil-water interface per unit of protein was found to be the greatest in the native gelatin. The EAI values obtained for the samples treated by different means (thermal, ultrasonic, mechanochemical)

remained relatively similar to one another, varying between 56.01 and 58.22 $m^2/g$. The ESI, which is expressed as the time required to achieve a turbidity of an emulsion that is half of its original value was highest in the mechanochemically glycated samples.

Protein solubility plays an important factor contributing to a protein's emulsifying properties. With increasing protein solubility, it is expected to see an improvement in emulsion stabilization (Li, Huang, et al., 2014) . Although it is not necessary to obtain 100% solubility of a protein, solubility of the protein is necessary as emulsion stability is dependent on the favorable interactions between the oil-water interface (Fennema, 1996). Partially dissolved protein will not be cable of exhibiting high emulsifying properties as they will not be able to migrate to the oil-water interface (Zayas, 1997). The protein must be soluble to be able to diffuse and concentrate at the oil-water interface. The greater ESI demonstrated by the mechanochemical conjugate can be attributed to its high solvation properties compared to the thermal and ultrasonic conjugates, as indicated in section 4.5.1. In theory, a low particle size will help obtain good emulsifying properties, as the increased surface area of the particles will allow greater solvent contact (McClements, 1998). Mechanochemical milling is a method known to physically breakdown the particles into finer ones through mechanical energy (Loh et al., 2015). Therefore the reduced particle size of the milled protein exhibits greater solubility, thus contributing to greater emulsifying properties. It is well known that a protein/saccharide conjugate holds the combined characteristic of saccharides which dissolve into the aqueous phase medium and the proteins which adsorb to the oil-water interface (Dickinson & Izgi, 1996). The combined effect was well established by the mechanochemically derived conjugates, where its improved solubility denotes a major contribution.

Table 4.6. Emulsifying properties (EAI and ESI) of native gelatin and gelatin/glucose conjugates obtained by different glycation methods

| | **ESI (min)[a]** | **EAI ($m^2/g$)[a]** |
|---|---|---|
| Commercial gelatin | 35.83 ± 4.09 | 58.22 ± 5.22 |
| Freeze dried gelatin | 32.80 ± 5.58 | 58.13 ± 6.03 |
| Thermally glycated conjugate | 36.14 ± 3.33 | 56.99 ± 4.53 |
| Ultrasonically glycated conjugate | 31.26 ± 1.48 | 56.25 ± 5.16 |
| Mechanochemically glycated conjugate | 42.54 ± 2.85 | 56.01 ± 4.17 |

[a]Experimental result are average of triplicates±standard deviation

In relation to emulsifying properties, it can be noted that the thermally and ultrasonically glycated conjugates did not exhibit the greatest emulsifying properties with respect to the native gelatin. The emulsifying properties of glycoconjugates are known to be dependent on reaction time, however this phenomenon is protein specific. Some proteins have shown increasing emulsifying properties with increasing glycation, while others obtain a maximum ESI and EAI at specific extent of glycation. Extensive degree of glycation has been reported to reduce the emulsifying properties (Oliver et al., 2006). Similar to studies by Kato et al. (1993), Nakamura, Kato, and Kobayashi (1991) showed negative impacts on emulsifying properties after a certain period of glycation. The thermally glycated gelatin/glucose conjugates did not show any significant improvement in ESI (36.14 min) compared to native gelatin (35.83 min), while the ultrasonically glycated conjugates showed reduced ESI (31.26 min). The positive or negative impacts can be related to either the extent of glycation or perhaps the degradation of the protein. In this case however, the reduced emulsifying properties of the sonicated conjugate can be attributed to its low solubility properties seen in earlier sections, due to the formation of aggregates by the ultrasound treatment, which limits protein solubility.

Although we associate the improvement in ESI or EAI to the conjugation of sugars to the protein, Xue et al. (2013) raise an interesting observation through their study. After the conjugation reaction, alongside conjugates, there exist un-reacted protein and saccharides. This mix or perhaps ratio of the mix can result in the formation of a mixed protein/sugar film at the oil-water interface responsible for the enhancement in the emulsifying properties. Therefore, the emulsifying ability of the glycated conjugates cannot be attributed to the degree of glycation or the conjugate formed alone, but also to the presence of unreacted protein and saccharide that may work synergistically to contribute to these favorable emulsifying properties. Comparing the degree of glycation and percent residual sugar for the conjugates studied can help establish a correlation with respect to the ratio beneficial for obtaining enhanced emulsifying properties. Table 4.3 shows the degree of glycation obtained along with the residual sugar content for each method studied. As all glycated samples used for studying the functional properties contained a relatively similar degree of glycation (~35%), this did not influence the emulsifying properties. The noticeable difference between the conjugates used, was with respect to the residual sugar content. The mechanochemical conjugates which expressed greater emulsifying properties had lower residual sugar content

(69.21%) compared to the thermal (81.47%) and ultrasonically treated samples (74.31%) as seen in Table 4.3. The results therefore show that the lower residual sugar content presents favorable emulsifying properties. In addition, despite the protein having a similar degree of glycation as estimated from the fluorescamine assay, the decrease in fluorescence may have been due to cross-links and aggregations, therefore there exists discrepancies in the values presented and hence accurate conclusions cannot be drawn with respect to the ratio of conjugate on the emulsifying properties. This observation is confirmed through the varying residual sugar content despite the assumed similar glycation efficiency from the fluorescamine assay. The greater residual sugar content implies either the lower degree of glycation or greater presence of aggregates or cross-links in the protein matrix, which affects the emulsifying properties.

### 4.5.5 Effect of Sonochemical Glycation on Emulsifying Properties

To further study the reasoning behind the low emulsifying properties of the sonochemically generated conjugates seen above, the 5, 10 and 15 minute sonicated conjugates were evaluated for their emulsifying properties. Table 4.7 shows the emulsifying stability index (ESI) and emulsifying activity index (EAI) of the sonicated samples at various reaction times. The native gelatin samples, without any treatment has an ESI and EAI of 35.83 and 58.22 as shown in Table 4.6. The sonication of gelatin/glucose samples at different ultrasonic treatment times showed an increase in the native gelatin's ESI of 35.83 to 44.75, 50.58 and 35.58 min respectively at 5, 10 and 15 minutes of ultrasonic treatments. The EAI remained relatively the same between the three ultrasonic treatment times ranging between 55.19 - 56.30 $m^2/g$. It can be observed that with increased glycation, the emulsion stability and emulsion activity increased as well. The ESI values were greater in the 5 and 10 minute treated samples in comparison with the sample treated for 15 minutes. It is observed that the ESI decreases significantly from 44.75 and 50.58 min at 5 and 10 min treatments to 31.26 after 15 minute of ultrasonic treatment. It is believed that the adsorbability of the protein in combination with the hydrophilicity of the saccharide moiety will generate a strong solvated layer at the oil-water interface thus aid in conferring steric stabilization for the oil droplets (Nakamura et al., 1991). This implies that the solubility of the protein plays a vital role in the emulsifying properties. As seen in the solubility section (4.5.1), the 15 minute treatment resulted in reduced solubility and was likely due to extensive crosslinking formation, aggregation or unstable reaction

conditions induced by ultrasonic glycation. The solubility of the 15 minute conjugates was much less than the 5 and 10 minute conjugates hence explained the observed results.

Table 4.7. Effect of sonication time and the degree of glycation on the resulting gelatin/glucose conjugates and its emulsifying properties

| Sonication time (min) | Degree of Glycation (%) | ESI (min)[a] | EAI ($m^2/g$)[a] |
|---|---|---|---|
| 5 | 15.36±1.71 | 44.75 ± 1.02 | 55.19 ± 0.78 |
| 10 | 27.91±3.44 | 50.58 ± 2.80 | 56.20 ± 0.91 |
| 15 | 35.42±2.35 | 31.26 ± 1.48 | 56.25 ± 5.16 |

[a] The experimental results are averages of triplicates

The ultrasonic treatment in general is expected to improve emulsifying ability as the treatment induces changes in conformation and structure of the protein. In addition, the partial denaturation of the protein gives rise to a disordered structure that can better adsorb to the oil-water interface (Jambrak et al., 2008). Guzey and Weiss (2001) found an improvement in the emulsifying properties of whey protein isolates after high intensity ultrasonic treatment. Similarly Jambrak et al. (2008) found that the ultrasonic treatment of soy proteins allowed unfolding of the 7S and 11S fractions of the soy protein and allowed aggregation of the proteins thus allowing enhanced emulsifying properties. In agreement with these studies, ultrasonic treatment of gelatin at specific times were found to improve emulsifying properties. The degree of glycation and stability of the reaction play an important role on the respective functional properties.

### 4.5.6 Foaming Properties

The foaming properties of the native and glycated gelatin is presented in Table 4.8. Foaming ability of a protein is related to the capacity of the protein to reduce surface tension at the air-liquid interface, while foam stability refers to stabilizing the air-liquid interface (Zayas, 1997). The native and glycated gelatins were capable of producing foam when vortexed to allow the incorporation of air. The foam capacity ($F_{max}$) of the native and glycated gelatins were determined and were found to be relatively similar in volume for all samples studied, ranging between 200-250$\mu$L of foam per $\mu$L of protein. Despite the similar foam capacity, the foam stability varied amongst the samples. The glycated samples showed higher foam stability in comparison to the

native protein. To ensure the freeze drying process of the wet-Maillard reaction (thermal and ultrasonic) did not interfere with the functional properties of the conjugates, the foaming properties of the freeze dried gelatin was also determined as a control. Table 4.8 shows similar foam volumes recorded as a function of time, for both native and freeze dried gelatin. This indicated that the freeze drying process had no effect on the foaming properties.

Table 4.8. Foaming properties of the native and glycated gelatins

| | Volume of foam generated at specific times ($\mu$L of foam/ $\mu$L of protein) | | | Foam Stability[b] (hour) |
|---|---|---|---|---|
| | Immediate ($F_{max}$)[a,b] | 1 minute[b] | 24 hours[b] | |
| **Native Gelatin** | 250 | 200 | 100 | 40.0 |
| **Freeze Dried gelatin** | 240 | 200 | 100 | 41.1 |
| **Thermally glycated** | 200 | 160 | 100 | 48.0 |
| **Ultrasonically glycated** | 200 | 200 | 160 | 120.0 |
| **Mechanochemically glycated** | 250 | 220 | 200 | 120.0 |

[a]The immediate foam volume corresponds to the foam capacity of the protein or conjugate
[b]Experimental results are average of duplicates with a standard deviation of < 5%

Generally, the addition of a sugar moiety to a protein solution is found to weaken foaming ability but in turn increases foam stability of the protein (Fennema, 1996). Such was found to be in agreement with the obtained result where the foaming capacity of the glycated conjugates were similar in relation to the native gelatin, while the foaming stability was enhanced with sugar conjugation. Amongst the glycation methods studied, the foaming stability was greatest in the ultrasonically and mechanochemically produced conjugates, while the thermally produced conjugates showed similar values to the native gelatin.

Several authors have stated that the ultrasonically induced glycation can improve foaming properties of the conjugates. Martínez et al. (2007) reported that the modifications of the protein structure as a result of ultrasonic treatment leads to increased conformational flexibility thus allowing significant improved foaming properties. Similarly Jambrak et al. (2008) reported that mechanical homogenization process induced by sonication tends to increase the foaming power. They further explain that the ultrasound treatment causes partial unfolding of the protein consequently exposing more hydrophobic moieties. The hydrophobic regions are important in

foaming ability as they are crucial for adsorption at the air-liquid interface. Thus explaining the improved foaming properties by the ultrasonically treated conjugates.

As mentioned, the function of the protein in foam formation is to decrease interfacial and surface tension at the air-liquid interface by adsorbing to the interface. To do so, solubility of the protein is important. The greater foaming stability imparted by the mechanochemically glycated conjugates compared to the native protein can be attributed to its higher solubility. The greater is the solubility of a protein, the greater is its ability to reduce the surface tension and form a strong film at the air-water interface (Zayas, 1997). In addition, the attachment of glucose molecule to the gelatin perhaps induced favorable structural changes such as exposing greater hydrophobicity and inducing protein unfolding which helped adsorb better at the air-water interface during bubbling (Jambrak et al., 2008). Sugars are expected to add viscosity and help the protein structure stay intact and avoid unfolding when adsorbed to the air-liquid interface (Fennema, 1996). Although this enhances the stability, it reduces the protein's ability to produce large foam volumes. The method of glycation however could change the glycation sites, change the configuration of the molecule amongst other aspect rendering one method superior to the another in relation to foam stability, thus explaining the lower foam stability imparted by the thermally glycated conjugates.

### 4.5.7 Film Forming Properties

With increasing concerns regarding the solid waste aspect of food packaging, biodegradable and edible food packages are of high interest (Schmitt et al., 1998). Packaging specialist have proposed and shown interest in the use of biopolymers including proteins and polysaccharides. The combined use of proteins and polysaccharides in the form of glycated proteins can perhaps serve for such purposes.

The Maillard reaction induced by thermal, ultrasonic and mechanochemical means was used to modify the properties of gelatin-based films and study the differences imparted in film properties as a result of the conjugation under different conditions. The mechanical strength and flexibility of the film are highly important properties especially in regards to maintaining the integrity of a packaged product. For such reasons the mechanical properties of the films were tested. Figure 4.12

shows the elongation at break (EB) and tensile strength (TS) of the films obtained with native and glycated gelatin. The thickness of the films were measured and ranged between 0.010 to 0.030mm, corresponding to no significant difference amongst the film thicknesses. The method of glycation or the conjugation of sugar to the protein did not contribute to the enhancement of the EB values of the films generated. On the contrary, significant differences were observed in the tensile strength values (TS) of the 4 film samples developed as shown in Figure 4.12. The TS was lowest in the thermally glycated films (16.94±1.81) and highest in the films produced mechanochemically (39.73±0.66). The TS of the native and ultrasonically generated films were similar with values ranging between 20.62±0.32 and 23.42±0.35 respectively. It is noted that with the incorporation of glucose, the elongation at break of the films remained the same while the tensile strength increased, suggesting that the films were as stretchable as the control films, however exhibited greater strength.

The greater TS exhibited by the glycated gelatin films, specifically the mechanochemically glycated samples can be due mainly to the structural changes induced by the glycation reaction. The mechanochemical conjugate based films showed nearly a two-fold increase in the TS relative to the native gelatin based films. The conjugation of glucose with the gelatin evidently posed changes in the three dimensional structure of the protein. As a result of increased cross linking and increased exposure of hydrophobic and sulfhydryl groups, greater hydrophobic interactions and formation of disulfide linkages will be observed during the drying step of the film preparation (solvent evaporation) (Lin et al., 2015; Liu, Tellez-Garay, & Castell-Perez, 2004). This alteration in structure will contribute to the formation of a stronger protein network hence improve the strength of the films. Although similar observations are expected from the thermal and ultrasonically conjugated films, it was noted that the TS values of those respective conjugates were closer to that of the native gelatin based films.

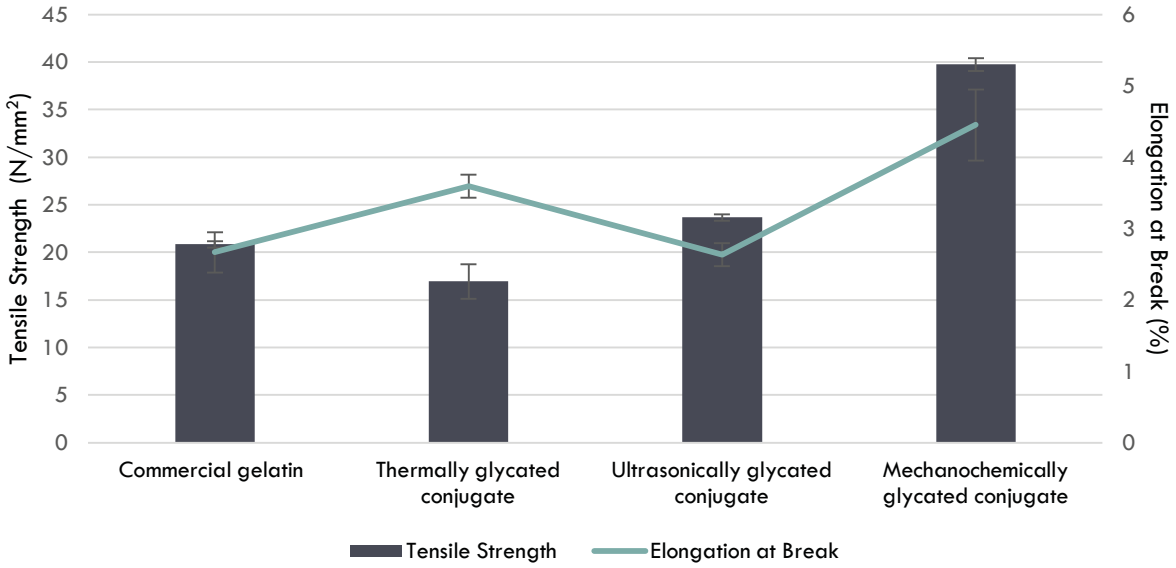


Figure 4.12. Physical and mechanical properties of gelatin films derived using native and glycated gelatin

The existing literature show conflicting reports on the effect of glycation on the TS and EB of gelatin based films. The existing literature however focus on the thermal glycation of protein and saccharides for the generation of films. While our study showed significant improvement in TS, and no effect on EB, Samira, Thuan-Chew Tan, and Azhar (2014) found that the conjugation of gelatin with ribose showed a 2 fold improvement in EB, and no significant effect on TS. Further, Etxabide et al. (2015) studied the effect of the conjugation reaction between gelatin and lactose on film properties, where the study reported no improvement in either TS or EB. The existing literature indicates an absence of correlation between protein glycation and its effects on the mechanical properties of the film. It seems as numerous factors including the type of saccharide, degree of glycation, and protein concentration may all have an effect on the TS and EB.

## 4.6 Overall Observations of Functional Properties

A compilation of the functional properties of the conjugates obtained through thermal, ultrasonic and mechanochemically induced glycation is presented in Table 4.9. As revealed by the table, the mechanochemically glycated conjugates exhibited exceptional functional properties relative to not only the native gelatin but also to the other glycated conjugates. The conjugates with enhanced

functionality is as follows: mechanochemically glycated conjugates > ultrasonically glyated conjugates > thermally glycated conjugates > native gelatin. The greater functionality of the conjugates generated mechanochemically is mainly due to its enhanced solubility through the milling reaction. The reaction induces repeated collision effect which promotes particle refinement by the breakdown of the molecule. This phenomenon not only benefits the glycation reaction but also makes favorable contributions to the conjugates functional properties where solubility is a major contributor to other functional properties. Solubility is in fact an excellent indicator of protein functionality (Kinsella & Melachouris, 1976). Despite the formation of aggregates by the sonochemical method, which is expected to greatly impose negative effects on the functional properties, lower sonication treatment times made favorable contributions to the solubility and emulsifying properties. In fact, in relation to the emulsifying properties, the ultrasonically glycated samples for 10 minutes, showed greater EAI and ESI relative to the mechanically glycated conjugates. Both ultrasonic and mechanochemical glycations were found effective in imparting enhanced foaming properties. These two methods were always superior to the thermally induced glycation method in relation to making favorable contributions to the functional properties of the glycated gelatin. Overall, the thermally glycated conjugates showed similar functional properties to that of the native gelatin. It is highly possible that the thermally glycated conjugates showed increased aggregation and crosslinks as a result of prolonged heating, thus having an impact on the resulting functional properties.

Table 4.9. Functional properties of gelatin & thermal, ultrasonic and mechanochemical conjugates

| | **Commercial gelatin** | **Thermally glycated conjugate[a]** | **Ultrasonically glycated conjugate** | **Mechanochemically glycated conjugate[d]** |
|---|---|---|---|---|
| **Solubility** | ++ | ++ | +++[b] | ++++ |
| **Heat Stability** | +++ | ++ | +[c] | ++++ |
| **Emulsion properties** | + | ++ | ++++[b] | +++ |
| **Foam properties** | + | ++ | ++++[b] | ++++ |
| **Film Forming Properties** | + | ++ | +++[c] | ++++ |

[a]gelatin/glucose saccharide conjugated for 24 hours
[b]gelatin/glucose saccharides conjugated through ultrasonic means for 10 minutes
[c]gelatin/glucose saccharides conjugated through ultrasonic means for 15 minutes
[d]gelatin/glucose saccharides conjugated through mechanochemical means for 30 minute

## GENERAL CONCLUSIONS

The objective of the study was to evaluate the effect of traditional heating, sonochemical and ball-assisted mechanochemical milling on the rate of glycation, production of stable conjugates and their effect on the functional properties of the glycated gelatin. Gelatin was used as a model protein for the purpose of this study, despite its non-uniform molecular weight distribution which rendered it difficult for MS-based analysis. Wen studying the effect of the carbohydrate type on thermal glycation with ribose and glucose, it was found that the glycation reaction with ribose occurred much faster than with glucose. The reactivity of ribose therefore rendered it difficult to control the glycation reaction under accelerated conditions of ultrasound and mechanochemical treatments and as a result only glucose was used for the interpretation of the efficiency of the new techniques of glycation. In general, the study demonstrated that the thermal, ultrasonic and mechanochemical means were able to produce gelatin/glucose conjugates through the Maillard reaction. The reaction progression was confirmed by monitoring the available amino groups in combination with the consumption of sugar over the reaction time. Studying the effect of the method on the protein in the absence of sugar, showed that the sonochemical treatment causes great aggregation of the protein with short reaction times. The thermal and mechanochemical methods however showed increased fluorescence indicating greater hydrolysis promoted by the respective methods. When studying the effect of the method on glycation, the ultrasonic and mechanochemical treatments were found to be more effective in accelerating the conjugation reaction between gelatin and glucose and attaining similar degree of glycation as the classical heating method within shorter reaction times. The traditional heating method however was able to attain a slightly higher degree of glycation compared to sonochemical and mechanochemical methods. This study further demonstrated that the functional properties of gelatin can be improved significantly through the conjugation reaction. The improvement of the functional properties was dependent on the method of glycation used. Taking into account the results of the study, the mechanochemically induced glycation showed a great potential for the food industry, mainly due to its efficiency in inducing the conjugation reaction but also due to its enhanced rate of reactivity. The reaction can be carried in the absence of solvent, requires shorter reaction time, produces stable conjugates and most importantly it can significantly improve the functional properties of the protein/saccharide conjugates. Although the classical heating method is also efficient in creating protein/saccharide

conjugates it required longer reaction times. Comparatively, sonication can be as beneficial as the mechanochemical method, however careful monitoring of the reaction and stability of the conjugate is important to obtain desirable effects on the functional properties of the conjugates. Further research is needed to elucidate the effective reaction conditions for the development of stable conjugates through sonication. Alongside extensive degree of glycation, the extent of protein aggregation and increased formation of cross-links was found to be an important variable in producing conjugates with reduced functionalities. Modification of the functional properties of gelatin as a result of glycation were attributed mainly to the changes in the coiled and α-helical structure of gelatin to an disordered random chain facilitating the improvement of functional properties with greater exposure to hydrophobic and hydrophilic counter parts.

Compilation of the experimental finding through this study demonstrates the scientific basis for the modification of proteins with enhanced functional properties through not only the traditional heating method but also through new techniques of glycation; where mechanochemistry seems to be the most promising. The results further show that protein glycation through the Maillard reaction is an effective method for the development of novel ingredients for the food industry. With controlled reaction parameters, the glycation reaction can be limited to its earlier stages thus having beneficial improvement on the functional properties of the protein. As the conjugates formed through the Maillard reaction are seen to have improved functional properties, the use of a smaller quantity of this modified protein will be sufficient in providing equivalent or enhanced functional properties relative to the native protein. This will reduce the production costs in an industrial scale. Additionally, the resulting conjugate with these improved functionalities can be promoted as a functional food ingredient with not only added value but also safe as it is produced with sugars and no extraneous chemicals. This method of protein modification will be of utmost benefit in regards to food waste. Proteins that are seen to lack functional properties or perhaps have no specific function in a given food matrix, can be easily modified with this promising approach to render safe, low cost and value added functional ingredients.

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