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Understanding the Impact of Physicochemical Modifications on the Cold Gelling Behavior of Micellar Casein Concentrate Dispersions

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UNDERSTANDING THE IMPACT OF PHYSICOCHEMICAL MODIFICATIONS ON THE COLD GELLING BEHAVIOR OF MICELLAR CASEIN CONCENTRATE DISPERSIONS

by

Nathan Pougher

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Approved

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ABSTRACT

Understanding the Impact of Physicochemical Modifications on the Cold Gelling Properties of Micellar Casein Concentrate Dispersions

by

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Highly Concentrated-Micellar Casein Concentrate (HC-MCC) is a dairy ingredient comprised of 17-23% protein. Obtained via microfiltration and vacuum evaporation, it can form a gel in cold conditions without any physicochemical modifications. With consumer preferences moving away from polysaccharide-based stabilizers in dairy products, there is potential for the gelling properties of HC-MCC to be applied in industry. This study investigates the gelling properties of HC-MCC various states to better understand the mechanism behind cold gelation.

The first study examined combinations of physicochemical modifications (dilution, calcium chelation, pH adjustment) to optimize gel strength. The second study examined certain treatments in further detail, i.e., examining additional physical properties. Lastly, the third study examined the cold gelling ability of diluted MCC and kappa carrageenan gels. A three-stage multiwave rheological protocol was applied to HC-MCC samples to observe gel strength and the temperature of cold gelation alongside texture analysis, particle size, and zeta potential measurements to observe additional

characteristics in response to treatments. Ultrastructure analysis was conducted on selected treatments using transmission electron microscopy (TEM) to observe morphological changes in casein micellar structure.

We observed that pH adjustment resulted in an exponential increase in gel strength as pH increased from 6.2 to 6.8 (R^2=0.99), along with significantly higher gelation temperatures. The addition of a calcium chelating salt (trisodium citrate, or TSC) significantly increased gel strength in most combinations of treatments ($P<0.05$), with 25mM concentrations consistently yielding the strongest gels. Ultrastructure analysis of samples showed that alkalization to pH 6.8 and 7.0 increasingly disintegrated the micellar structure of casein and released individual fragments into the aqueous phase, which was observed alongside a significantly increased gel strength and gelation temperature. The use of TSC at 25mM partially disintegrated micelles, whereas 50mM resulted in the formation of large aggregates with a concomitant decrease in gel strength. Kappa carrageenan addition resulted in gelation temperatures similar to modified higher protein samples, but gel strength was lower. TEM micrographs depicted minimal interaction between kappa carrageenan and casein, creating a biphasic solution. Overall, the gelling properties of HC-MCC can be significantly improved in response to physicochemical modification with significant changes in protein structure taking place.

(152 Pages)

PUBLIC ABSTRACT

Understanding the Impact of Physicochemical Modifications on the Cold Gelling Properties of Micellar Casein Concentrate Dispersions

Nathan Pougher

When skim milk is filtered via microfiltration, the amount of casein (one of the major milk proteins) in solution can be concentrated. When casein content is high enough (>15%), the solution forms a gel at cold temperatures. With growing trends in the food industry towards simplistic ingredient labels, commonly used gums and stabilizers in the dairy industry are becoming less preferred. In the future, there is potential for the gelling properties of micellar casein to be applied to dairy products as a thickener or stabilizer, but the mechanism behind gel formation isn't understood well. In this study, the gel strength, gelation temperature, and structural changes of casein in response to modifications were studied to understand how they may affect gelation. These modifications included reductions in protein content (from 18.5 to 10%), pH adjustment (from 6.2 to 6.8), addition of a calcium chelating salt (sequesters calcium, a structural component of casein), and addition of a common dairy stabilizer: kappa carrageenan. Protein content was the main determinant of gel strength; reductions from the original protein content of 18.5% to less than 15% resulted in weaker gels that required lower temperatures to form a gel. We found that as the pH increased from 6.2 to 6.8, stronger

gels can be formed at a higher temperature. The addition of calcium chelating salts improved these qualities as well but increasing concentrations from moderate (25mM) to high (50mM) resulted in a reduction in gel strength. Microstructure analysis of gels via transmission electron microscopy revealed that with increasing pH, the micellar structure of casein was disintegrating, forming a dispersion of free casein fragments. Calcium chelation at moderate concentrations partially disintegrated the protein structure, but high concentrations led to the formation of large casein aggregates causing a reduction in gel strength. When kappa carrageenan was added, it allowed samples diluted to 10% protein to form a gel which was not previously possible. Kappa carrageenan had minimal interaction with casein, but it was responsible for a stronger gel. Overall, modifications of casein can increase the gel strength and temperature of gelation due to the structural changes in casein.

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LIST OF ABBREVIATIONS

HC-MCC – Highly Concentrated-Micellar Casein Concentrate

MCC – Micellar Casein Concentrate

CCP – Colloidal Calcium Phosphate

MF – Microfiltration

GDL – Glucono-Delta Lactone

TSC – Trisodium Citrate

SMUF – Simulated Milk Ultrafiltrate

KC – Kappa Carrageenan

CGT – Cold Gelling Temperature

G’ – Storage Modulus

G” – Loss Modulus

LT – Loss Tangent

TEM – Transmission Electron Microscopy

CHAPTER 1

INTRODUCTION

In addition to manufacturing consumer products such as fluid milk, ice cream and cheese, the dairy industry is known for producing large amounts of commodity products. Many of these commodity products are utilized as ingredients in other food products. For example, non-fat dry milk powder is a frequent addition to ice-cream to increase the dairy solids content, similarly, mozzarella cheese made by a dairy industry for another company to use in a pre-made frozen pizza. One such commodity product is micellar casein concentrate (MCC), which is casein-rich product (9-10% protein), concentrated by using a membrane processing technique known as microfiltration (MF) and produced in either a liquid or powder form. MCC has the potential to be used in the production of cheese to adjust the casein to fat ratio to increase the cheese yield and to produce a consistent quality product throughout the year (Lu et al., 2017). A downside of using liquid forms of MCC in cheese making is that it forms a gel at cold temperatures, which slows down cheese production, because in order to make cold MCC usable, it requires a heating step to bring it back to a liquid state prior to addition to the cheese milk (Lu et al., 2017). This effect is amplified due to a higher concentration of casein micelles during evaporation of regular MCC, resulting in Highly Concentrated-Micellar Casein Concentrate (HC-MCC). While cold gelling behavior of HC-MCC is considered

disadvantageous in the production of cheese and beverages, it could be seen as a positive attribute if used as a stabilizer/thickener in ice-cream and yogurt manufacture.

Many consumer dairy products, such as yogurt and ice cream, utilize polysaccharide-based gums derived from plants as a stabilizer. These gums prevent whey syneresis in the products, as well as increase consumer preference in categories such as texture, viscosity, and mouthfeel (Rafiq et al., 2020). With increasing awareness of clean label practices within the dairy industry, these gums and stabilizers are negatively perceived by conscious consumers (Maruyama et al., 2021). With growing reservations against these gums, potential utilization of HC-MCC to stabilize dairy products could yield a viable clean label alternative in high protein dairy products.

This research investigated the impact of physicochemical modifications of HC-MCC on the cold gelling properties of HC-MCC and focuses on finding optimum conditions to enhance these characteristics. Advanced rheological testing was conducted in order to observe the effects of these modifications on gelling behavior and overall physical properties. Additional testing such particle size, zeta potential and transmission electron microscopy (TEM) was conducted to determine the effects of these modifications at a microscopic scale. Results from this study will serve as a basis for additional research into HC-MCC and potential applications within the dairy industry.

Research Hypothesis

Overall hypothesis: Physicochemical modifications of HC-MCC will affect gel strength and gelation temperature during cooling because of changes to protein-protein and protein-water interactions.

Objectives

Study the effects of changes in protein content, pH, and the addition of calcium chelating salts on the cold gelling properties in HC-MCC.

Understand the impact of kappa carrageenan addition on the cold gelling behavior of micellar casein concentrate solutions.

Study the changes in protein structure in response to physicochemical modifications and how they relate to gelling properties.

CHAPTER 2

LITERATURE REVIEW

2.1: HC-MCC Manufacture

Highly Concentrated-Micellar Casein Concentrate (HC-MCC) is an emerging bovine dairy ingredient notable for its high casein content, ranging from 17-23% in solution. Casein is one of the major proteins found in milk which provides unique structure in various dairy products, such as cheese and yogurt. HC-MCC is produced through microfiltration (MF) of milk. The casein in its native micellar state is retained by the MF membrane (pore size 0.145 μm), resulting permeation of the whey proteins, lactose, minerals, and much of the moisture. MCC can also undergo a diafiltration step as well; these repeat filtration cycles result in a purified form of MCC retentate. The resulting MF retentate (or MCC) is then subjected to vacuum evaporation which reduces additional moisture content. The evaporation process can continue until the desired level of casein is reached. Traditional MCC powders are produced using drying methods such as spray drying, but liquid forms of HC-MCC require less energy input to meet final specifications. There are tradeoffs however, as the casein is in solution, there is additional weight to the product, and refrigeration is still required.

Liquid HC-MCC is unique compared to other sources of casein since it is retained in the native micellar state (Saboyainsta & Maubois, 2000). This offers a unique advantage in terms of functionality compared to many other dry sources of casein where solubility is

drastically reduced due to heat or acid induced irreversible changes in the protein structure. In addition, the use of MF in HC-MCC manufacturing results in relatively lower levels of whey (serum) proteins, which are sensitive to heat. The reduction in these proteins can lead to higher stability at elevated temperatures (Lu et al., 2015). Due to high levels of casein within the solution, a thermo-reversible gel is observed at lower temperatures. The gelling capability of this solution is dependent on the composition and physical properties of the HC-MCC produced.

2.2: Casein Structure and Gel Formation

The four main casein proteins (αS1, αS2, β, κ) can form a micellar structure with diameters ranging from 100-200 nm that is colloidally dispersed within milk solution (McMahon & Brown, 1984). The most important protein that protects the structure of the micelle is kappa casein, which is found on the exterior of the micelle. Kappa casein forms many of the protruding hairlike structures (strands) on the micelle surface and acts as the hydrophilic exterior of the micelle as it interacts with the aqueous phase while also binding to the hydrophobic casein proteins on the inside of the micelle (Dalgleish, 1998). Another notable component that holds the casein micellar structure intact is colloidal calcium phosphate (CCP). CCP is a calcium salt that tends to form nanoclusters within the micelle and helps binding two sub-micelles together (Broyard & Gaucheron, 2015). These clusters are the major source of stability for the inter-protein complex due to the crosslinking observed between calcium ions and phosphoserine residues in casein proteins. The structure of the micelle itself has an outer shell with a strong net negative charge. Casein micelles are unique compared to other known micelles such as detergents. The interior of

the casein micellar structure contains the hydrophobic portions of the micellar proteins. In addition, the structure contains considerable amounts (more than twice the weight of the protein molecule) of bound water (Huppertz et al., 2017). The hydrophobic interactions between proteins are the secondary source of stability within the micelle. However, protein cross-linking through CCP remains the strongest force that contributes towards stabilization of the micellar structure.

Casein micelles are best known for their ability to form a gel under specific conditions, particularly when acid or rennet is introduced to milk. In the case of HC-MCC however, cold gel formation is observed with casein in an unmodified state (Lu et al., 2015). When caseins are reduced in temperature, the energy within the system is reduced which leads to various conformational changes. As the temperature is lowered, CCP solubilizes into the aqueous phase; in return, water within the system begins to enter the micelles, causing swelling and an increase in particle size. As described by Dunn et al. (2021), gelation can be attributed to the increase in the voluminosity of casein micelles as the increase in particle size at native pH levels results in a vast reduction in space between the micelles. This is combined with reduced energy within the system from lowered temperatures (Dunn et al., 2021). When the micelles are in very close proximity to one another, the friction between particles is heightened, leading to the occurrence of gelation via the excessive packing of particles. Lu et al. (2015) described another situation in which this gelation behavior is amplified, that is via casein strands interlinking and forming a matrix (Lu et al., 2015). The role of calcium in this interaction is important as it can cause the linking of the casein strands with one another (Figure2-1). When added to HC-MCC,

this can enhance the cross-linking ability of casein micelles, resulting in stronger gels and higher gelling temperatures.

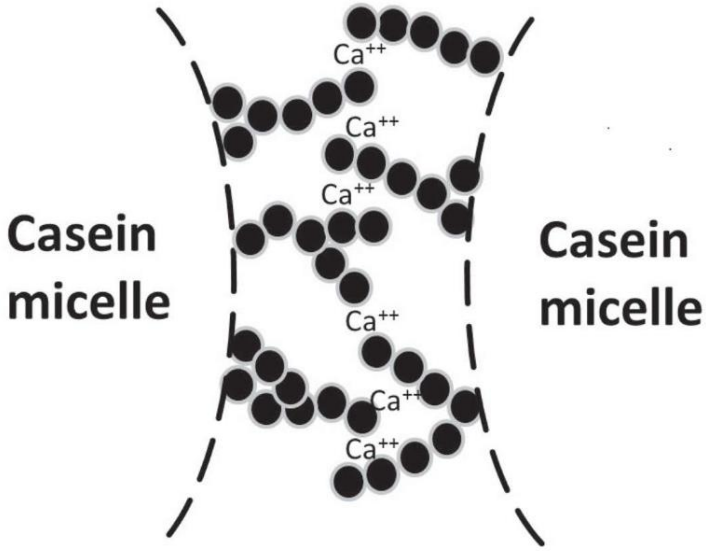

Figure 2-2: A proposed model of interaction from casein micelles with calcium ions forming a bridge between individual protein strands (adopted from Lu et al., 2015)

2.3: Effect of pH on Casein

Casein micelles have a net negative charge while in a milk solution. This charge remains on the micelle until the pH is reduced to the isoelectric point of 4.6. As the pH of the solution declines from the initial milk pH of 6.6, the negatively charged sites on the molecule begin to neutralize. When reaching the isoelectric point, casein proteins aggregate

and undergo a process known as acid coagulation. The reduction in pH to near the isoelectric point can result in increases in particle sizes, as well as in forming a matrix within the solution (Eshpari et al., 2016). Minor reductions in pH also have similar but less drastic effects on the micellar structure. As the pH declines within the solution, the negative charge on the kappa casein protein is reduced, the kappa casein layer partially collapses due to weakened submicelle interactions, which results in a reduction in stearic hinderance between micelles (Li & Zhao, 2019). Prior to reaching the isoelectric point, this minor reduction in pH can result in the reduction of the casein micelle size (Sinaga et al., 2017). When considering this reduction in particle size with the packing effect described in Dunn et al. (2021), gel strength could potentially be reduced by lowering the pH from 6.6 to 6.0. As the casein micelle size is reduced, there is more space between micelles allowing for a weaker gel formation (Dunn et al., 2021).

As the pH declines, CCP solubilizes and exits the micelle into the serum phase; the inorganic phosphate ions are solubilized near a pH of 5.2 while the remaining calcium ions are fully solubilized at the isoelectric point of casein, which is at pH 4.6 (Li & Zhao, 2019). Collapse of kappa casein with concomitant solubilization of CCP results in the aggregation of micelles most typically found in many dairy foods, either through direct acidification or the use of lactic acid generating bacteria. Compared to cow's milk, HC-MCC has a much higher casein content; this would mean that the effect of acidification would have a stronger effect on physical properties compared to milk.

While the reduction in pH can result in coagulation and CCP solubilization, the increase in pH results in changes in physical properties as well. Adjusting the pH of casein within a solution higher than 6.6 yields an increase in particle size (Y. Liu & Guo, 2008).

The reason for this effect is the opposite of acidification: as the pH increases, the net charge on the casein micelle continues to grow more negative, increasing the repulsion effect within the micelle, promoting protein-water molecule interactions. This effect results in the micelle swelling until it fully dissociates, at a pH of around 8.5 (Sinaga et al., 2017). In addition, since CCP solubility in the aqueous phase decreases with increases in pH, there is an increase of CCP bound to the micellar structure. While this can increase the calcium content within the micelle, it can also reduce the hydrophobic interactions between proteins, which can contribute to micellar dissociation at elevated pH levels (Madadlou et al., 2009).

2.4: Calcium Chelating Salts on the Casein Micelle

The use of calcium chelating salts is a common practice in the dairy industry, particularly in products such as processed cheese. These salts encourage the sequestration of calcium from the casein micelles, resulting in the release of non-sedimentable casein fragments (Broyard & Gaucheron, 2015). When specific emulsifying salts, such as trisodium citrate (TSC) and disodium hydrogen phosphate (Na_2HPO_4), are added to the casein solution, they function by chelating calcium molecules found in CCP. This sequestration of calcium from the micelle destabilizes the quaternary structure of the micelle and causes the dissociation of casein micelles (Culler et al., 2017). The excessive buildup of free caseins in the solution creates a situation where these proteins bind the remaining portions of micelles together, creating a stronger gel matrix.

When TSC is added to dairy products such as yogurt, there is a notable increase in gel strength (Ozcan-Yilsay et al., 2007). There appears to be an optimum concentration of

TSC to add to a sample though, with higher quantities reducing gel strength. One potential explanation is that while a lower concentration of TSC may cause the dissociation of caseins into the solution, a high enough concentration has the potential to chelate all CCP within the solution which could completely disrupt the micellar structure and yield a weaker yogurt gel (Ozcan-Yilsay et al., 2007). Since HC-MCC has a concentrated amount of casein micelles dispersed within the solution, it has a much higher calcium and overall ash content compared to regular milk (Lu et al., 2016). Because of this, HC-MCC solutions would likely require a higher amount of a given calcium chelating salt to result in higher gel strengths. The effect of high concentrations of TSC, or "over-salting" the product, is assumed to be similar. Introducing calcium chelating salts to milk solutions can also result in increases in pH levels, meaning that some of the effects of elevated pH may be observed when some of these salts are introduced to the solution. There is a lack of research observing the effect of calcium chelation compared to the effect of alkalization to a similar pH level.

2.5: Carrageenan-Casein Interactions

Carrageenan is one of the most commonly used stabilizers in dairy products; especially since the three main forms (κ, ι, and λ) offer different results for specific uses (Campbell & Hotchkiss, 2017). κ and ι forms of carrageenan are best known for their ability to create a cold gel in the presence of proteins while the lambda form serves only as a thickener (Langendorff et al., 2000). The Kappa form of carrageenan facilitates cold gel formation with the sulfate group on each disaccharide portion of the chain: this sulfate group then bonds with the positively charged section of the κ-casein molecule, effectively

increasing the size of the casein micelle, and reducing the space between micelles. This effect can be observed with the employment of particle size analysis (Spagnuolo et al., 2005). In addition, temperature changes result in the conformational change of the polysaccharide chain as well: above temperatures of 50^{o}C the chain exists in a coiled shape, but this conformation changes to a helix structure as the temperature is reduced (Bourriot et al., 1999). This newly formed helix shape also helps facilitate the gelation of solutions as well (Drohan et al., 1997). Spangunolo et al. (2005) conducted an analysis of the carrageenan bound to micelles at 25^{o}C, noting that the carrageenan was in a helix form.

As Pang et al. (2015) find, the addition of a 50:50 mixture of κ and ι carrageenan in small quantities (0.02%) to an acid milk gel yields a gelation point at a higher temperature but a weaker overall gel. In higher concentrations (0.2%), the carrageenan inhibits the gelation of the milk. This is because of the aggregation of carrageenan on the casein molecules: this casein-carrageenan aggregate is inhibiting the normally present casein-casein aggregation that happens under acidic conditions, resulting in a decrease in storage modulus with increasing carrageenan content (Pang et al., 2015). While there has been sufficient rheological research on casein with respect to carrageenan and acidity, there has been minimal research conducted on the combination of calcium chelating salts and carrageenan to the casein dispersions.

2.6: Rheology of MCC dispersions

HC-MCC is considered a viscoelastic material at low temperature, meaning that it exhibits both viscous (liquid) and elastic (solid) properties (Zad Bagher Seighalani et al., 2021). Rheological measurements on viscoelastic materials consist of measurements for

these two properties. The storage modulus, or G', is the measurement of elastic properties (gel strength) in a sample. The loss modulus, or G", is the measurement of viscous properties within a sample, meaning that it is an indicator of liquid-like properties within a measured sample. Calculating the ratio of G" to G' results in a measurement known as the loss tangent which indicates the relative proportion of viscous and elastic behavior of a material and the time dependence.

There have been multiple extensive rheological studies conducted on dairy products (Bouchoux et al., 2009; Keogh & O'kennedy, 1998; Muliawan & Hatzikiriakos, 2007; Ozcan-Yilsay et al., 2007), but there are only few publications on the products like HC-MCC. In these studies, only preliminary measurements have been conducted. Lu et al. (2015) measured the temperature at which the crossover from viscoelastic liquid to viscoelastic solid takes place during cooling of HC-MCC. Using the crossover point, where the storage modulus becomes greater than the loss modulus, to determine the gelation temperature has a drawback as the sample often exhibits properties of a viscoelastic liquid after the point in question. Utilizing this method is a pseudo estimate of the gelling point because the crossover point is greatly dependent on the frequency applied during rheological testing as well as other factors such as the rate of sample cooling (Zad Bagher Seighalani et al., 2021). A method utilizing measurements at multiple frequencies (simultaneously) shows an improvement in determining a more accurate gelation temperature. Rather than measuring the crossover point, this method measures the loss tangent at multiple frequencies; the point where these loss tangents converge is considered the gelation point (Zad Bagher Seighalani et al., 2021). The former system fails to take into

consideration the measurement at different frequencies, while the ladder relies on these different frequencies to determine the gelation temperature.

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CHAPTER 3

EFFECT OF PROTEIN CONCENTRATION, CALCIUM CHELATION, AND PH ADJUSTMENT ON THE COLD GELLING PROPERTIES OF HIGHLY CONCENTRATED-MICELLAR CASEIN CONCENTRATE (HC-MCC)

ABSTRACT

Highly Concentrated-Micellar Casein Concentrate (HC-MCC) is a dairy ingredient with a high casein content, ranging from 17-23%. Under cold temperatures (<10^{o}C), it transforms from a liquid state to a viscoelastic solid state. However, the mechanism behind gelation is currently unknown. This chapter presents a body of work which helps to understand how the gelation of HC-MCC can be affected using physicochemical modifications such as changes in protein concentration, pH adjustment, and the addition of a calcium chelating salt. Viscoelastic properties such as storage modulus (G') and cold gelation temperature (CGT) were measured using a three-stage rheological protocol utilizing a multiwave technique. Additional metrics, such as particle size and zeta potential, were also analyzed in response to physicochemical modifications. In addition, transmission electron microscopy (TEM) was conducted on unmodified samples to understand the effect of temperature on cold gelation. Rheological results showed that the gel strength of cold gels was highly dependent upon protein concentration, with reduced protein samples being weaker than undiluted ones. Gel strength and CGT both increased in response to trisodium citrate (TSC) addition, but

there appeared to be a limit of TSC addition before a reduction in these qualities was observed. Reduction in pH from the native state of 6.6 resulted in a decrease in gel strength, gelation temperature, particle size and net charge. Alkalization of the sample however, increased strength, but the effect of calcium chelation in conjunction with higher pH improved gel qualities less than at native and acidic pH levels. Overall, this data can serve as a foundation for future research on HC-MCC to better understand what factors may lead to optimum gelling qualities for the product, leading to potential novel applications in the future.

3.1: Introduction

Casein is one of two major proteins found in milk alongside whey proteins. Made of four subunits (αS1, αS2, β, κ), casein is known to form a micellar structure that is colloidally dispersed within the aqueous phase of milk. As an important component of the micelle, colloidal calcium phosphate (CCP) functions as a structural base holding subunits together in the form of nanoclusters (Broyard & Gaucheron, 2015). Individual subunits of casein bind to the nanoclusters and enhance stability within the structure.

HC-MCC is produced via the microfiltration of skim milk. The size of the pores within the filter (0.145μm) is large enough for whey proteins, minerals, lactose, and additional moisture to pass through, effectively concentrating casein into a retentate phase in the form of MCC. Further concentration via vacuum evaporation yields HC-MCC, with a protein content ranging from 17% to 23%. Compared to other common

dairy ingredients, such as milk protein concentrate or powdered MCC, HC-MCC is unique as it is still in a liquid state, meaning no changes to the micellar structure of casein took place during the drying process (Saboyainsta & Maubois, 2000).

Under physicochemical modifications such as acidification or rennet addition, casein micelles undergo significant structural changes that cause the irreversible gelation of milk. Unlike other dairy products such as cheese and yogurt, gel formation in HC-MCC is possible due to cooling while the casein is still in a native micellar conformation. Holding HC-MCC at low temperatures ($<10^{o}C$) results in the formation of a thermoreversible cold gel (Lu et al., 2015). Multiple studies have examined the potential causes behind gelation. Authors from one of these studies propose a packing transition as the reason where the space between micelles is so small that stearic hinderance was a likely driving force for the transition into a gel (Lu et al., 2015). Another potential cause for this effect could be the increase in micellar size in response to CCP migration from colloidal phase to the soluble phase, resulting in swelling of the casein micelle structure and further reduction in space between micelles (Dunn et al., 2021).

When casein micelles are in solution, they have a net negative charge. Changes in the pH of the solution can affect the stability of the micelle by changing the net charge of the structure, and this in turn may affect the strength of a cold gel. During acidification, the net negative charge on the micelle is neutralized due to the excess of H^{+} ions in solution until the isoelectric point (IP) is reached (Francis et al., 2019). At the IP, there are not enough negative charges remaining on the caseins to maintain their micellar stability, therefore an acid gelation, or precipitation occurs (Eshpari et al., 2016). In addition to this, CCP solubility increases with increasing acidity, further destabilizing the

micelle. A reverse effect is expected if pH is adjusted towards an alkaline solution. The negative charge on casein increases in response to increases in pH, which promotes protein-water interactions rather than hydrophobic interactions (Y. Liu & Guo, 2008). As the solubility of CCP decreases with increases in pH, there is also more available calcium for casein subunits to bind to each other (Madadlou et al., 2009)

In order to improve the heat stability of casein micelles, it is common practice in the dairy industry to use a calcium chelating salt such as tri-sodium citrate (TSC) which is able to sequester CCP within the micellar structure. TSC and other calcium chelating salts are often referred to as emulsifying salts in the processed cheese industry due to its ability to open the micellar structure of casein due to the removal of structural CCP, releasing individual casein fractions at higher concentrations. These free caseins can act as an emulsifier by interacting with fat globules within the system, effectively stabilizing fat in molten protein matrix (Culler et al., 2017; Deshwal et al., 2023). Some studies suggest that the addition of calcium chelating salts can result in an increase in the gel strength of yogurt, but higher concentrations can reduce strength (Ozcan-Yilsay et al., 2007). This outcome shows that while there is an improvement on casein strength using calcium chelating salts, there is a limit to the amount of increase possible while utilizing them.

While previous studies have proposed models of gelation, the formation of a cold gel is still not known. The aim of this study is to better understand the mechanism behind the cold gelation of HC-MCC and the factors impacting this phenomenon. In addition, gathering knowledge on the gelation properties of physicochemically modified samples will help us understand how HC-MCC will behave in response to different treatments.

This will help optimize the cold gelling properties of HC-MCC and potentially create a gel requiring lower protein content, reducing the quantity of material required to encourage a desired effect within a product.

3.2: Materials and Methods

3.2.1: HC-MCC Manufacture and Storage

HC-MCC used in the study was produced at South Dakota State University as described in Lu et al., (2015). The MF system consisted of a four-vessel continuous design utilizing polyvinylidene fluoride membranes with a combined surface area of 57.4m^2. The subsequent vacuum evaporation of the MCC was conducted at 63^oC at a pressure of -680mbar. The samples were held in large pails at -20^oC. Frozen samples were thawed and melted into a liquid state in a water bath at 50^oC. The liquid HC-MCC was thoroughly mixed and poured into screw-cap plastic containers in ~120g quantities. Spoilage of the samples due to potential microbial growth was prevented by the addition of a chemical preservative (0.05% wt/wt sodium azide). Samples were kept frozen at -20^oC until ready for use. Sample cups were thawed in a water bath and stored at 4°C between tests. All tests were performed in triplicate.

3.2.1.1: HC-MCC Modifications

In this study, HC-MCC samples were treated in three ways: protein concentration was varied (18.5% and 15%), pH adjusted (6.2, 6.4, 6.6, 6.8), and TSC added (0, 10, 25,

50 mM). The HC-MCC used in the study had an original protein content of 18.5% and a pH of 6.6 at 60^{o}C. Adjustment of protein content was performed with deionized water. Adjustment of pH was accomplished utilizing glucono-delta-lactone as an acid and sodium hydroxide (NaOH) as a base. For pH adjustment, samples were first homogenized using an overhead mixer for two minutes, followed by the addition of GDL/NaOH and further mixing for three minutes. The samples were then held at room temperature for two hours to allow for the additions to dissolve. The calcium chelating salt used in this study was food grade trisodium citrate dihydrate (TSC), sourced from Cargill (Eddyville, IA). TSC at 10mM, 25mM, and 50mM levels was added to the samples at 60^{o}C with constant stirring using a glass rod immediately prior to loading into the rheometer. For some samples, additional heating for five seconds in a microwave was required to fully incorporate the salt.

3.2.2: Simulated Milk Ultrafiltrate (SMUF) Preparation

SMUF was prepared using ingredients listed in Table 1 for diluting HC-MCC sample for particle size and zeta potential analysis. Reagent salts were measured and mixed in proportion to create SMUF in one-liter quantities. The salt solution was filtered using a vacuum flask and a Millipore 1.2μm filter (Bedford, MA). To prevent precipitation of phosphates in the completed buffer, the SMUF was stored in a refrigerator at 5^{o}C in between uses.

Table 3-1: SMUF reagent concentrations

Reagent	Concentration (mM)
KH_2PO_4	11.61
K_3 citrate* · H_2O	3.70
Na_3 citrate* · $2H_2O$	6.09
K_2SO_4	1.03
K_2CO_3	2.17
KCl	8.05
$CaCl_2$ · $2H_2O$	8.98
$MgCl_2$ · $6H_2O$	3.21

*Citrate=$C_6H_8O^-_3$ Table adopted from (Rosmaninho et al., 2007)

3.2.3: Experimental Design

Factorial design was used for this study, with two protein concentration levels, four pH levels, and four TSC concentration levels for each pH level. In addition, unmodified MCC (18.5% protein, pH 6.6, and 0mM TSC) acted as a control sample. Each sample was tested in triplicate.

3.2.4: Rheological testing

Rheological measurements were performed using an Anton Paar MCR 302 rheometer (Anton Paar GMBH, Graz, Austria) using a concentric cylinder geometry setup (model no. CC27). For sample loading, 20ml of sample was heated to 60^{o}C and poured into the bottom of the sample cylinder. An oil layer was added on the top via a pipette to prevent dehydration during the rheological protocol. Rheological testing was conducted in three stages (Figure 3-1). In the first stage a time sweep was conducted by

holding the sample at 60ºC for one hour taking measurements every 30 seconds for a total of 120 data points. The second stage was a temperature sweep where the temperature was decreased from 60ºC to 5ºC over the course of 56 minutes, taking a measurement every 41.25 seconds for a total of 81 data points. Lastly, the third stage held the sample at 5ºC for at least ten hours, making measurements every minute. Rheological testing was conducted by applying simultaneously multiple angular frequencies (3, 6, 12, 24, and 48 rad/s), and the gelation point was determined by using the criteria described in Zad Bagher Seighalani et al., (2021). Here, the gelation point was defined as the point where the loss tangent of the sample is independent of frequencies applied (Winter & Chambon, 1986). Storage modulus (G') values obtained during the temperature sweep at 20ºC, 8ºC, and 5ºC at the end of the temperature sweep, and holding 5ºC for ten hours during the time sweep was used to compare the gel strength between different treatments.

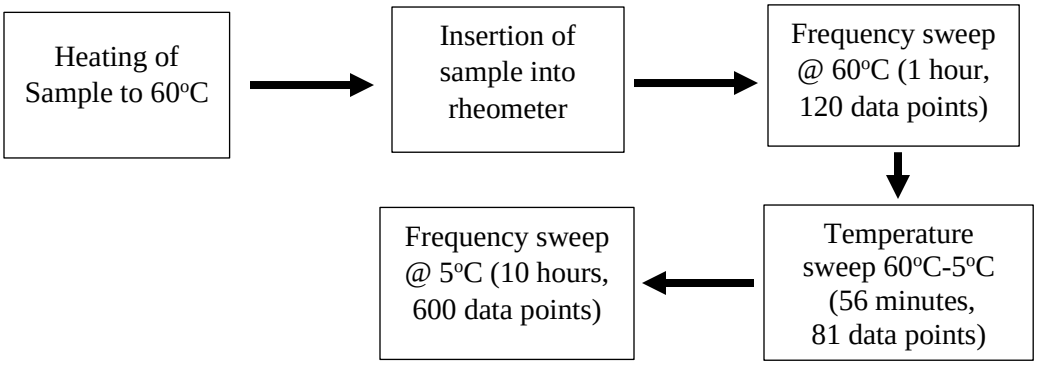

Figure 3-1: Scheme of rheological testing protocol

3.2.5: Particle Size Analysis and Zeta Potential Measurements

Tests examining casein particle size and zeta potential was measured in Sarstedt disposable cuvettes (Nümbrecht, Germany) using an Anton Paar Litesizer 500 (Anton Paar GMBH, Graz, Austria). Changes in the particle size of unmodified HC-MCC during cooling were measured using a temperature series set up; with readings taken starting from 60^{o}C every 5 degrees until 5^{o}C is reached. HC-MCC was diluted by 1000x in simulated milk ultrafiltrate (SMUF) buffer. Zeta potential measurements on unmodified HC-MCC were conducted in the same manner utilizing a model no. 225288 zeta potential cuvette (Anton Paar GMBH, Graz, Austria). All zeta potential readings were recorded at 18V with a maximum of 300 measurements for each reading. Zeta potential data was analyzed using Smoluchowski approximation in the Kalliope software version 2.26.3 (Anton Paar, Graz, Austria).

Testing with respect to pH adjustment were conducted using a Metrohm AG model 867 pH module as a dosing system with model 800 Dosinos (Herisau, Switzerland) in conjunction with the particle size analyzer utilizing the same Anton Paar model no. 225288 zeta potential cuvette (Graz, Austria). For testing, 25ml of HC-MCC in SMUF solution was poured into the dosing system mixing cup, which is connected to the zeta potential cuvette. The mixing cup was also connected to solutions of 0.1M HCL and NaOH solutions controlled by testing software. Acid or base solutions were added to the solution within the mixing cup via the Dosinos to adjust the pH to a desired level, followed by a fraction of the sample solution being transferred to the cuvette for testing. The dosing system was used to adjust the pH of the sample in a range from 6.6 to 5.8, taking a reading of both particle size and zeta potential every 0.1±0.02 pH change. These

measurements were conducted at three temperatures: 5ºC, 20ºC, and 45ºC. Samples with 10, 25, and 50mM TSC were prepared prior to testing and diluted into SMUF 1000x where it will undergo the same pH range testing.

3.2.6: Transmission Electron Microscopy (TEM)

Ultrastructure analysis of HC-MCC mixtures was performed using the TEM method described by (Lu et al., 2015). Samples underwent rheological testing using the sample loading and testing method described above. Testing occurred within the rheometer but stopped at certain points (25ºC, 11ºC, 5ºC at the end of the temperature sweep) to observe the effect of temperature on the micellar structure. After testing was complete, the CC27 geometry was removed from the rheometer. The bottom of the geometry was removed to reveal the sample which was used for TEM analysis. Samples were fixed using 2% glutaraldehyde and formaldehyde for at least two hours. Samples were then rinsed in a sodium cacodylate buffer and postfixed in 2% osmium tetroxide solution for 1 hour, followed by rinsing with distilled water in two 10-minute stints. After dehydration of the samples via a progressive ethanol series (20 min each in 50%, 70%, twice in 95%, and three times in 100% EtOH) samples were transitioned into plastic resin and infiltrated with a resin-acetone mix. Infiltration with pure resin followed, and polymerization of sample blocks occurred overnight at 65ºC. Sections (70-100nm) were cut on a Leica EM UC6 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL) using a diamond knife (Diatome, Hatfield, PA). Sections were double stained for 20 minutes with saturated aqueous uranyl acetate followed by 10 minutes with Reynold's lead citrate. Sections were then analyzed using a transmission electron microscope (TEM,

JEM 1400 Plus, Jeol USA Inc., Peabody, MA) operated at 120kV, and digital images were captured using a Gatan camera (Gatan Inc., Pleasonton, CA).

3.2.7: Statistical Analysis

Data points from rheological tests were compared using a GLM model in SAS to compare the effects of modifications. Differences were evaluated using ANOVA and Tukey's HSD adjustment with $P \leq 0.05$ considered statistically significant. Statistical comparisons against pH levels were accomplished via 95% confidence intervals.

3.3: Results and Discussion

3.3.1: Particle Size

HC-MCC at an unmodified pH of 6.6 (at 60^{o}C) diluted 1000x into a SMUF buffer exhibited a nonsignificant ($P>0.05$) but general increase in particle size from 165nm to 209nm in response to lowering temperatures from 60^{o}C to 5^{o}C (Figure 3-2). This phenomenon can be attributed to the fact that micellar structures gain additional hydration in cold temperatures due to the release of CCP into solution (Broyard & Gaucheron, 2015).

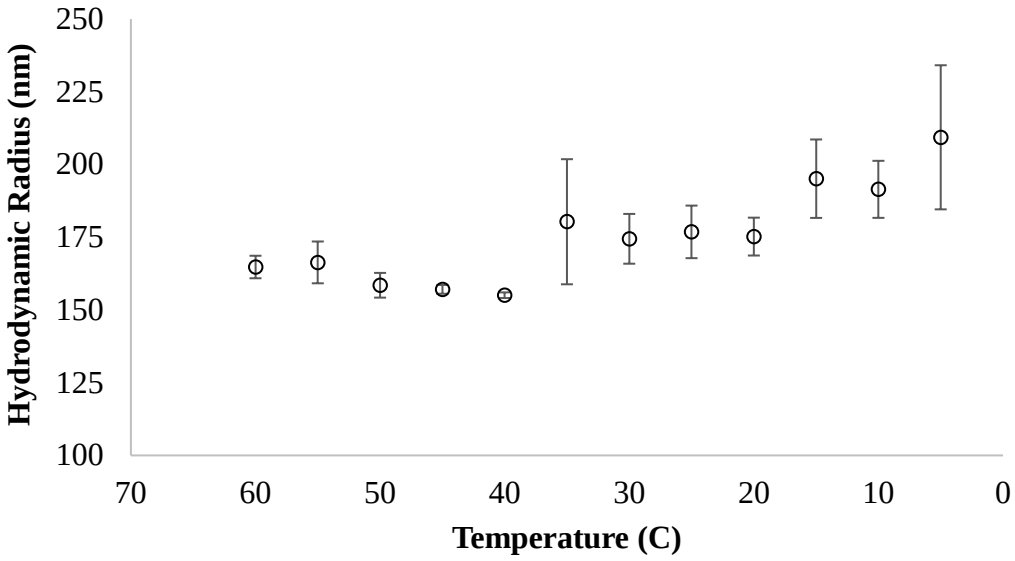

Figure 3-2: Effect of temperature on casein micelle size. Sample used was unmodified HC-MCC at natural pH, diluted with SMUF. Each data point represents mean of triplicate measurements and error bars indicates standard error of mean.

Decreasing pH from 6.6 to 5.8 led to a consistent, linear decrease in the particle size of MCC at all three TSC treatment levels (10, 25, and 50mM) (Figure 3-3). Higher pH is usually associated with larger size of the casein micelle due to increased negative charge and therefore, increased hydration. Conversely, acidification (decline in pH), leads to a decline in net negative charge on the casein micelles, causing increased protein and protein interaction which results in a tighter micellar structure, reduction in particle size (Figure 3-4) and expulsion of the aqueous phase (Sinaga et al., 2017). The reducing effect of pH on particle size was more pronounced for samples added with 25mM TSC. Samples added with 25mM TSC exhibited higher values of hydrodynamic diameter

(315nm to 254nm) followed samples added with 50mM (234nm to 208nm) and 10 mM (199nm to 168nm). The effect of TSC on particle size indicated that calcium chelation may cause changes in the micellar structure, such as swelling (de Kort et al., 2011).

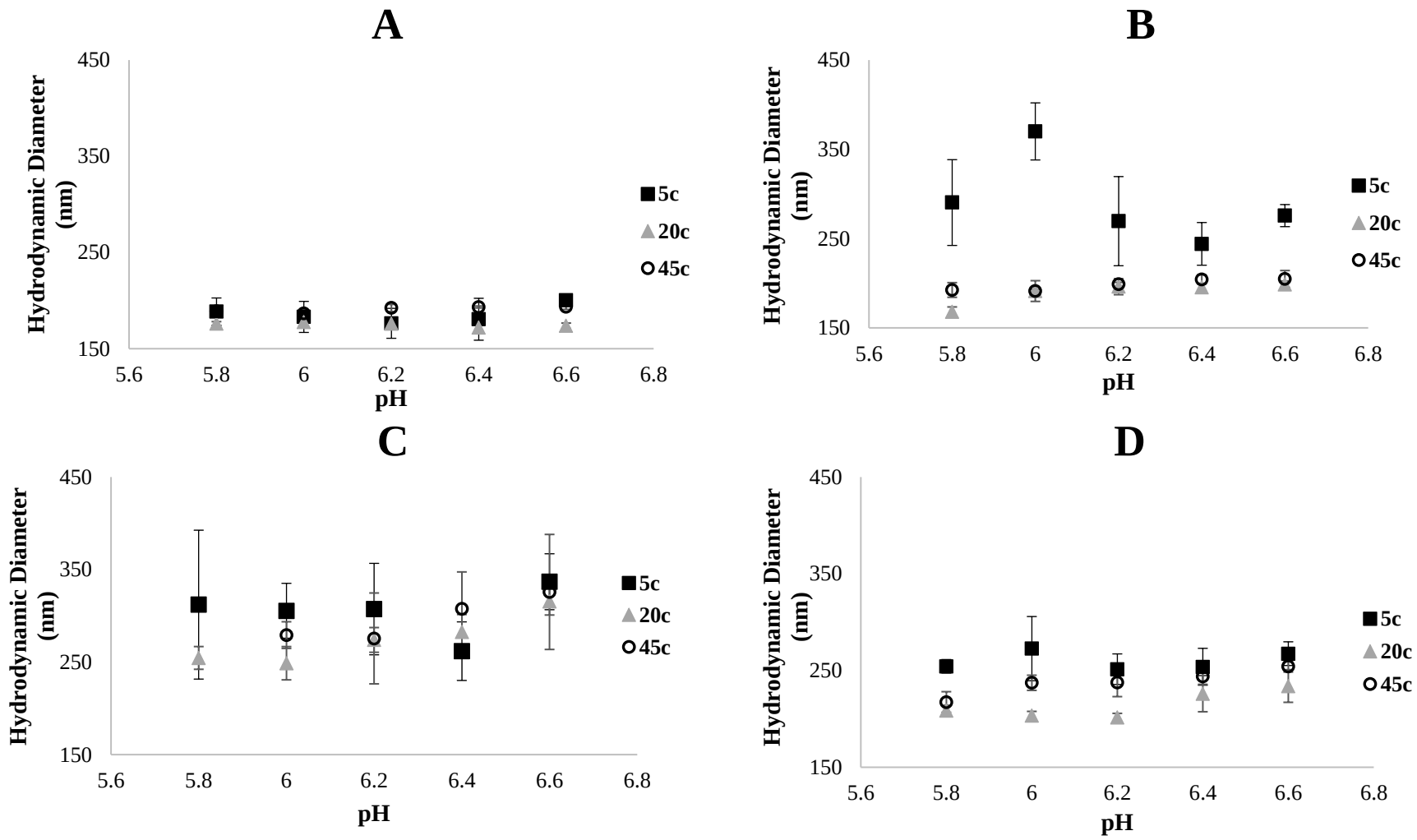

Figure 3-3: Particle size data of TSC and temperature levels during pH testing. A: Unmodified, B: 10mM TSC, C: 25mM TSC, D: 50mM TSC

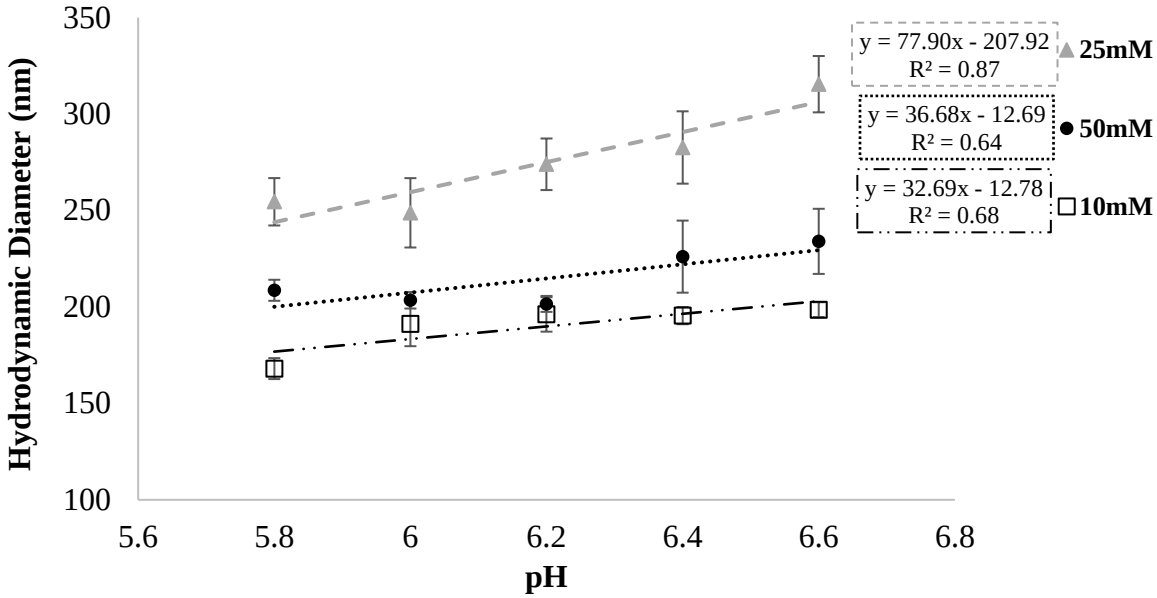

Figure 3-4: Effect of pH and TSC on particle size. Average Particle sizes during pH sweep testing at 3 different concentrations of TSC. All samples were tested at 20^{o}C.

At higher temperatures (20 and 45^{o}C), the effect of pH on particle size of HC-MCC was not as pronounced compared to samples at 5^{o}C (Figure 3-3). Samples kept at 5^{o}C exhibited a slight trend but not very consistently. At pH 6.0 and 5^{o}C, HC-MCC samples (10mM and 50mM TSC content) exhibited the smallest particle size; these variations could be attributed to combined effect of calcium chelation and pH decline. We are unsure of the exact mechanism causing this phenomenon, but the water binding capacity of calcium phosphate within the micelle could be a potential reason.

3.3.2: Zeta Potential

Cooling unmodified HC-MCC (pH 6.6) from 60ºC to 5ºC changed the zeta potential from -16.8mV to -19.7mV (Figure 3-5), indicating that even at low temperature there was still sufficient repulsion between micelles. Decreasing pH 6.6 to 5.8 reduced the net negative charge on the casein micelles, linearly, from -13.2mV to -7.2mV for samples treated with 10mM TSC (Figure 3-6). Figure 3-6 displays the linear relationship of the zeta potential change that occurs irrespective of temperature or TSC concentration of the sample. This effect has been described in previous studies; as casein approaches its isoelectric point of 4.6, the charge on the proteins molecules become neutralized due to increased H+ ions (Anema & Klostermeyer, 1996). The gradual movement towards 0mV as the pH moves in the direction of the IP in this test is further evidence of this phenomenon.

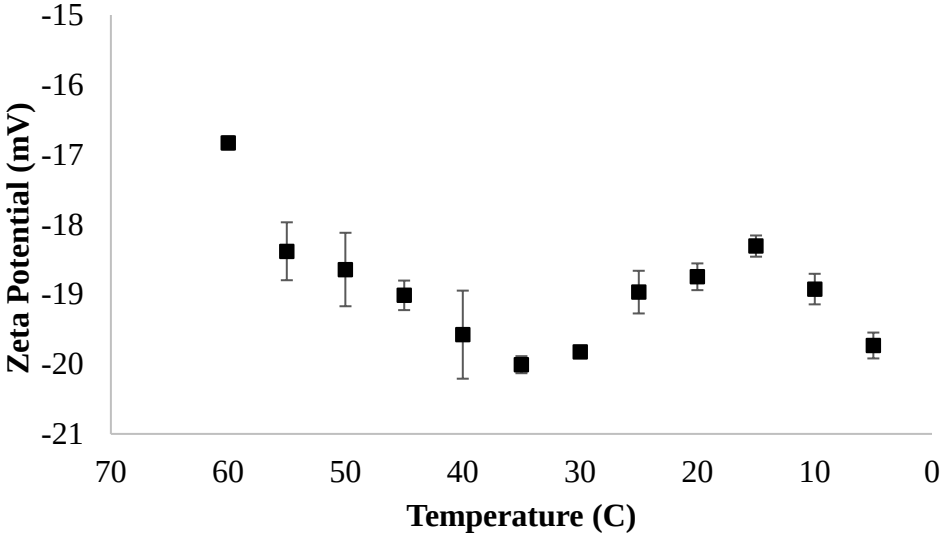

Figure 3-5: Zeta potential of unmodified HC-MCC while reducing from 60ºC to 5ºC.

A similar but less intense trend was observed at 50mM TSC level (Figure 3-6). These results also indicate that more calcium chelation led to more retention of net negative charge on the casein micelle, suggesting that structural integrity is inversely linked to robustness against pH change. Addition of TSC to a milk system may lead to an increase in pH because of its buffering effect and/or calcium chelation. The impact of these two processes on zeta potential may be contradictory, which may lead to inconsistent trends across different pH and TSC levels. However, it was evident that the effect of pH decline on reduction of net negative charge was less pronounced with increasing amounts of TSC regardless of temperature. Interestingly, unmodified MCC demonstrated a higher buffering tendency and a greater charge than TSC added samples at the selected temperatures, which could be attributed to the fact that in native state, more CCP was attached with proteins. Greater amounts of CCP bound to protein help exhibit a stronger buffering effect and retention of charge. However, addition of TSC would cause chelation of calcium i.e., mobilizing calcium from the colloidal phase to soluble phase. With calcium mobility, some of the phosphate groups may be released into the aqueous phase, causing a decrease of the net charge on the micelles (Horne, 2017). With increasing TSC concentration, this phenomenon may be reversing, therefore, increasing net negative charge at a given pH (Figure 3-6).

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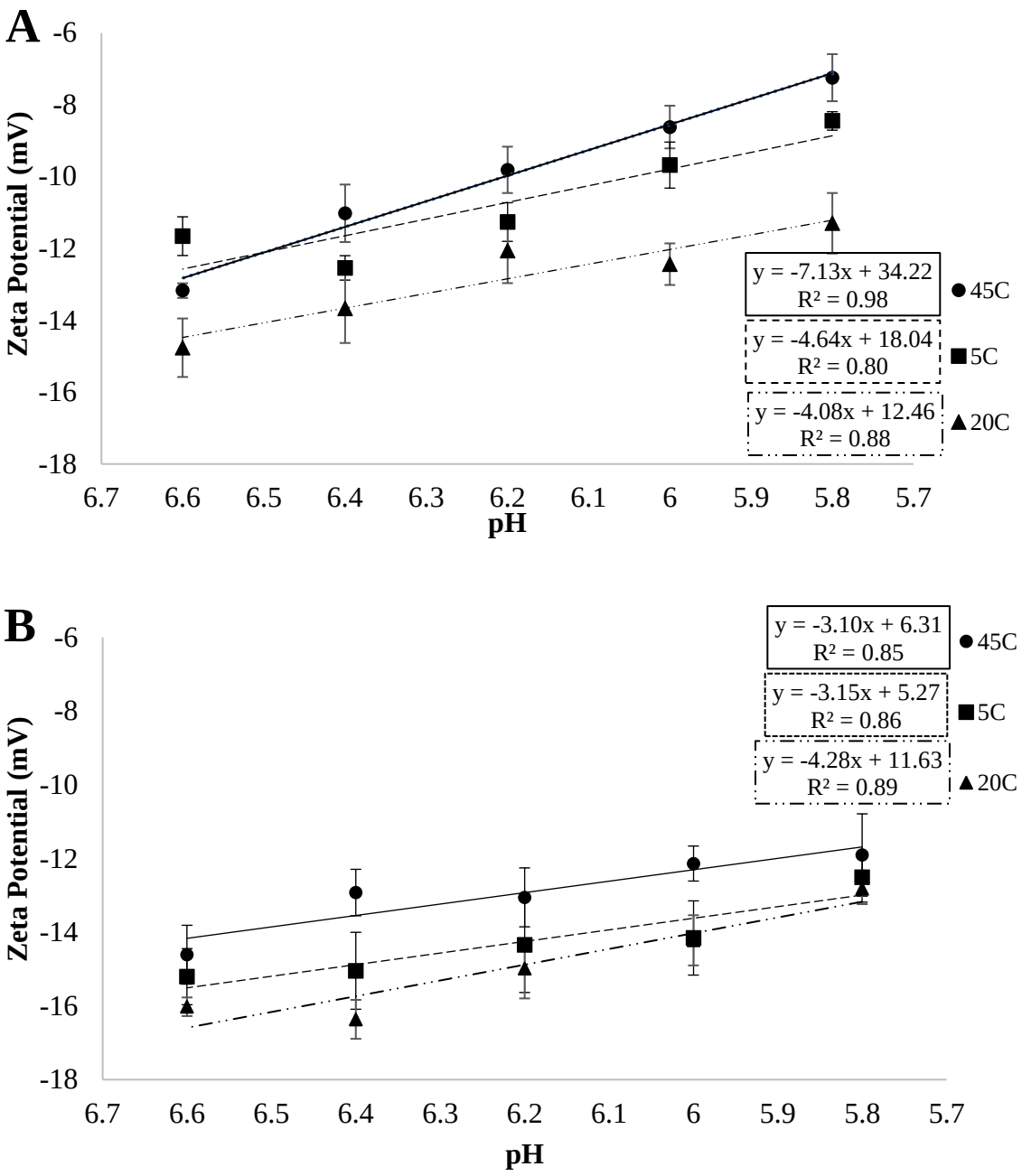


Figure 3-6: Zeta potential in response to temperature and pH. A: 10mM TSC added, B: 50mM TSC added.

3.3.3: Transmission Electron Microscopy

TEM micrographs of unmodified HC-MCC samples harvested at the three temperatures i.e., above (25^{o}C), at (11^{o}C) and below the gelling point (5^{o}C) exhibit clear differences in micellar structure, particularly at higher magnification level (20,000x) (Figure 3-7). Based upon visual observations, it was apparent that the concentration of dense particles (casein micelles) and their distribution was similar at all three temperatures. At temperatures above gelation (25^{o}C), the overall shape of casein micelles appears to be more irregular and distorted, indicating a structural change in response to higher temperatures (Figure 3-7). Additionally, at elevated temperatures, there is evidence of free casein fragments forming less dense pockets within solution rather than maintaining a micellar structure (Figure 3-8). This could be related to several factors, such as the shear forces acting during microfiltration of skim milk to produce HC-MCC (Gebhardt et al., 2012) or phase separation arising from higher hydrophobic interaction at elevated temperatures (Lucey & Horne, 2018) or simply the fluid-like nature of the material at 25^{o}C (Zad Bagher Seighalani et al., 2021). The casein micelles from samples harvested at the gel-sol transition point at 11^{o}C had a spherical, round shape and defined boundary structures. In addition, they appeared to be swollen and more densely packed and possibly interactive with water compared to the higher temperature samples. Below the gelation temperature (5^{o}C), this phenomenon was more prevalent, giving the impression of the typical cold gel structure observed by others (Lu et al., 2015).

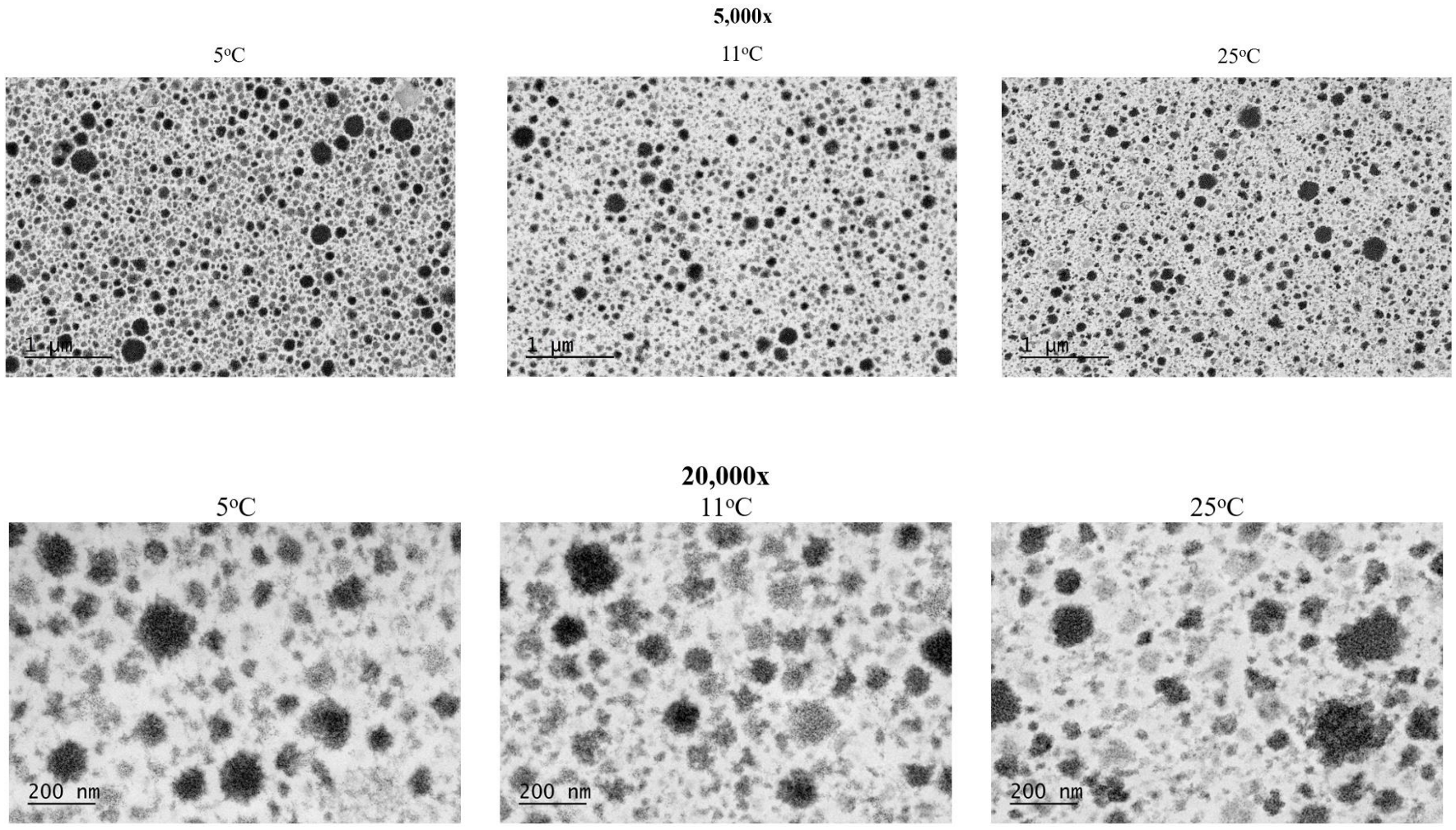


Figure 3-7: TEM micrographs of HC-MCC at different temperatures at 5,000x and 20,000x instrumental magnification. Casein micelles can be seen as electron-dense (dark), more or less spherical structures.

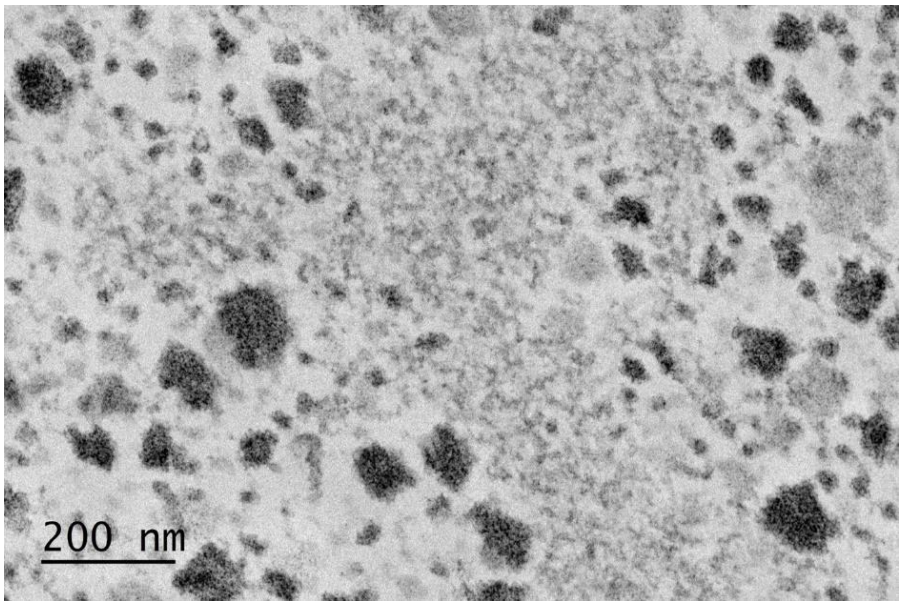


Figure 3-8: TEM Micrograph Showing Presence of Fragmented Casein Structures. The sample was harvested at 25^{o}C, and the micrograph was taken at 20,000x magnification.

3.3.4: Rheology

Figure 3-9 represents the dynamic moduli (G' and G") of HC-MCC at different pH levels (6.2, 6.6, 6.8) while holding at 60^{o}C, cooling to 5^{o}C to form a cold and curing of the gel at 5^{o}C. At 60^{o}C, all the samples behaved as a viscoelastic liquid (G'<G"). At a higher pH, both G' and G" had higher values, indicating that the pH had a large effect on the viscoelastic characteristics of the material. This could be attributed to the fact that at higher pH, casein micelles have a greater net negative charge and particle size (Figure 3-6, Figure 3-4). During cooling, the material went through a phase transition from liquid like (G'<G") to a solid like (G'>G") material state, exhibiting a cross over point for G' and G". The strengthening of HC-MCC network during cooling was attributed to the

morphological changes in the micellar casein concentrate electrostatic charge and swelling (Table 3-3). With an increase in pH, the cross over point between dynamic moduli (G' and G") was occurring at a higher temperature during the temperature sweep stage of testing (Figure 3-9). This could also be related to an increase in micellar hydration and swelling of the structure at a higher pH. True cold gelling temperature (CGT) as determined using the protocol described by Zad Bagher Seighalani et al., (2021) also indicated a similar pattern (at pH 6.2, CGT 7.80^{o}C; pH 6.8, CGT 26.99^{o}C) (Table 3-2). Protein concentration had an impact on the CGT (Table 3-2). Samples diluted to 15% casein content formed cold gels at a lower temperature compared to samples with 18.5% protein. A similar effect was observed with G' values, with 15% protein samples forming weaker gels. (Table 3-3, Table 3-4). The effect of protein content was significant ($P<0.05$) on the G' values of the MCC during cooling from 60^{o}C to 5^{o}C and holding at 5^{o}C (Table 3-3, Table 3-4). At natural pH (6.6), diluting protein content from 18.5% to 15% decreased G' at 20^{o}C by ~4 fold (from 6.95Pa to 1.69Pa).

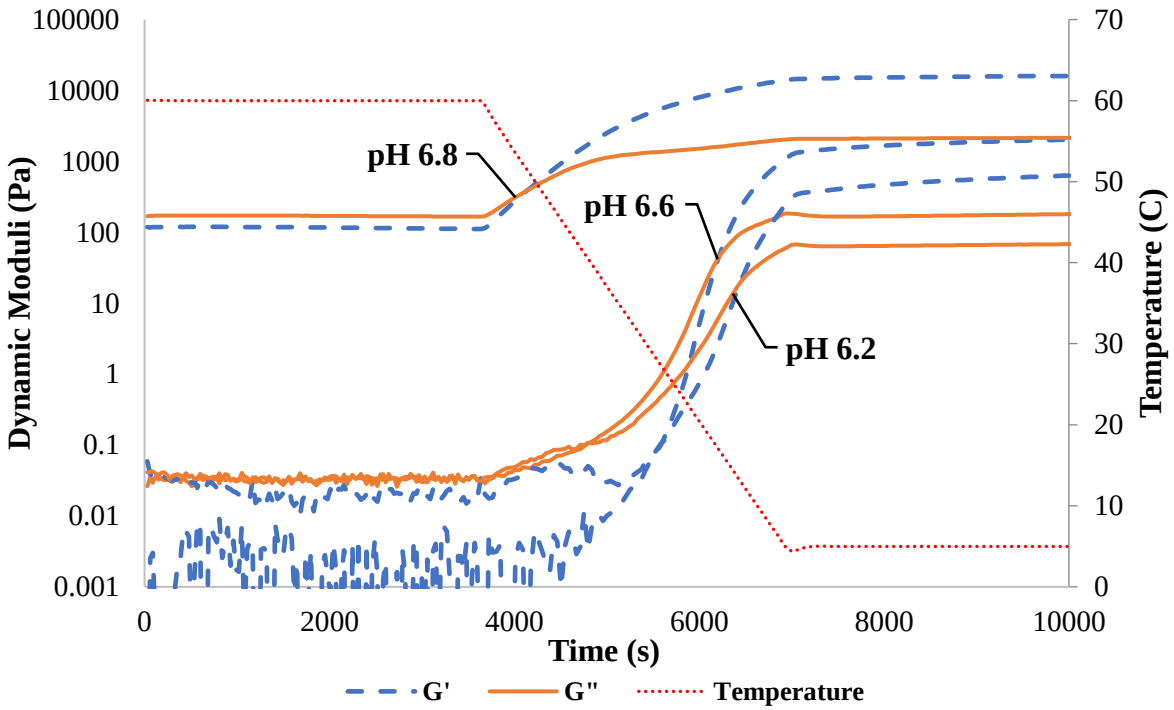


Figure 3-9: Dynamic moduli of select pH levels during temperature sweep testing. Black arrows designate pH level and crossover point of G' and G". The testing was conducted using 1% amplitude strain and 1rad/s frequency.

At the end of the temperature sweep, the strength of the cold gels increased by approximately an order of magnitude from pH 6.2 (G'=261 Pa) to 6.6 (G'=901 Pa) to 6.8 (G'=12100 Pa) (Table 3-3). The reinforcement of the gel structure due to pH modifications could be attributed to the increase in net charge and changes in the dynamics of CCP mobility from colloidal phase to soluble phase (Gonzalez-Jordan et al., 2015). Holding cold gels at 5°C for 10h further increased the gel strength. The majority of increases in dynamic moduli appeared to take place within the first 2 hours of holding,

with minimal increases after. The highest ($P<0.05$) relative increase was for pH 6.2 (from 261Pa to 1490Pa), and smallest ($P>0.05$) relative increase was for pH 6.8 (from 12100Pa to 15800Pa) (Table 3-3). Samples at pH 6.8 appear to maintain a high gel strength even at elevated temperatures such as 20^{o}C (Table 3-3). We attribute these differences to the fact that soluble calcium levels vary based on pH values and there is a limit of volume fraction casein micelles can take up in the given circumstances due to the high concentration of protein in solution (Lu et al., 2015). The relationship between pH and the dynamic moduli (G' and G") of the cold gels held at 5^{o}C for 10 hours followed an exponential equation, indicating a slow increase in the moduli at low pH values, followed steeper increase at higher pH levels (Figure 3-10).

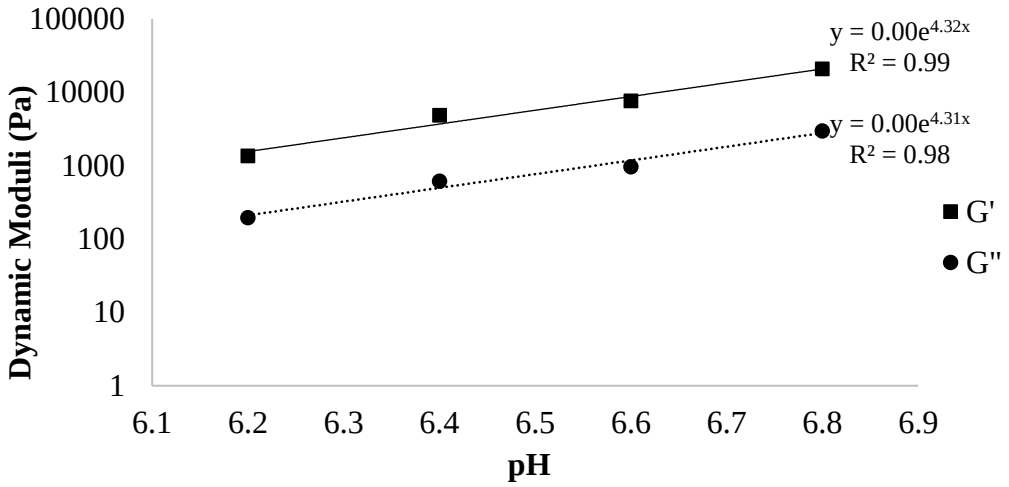


Figure 3-10: Changes in dynamic moduli with respect to pH after holding MCC sample at 5°C for 10 hrs. Average modulus values of HC-MCC with respect to pH adjustment. The samples used in the following tests were 18.5% protein and had 10mM of TSC added.

The impact of TSC addition on the viscoelastic properties of final cold gels (after holding at 5°C for 10hrs) prepared at both protein content levels can be seen in Figure 3-11. It was clearly evident that diluting the protein content from 18.5% to 15% decreased G' values by 4-fold at all TSC levels. Addition of TSC caused an increase in gel strength at all levels (Figure 3-11) and at all pH treatments (Table 3-3). However, the maximum increase in gel strength was observed at 25mM for both protein levels (Figure 3-11). This was due to calcium chelation activity of TSC, possibly causing changes in the micellar structure (Kaliappan & Lucey, 2011). On the other hand, excessive amounts of TSC (e.g., 50mM), led to a decline in gel strength and CGT (Figure 3-11, Table 3-2, and Table 3-3).

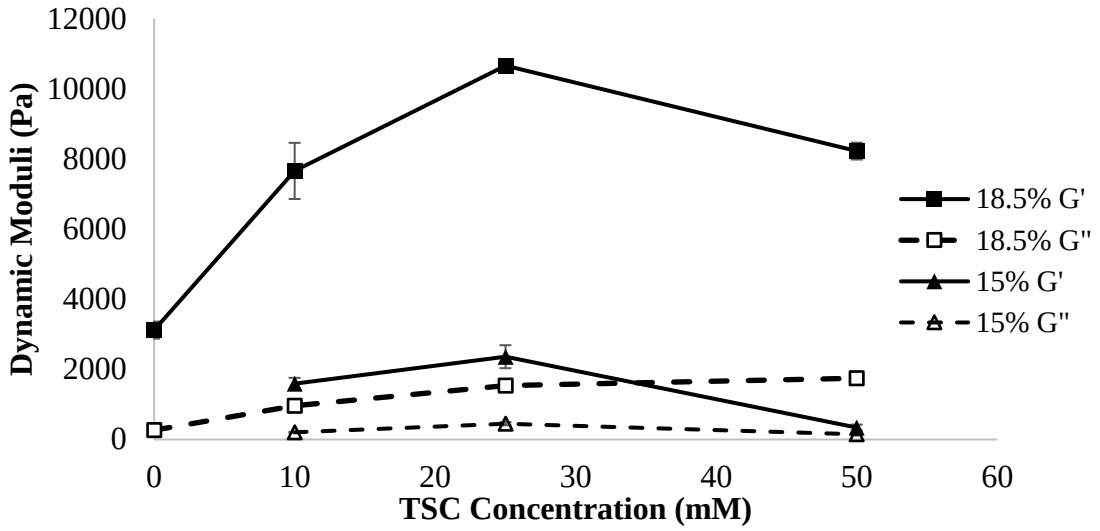


Figure 3-11: Changes in dynamic moduli with respect to TSC concentration. Average modulus (G' and G") values of HC-MCC with respect to TSC concentration. The samples used in these tests were at a native pH of 6.6 prior to the addition of TSC.

Samples with 15% protein content and no modifications formed a very weak structure, not strong enough to hold its shape. With lower pH values, the required amount of TSC to obtain the maximum G' value increased (Figure 3-12). This can be attributed to the fact that at low pH, more soluble calcium is released from the micelle, requiring a larger amount of the TSC to chelate excess calcium. It is likely that TSC interacts first with the soluble calcium and then it starts interacting with casein bound calcium (CCP). We hypothesize that changes in CCP due to TSC addition contributes to the increase in the strength only after it is able chelate all the solubilized portion of CCP and remaining portion disturbs the micellar integrity. More work is needed to test this hypothesis.

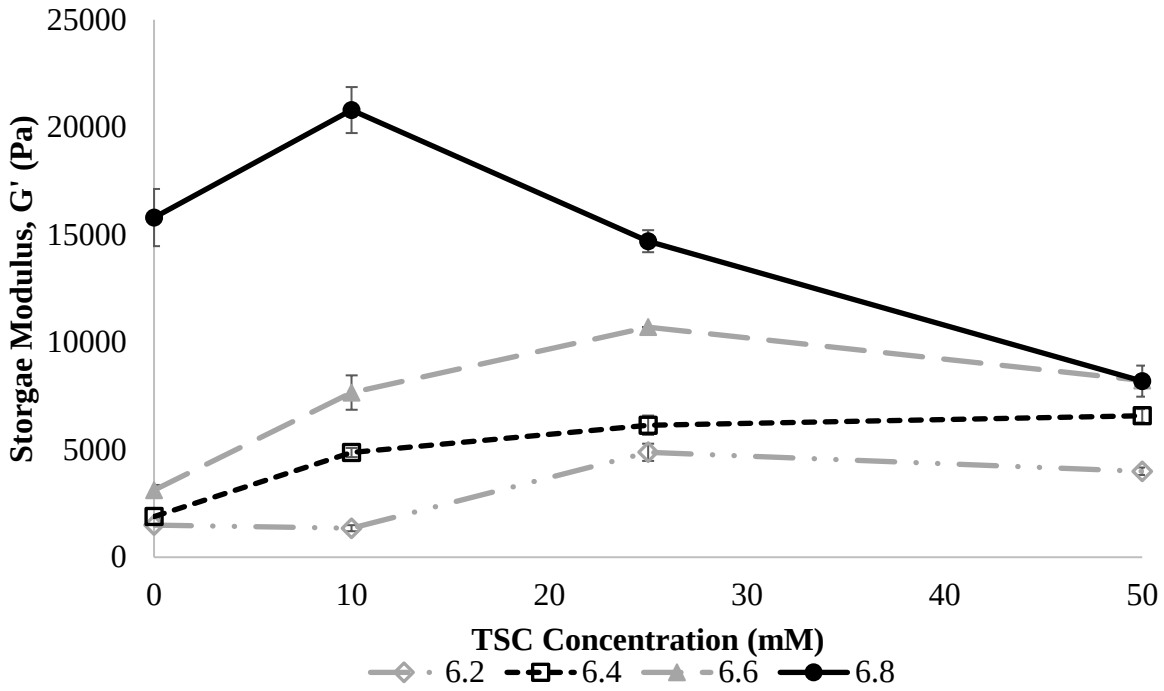
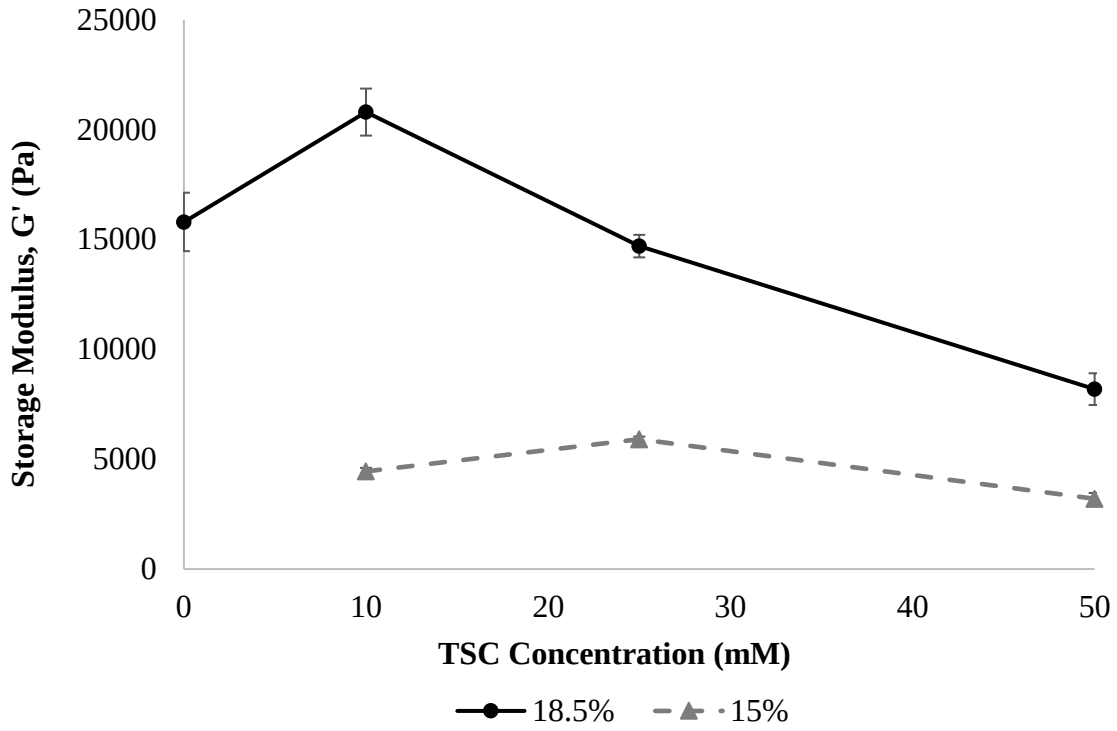


Figure 3-12: Effect of TSC addition on the storage modulus of 18.5% protein gels adjusted to different pH levels.

At 25mM TSC levels, the impact of protein content on CGT was slightly diminished, indicating that there is an optimum concentration of TSC (25mM) needed to increase CGT. Interestingly, at lower pH levels (<6.8) and 10mM TSC concentrations, gel formation was not observed during cooling MCC from 60ºC to 5ºC (Table 3-2). This can be attributed to the fact that excessive calcium dissociation from the micelle and its sequestration by TSC made less calcium available for participating in forming protein-protein bridges. On the other hand, complete removal of CCP (without a drastic pH

change) may lead to formation of irreversible aggregates, losing ability to form a thermoreversible gels. However, to prove this hypothesis more work is needed.

In response to alkalized samples, the amount of TSC needed to form a stronger cold gel at pH 6.8 decreased with higher concentration of protein (10mM at 18.5% and 25mM at 15%) (Figure 3-13). We hypothesize that there is a dependency between the spacing of casein micelles and amount of soluble calcium needed to form cross-links between structures. At higher protein concentrations, spacing between casein micelles reduces; this necessitates less quantity of TSC needed to solubilize calcium from the colloidal phase and make bridges between protein structures. There are some indications in literature on the role of calcium in forming bridges during cold gelling (Lu et al., 2015; Zad Bagher Seighalani, 2021). However, there is more work needed to test our hypothesis.



198

199 **Figure 3-13:** The effect of TSC on storage modulus values of pH 6.8 samples at different

200 protein concentrations.

201

Table 3-2: Cold gelation temperature (CGT) values of HC-MCC with different protein and TSC content.

pH	TSC Concentration (mM)	Protein concentration, w/w	
		15%	18.5%
6.8	0	X	26.99 ± 0.23^{AB}
	10	19.90 ± 0.40^{ABb}	27.68 ± 0.23^{Aa}
	25	21.50 ± 0.61^{Ab}	25.39 ± 0.40^{Ba}
	50	17.84 ± 0.40^{Bb}	20.81 ± 0.61^{Ca}
6.6	0	X	8.01 ± 0.23^{C}
	10	No Gel*	20.58 ± 0.69^{B}
	25	20.94 ± 1.62^{Ab}	25.84 ± 0.46^{Aa}
	50	13.72 ± 0.68^{Bb}	21.27 ± 0.40^{Ba}
6.4	0	X	7.79 ± 0.23^{C}
	10	No Gel*	17.61 ± 0.91^{B}
	25	17.38 ± 0.83^{Bb}	21.72 ± 0.61^{Aa}
	50	24.92 ± 0.83^{Aa}	19.21 ± 0.69^{ABb}
6.2	0	X	7.80 ± 0.23^{C}
	10	No Gel*	7.78 ± 0.23^{BC}
	25	No Gel*	21.49 ± 0.82^{A}
	50	12.81 ± 1.65^{b}	14.63 ± 0.23^{Ba}

A comparison of gelation temperatures in response to different protein concentrations and TSC content. * Designates no gel formation during the temperature sweep. Values are mean ± standard error. Capital letters denote significance within the same protein concentration, while lower case denotes significance within the same TSC concentration. Statistics were not conducted across different pH levels. Significance is at $P<0.05$.

Table 3-3: Storage Modulus Values of 18.5% protein HC-MCC with different pH and TSC content.

18.5% Protein		Storage Modulus (G') Value (kPa)			
pH	TSC Concentration (mM)	20°C	8°C	5°C	5°C + 10 Hours
6.8	0	6.95 ± 0.633^{Bc}	11.0 ± 0.897^{Bbc}	12.1 ± 0.969^{Bab}	15.8 ± 1.33^{Ba}
	10	10.7 ± 0.657^{Ac}	16.0 ± 0.937^{Ab}	17.4 ± 1.01^{Aab}	20.8 ± 1.07^{Aa}
	25	7.79 ± 0.251^{Bc}	11.9 ± 0.342^{Bb}	13.0 ± 0.362^{Bb}	14.7 ± 0.512^{Ba}
	50	3.77 ± 0.366^{Cb}	6.87 ± 0.581^{Ca}	7.76 ± 0.640^{Ca}	8.19 ± 0.723^{Ca}
6.6	0	0.00419 ± 0.00180^{Dc}	0.519 ± 0.0912^{Ccb}	0.901 ± 0.127^{Cb}	3.12 ± 0.242^{Ca}
	10	1.12 ± 0.236^{Cc}	3.18 ± 0.477^{Bcb}	3.94 ± 0.548^{Bb}	7.66 ± 0.800^{Ba}
	25	3.59 ± 0.0635^{Ad}	6.62 ± 0.0665^{Ac}	7.57 ± 0.00652^{Ab}	10.7 ± 0.00751^{Aa}
	50	2.83 ± 0.0668^{Bd}	5.80 ± 0.141^{Ac}	6.76 ± 0.164^{Ab}	8.22 ± 0.240^{Ba}
6.4	0	$0.000864\pm 0.0000388^{Cd}$	0.161 ± 0.00874^{Cc}	0.352 ± 0.0170^{Cb}	1.89 ± 0.0586^{Ca}
	10	0.395 ± 46.1^{Bc}	1.71 ± 0.109^{Bb}	2.29 ± 0.134^{Bb}	4.87 ± 0.212^{Ba}
	25	1.34 ± 107^{Ac}	3.28 ± 0.207^{Ab}	3.98 ± 0.241^{Ab}	6.14 ± 0.450^{ABa}
	50	1.29 ± 101^{Ac}	3.51 ± 0.176^{Ab}	4.32 ± 0.195^{Ab}	6.58 ± 0.311^{Aa}
6.2	0	$0.000610\pm 0.000142^{Bd}$	0.109 ± 0.00289^{Bc}	0.261 ± 0.00801^{Bb}	$1.49\pm0.045.8^{Ba}$
	10	0.0018 ± 0.000944^{Bb}	0.118 ± 0.0347^{Bb}	0.248 ± 0.0547^{Bb}	1.35 ± 0.142^{Ba}
	25	0.494 ± 0.113^{Ac}	1.76 ± 0.289^{Acb}	2.43 ± 0.343^{Ab}	4.88 ± 0.400^{Aa}
	50	0.545 ± 0.0377^{Ad}	2.00 ± 0.0980^{Ac}	2.64 ± 0.115^{Ab}	4.00 ± 0.174^{Aa}

A comparison of storage modulus values for 18.5% protein samples in response to different TSC concentrations. Values are mean ± standard error. Capital letters denote significance within the same protein concentration, while lower case denotes significance within the same TSC concentration. Statistics were not conducted across different pH levels. Significance is at $P<0.05$.

Table 3-4: Storage Modulus Values of 15% protein HC-MCC with different pH and TSC content.

15% Protein		Storage Modulus (G') Value (kPa)			
pH	TSC Concentration (mM)	20°C	8°C	5°C	5°C + 10 Hours
6.8	10	1.69 ± 0.0786^{Bc}	3.15 ± 0.116^{Bb}	3.34 ± 0.129^{Bb}	4.45 ± 0.156^{Ba}
	25	2.64 ± 0.0507^{Ad}	4.40 ± 0.0688^{Ac}	4.86 ± 0.0740^{Ab}	5.91 ± 0.121^{Aa}
	50	0.899 ± 0.0579^{Cc}	2.26 ± 0.154^{Cb}	2.72 ± 0.189^{Cab}	3.20 ± 0.265^{Ca}
6.6	10	0.00235 ± 0.00141^{Bb}	0.135 ± 0.0354^{Bb}	0.273 ± 0.0588^{Bb}	1.60 ± 0.163^{Aa}
	25	0.358 ± 0.0818^{Ac}	1.12 ± 0.191^{Abc}	1.40 ± 0.226^{Aab}	2.36 ± 0.328^{Aa}
	50	0.0164 ± 0.00683^{Bb}	0.0860 ± 0.0247^{Bb}	0.129 ± 0.0344^{Bab}	0.340 ± 0.0851^{Ba}
6.4	10	$0.00000154\pm 0.000000123^{Bb}$	0.00179 ± 0.293^{Bb}	0.0104 ± 0.00144^{Bb}	0.566 ± 0.0191^{Ca}
	25	0.0456 ± 0.00217^{Ad}	0.332 ± 0.0119^{Ac}	0.480 ± 0.0169^{Ab}	1.41 ± 0.0467^{Aa}
	50	0.0479 ± 0.00216^{Ad}	0.307 ± 0.0133^{Ac}	0.466 ± 0.0198^{Ab}	1.10 ± 0.0465^{Ba}
6.2	10	$0.000000291\pm 0.000000162^{Bb}$	$0.00000677\pm 0.00000139^{Bb}$	$0.000317\pm 0.000103^{Bb}$	0.143 ± 0.0312^{Ca}
	25	$0.000252\pm 0.0000735^{Bb}$	0.0412 ± 0.0121^{Ab}	0.105 ± 0.0247^{Ab}	0.957 ± 0.104^{Aa}
	50	$0.00679\pm 0.0000970^{Ac}$	0.0742 ± 0.00785^{Abc}	0.129 ± 0.0118^{Ab}	0.479 ± 0.0400^{Ba}

A comparison of storage modulus values for 15% protein samples in response to different TSC concentrations. Values are mean ± standard error. Capital letters denote significance within the same protein concentration, while lower case denotes significance within the same TSC concentration. Statistics were not conducted across different pH levels. Significance is at $P<0.05$.

3.4: Discussions

In this work we found that there are several factors which can influence the formation of cold gels. For example, cooling MCC from 60^{o}C to 5^{o}C causes an increase in particle size and net negative charge on the casein micelle, as well as solubilizes CCP so that it can act as bridging element during formation of cold gels.

The rheology of a system depends upon the volume fraction of the dispersed phase (in our case casein micelles), rigidity of dispersed particles, interaction between particles, viscosity of the solvent, and temperature of the system. Swelling of the casein micelles at low temperatures increases the volume fraction, therefore viscosity and gel strength of the system (D.Z. Liu et al., 2013). Increasing concentration of dispersed phase or volume fraction increases the interaction between particles. When the volume fraction reaches a maximum packing effect, jamming of particles takes place where particles exert steric hindrance to the extent where they cannot move. On the other hand, at low temperatures, the kinetic energy of the system decreases to the extent where molecules cannot move past each other and get arrested (Lu et al., 2015). Both concepts offer a potential explanation behind cold gelation.

The Arrhenius equation is widely used for explaining temperature dependence of a system based upon the kinetic theory of the molecular movement. In our case, we are taking G' as a factor dependent upon mobility of particles influenced by temperature. Figure 3-14 shows the storage modulus of 4 selected treatments during the temperature sweep fitted to an Arrhenius equation (Eq 1). Using the following equation, we can obtain the activation energy (E_a) of deformation for each sample and temperature

dependence of storage moduli (G') (Tunick, 2010). Lower activation indicates ease of gel formation and stability of the gel.

$$k = Ae^{\frac{-Ea}{RT}} \quad (1)$$

As expected, since both of the strongest samples (18.5% protein with 10mM TSC at pH 6.8, and 15% protein with 25mM TSC at pH 6.8) for their respective protein concentrations had formed a gel by 20^{o}C (0.00341 in 1/Kelvin), their activation energy is a lower value. The control sample and weaker 15% protein sample, however, did not form a gel by 20^{o}C, and as such they have a higher activation energy (>200 kJ/mol.K) and more temperature dependence in the given temperature range.

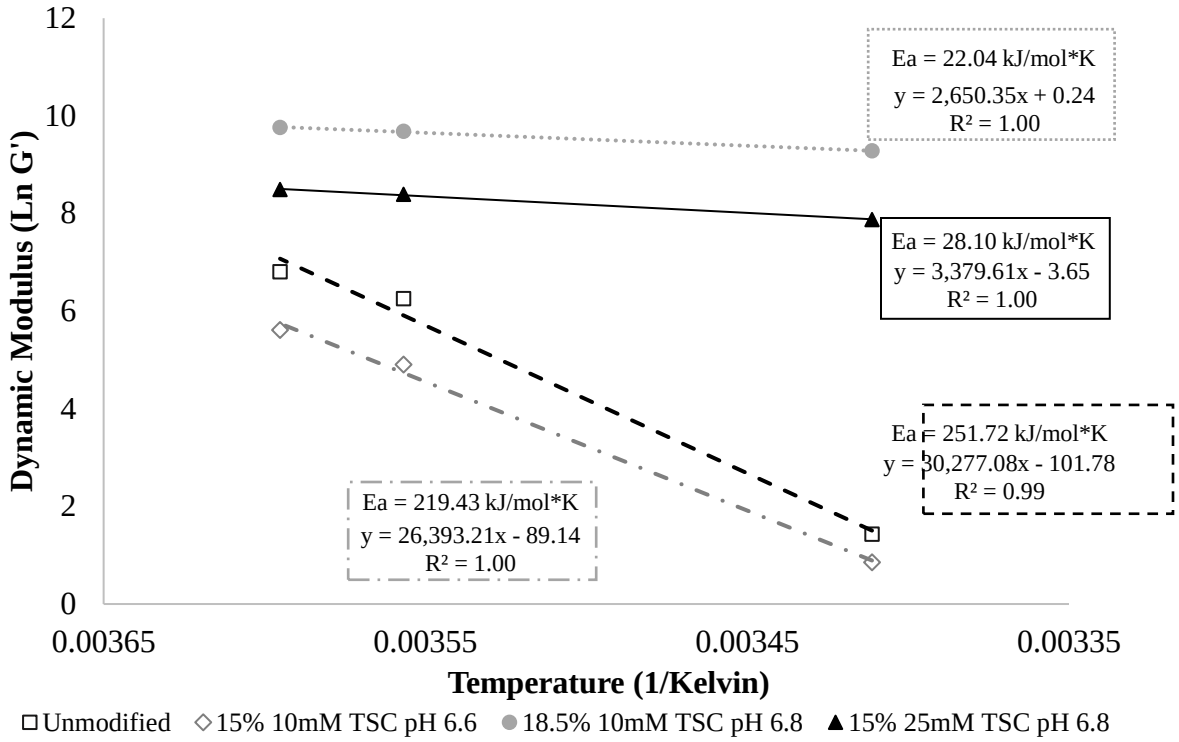


Figure 3-14: Storage modulus values of select treatments in response to temperature change fitted to an Arrhenius equation. Control samples are 18.5% protein at pH 6.6 with 0mM TSC added.

The initial increase and subsequent decrease in particle size in response to increasing the TSC content (Figure 3-4) follows the same pattern observed for gel strength with the same quantities of TSC, indicating that there could be a potential link between size of particles in solution and gel strength, conforming the hypothesis related to the volume fraction. A similar effect can be observed in the TEM results, where micrographs of samples taken well above gelation (25^{o}C) exhibited free hydrated casein

pockets released from casein micelle where particle size is minimal (Figure 3-8). This observation was concomitant with the irregular structures exhibited, indicating a less homogeneous and less interactive dispersed phase, possibly due to residual hydrophobic interactions of casein micelle. The presence of these fragmented casein pockets at slightly higher temperature was contradictory to the behavior of β-casein, which is known to migrate from the micelle to the aqueous phase in response to low temperature (<15^{o}C) (O'Connell et al., 2003). Such pockets were not observed at CGT (11^{o}C) and below; instead, there was evidence of protein structures smaller than those seen for the typical micelle size. The formation of these smaller structures indicates that there are entropic forces in response to changes in temperature causing the formation of regular, well hydrated and interactive structures observed in the sample (Holt et al., 2013).

The effect of TSC on samples falls in line with previous rheological research conducted on dairy samples (de Kort et al., 2011; Ozcan-Yilsay et al., 2007). As TSC can remove the CCP from the interior of the casein micelle, the resulting destabilization can open the structure, releasing individual casein fractions at higher concentrations (Deshwal, 2017). In addition to this release of casein fractions, the remaining structure gains additional size due to increased hydration (de Kort et al., 2011). Increases in casein hydration are the likely cause of increases in gel strength and CGT, but there is additional research required to better understand how the release of casein fractions may impact rheological properties.

The decrease in zeta potential values in response to alkalization is a possible explanation for increased CGT and G' values in these samples. With a greater negative charge on caseins within solution, there is a potentially higher degree of protein-water

interaction than what is observed at native or acidified pH levels (Vaia et al., 2006). Higher protein-water interactions could prove beneficial for gel strength by preventing syneresis and long-term stability in a sample. Another potential explanation can come from particle size data: with higher pH, there is a greater average hydrodynamic diameter (Figure 3-4). If the pH is high enough to encourage swelling of the micellar structure, it could encourage the packing transition proposed in prior literature (Dunn et al., 2021; Y. Liu & Guo, 2008; Lu et al., 2015). More analysis is required to better understand the effect of alkalization on both the structure of casein, and how it impacts gelling behavior.

3.5: Conclusions

The effects of the protein concentration, pH adjustment and calcium chelation had a significant impact on the cold gelling behavior of HC-MCC. We propose that cold gel formation in MCC takes place due to increased hydration, net negative charge and solubilization of calcium. Acidification of the sample resulted in the reduction of gel qualities and particle size, whereas alkalization improved gel strength and increased CGT, owing to increased particle size and zeta potential. Addition of TSC at both protein levels (18.5% and 15%) led to formation stronger gels, indicating significant role of calcium in cold gel formation. The quantity of TSC needed to form stronger gels is dependent on the pH, and protein content of the MCC samples. Additional research is needed to get further insights into understanding the mechanism of cold gel formation.

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CHAPTER 4

RHEOLOGICAL AND ULTRASTUCTURAL ANALYSIS OF MODIFIED MICELLAR CASEIN COLD GELS

ABSTRACT

The purpose of this study was to examine the effect of calcium chelation and alkalization on the cold gelling properties of Highly Concentrated-Micellar Casein Concentrate (HC-MCC). Rheological measurements of dynamic moduli (G' and G") were measured on HC-MCC gels at multiple frequencies to determine gelation via Winter–Chambon criterion. In addition, Transmission Electron Microscopy (TEM) micrographs for each of the treatments were produced to understand the effect of modification on the micellar structure of casein. All modifications performed resulted in a significant increase ($P < 0.05$) in gel strength and gelation temperature, with samples adjusted to pH 7.0 yielding the strongest gels. Both forms of treatments raise sample pH, but TEM micrographs show evidence of different mechanisms causing morphological changes to casein micelles in solution. The addition of 25mM of TSC resulted in the partial disintegration of the micellar structure whereas samples treated with 50mM (TSC) show formation of large aggregate structures. pH adjusted samples show an increasingly disintegrated micellar structure, with pH 7.0 samples displaying complete micellar dissolution and the formation of a casein matrix. These findings show that the effect of

calcium chelation and alkaline pH adjustment greatly affect both the structure of casein micelles and the rheological properties of HC-MCC. The mechanism behind both forms of modification appear to be different, additional research is required to better understand the effect of these modifications on micellar structure.

4.1: Introduction

Highly Concentrated Micellar Casein Concentrate (HC-MCC) is a dairy product with the unique capability of creating a gel without modification (Lu et al., 2016). HC-MCC is produced in a similar manner to MCC via the microfiltration and diafiltration of milk; the difference between the two being that HC-MCC undergoes an additional stage of vacuum evaporation to increase the solids content. The final protein content ranges from 17% to 23% while maintaining the native conformation of the casein micelle (Saboyainsta & Maubois, 2000). The cold gelling nature of HC-MCC has been documented and is the subject of research in order to understand it better (Dunn et al., 2021; Lu et al., 2016; Zad Bagher Seighalani et al., 2021). Lu et al. (2015) proposed a mechanism of gelation behavior where the high amount of casein micelles within solution created a packing effect where there is little viscoelastic flow due to the minimal space between protein supramolecules. In conjunction with this, additional gel strength was attributed to bridging between micelles via bridging of Colloidal Calcium Phosphate (CCP).

The effect of calcium bridging between micellar structures can be modified by utilizing a calcium chelating salt such as trisodium citrate (TSC). CCP is also considered

an important molecule for the overall stability of a casein micelle (Younes, 2017). Calcium chelating salts have the ability to remove CCP from the micelle resulting in conformational changes in the tertiary structure, potentially resulting in crosslinking between strands of caseins. Previous rheological studies have shown that TSC in casein gels has an effect on gel strength, likely due to this effect (Ozcan-Yilsay et al., 2007). When TSC is added to casein solutions, there is a notable increase in gel strength. In the event of a high concentration of TSC added to casein, there is a notable decrease in gel strength compared to a lower concentration of salt. One potential explanation is that while a lower concentration of TSC may cause the dissociation of caseins into the solution, a high enough concentration has the potential to chelate all CCP within the solution which could completely disrupt the micellar structure and yield a weaker gel (Ozcan-Yilsay et al., 2007).

Adjustment in sample pH can also affect calcium equilibrium within casein. Increases in pH result in a decrease in CCP solubility, and by extension a reduction in hydrophobic interactions between caseins (McMahon & Oommen, 2008). In the event of alkalinization, the charge on individual caseins changes in response to the increase in OH^- within solution (Touhami et al., 2022). This reduction in the hydrophobic interactions can result in the dissolution of the micelle, leading to the release of free casein proteins within solution (Vaia et al., 2006). If calcium bridging between individual caseins were to occur in a highly dissociated casein solution, there is potential for strong gel formation. Mild increases in pH (as in not alkalized enough for micellar destabilization) have been found to result in particle size increases as well, potentially increasing gel strength due to a heightened version of the packing effect proposed in Lu et al. (2015).

As with many other food gels, HC-MCC exhibits viscoelastic behavior where it acts more like a viscoelastic fluid at elevated temperatures and transitions into a viscoelastic solid as temperature within the system decreases. Applying rheological techniques to measure the properties of an HC-MCC gel offers an excellent opportunity to better understand the gelling characteristics of casein, and the effect of modification on casein gels. To determine the temperature where a viscoelastic material transitions to a gel requires the use of a technique based on the data recorded during testing. A common method utilized is the crossover point method, where the storage modulus (G'), or measurement of elastic properties yields a greater value than the loss modulus (G") or measurement of liquid properties. This is seen as a pseudo-gelation point as the determined gelation point is highly dependent on the frequency used during testing (Liu et al., 2016). Zad Bagher Seighalani et al., (2021) utilized a multiwave method originally devised by Winter & Chambon, (1986) where gelation was defined as the point where the loss tangent of the sample was independent of the frequency that oscillatory shear was applied to the sample. This method resulted in a significantly lower temperature of transition, but samples at gelation point behaved more accurately as a gel.

Transmission Electron Microscopy (TEM) is an imaging method that allows the direct observation of protein structures at high details and has been used to image the structure of dairy products in multiple forms (Lu et al., 2015; Marchin et al., 2007; Vollmer et al., 2021). Imaging of unmodified casein micelles in milk and concentrated dairy products has shown that the casein micelle structure is consistently spherical in shape and generally measures 100-200nm in diameter with fine protein strands of casein on the exterior (McMahon & Oommen, 2008). In conjunction with the strong imaging

potential it offers, TEM can also be used effectively in conjunction with rheological testing. A rheological testing protocol can be conducted on a sample and then can be used as a sample for TEM imaging, allowing two sources of data on a singular sample. Comparison of TEM micrographs to rheological data allows us to observe interactions on a micro scale while offering insight on how this can affect rheological data on the macro scale. In this study, we will observe the gelling properties of HC-MCC using oscillatory rheological protocols in conjunction with TEM imaging. Our goal is to find the strongest possible gel and the highest gelation temperature for HC-MCC using rheological testing, and to use TEM to understand the effect of modifications on the structure of casein micelles on a microscopic scale.

4.2: Materials and Methods

4.2.1: Sample Preparation

4.2.1.1: HC-MCC Production

HC-MCC used in the study was produced at South Dakota State University in the same manner as described in Lu et al., (2015). The MF system was a 4-vessel continuous design utilizing polyvinylidene fluoride membranes with a combined surface area of 57.4m^2. The subsequent vacuum evaporation of the MCC was conducted at 63^{o}C at a pressure of -680mbar. The samples were held in large pails within a -20^{o}C freezer. Frozen samples were taken out and molten to a liquid state in a water bath at 50^{o}C. The liquid HC-MCC is thoroughly mixed and poured into screw cap plastic

containers in ~120g quantities. Spoilage of the samples via microbial growth was prevented by the addition of a chemical preservative in the form of 0.05% wt/wt sodium azide. Samples not in use will return to the -20^{o}C freezer until ready for use. Sample cups will be thawed in a water bath and stored in refrigerators in between tests. All tests will be performed in triplicate.

4.2.1.3: Sample modification

In this study, HC-MCC gels were given two forms of modifications in two different treatment levels: pH adjustment from a native pH of 6.6 to 6.8 and 7.0, and the addition of TSC in 25mM and 50mM quantities. For pH adjustment, samples were first homogeneously mixed using an overhead mixer for 2 minutes, followed by the addition of NaOH and were mixed further for 3 minutes. The samples were then held at room temperature for 2 hours to allow for the additions to dissolve. TSC at 25mM and 50mM levels was added to the samples at 60^{o}C with constant stirring using a glass rod immediately prior to loading into the rheometer. For some samples, additional heating for 5 seconds in a microwave was required to fully incorporate the salt. pH testing was performed using an ORIONSTAR A111 model pH meter (Thermoscientific, USA. CA) and sample pH was measured immediately prior to sample testing at 60^{o}C.

4.2.2: Rheological Testing

4.2.2.1: Sample loading

Rheological measurements were performed using an Anton Paar model 302 rheometer (Graz, Austria) using a CC27 concentric cylinder geometry setup. 20ml of sample was heated to 60^{o}C using a heated water bath and mixed using a glass stirring rod to ensure a homogenous sample. Heated HC-MCC was then loaded into the test cylinder heated to the same temperature. A mineral oil layer was added to the top of the sample and solvent trap via a pipette to prevent dehydration during the rheological protocol.

4.2.2.2: Rheological Protocol

Testing took place in three stages and automatically advances to the next stage once the previous stage is complete (Figure 3-1). The first stage held the temperature of the geometry and sample at 60^{o}C for one hour taking measurements every 30 seconds for a total of 120 data points. The second test was a temperature sweep where the temperature will decrease from 60^{o}C to 5^{o}C over the course of 56 minutes, taking a measurement every 41.25 seconds for a total of 81 data points. Lastly, the third stage held the sample at 5^{o}C for 10 hours, making measurements every minute. Proper temperature of the sample was maintained using a Peltier temperature control system attached to a refrigerated water bath (CORIO CP-200F, Julabo Gmbh, Seelbach, Germany).

4.2.2.3: Multiwave Measurements

The testing protocol conducted measurements at multiple angular frequencies simultaneously (3, 6, 12, 24, and 48 rad/s with a strain of 0.18, 0.16, 0.14, 0.11, 0.09, and 0.07% respectively) during all three testing phases. Lower strain values at higher frequencies were chosen to ensure the LVR limit of the sample was not exceeded according to the findings reported by Zad Bagher Seighalani et al., (2021). Signals from multiple frequencies were separated into individual signals using the Fourier transform package within RheoCompass software 1.31 (Anton Paar, Graz, Austria) connected to the rheometer. Gelation point was determined by using Winter and Chambon criteria described in Winter & Chambon, (1986) and Zad Bagher Seighalani et al., (2021), where gelation is defined as the point where the loss tangent of 3, 6, 12, 24, and 48 rad/s frequencies converge. This criterion can be defined in the following equations:

$$G' \propto \omega^n \; ; \qquad G'' \propto \omega^n \tag{2}$$

and;

$$\tan \delta = G'' / G' = \tan(n\pi / 2) \tag{3}$$

where n $0<n<1$ is the slope of dynamic moduli within a frequency spectrum (Higham et al., 2014). Measurements of 6 rad/s storage and loss modulus (G') at 20°C, 8°C, 5°C at the end of the temperature sweep, and 5°C at the end of the frequency sweep was used to compare gel strength between the different treatments.

4.2.4: Transmission Electron Microscopy

Ultrastructure analysis of HC-MCC mixtures was performed using the TEM method described by (Lu et al., 2015). Samples underwent rheological testing using the sample loading and testing method described above. After testing was complete, the CC27 geometry was removed from the rheometer. The bottom of the geometry was removed to reveal the sample which were harvested for TEM. Samples were fixed using 2% glutaraldehyde and formaldehyde for at least two hours. Samples were then rinsed in a sodium cacodylate buffer and postfixed in 2% osmium tetroxide solution for 1 hour, followed by rinsing with distilled water in two 10-minute stints. After dehydration of the samples via a progressive ethanol series (20 min each in 50%, 70%, twice in 95%, and three times in 100% EtOH) samples were transitioned into plastic resin and infiltrated with a resin-acetone mix. Infiltration with pure resin followed, and polymerization of sample blocks occurred overnight at 65^{o}C. Sections (70-100nm) were cut on a Leica EM UC6 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL) using a diamond knife (Diatome, Hatfield, PA). Sections were double stained for 20 minutes with saturated aqueous uranyl acetate followed by 10 minutes with Reynold's lead citrate. Sections were then analyzed using a transmission electron microscope (TEM, JEM 1400 Plus, Jeol USA Inc., Peabody, MA) operated at 120kV, and digital images were captured using a Gatan camera (Gatan Inc., Pleasonton, CA).

4.2.5: Gel Electrophoresis

Molecular changes in the casein proteins was analyzed using a Urea-PAGE and native PAGE technique using the protocol from Lamichhane et al. (2019). Control, 25 and 50mM TSC, pH 6.8 and pH 7.0 treated MCC samples were centrifuged at 50,000g for 4 hours at 30^{o}C using a Beckman model LE-80 Ultracentrifuge (Brea, CA) to sediment intact casein structures. The supernatant of each treatment served as the protein sample for electrophoresis. Supernatant containing 1mg of protein was mixed into sample buffers and heated at 70^{o}C for 30 minutes 5µl of sample (containing 5µg of protein) was loaded into each well of a Biorad 15% Mini-PROTEAN TBE Urea gel (Hercules, CA) where it was then run at 120V for 2.5 hours. Sample staining was accomplished using Coomassie blue stain for 1 hour, followed by de-staining using distilled water. Imaging of the finished gels was accomplished in red light with UV conditions and processed within Bio-Rad Image Lab software (Hercules, CA).

4.2.6: Texture Analysis

Texture analysis of treatments was conducted using a TA-XT Plus texture analyzer (Stable Micro System ltd., Surrey, UK) with a texture profile test using a two-bite test with 25% compression. Samples of HC-MCC treatments were poured into 30mm diameter cylindrical molds and were allowed to solidify overnight in a 5^{o}C refrigerator prior to testing. Fracture properties were tested in compression deformation modes using a 1.5cm thick section of sample. Force and compressional data from testing was converted into Hencky strain (ε) using equation 5 where L(t) is the height of the compressed sample and L_o is the initial height. A metric to observe the strain hardening

potential of a sample is the strain hardening ratio (SHR), or the ratio of the slope at the beginning of testing compared to the end (Sharma et al., 2018)(Bast et al., 2015). As the sample continues to harden in response to strain, the slope will increase, and the ratio can describe the level of hardening possible.

$$\text{SHR} = \frac{\text{Maximum modulus}}{\text{Initial modulus}} \quad (4)$$

$$\varepsilon = ln\frac{L(t)}{L_o} \quad (5)$$

4.2.7: Statistical Analysis

Values for storage modulus measured at 6 rad/s, gelation temperature using the multiwave method, loss tangent values at the end of the temperature sweep and the slope of frequency dependent G' measurements were analyzed using SAS Studio (version 3.8) with analysis of variance (ANOVA) accompanied with Tukey's HSD to determine significant difference at ($P < 0.05$).

4.3: Results and Discussion

4.3.1: pH Measurements Pre- and Post-Rheological testing

Utilization of TSC from 25mM to 50mM levels yielded an expected increase in pH, for pre-test pH of 6.78 and 6.93, and a post-test pH of 6.91 and 7.05 respectively

Samples adjusted via NaOH yielded pre-test pH values of 6.82 and 7.04, and post-test values of 7.04 and 7.65 respectively. Since the post-test pH increased further, it indicates the role of CCP solubilization on the final pH in response to temperature (Wang & Ma, 2020). The more pronounced increase in post-test pH values for NaOH samples as compared to TSC samples could be due to the additional buffering effect of TSC; as there is more calcium bound to TSC, there is less CCP to affect the pH in response to temperature changes (On-Nom et al., 2010). Also, TSC itself acts as buffering agent, manipulating final pH. In addition to this, caseins themselves also act as buffering agents. The inherent buffering capacity of casein is at max around natural milk pH i.e., 6.6. If the pH is near 7.00, the tendency to resist the pH decreases. This could be one of the potential reason for a further rise in the pH while holding sample for 10hrs at 5^{o}C (Salaün et al., 2005).

4.3.2: Rheological Testing

4.3.2.1: Effect of pH and Calcium Chelation on Cold Gel Formation

The effects of TSC addition and pH modifications (by adding NaOH) to HC-MCC dynamic moduli are presented in Figure 4-1 and Table 4-1. Both methods of modification resulted in higher values of G' throughout cooling of HC-MCC (Table 4-1), indicating strengthening of the matrix with these modifications. Samples with TSC treatments reached a peak in gel strength and gelation temperature at 25mM TSC, whereas 50mM resulted in a reduction in these metrics (Figure 4-1). It is evident that the effect of pH modifications (6.8, 7.0) dominated over the TSC addition (25mM and 50mM), with G' values being significantly higher ($P<0.05$) for the former treatment

(Table 4-1 and Figure 4-1). This effect is observed with increasing alkalization, with 7.0 samples being stronger than 6.80 samples (Figure 4-1). CGT values for pH 7.0 treatments were significantly higher (P>0.05) than either concentration of TSC treatment. This indicates that the mechanism for gel formation in both treatments leads to a heightened degree of protein interaction. More work is needed to establish the nature of these interactions.

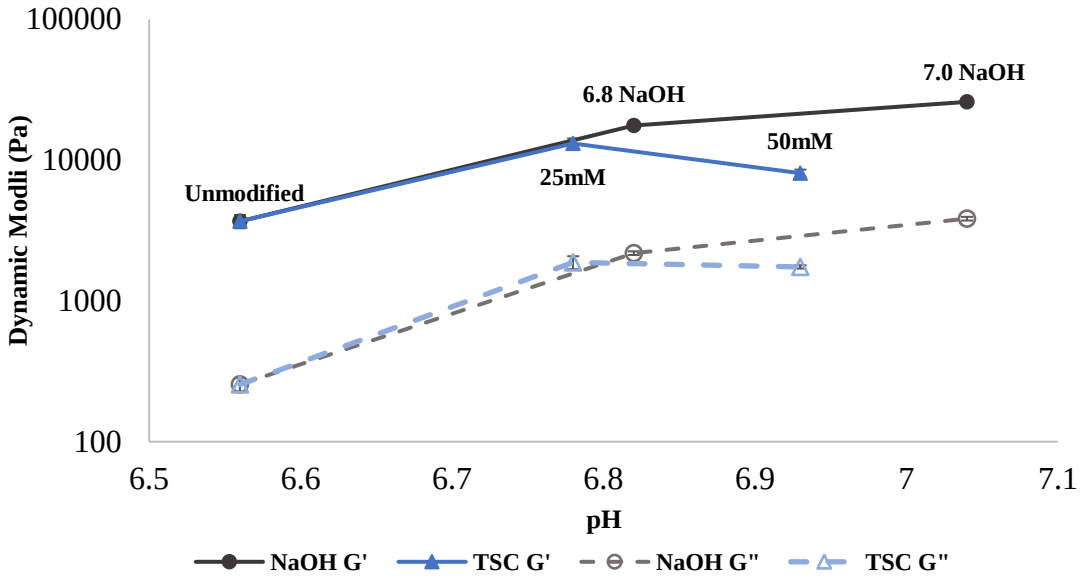


Figure 4-1: A comparison of dynamic moduli (G' and G") between the different treatments of HC-MCC and their effect on pH.

688 **Table 4-1:** Gel Strength Measurements and CGT Values for HC-MCC treatments.

	G' Value (kPa)				Loss Tangent	CGT
Treatment	20ºC	8ºC	5ºC	10hr @ 5ºC	5ºC (48 rad/s)	(ºC)
Control	0.0123 ± 0.00620^{Eb}	0.671 ± 0.147^{Eb}	1.13 ± 0.197^{Eb}	3.68 ± 0.388^{Ea}	0.123 ± 0.011^{B}	8.93 ± 0.69^{D}
TSC 25mM	4.56 ± 0.532^{Cb}	8.16 ± 0.794^{Cb}	9.29 ± 0.863^{Cab}	13.1 ± 1.14^{Ca}	0.141 ± 0.0025^{B}	26.08 ± 0.69^{B}
TSC 50mM	2.53 ± 0.348^{Db}	5.50 ± 0.480^{Db}	6.47 ± 0.505^{Db}	8.10 ± 0.470^{Da}	0.220 ± 0.0035^{A}	21.61 ± 0.34^{C}
NaOH 6.8	7.23 ± 0.260^{Bc}	11.7 ± 0.298^{Bb}	12.9 ± 0.294^{Bb}	17.6 ± 0.0730^{Ba}	0.127 ± 0.005^{B}	27.79 ± 0.34^{AB}
NaOH 7.0	15.1 ± 0.0795^{Ad}	21.4 ± 0.211^{Ac}	22.9 ± 0.248^{Ab}	26.0 ± 0.271^{Aa}	0.144 ± 0.023^{B}	29.16 ± 0.34^{A}

689 Letters denote significant difference at ($P<0.05$), capital letters denote significant difference across treatments in the same column, and

690 lowercase letters denote significant difference within the same row.

Rheological testing of the HC-MCC samples involved three steps: holding at 60^{o}C for 1hr, followed formation of cold gels by cooling to 5^{o}C in around 56 minutes and holding the cold gels at 5^{o}C for 10hrs for curing. While holding samples at 60^{o}C, control samples exhibited a higher variance in both G' and G" which is attributed to low viscoelastic strength nearing the limit of reliable measurement by the rheometer (Sharma et al., 2016). Having a low material strength that is near and below the inertia effect of the geometry affects sensitivity of the instrument and can create large jumps in the data, especially when on a logarithmic scale as seen in Figure 4-2. The material strength of the samples added with 25mM TSC (G'=80 Pa) and pH adjusted 7.0 (G'=950) was much higher than the control (~0.5 Pa) at 60^{o}C. Higher G' values obtained for the treatment samples significantly improved the reliability of the data. Impact of pH adjustment on G' values was significantly higher ($P<0.05$) than 25mM TSC samples. Overall, minimal changes were observed during holding these samples at 60^{o}C for 1hrs, suggesting that the samples were stable under these conditions.

As the temperature of the sample was reduced during temperature sweep, the values of both dynamic moduli increased, resulting in less noisy data (Figure 4-2). Cooling samples from 60^{o}C to 5^{o}C increased gel strength significantly for all four treatments (Table 4-1). At the end of the temperature sweep, G' values obtained for the samples were pH 7.0 (22.9kPa) > pH 6.8 (12.9kPa) > 25mM TSC (9.29kPa) <50mM TSC (6.47kPa) > control (1.13kPa) (Table 4-1). This indicates the pH 7.0 treatment was most effective in manipulating gel strength of HC-MCC samples. Rate of increase in G' with cooling was more pronounced with control samples, possibly due to the fact in the

absence of other modifications (pH and TSC), more calcium is available to interact with casein micelles which are relatively less hydrated than modified samples (Chapter 3.3.1).

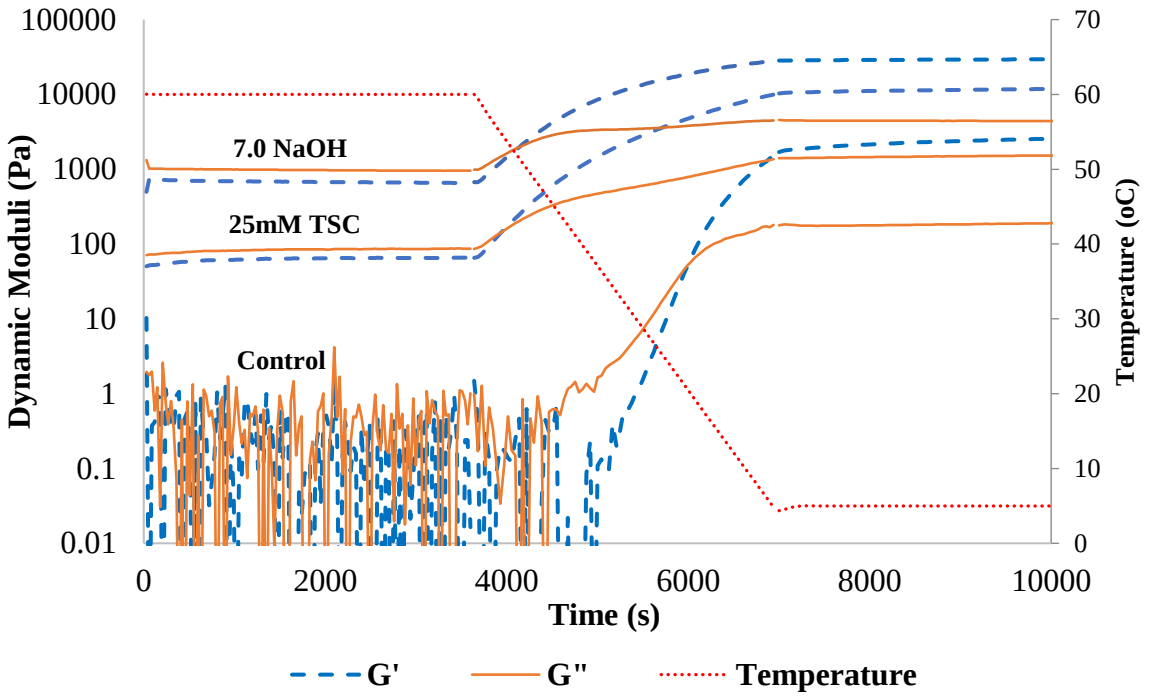


Figure 4-2: Temperature sweep data for select treatments. A comparison of dynamic moduli in response to the temperature sweep in the rheological protocol using 1 rad/s frequency

4.3.2.2: Gelation Measurements

During cooling, we observed that the material was going through a phase transition from a viscoelastic liquid (G'<G") to a viscoelastic solid (G'>G") state with occurrence of a pseudo gelling point, or cross over temperature where G'=G" (Figure 4-2). This was later accompanied by a true gelling point, where the LT from multiple frequencies converged to one single point, where the values are independent of the frequency measured (Figure 4-3). The crossover temperature for highest gelling samples, i.e., pH 7.0 and 25mM TSC were almost similar (42.5^{o}C and 46.7^{o}C at 1 rad/s respectively), but both were higher than control sample, which crossed over at 15.8^{o}C (Figure 4-2). Similarly, true gelling points were significantly higher for treatments (21.61^{o}C and 29.16^{o}C) than control (8.93^{o}C) for both 25mM TSC and pH 7.0 treatments. After the occurrence of the true gelation point (CGT), the LT reduces further but at slower rate, denoting a further relative increase in elastic properties within the sample and changes in the gel structure. This phenomenon may be related to continuous migration of CCP at slow rate and reduction in kinetic energy and mobility of protein particles (Chapter 3.4).

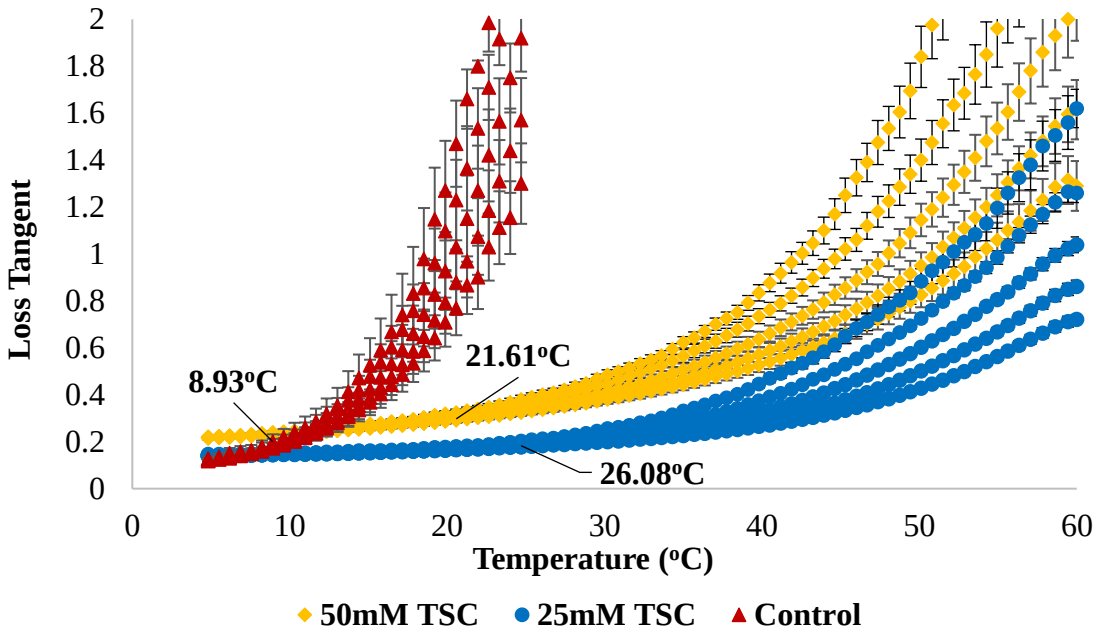


Figure 4-3: Gelation curves of select treatments. A comparison of multiwave gelation curves between three different treatments. Each sample has the loss tangent of 3, 6, 12, 24, and 48 rad/s measured to observe the convergence of each frequency. The gelation point for each treatment was denoted with a temperature value.

4.3.2.3: Frequency Dependence

Frequency sweeps are useful and alternate tools for tracking the phase transition and exact state of material from the rheology data (Sharma et al., 2016). Frequency dependence can indicate the type of bonding between various structural elements. Above CGT, G' values for control samples had more frequency dependence (n>0.9) than TSC and pH treated samples (n>0.5) (Figure 4-4). Higher frequency dependence of G' is linked with weak interaction such as entanglement of polymer units, particularly at higher

frequencies (Gaspard et al., 2021; Nordby et al., 2003; Sharma et al., 2016; Tunick, 2011; Zad Bagher Seighalani et al., 2021). All samples behaved as a weak entangled polymer network above CGT, because colloidal casein micelles are relatively free to move around at these temperatures. However, at and below CGT, all samples behaved as crosslinked stronger gels as indicated by almost no frequency dependence ($n<0.2$) of G' (Table 4-2) (Zad Bagher Seighalani et al., 2021). At the sol-gel transition point, these samples exhibited almost equal frequency dependence both for G' and G", conforming to the Winter and Chambon criteria of being LT independent of frequency (Zad Bagher Seighalani et al., 2021; Winter and Chambon, 1986).

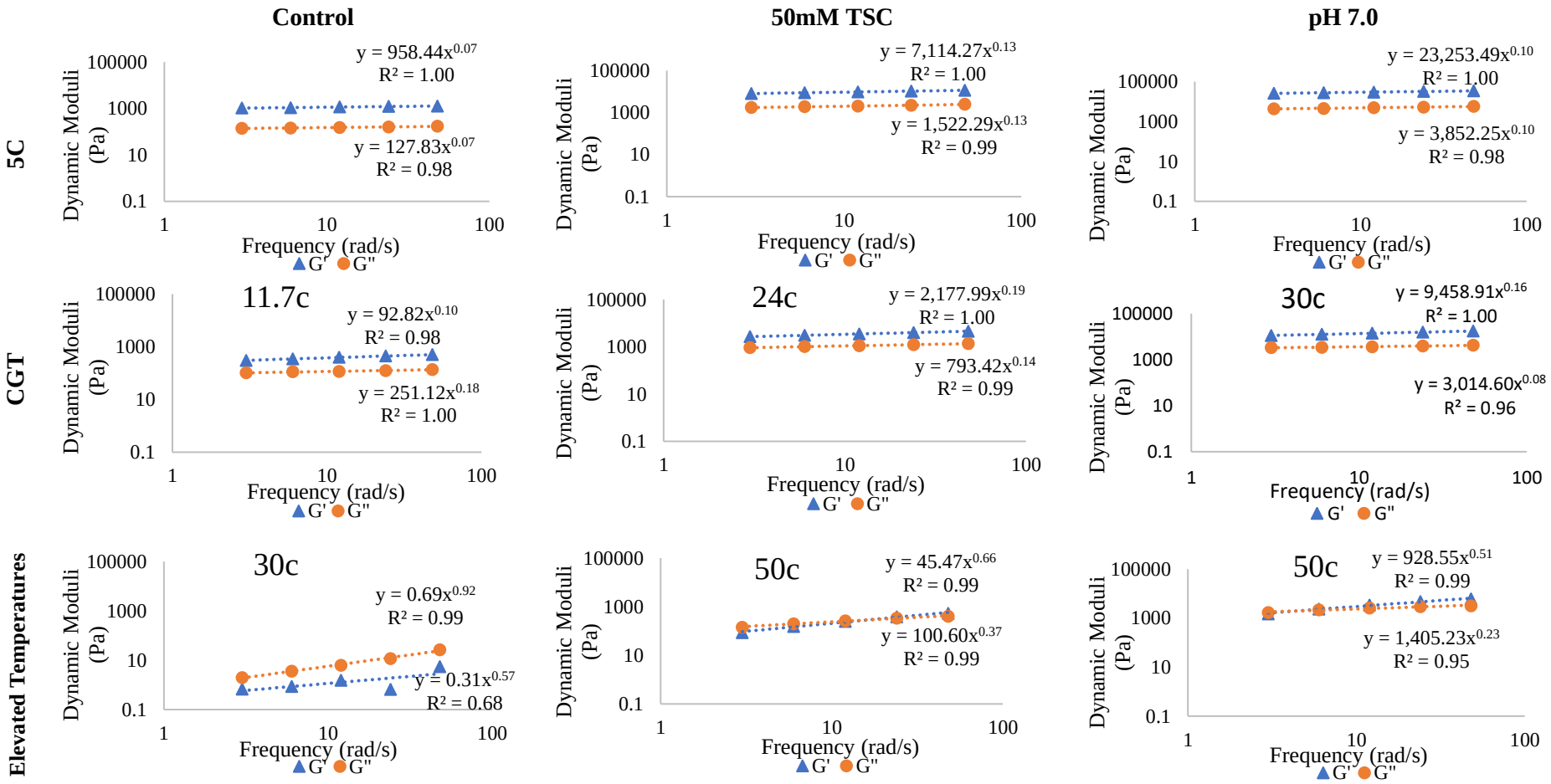


Figure 4-4: Frequency dependency of selected treatments. A comparison of dependance is presented at below CGT (5°C), CGT, and above CGT

Table 4-2: Tables for Frequency Dependance of HC-MCC Treatments at Different Temperatures.

	5°C				(CGT)				Temperatures above CGT			
TREATMENT	LG'	nG'	LG''	nG''	LG'	nG'	LG''	nG''	LG'	nG'	LG''	nG''
Control	1150±196^{B}	0.065±0.005^{B}	147±19.6^{B}	0.045±0.03^{B}	350±99^{B}	0.150±0.03^{A}	103±10.1^{A}	0.08^{AB}	0.195±0.12^{B}	0.955±0.39^{A}	0.88±0.19^{A}	0.90±0.02^{A}
25mM TSC	9370±935^{AB}	0.085±0.005^{B}	1390±160^{AB}	0.07^{AB}	2340±350^{AB}	0.135±0.005^{A}	635±81.5^{A}	0.045±0.005^{B}	186±45.5^{AB}	0.405±0.02^{A}	213±38^{A}	0.150^{C}
50mM TSC	6600±510^{B}	0.130^{A}	1450±70^{AB}	0.130^{A}	1880±300^{AB}	0.205±0.02^{A}	720±73^{A}	0.150±0.01^{A}	32.8±12.7^{B}	0.695±0.02^{A}	76.9±24.1^{A}	0.405±0.04^{B}
6.8 NaOH	13100±350^{AB}	0.080^{B}	1730±115^{AB}	0.065±0.005^{AB}	4290±220^{AB}	0.11^{A}	981±69.5^{A}	0.030±0.01^{B}	500±21.5^{AB}	0.335±0.02^{A}	470±22.5^{A}	0.080±0.02^{C}
7.0 NaOH	18800±4550^{A}	0.09±0.01^{B}	2860±970^{A}	0.080±0.02^{AB}	7020±2440^{A}	0.135±0.03^{A}	2065±945^{A}	0.055±0.03^{B}	711±219^{A}	0.430±0.08^{A}	960±451^{A}	0.170±0.06^{C}

Letters denote significant difference at ($P < 0.05$) between values within the same column.

4.3.2.4: Effect of Holding on Gel Strength

The last stage of rheological testing involved holding gelled samples at 5^{o}C for 10hrs. The purpose of this step was to provide enough time for the sample to attain calcium equilibrium. G' increased by 300% for the control sample and 10-40 % for TSC and pH treated samples over the 10hrs of storage time (Table 4-1). The increase in G' for control sample was much steeper compared to the treated samples. This was attributed to the fact that control samples had more available calcium than treated samples, therefore was continuously participating in bridging links between hydrated casein micelles. Another reason for this steeper increase could be related to the fact that treated samples had a CGT, which meant that further cooling post gelling point kept decreasing temperature of the treatment gels and reducing the kinetic energy of the system. This can then result in less mobility of structural components including CCP (Chapter 3.3.3).

4.3.3: Gel Electrophoresis

Urea PAGE conducted on supernatant of each of the 5 samples yielded differences in bands based on the treatment. There were clear indications of leaching out individual casein fractions in the aqueous phase of MCC. All MCC samples show a defined band found near the top of the wells which is absent from the sodium caseinate control. This is assumed to be a residual serum protein which remained in solution after microfiltration. A distinct κ-casein was evident in all lanes, meaning that regardless of treatment, there are free κ-casein strands within solution that were not sedimented during centrifugation (Figure 4-5). Like with κ-casein, there is a distinct and easily visible α-casein band observable within all treatments and within the sodium caseinate control. The

ratio of α-casein does not appear to change based off treatment, indicating that both methods of modification do not affect the release of α-casein into solution. The main difference between treatments is evidenced within β-casein and its variants; control and sodium caseinate had a singular β-casein band (C), while pH adjusted and TSC added samples had the presence of an additional β-casein band (D) (figure 4-5). The concentration of this β-casein variant may be higher within the supernatant of treatment groups, resulting in a stronger band. Migration of beta-casein from casein micelles at low temperatures is a known phenomenon (O'Connell et al., 2003). However, the migration of individual casein fractions and their impact on cold gelling behavior is still unknown.

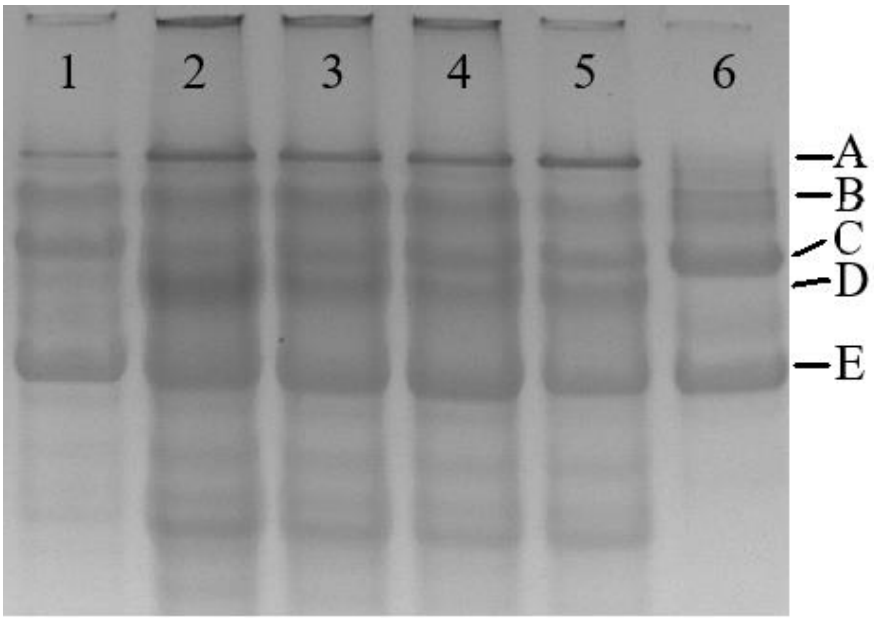
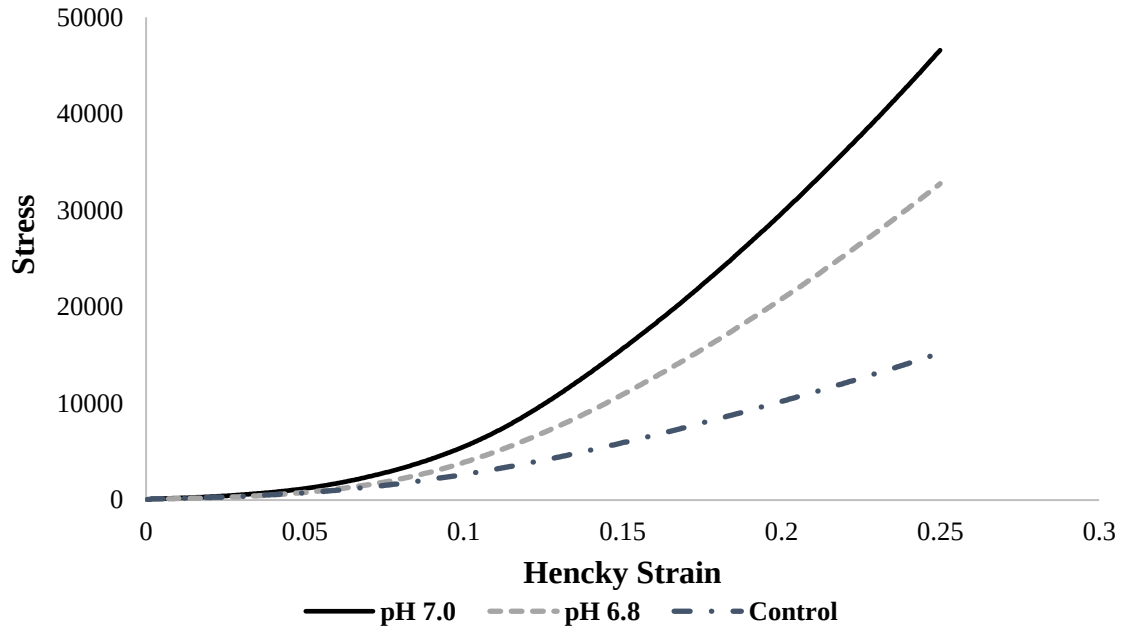


Figure 4-5: Urea PAGE of modified HC-MCC supernatants. 1: Unmodified HC-MCC 2: 25mM TSC 3: 50mM TSC 4: pH 6.8 5: pH 7.0 6: Sodium caseinate. A: Serum protein B: κ-casein C: β-casein D: β-casein E: α_{s1}-casein

4.3.4: Texture Analysis

Samples under texture profile analysis exhibited a strain hardening behavior, meaning that under deformation, the strength of the material increases with incremental increase in strain. pH adjusted samples had a higher degree of strain hardening compared to control and TSC samples, with pH 7.0 displaying the stronger tendency of strain hardening (SHR=7.0) (Figure 4-6). Strain hardening is a result of internal friction of structural elements and formation of new bonds during deformation (Sharma et al., 2018). Both maximum stress and hardness values were higher in treatment samples compared to the control. Highest hardness and maximum stress were observed with pH 7.0 adjusted samples (Table 4-3). These results are in line with the linear viscoelastic parameters, such as G' as observed in rheological testing. TSC added groups had significantly lower values of mechanical properties compared to pH adjusted samples suggesting that the mechanism behind structural changes plays an important role in the textural properties of the gels.



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827 **Figure 4-6**: Compressional testing of pH adjusted gels. Values are until 25% maximum

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831 **Table 4-3:** Texture Analysis Results

Treatment	Control	25mM TSC	50mM TSC	pH 6.8	pH 7.0
Maximum Stress (Pa)	25500 ± 1860^{C}	32100 ± 1510^{B}	37300 ± 2480^{B}	62500 ± 1050^{A}	71200 ± 3080^{A}
Strain Hardening Ratio (SHR)	2.35 ± 0.110^{D}	2.91 ± 0.125^{CD}	4.79 ± 0.450^{BC}	6.32 ± 0.660^{AB}	7.00 ± 0.575^{A}
Hardness (g)	263 ± 19.0^{C}	369 ± 7.27^{BC}	452 ± 33.8^{B}	715 ± 12.3^{A}	799 ± 30.7^{A}

832 Values are mean ± standard error. Letters designate significant difference between

833 treatments at a 0.05 significance level.

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4.3.5: Transmission Electron Microscopy

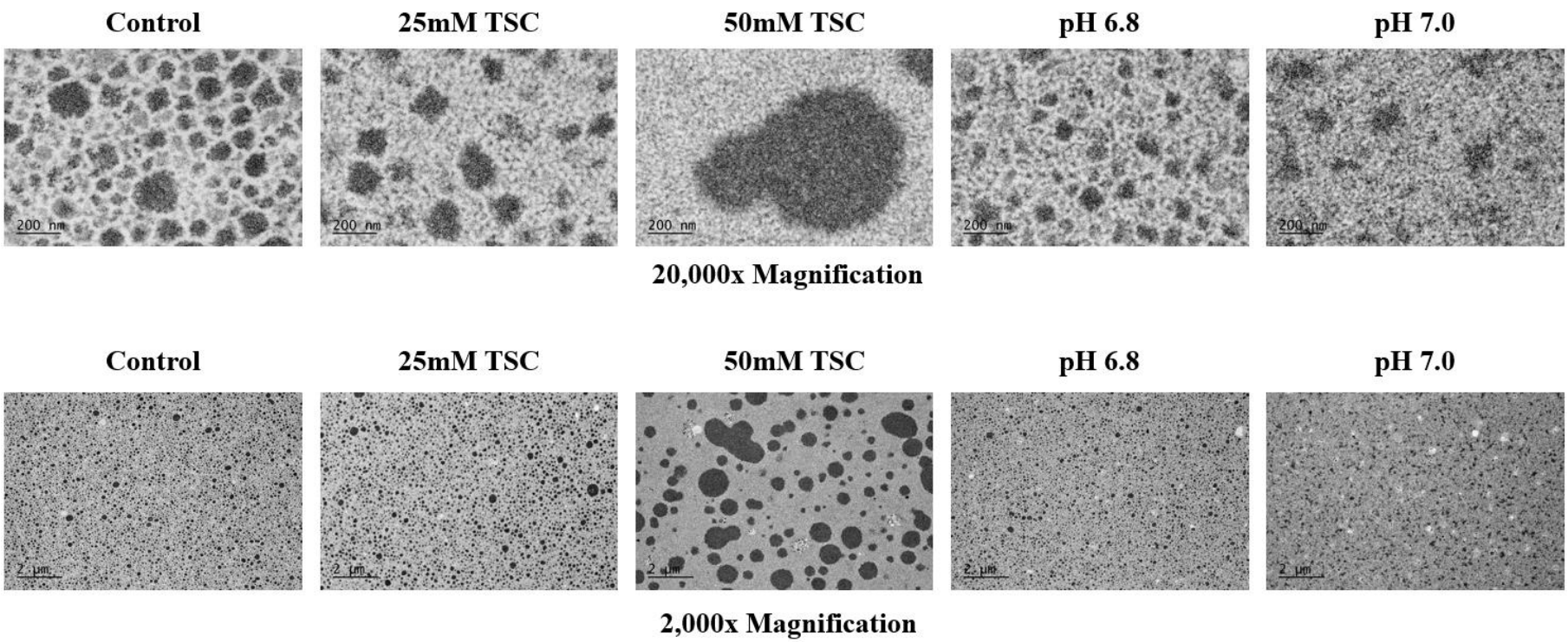
TEM images obtained from control samples indicated clear and distinctive micellar structures that had minimal space between structures exhibiting a potential jamming effect as observed by Lu et al., (2015). The effect of calcium chelation via TSC however, resulted in distinctive morphological changes in the micellar structure of casein and its fragments. At lower magnification (2000x), the structure from control samples, 25mM TSC and pH 6.8 gels appeared to have only minor variations (Figure 4-7). At pH 6.8, samples exhibited a slightly smaller particle size with more dispersion of casein in the aqueous phase. This phenomenon became more pronounced at pH 7.0 with even smaller size and more dispersion of casein fractions. This indicates the casein micelles may be undergoing a fragmentation of structure (dissociation) at higher pH levels, and the released fragments interact with the aqueous phase to a greater extent (more protein-water interactions). On the other hand, a very slight increase in the number of larger-size particles was observed for 25mM TSC samples compared to control samples, indicating relatively more protein-protein interactions compared to control and pH treatments. Samples with 50mM added TSC formed larger aggregates, often in sizes >1 μm. We believe these aggregates are formed after casein micelles are disintegrated due to calcium chelation action of TSC.

It is clear from the high magnification images (20,000x) that control samples exhibited a distinctive border between micellar structures, which was also the case for residual intact micelles at pH 6.8, suggesting a better integrity of the casein micelles. However, TSC samples exhibited the disappearance of distinctive boarders of intact micelles, and many of the smaller structures evident in the control sample were no longer

present. Rather than exhibiting a distinct border, the remaining structures were instead mixing with the adjacent aqueous phase, indicating release of CCP and by extension, casein fragments. The increased interactivity due to calcium chelation may occur first at the exterior surface of the micelle and then in the interior. The release of these individual casein fractions was confirmed through Urea-PAGE (Figure 4-5). We believe that the smaller micelles were disintegrating first due to the calcium chelating action of TSC. At 50mM concentrations, the complete collapse of micellar structure was accompanied with the formation of new type of aggregated structure dispersed in a unique continuous protein network (hydrated matrix formed from fragmented casein fractions). The formation of the new aggregate structures indicates that the new structures were not interacting strongly with the aqueous phase, therefore causing a decline in the G' (Figure 4-1; Table 4-1).

At pH 7.0 casein micelles and their fragmented structures interacted with the aqueous phase at a higher rate, therefore exhibited higher gel strength (Figure 4-1 and Table 4-1). Samples at pH 7.0 had an overall darker appearance in micrographs due to the dissociation of all micelles within solution. These individual caseins created a complete network which encompasses the sample. This dense network offers a good explanation as to why 7.0 pH samples formed the strongest gels within the study; the complete dissolution of micellar structures coupled with a high degree of crosslinking between caseins show much more interaction than control and TSC samples. While there is little research on alkalized casein micelle structure, the micrographs exhibiting this casein matrix are novel, allowing additional research to be conducted on casein matrix formation in alkaline conditions.

In the TEM micrographs, white spots were considered remnants of fat globules. We observed some indication of emulsified fat globule structures in the samples with 50mM TSC via the presence of protein structures around fat globules (Figure 4-8). It is likely that these proteins are being released due to calcium chelation activity of TSC and acting as an emulsifier (Lazzaro et al., 2017).



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889 **Figure 4-7:** TEM micrographs of treatments at 20,000x and 2,000x magnification. Samples were harvested after holding at 5°C for 10

890 hours.

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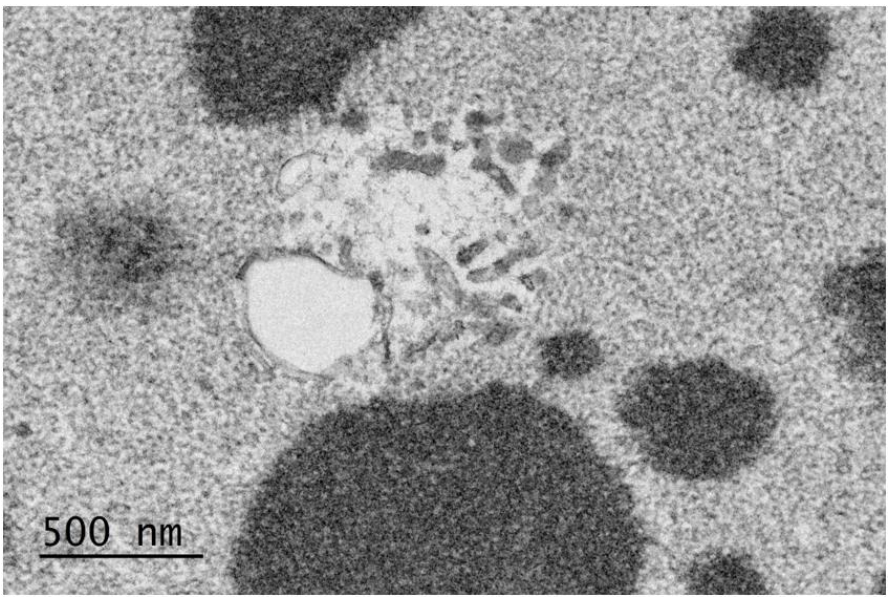

Figure 4-8: Emulsification effect observed in 50mM TSC micrographs. Magnification level is 10,000x

4.4: Discussions

Comparisons of gelling points obtained from the multiwave technique (Figure 4-3) and cross over temperature (Figure 4-2) indicated discrepancy which was dependent on the frequency measured. The discrepancy increases as dynamic moduli increases, suggesting that crossover point methodology is less accurate with stronger gels.

With a high strength gel, the G' must increase at the same proportional rate as a weaker gel in order to result in a lower LT value. However, for a strong gel with a high starting G' value, this means that an increase in the G' value must be far higher than a weak sample that is more sensitive to temperature. For reference, the G' values for pH

7.0 samples increased from 15100Pa to 26000Pa while cooling from 20^{o}C to 5^{o}C, whereas control samples raised from 12.3Pa to 3680Pa in the same timeframe. While control samples reached a higher G' value in proportion to its starting value, the samples at pH 7.0 gained more in plain numbers, which results in a larger difference between the gelling point observed from both methods. These results are in line with the observations made by Zad Bagher Seighalani et al. (2021)

Elevated temperatures (e.g. 40^{o}C) in conjunction with elevated pH levels (pH 8) are known to encourage micellar destabilization (Vaia et al., 2006); the step where the sample was held at 60^{o}C for 1hr might have encouraged the destruction of the micellar structure and was a major factor in catalyzing the formation of the matrix of individual caseins shown in 7.0 pH samples. TEM micrographs exhibiting the morphological changes in casein micelles with either type of treatment indicate the destabilization of casein. Both of these modifications indicated that the increase in gel strength was due to increasing interactions between casein fragments and the aqueous phase. It is still unclear how does these fragments interact among each other and water to form a stronger gel. Additional research into the development of these new structures is required to gather a better understanding of the destabilizing effect of calcium chelation and pH modifications.

TSC within solution appears to chelate the structural CCP from the micelle leading to partial structural dissociation resulting in a network seen in the micrographs (de Kort et al., 2011). Evidence of potential crosslinking between caseins supports rheological data (Figure 4-1), as 25mM TSC samples were significantly stronger and had a significantly higher gelation temperature ($P < 0.05$).

When comparing findings from the TEM micrographs with rheological data, the formation of aggregates in 50mM TSC samples could offer insight as to why the gel strength is reduced at this concentration. As there is greater space (reduced volume fractions of new denser protein structures) between aggregates compared to the spaces between micelles in the control sample, there is potential for higher mobility within solution. As such, there is a greatly reduced packing and particle interaction effect between structures, which results in lower strength compared to other treatment samples. 50mM samples also had significant rheological differences compared to other samples within the study; at 5^{o}C the frequency dependence of G' of 50mM samples ($n>0.1$) was found to be significantly higher than other samples ($n<0.1$) within the study (Table 4-2).

The main determinant of gel strength in this study appears to be based on protein interactivity, with increased protein-water and protein-protein (outside of the micellar structure) resulting in increased strength. Control samples exhibited minimal interaction outside of the micellar structure, and as such, they had the lowest G' and CGT values. However, the disintegration of the micellar structure seen in pH 7.0 samples and the formation of one continuous phase resulted in the strongest samples measured. The same can be said between 25mM and 50mM TSC samples; while 50mM resulted in the disintegration of the original micellar structure, the new aggregates had less interaction with the aqueous phase due to the larger size of the structures compared to 25mM TSC. As such, the gel strength and CGT is significantly lower.

4.5: Conclusions

pH and TSC modifications at all levels of treatment resulted in an increase in gel strength and temperature of gelation. pH had larger impact on increase of gel strength than TSC treatments. TSC samples at 25mM concentrations formed stronger gels that transitioned earlier in rheological testing compared to 50mM TSC samples. 50mM TSC concentrations resulted in creation of unique aggregated structures which were less interactive with aqueous phase, slightly weakening gel matrix. The effect of calcium chelation and pH treatment resulted in the dissolution of micellar casein structure as observed through TEM and Urea-PAGE analysis. pH adjustment to 7.0 resulted in the strongest gels of all treatments. While both methods of treatment exhibited elevated material properties and changes in morphology, it is likely that the mechanisms for formation of cold gels may be different. Therefore, more research is needed to elucidate the exact mechanism of cold gel formation.

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CHAPTER 5

EFFECT OF KAPPA CARRAGEENAN ADDITION ON COLD GELLING BEHAVIOR OF MICELLAR CASEIN DISPERSIONS

ABSTRACT

This study aims to study the gelation properties of Micellar Casein Concentrate (MCC) in conjunction with the addition of κ-carrageenan and physicochemical modifications in the form of calcium chelation and alkalization via trisodium citrate and sodium hydroxide respectively. Using a 3 stage multi-frequency testing protocol, we observed the rheological properties of modified MCC in response to declining temperature. Ultrastructure analysis via TEM was conducted to observe the effect of κ-carrageenan (KC) inclusion in conjunction with treatments. In addition to these, particle size, zeta potential, and texture profile analysis methods were conducted in order to understand more about the gelling properties of MCC. Alkalization of the sample raised gel strength compared to control samples, although κ-carrageenan inclusion into HC-MCC was the main source of increases in gel strength and gelation temperatures. TEM micrographs depict 2 separate phases within the solution despite high shear homogenization, as well as minimal interaction between these two phases. This study

lays the groundwork for additional work on HC-MCC on understanding cold gel formation.

5.1: Introduction

Modern trends in the food industry have moved towards a clean label approach during the formulation of a product. In the dairy industry, many polysaccharide-based gums are utilized in various products to improve textural aspects and reduce syneresis (Rafiq et al., 2020). While improving these aspects of a food are important, so is taking into consideration the concern of consumers. Studies have found that many of these gums are perceived negatively by consumers, meaning that reduction in use could lead to better product perception (Maruyama et al., 2021). Highly Concentrated-Micellar Casein (HC-MCC) is a concentrated form of micellar casein concentrate (MCC), created via the microfiltration of milk to concentrate casein within solution. Previous studies have observed the formation of a cold gel in HC-MCC or conducted research on its material properties (Dunn et al., 2021; Lu et al., 2016; Zad Bagher Seighalani et al., 2021). These studies propose that gelation comes from a packing or jamming transition, where an increase in entropy within the system combined with stearic hinderance between micellar structures limit flow and deformation of the sample in cold temperatures. While the exact mechanism behind the formation of a cold gel is still not known, there is a potential application in the use of HC-MCC as an alternative ingredient in dairy products to increase viscosity and stabilize solutions. Due to its composition (17-23% casein in solution), it offers a clean label option by utilizing dairy proteins for stabilizing properties

rather than gum-based products currently used. To consider HC-MCC as a potential ingredient in the future, a better understanding of its gelation is required to apply it to specific foods.

Previous studies have shown that physicochemical modifications such as calcium chelation via trisodium citrate (TSC) and alkalization of HC-MCC yield increases in gel strength and raise the temperature in which the sample forms a gel (Ozcan-Yilsay et al., 2007). The reason behind this change in properties is due to the destabilization of the micellar structure in response to the modification applied. While the methods of treatment create a different effect on the morphology of casein, both are able to increase gel qualities. Calcium chelation removes the structural colloidal calcium phosphate (CCP) within the micelle (Udabage et al., 2000). Such a reaction can result in the release of free casein fragments into a solution, increasing interactivity between structures. On the other hand, alkalization of the sample is thought to increase the negative charges on caseins promoting further interaction between protein and water, culminating in the dissociation of the micellar structure (Sinaga et al., 2017). Both treatments encourage interaction among proteins outside of the micellar structure, which is thought to be the reason why gel properties increase compared to unmodified samples. This increased rate of interaction, however, is the antithesis of the packing/jamming effect previously described, indicating that further research is required to fully understand the mechanisms behind gelation.

While polysaccharide-based stabilizers have had declining preference, κ-carrageenan (KC) is still a commonly used product within the dairy industry (Campbell & Hotchkiss, 2017). KC is well known for its variable conformation in response to

temperature; in temperatures above 50^{o}C, the structure converts from a helix to a coil (Bourriot et al., 1999). The helix form of KC assists in the gelation of protein solutions by binding to micelles and encouraging interaction with the newly bound polysaccharide chains (Drohan et al., 1997; Spagnuolo et al., 2005).

While there have been multiple studies exhibiting the interactions between casein and carrageenan mixtures (Bourriot et al., 1999; Spagnuolo et al., 2005; Pang et al., 2015), there has been minimal research exhibiting the interaction between KC and casein with the addition of calcium chelating salts. This study aims to study the potential interactions between KC and modified casein micelles, be it from calcium chelation or from pH adjustment. While the addition of KC or a calcium chelating salt to a casein gel may not be considered clean label, it will be used to better understand the gelation properties of HC-MCC. We also intend to observe how the addition of KC to HC-MCC can reduce the minimum amount of protein required to form a gel, and how it may impact the other qualities of the gel such as texture and gel strength.

5.2: Materials and Methods

5.2.1: HC-MCC Manufacture and Storage

HC-MCC used in the study was produced by South Dakota State University in the same manner as described in Lu et al., (2015). The MF system was a 4-vessel continuous design utilizing polyvinylidene fluoride membranes with a combined surface area of 57.4m^{2}. The subsequent vacuum evaporation of the MCC occurred at 63^{o}C at a pressure of -680mbar. The samples will be held in large pails within a -20^{o}C freezer, where they

were taken out and molten to a liquid state via water bath. The liquid HC-MCC is thoroughly mixed and poured into screw cap plastic containers in ~120g quantities. To prevent microbial spoilage, all HC-MCC used had 0.05% wt/wt sodium azide added. Samples not in use returned to the -20^{o}C freezer until ready for use. Sample cups were thawed in a water bath and stored in refrigerators in between tests. All tests were performed in triplicate.

5.2.1.1: HC-MCC Modifications

Modifications to the HC-MCC were in 4 categories: dilution, pH adjustment, calcium chelating (TSC) salt addition, and KC addition. Adjustment of protein content was performed with deionized water. Adjustment of pH was accomplished utilizing sodium hydroxide (NaOH) as a base. For pH adjustment, samples were first homogeneously mixed using an overhead mixer for 2 minutes, followed by the addition of NaOH and were mixed further for 3 minutes. The samples were then held at room temperature for 2 hours to allow for the additions to dissolve. The calcium chelating salt used in this study was food grade trisodium citrate dihydrate (TSC), sourced from Cargill (Eddyville, IA). TSC at a 25mM concentration was added to the samples at 60^{o}C with constant stirring using a glass rod immediately prior to loading into the rheometer. For some samples, additional heating for 5 seconds in a microwave was required to fully incorporate the salt.

Treatment samples from each group were diluted with DI water from 18.5% to 10% protein and will serve as a baseline for further testing. MCC then had 0.1%, 0.2%, or 0.3% wt/wt KC added. The sample was then vortexed within a centrifuge tube at 2800

rpm for 30 seconds and warmed in a water bath to 40°C. Lastly, the sample was homogenized using an Omni GLH (Kennesaw, GA) tissue homogenizer to ensure a lack of κ-carrageenan aggregation.

5.2.2: SMUF Preparation

Reagent salts found in Table 3-1 were measured and mixed in proportion to create SMUF in 1-liter quantities. The remaining volume is filled with deionized water and stirred until the salts are solubilized. The salt solution was filtered using a vacuum flask and a Millipore 1.2μm filter (Bedford, MA). To prevent precipitation of phosphates in the completed buffer, the SMUF was stored in a refrigerator at 5°C in between uses.

5.2.3: Rheological testing

Rheological measurements were performed using an Anton Paar MCR 302 rheometer (Anton Paar GMBH, Graz, Austria) using a concentric cylinder geometry setup (model no. CC27). For sample loading, 20ml of sample was heated to 40°C and poured into the bottom of the sample cylinder. An oil layer is added on the top via a pipette to prevent dehydration during the rheological protocol. Rheological testing was conducted in three stages (Figure 3-1). In the first stage a time sweep was conducted by holding the sample at 40°C for 5 minutes, recording data every 30 seconds for a total of 10 data points. The following temperature sweep decreased from 40°C to 5°C and recorded data every 41.25 seconds resulting in 51 data points. Lastly, the third stage held samples at 5°C for at least 10 hours, making measurements every minute. Rheological

testing was conducted by applying simultaneously multiple angular frequencies (3, 6, 12, 24, and 48 rad/s), and gelation point was determined by using the criteria described in by Zad Bagher Seighalani et al., (2021), where gelation point was defined as the point where the loss tangent of the sample is independent of frequencies applied (Winter & Chambon, 1986). Storage modulus (G') values obtained during the temperature sweep at 20^{o}C, 8^{o}C, 5^{o}C at the end of the temperature sweep, and holding 5^{o}C for 10 hours during the time sweep will be used to compare gel strength between the different treatments.

5.2.4: Texture Analysis

Texture analysis of treatments was conducted using a TA-XT Plus texture analyzer (Stable Micro System ltd., Surrey, UK) with a texture profile test using a two-bite test with 25% compression. Samples of HC-MCC treatments were poured into 30mm diameter cylindrical molds and were allowed to solidify overnight in a 5^{o}C refrigerator prior to testing. Fracture properties were tested in compression deformation modes using a 1.5cm thick section of sample. Force and compressional data from testing was converted into Hencky strain (ε) using equation 5 where L(t) is the height of the compressed sample and L_o is the initial height. A metric to observe the strain hardening potential of a sample is the strain hardening ratio (SHR), or the ratio of the slope at the beginning of testing compared to the end (Sharma et al., 2018)(Bast et al., 2015). As the sample continues to harden in response to strain, the slope will increase, and the ratio can describe the level of hardening possible.

5.2.5: Transmission Electron Microscopy

Ultrastructure analysis of HC-MCC mixtures was performed using the TEM method described by (Lu et al., 2015). Samples from each treatment group will undergo TEM imaging with 0, 0.1, and 0.3% KC added. Samples underwent rheological testing using the sample loading and testing method described above. After testing was complete, the CC27 geometry was removed from the rheometer. The bottom of the geometry was removed to reveal the sample which were harvested for TEM. Samples were fixed using 2% glutaraldehyde and formaldehyde for at least two hours. Samples were then rinsed in a sodium cacodylate buffer and postfixed in 2% osmium tetroxide solution for 1 hour, followed by rinsing with distilled water in two 10-minute stints. After dehydration of the samples via a progressive ethanol series (20 min each in 50%, 70%, twice in 95%, and three times in 100% EtOH) samples were transitioned into plastic resin and infiltrated with a resin-acetone mix. Infiltration with pure resin followed, and polymerization of sample blocks occurred overnight at 65^{o}C. Sections (70-100nm) were cut on a Leica EM UC6 ultramicrotome (Leica Microsystems Inc., Buffalo Grove, IL) using a diamond knife (Diatome, Hatfield, PA). Sections were double stained for 20 minutes with saturated aqueous uranyl acetate followed by 10 minutes with Reynold's lead citrate. Sections were then analyzed using a transmission electron microscope (TEM, JEM 1400 Plus, Jeol USA Inc., Peabody, MA) operated at 120kV, and digital images were captured using a Gatan camera (Gatan Inc., Pleasonton, CA).

5.2.6: Particle Size

Particle size analysis was conducted in using an Anton Paar PSA 1190 micro particle size analyzer (Graz, Austria). Sample gels were cured overnight in a refrigerator, and mixed with 5^{o}C DI water to ensure the HC-MCC is still in a gelled state before loading the sample into the analyzer receptacle. Sample dispersion within the unit was accomplished using distilled water with a 5 second debubbling time, and measurements occurred over a 30 second period with a pump speed at 120 rpm.

5.2.7: Zeta Potential

Tests examining casein zeta potential were conducted using an Anton Paar Litesizer 500 particle size analyzer (Anton Paar GMBH, Graz, Austria). Testing HC-MCC in each treatment and each KC level was conducted using a temperature series measurement from 40^{o}C to 5^{o}C, conducting measurements at 40, 30, 20, 10, and 5^{o}C in descending order. Samples reached the target temperature for testing and equilibrated for 10 minutes prior to a measurement to ensure samples are uniformly cooled. The sample then sat overnight at 5^{o}C after the temperature sweep and was measured the following day to observe any potential difference due to additional holding time at 5^{o}C. HC-MCC was diluted in simulated milk ultrafiltrate (SMUF) buffer 1000x and loaded into a model no. 225288 zeta potential cuvette (Anton Paar GMBH, Graz, Austria). All measurements were conducted using 18V with a maximum of 300 measurements for each reading. Zeta potential data was calculated using Smoluchowski approximation which is part of the testing software.

5.2.8: Statistical Analysis

Data points from rheological, particle size, and texture analysis tests were pulled from their respective software programs. Statistical significance was measured using a GLM model in SAS to compare the effects of modifications. Differences will be evaluated using ANOVA and Tukey's HSD adjustment with $P \leq 0.05$ considered statistically significant.

5.3: Results and Discussion

5.3.1: Particle Size

With increasing KC content within samples, there was a growing issue during initial measurements using an Anton Paar model litesizer 500 nano particle size analyzer due to the formation of KC aggregates in micron size, causing issues with data interpretation. As KC content increased, the average particle diameter outputs grew increasingly erratic. Particle size distribution data showed the conversion from a monomodal dispersion in KC free samples to multimodal distributions at erratic sizes with 0.3% KC. In addition, the additional peaks observed from KC addition were near the limit of detection for the litesizer hardware, rendering the measurements incomplete. To correctly capture large particle size species, we conducted particle size analysis on the gelled particles using an Anton Paar model PSA 1190 particle size analyzer which was better equipped to observe the casein-KC gels on a micro-meter scale rather than on the nano-meter scale.

The increase from 0.1% to 0.3% KC within solution resulted in a significant decrease in average particle size regardless of treatment group (Table 5-1). Indicating that the size of casein within solution decreases as KC content increases. Increased KC content results in a multimodal distribution of particles (Figure 5-1), which is confirmed by TEM data (Figure 5-5). The effect of pH adjustment and calcium chelation significantly increases particle size compared to control groups; other studies have described an increase in micelle size in response to alkalization (Y. Liu & Guo, 2008). While TEM micrographs depict the disintegration of the micellar structure, alkalization could increase the ability of the caseins to hold more moisture. This effect would describe the significant increase in particle size at 0.3% KC content compared to control samples. In the case of TSC addition, the destabilization of the micelle brought on by calcium chelation could also encourage protein-water interactions, as TSC is also known to raise the pH of HC-MCC solutions (Chapter 4.3.1).

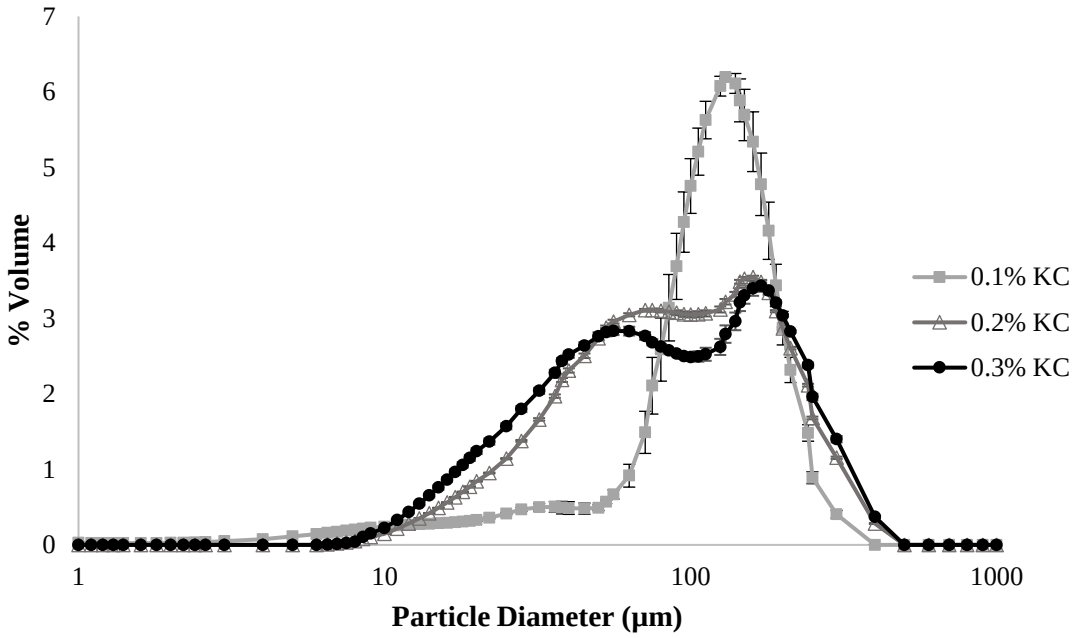


Figure 5-1: Comparison of particle size distributions across KC concentrations. All samples included are control samples, bars indicate standard error.

Table 5-1: Comparison of Particle Size ($d_{4,3}$) Against KC Content and Treatment.

KC Content (% wt/wt)	Control	25mM TSC	pH 7.0
0.1	116 ± 2.99^{Aa}	120 ± 2.22^{Aa}	112 ± 1.08^{Ba}
0.2	83.0 ± 0.464^{Bc}	116 ± 1.64^{ABb}	132 ± 1.27^{Aa}
0.3	76.1 ± 1.87^{Bb}	108 ± 2.59^{Ba}	103 ± 2.35^{Ca}

Capital letters designate significance across KC content levels, while lower case designates significance across treatment groups. Significance level is $P<0.05$

5.3.2: Zeta Potential

No significant changes in the zeta potential of HC-MCC were observed as a result of temperature sweep testing. In addition, no significant differences in values were observed after holding the sample overnight at 5^{o}C and resuming testing. It can be inferred from this result that the solubility of CCP in response to temperature does not have significant effect on the zeta potential of KC-casein mixtures. Increasing concentrations of KC within samples also produced an insignificant effect on zeta potential. While KC may simply not affect the zeta potential of casein, this finding in conjunction with TEM micrographs is further proof that there is minimal interaction between KC and casein in our samples. Sun et al., (2016) described a change in zeta potential values in response to starches binding to casein, however there was concrete evidence in the form of scanning electron microscopy micrographs depicting interaction between casein and starch. Treatment effects also produced minimal changes within zeta values, although as evidenced within particle size and texture tests, there were minimal differences observed as well. In future tests, conducting measurements at different concentrations may be recommended to ensure there is adequate sample within the SMUF solution.

5.3.3: Texture Analysis

Texture profile analysis was conducted only on the samples added with 0.3% KC, because they were able to hold their shape at 25% strain during testing. Maximum stress values achieved at 25% strain were higher (23.9 kPa) for control samples than pH (13.5 kPa) and TSC treated samples (10.1 kPa) (Figure 5-2). A similar pattern was found for

hardness and strain hardening ratio, with control samples being significantly higher than either form of treatment (Table 5-2). While comparing these results with small strain rheology, it is apparent that large strain rheology exhibits a distinctive behavior, because of the way deformation takes in the material.

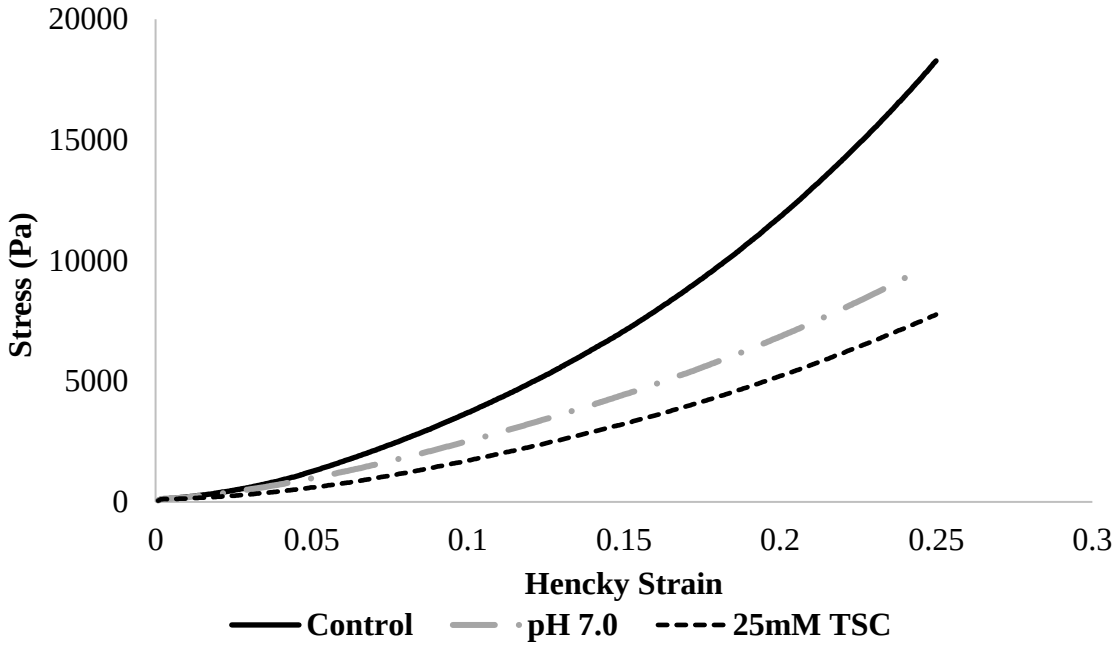


Figure 5-2: Compressional testing of KC-MCC gels. Values are until 25% maximum strain.

Table 5-2: Texture Analysis Results.

Treatment	Control 0.3% KC	25mM TSC 0.3% KC	pH 7.0 0.3% KC
Maximum Stress (Pa)	23900±1100^{A}	10100±741^{B}	13500±418^{B}
Hardness (g)	291±18.0^{A}	123±8.19^{B}	154±3.25^{B}
Strain Hardening Ratio	3.03±0.289^{A}	1.14±0.0755^{B}	1.30±0.0944^{B}

Capital letters designate significance across treatments at a significance level of $P<0.05$.

5.3.4: Rheology

5.3.4.1: Effect of KC addition

In this study, we diluted 18.5% protein HC-MCC to 10% protein, which did not form cold gels upon cooling to 5^{o}C (Table 5-4). Diluted samples then had KC added in 0.1%, 0.2 % and 0.3% quantities. As expected, increases in KC concentration from 0.2% to 0.3% led to a significant increase in gel strength, from 290 Pa to1300 Pa (Table 5-3). Rheological measurements captured during the temperature sweep exhibited a strong increase in storage modulus values consistently around 27^{o}C (Figure 5-3, Figure 5-6)). This increase was due to KC reaching critical sol-gel temperature within solution (Bui et al., 2019); while the values used in the study are not enough for KC to create a gel alone, they are enough to create a significant increase in dynamic moduli when mixed with HC-MCC. The effect of KC addition to MCC samples was evidenced on the gelling temperature (Table 5-4). Samples with 0.1% KC added did not form a gel during temperature sweep (40^{o}C to 5^{o}C) but formed a gel while holding at 5^{o}C for 10 hrs.

Increasing KC from 0.2% to 0.3% however, increased gelation temperature from 25.3^{o}C to 27.8^{o}C (Table 5-4).

Table 5-3: Comparison of storage modulus values between treatments and temperatures for kappa carrageenan added MCC samples

	G' (Pa)			
Treatment	20°C	8°C	5°C	5°C + 10hrs
Unmodified 0.1KC	2.23 ± 0.152^{Db}	3.81 ± 0.150^{Eb}	4.56 ± 0.281^{Eab}	7.94 ± 1.50^{Da}
Unmodified 0.2KC	113 ± 20.0^{CDb}	243 ± 41.0^{DCab}	290 ± 48.1^{DEab}	409 ± 71.2^{CDa}
Unmodified 0.3KC	396 ± 31.7^{BCc}	875 ± 72.1^{BCb}	1090 ± 102^{BCab}	1300 ± 33.3^{Ba}
25mM TSC 0KC	0.0640 ± 0.0134^{Db}	1.33 ± 0.377^{Eb}	1.62 ± 0.748^{Eb}	27.1 ± 5.59^{Da}
25mM TSC 0.1KC	17.8 ± 2.51^{Dc}	105 ± 6.13^{Eb}	137 ± 5.86^{Eb}	208 ± 11.6^{CDa}
25mM TSC 0.2KC	292 ± 42.2^{BCDb}	645 ± 80.7^{CDab}	758 ± 90.5^{CDa}	945 ± 116^{BCa}
25mM TSC 0.3KC	580 ± 49.6^{Bc}	1200 ± 94.4^{Bb}	1380 ± 109^{Bab}	1740 ± 141^{Ba}
7.0 0KC	0.00430 ± 0.00252^{Db}	0.00941 ± 0.00528^{Eb}	0.0262 ± 0.00614^{Eb}	3.53 ± 1.08^{Da}
7.0 0.1KC	13.8 ± 3.76^{Db}	106 ± 35.5^{Eb}	141 ± 47.8^{Eb}	414 ± 108^{CDa}
7.0 0.2KC	197 ± 41.8^{CDc}	758 ± 105^{BCb}	931 ± 117^{BCb}	1620 ± 162^{Ba}
7.0 0.3KC	986 ± 197^{Ab}	2070 ± 264^{Aab}	2340 ± 281^{Aab}	3530 ± 523^{Aa}

Capital letters designate significance between the same temperature, lower case designates significance between temperatures.

Significance level $P<0.05$

Table 5-4: Gelation Temperatures of Modified MCC Dispersions

Carrageenan Concentration (wt/wt%)	Treatment		
	Unmodified	25mM TSC	pH 7.0
0	NO GEL	NO GEL*	NO GEL*
0.1	NO GEL*	19.9 ± 0.46^{Ba}	14.6 ± 1.07^{Bb}
0.2	25.3 ± 0.46^{Bb}	29.0 ± 0.24^{Aa}	25.5 ± 0.47^{Ab}
0.3	27.8 ± 0.40^{Ab}	29.5 ± 0.23^{Aa}	27.2 ± 0.41^{Ab}

* Did not gel during the temperature sweep. Capital letters designate significant differences between the same treatment, lower case designates significance between KC concentrations. Significance level $P<0.05$.

5.3.4.2: Effect of Calcium Chelation and Alkalization

Physicochemical modifications (TSC and pH) on HC-MCC had less of an effect on the gel strength of samples compared to the effect of KC addition. TSC addition and pH modification resulted in gel formation while holding at 5°C without KC addition. When KC is added to the sample however, the CGT values are similar and appear to be largely dependent on the concentration of KC rather than the additional treatments. While not significant, the increase in KC content from 0.1% to 0.2% in TSC samples raised the storage modulus value of treatments by a factor of 4 and increased further when raised to 0.3% (Table 5-3). KC at all concentrations significantly improved the gel strength of cold gels, exhibiting maximum gel strength for pH 7.0 and 0.3% KC (G'=3500 Pa). This clearly indicates that combined the impact of pH and KC was the most effective at improving gel strength. The sudden increase in dynamic moduli around 27°C in response

to increasing KC indicates that KC may be forming a gel by itself. The gelling properties of KC are unaffected by pH adjustment from 6.6 to 7.0, or from the use of calcium chelating salts indicating that treatment group modifications only have an effect on the casein within the system.(Ould Eleya & Turgeon, 2000).

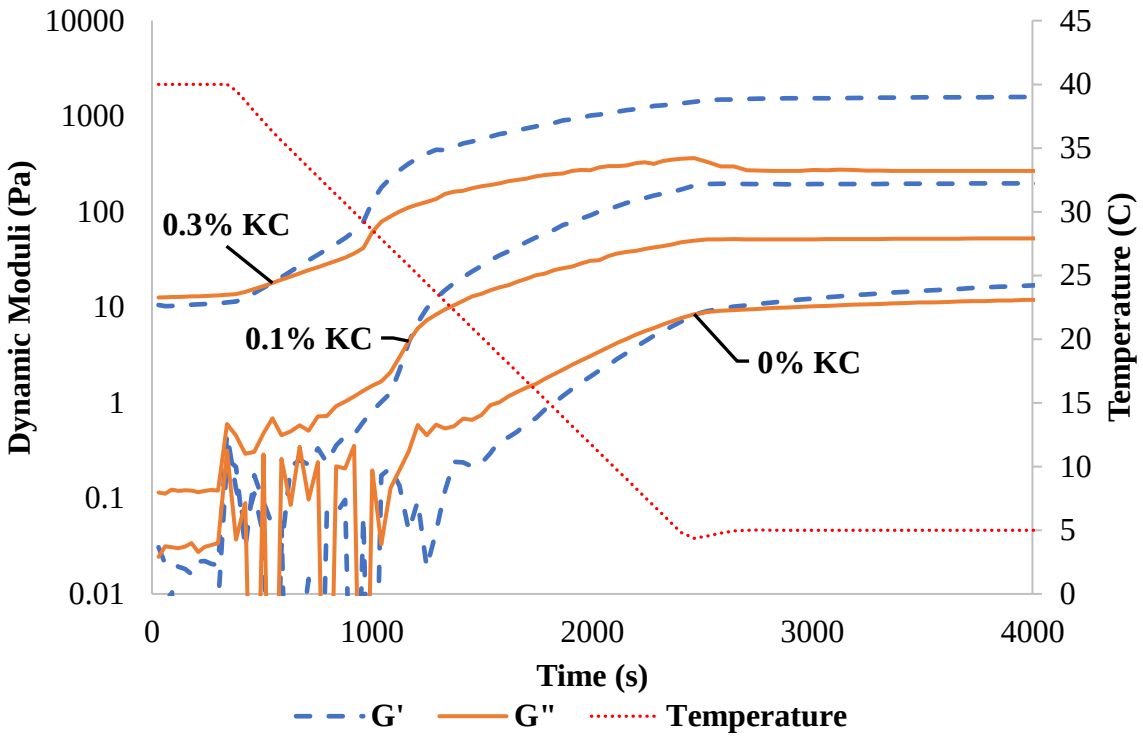


Figure 5-3: Temperature sweep data for 25mM TSC treatments. A comparison of dynamic moduli in response to the temperature sweep in the rheological protocol using 3 rad/s frequency.

5.3.5: TEM Micrographs

TEM micrographs of MCC samples treated with KC, pH and TSC are presented at two magnification levels (2,000x and 20,000x) in Figure 5-4 and 5-5. Without KC additions, TSC and pH treated samples had uniform, and monophasic structures. Even while utilizing high shear to homogenize the sample, the addition of KC led to the creation of a biphasic solution that was made of separate KC and casein phases. Samples with at 0.1% and 0.3% added KC exhibited minimal interaction with caseins regardless of treatment. In samples 0.3% KC, the casein phase appears to be more compact, indicating that KC is absorbing moisture more readily than casein, and compacting the casein phase into a denser state. Because of this reduction in moisture, the casein that appeared in the micrographs was darker than the other phase potentially due to a lower affinity to moisture holding compared to KC. The separate KC phase in the micrographs were less dense than casein, and appeared to create mesh like fragments that exhibit heavily crosslinking from individual strands. Any potential for interaction (mixing of two phases or bond formations) between caseins in any form and KC was observed within the border regions of KC and casein areas.

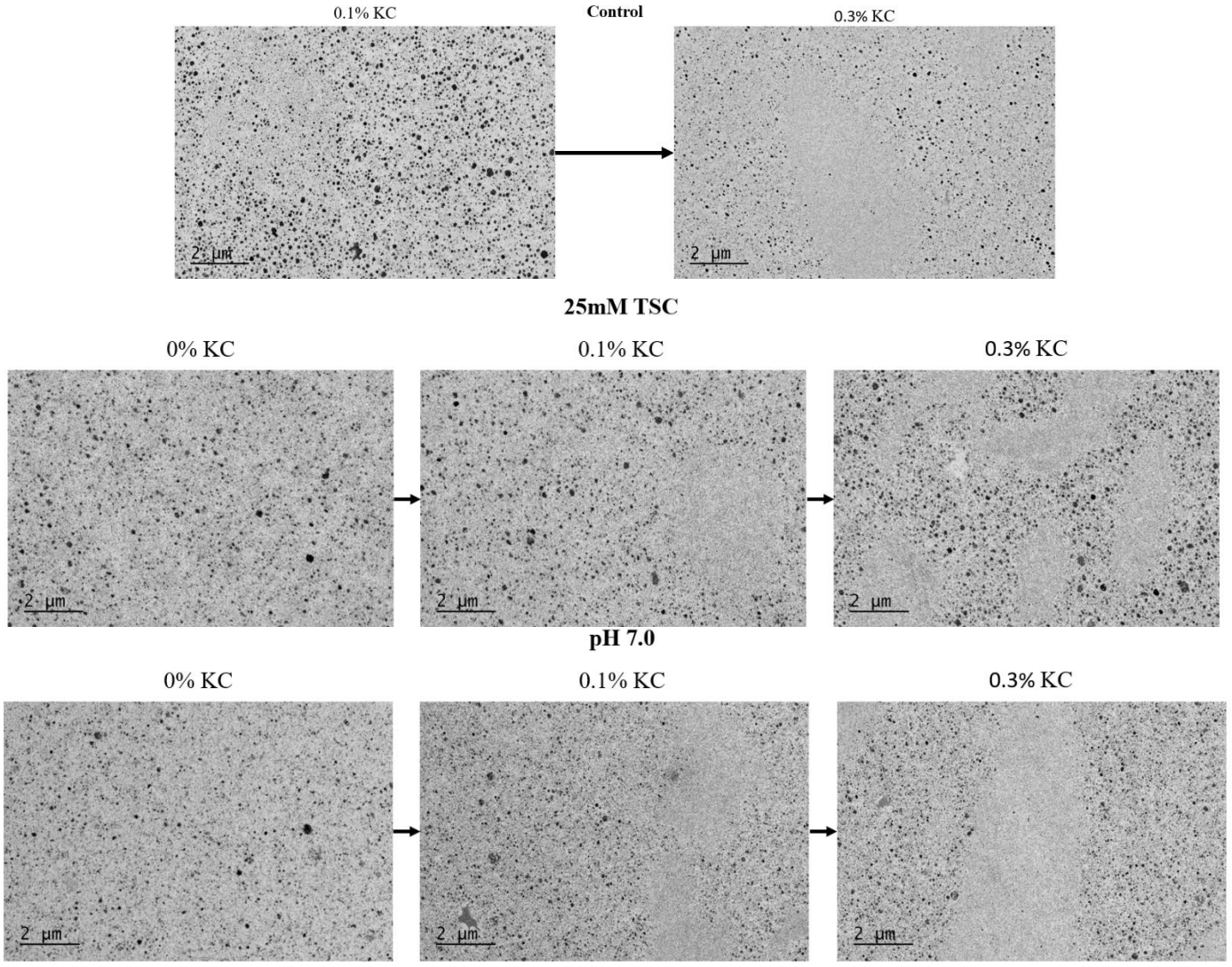


Figure 5-4: TEM micrographs of cold gels made from treated MCC samples (10% protein) at 2000x instrumental magnification

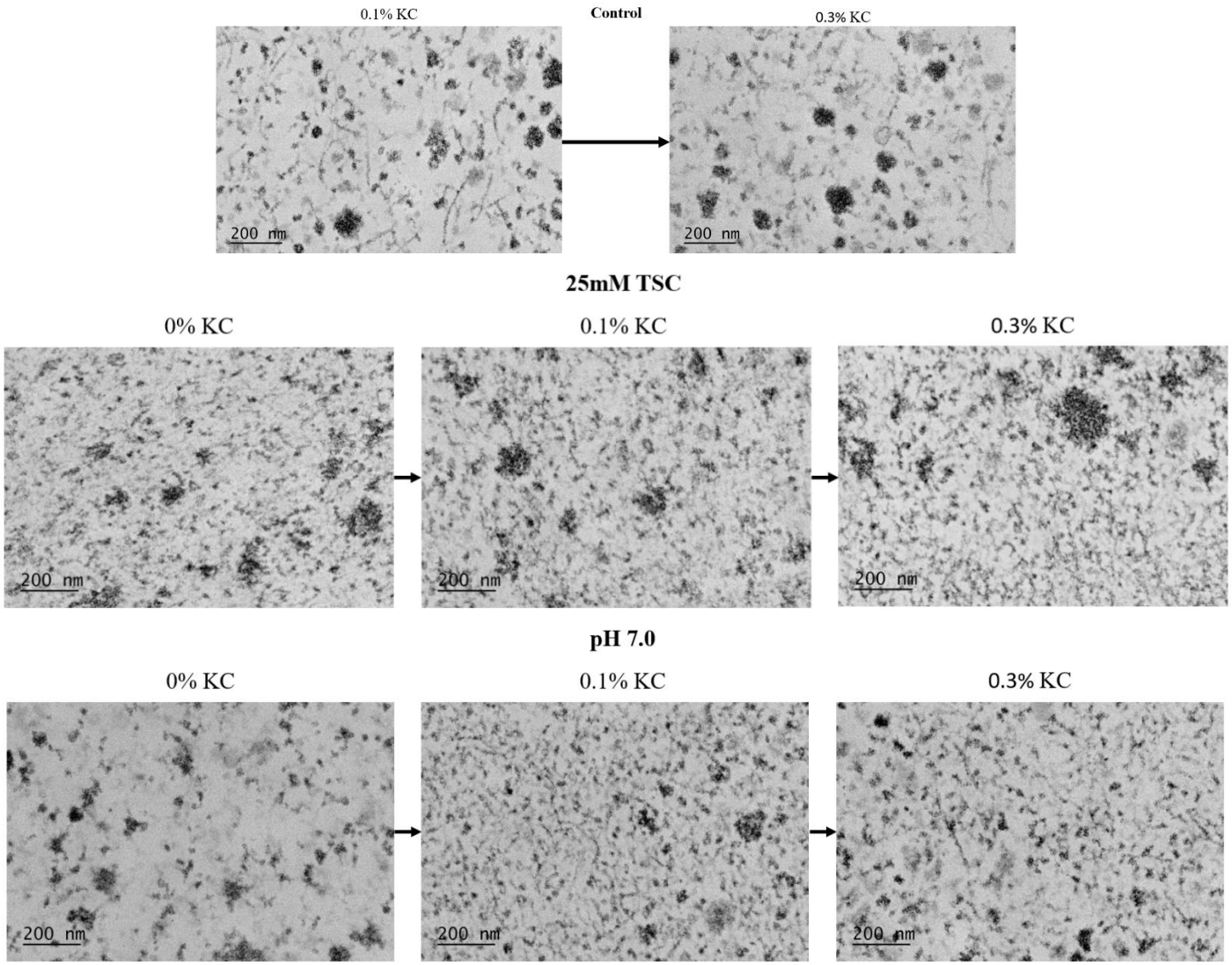


Figure 5-5: TEM micrographs of cold gels made from treated MCC samples (10% protein) at 20000x instrumental magnification

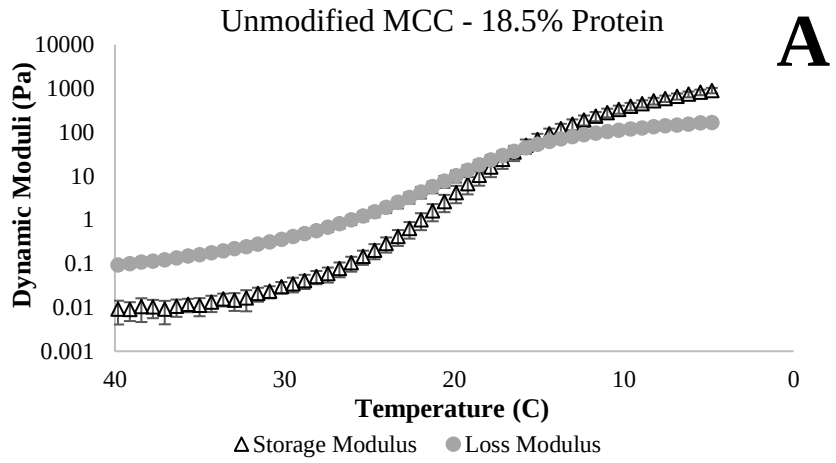
There were clear indications of changes in the micellar structures in response to modifications. Samples with 25mM TSC or adjusted to pH 7.0 at 10% protein exhibit similar morphological changes as TEM micrographs conducted on 18.5% protein samples (Figure 4-7). The disintegration of the micellar structure is clearly evident, and there is an abundance of free casein strands within solution compared to control samples. pH 7.0 samples appear to have an increased degree of structural disintegration similar to samples in the previous study (Figure 4-7); this greater fraction of free caseins is likely to interact well with water and the cause for the increase in gel strength over TSC and control samples. The pH at which micellar disintegration is occurring is lower than what was reported by Vaia et al., (2006), but is a similar result compared to the previous chapter (Chapter 4.3.5), which could mean that our MCC sample may have an increased sensitivity to pH adjustment. While still a minimal amount, samples with micellar collapse appear to have a higher degree of interaction between caseins and KC within solution, exhibited in the same border regions as control samples. As the structure of the casein micelle is weakened due to the effect of calcium chelation or alkalization (Touhami et al., 2022), the free caseins will have an increase potential for interaction with the KC phase.

5.4: Discussions

The aim of this research was to study the potential of KC to improve the gelling behavior of MCC at a lower protein concentration than previous chapters. When comparing studies on modified HC-MCC (18.5% and 15% protein) from previous

chapters (Chapter 3.3.4, Chapter 4.3.2) and diluted MCC samples (10% protein) from this study, the gel strength of samples from previous tests are an order of magnitude higher than the samples tested in this study due to differences in protein content. On the other hand, the highest CGT in this chapter was 29.5^{o}C (10% protein, 25mM TSC and 0.3% KC) which was only 3.5^{o}C lower than highest average CGT (18.5% protein, pH 6.8 with 10mM TSC) measured in the previous chapters (Table 3-2) While the addition of KC into diluted HC-MCC did not increase the gel strength enough to make up for reduced protein content, it was able to make a high CGT.

Figure 5-6 compares the temperature sweep and gelation curves of an unmodified 18.5% protein HC-MCC gel with a diluted, 10% protein gel with 0.3% KC. While the storage modulus values at the end of the temperature sweep are similar, the diluted sample has a significantly higher CGT. Due to this elevated CGT value, the frequency dependence on the sample with added KC did not change significantly from 20^{o}C through the end of testing (Figure 5-7). During this period, it maintained a frequency dependence ($n<0.2$) low enough to be considered a strong gel (Faber et al., 2017; Rafe & Razavi, 2017). The unmodified samples, however, had a significantly higher frequency dependence ($n\sim0.7$) at 20^{o}C in comparison, but significantly lower value at the end of the temperature sweep ($n=0.08$) and at the end of testing ($n=0.05$).



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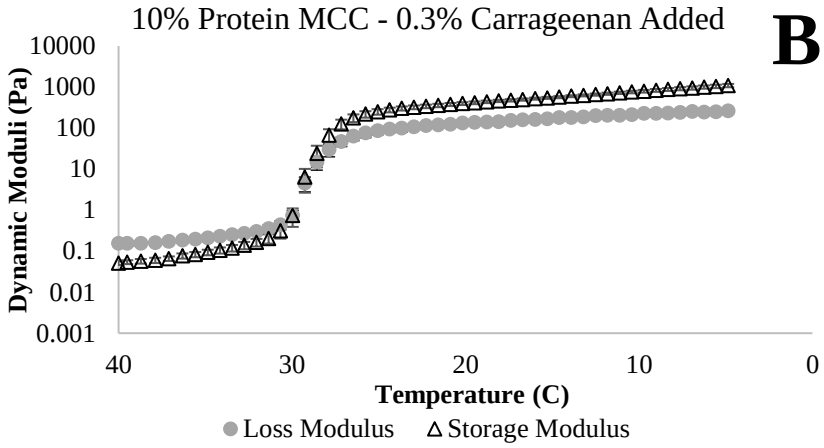

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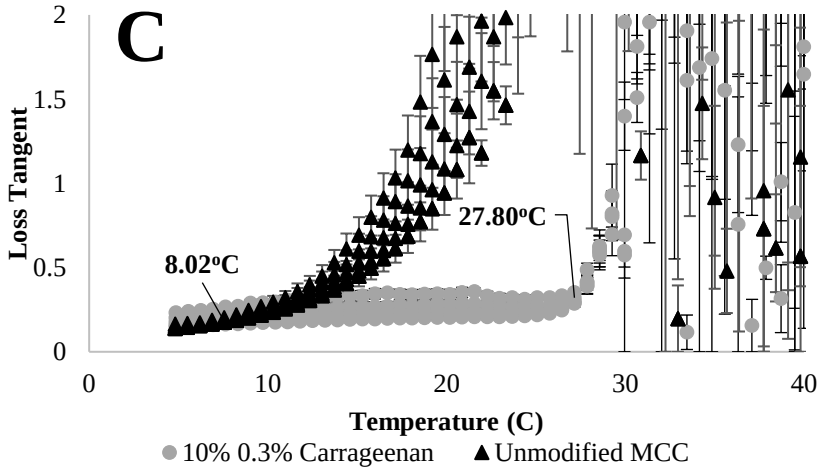

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1476 **Figure 5-6:** A and B: Dynamic Moduli of treatments during the temperature sweep. C:

1477 Gelation curves during the temperature sweep with their Respective Gelation Points.

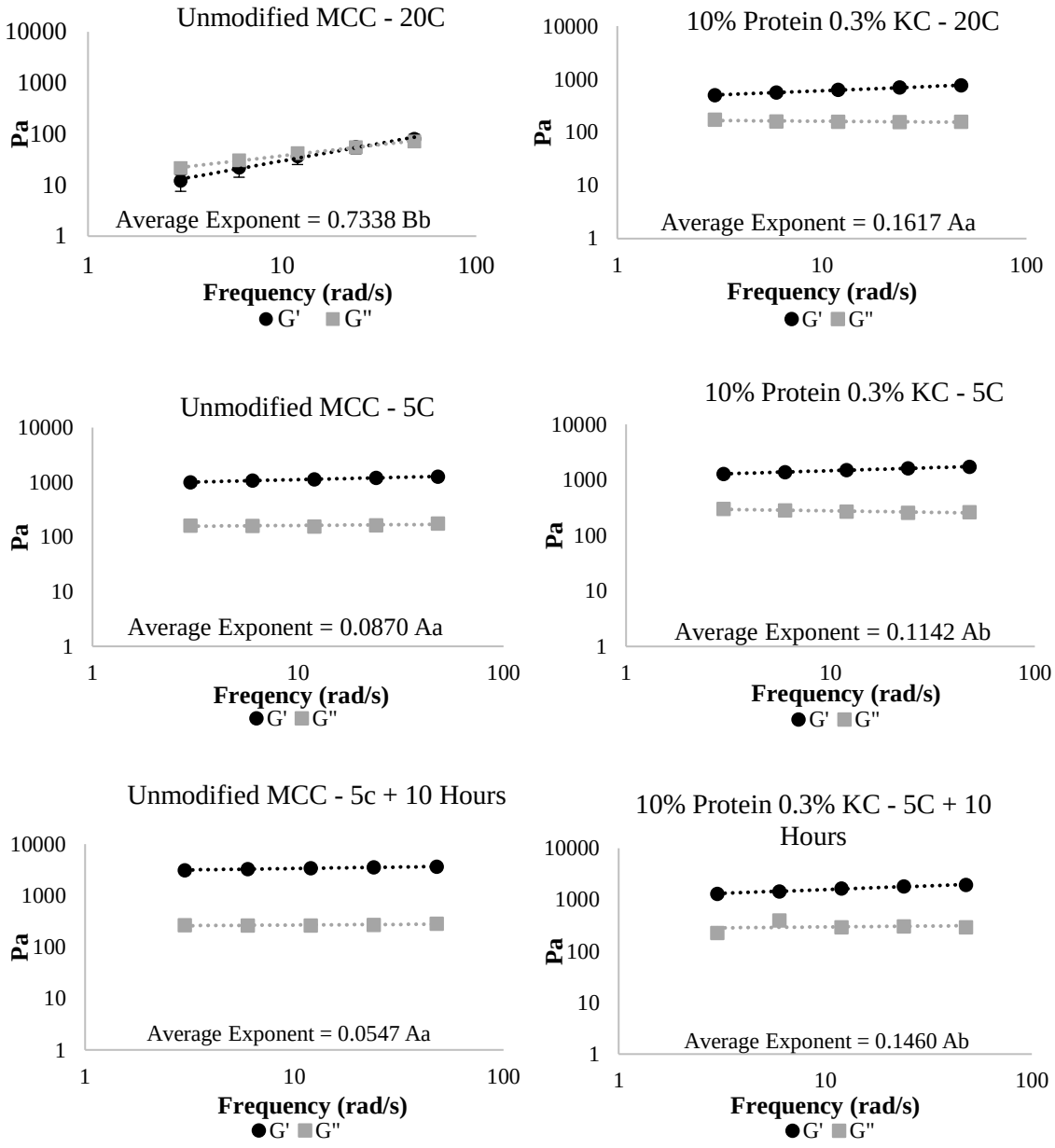

Figure 5-7: A comparison of frequency measurements at 20ºC, 5ºC, and 5ºC after 10 hours between unmodified HC-MCC and a diluted sample with 0.3% kappa carrageenan added. The exponent of the slope equation for G' can be used as a marker for gel strength, with a smaller exponent indicating a stronger gel and less frequency dependance. Capital letters denotes significant difference (P< 0.05) between temperatures and lower case denotes significance between treatments.

When comparing texture analysis results from previous chapters, we observed the opposite result i.e., pH and TSC modifications reduced the maximum stress, hardness and strain hardening ratio over the control. (Chapter 4.3.4). The reduction in protein content prohibits dense networks of casein compared to undiluted samples, resulting in less resistance to compression forces and a reduction in shear hardening qualities. Control samples still maintain a native protein conformation, which could offer more resistance in testing typically observed in other dairy samples (Sharma et al., 2018; Zad Bagher Seighalani & Joyner, 2019).

When considering the results from TEM micrographs, KC appears to bind moisture to a greater extent than casein in any form, reducing the moisture available to the casein phase within solution. This offers an explanation as to why a consistent increase in G' was observed in all rheology tests utilizing KC; as there is minimal interaction between casein and KC, the concentrated pockets of KC were able to absorb available moisture within the system and create a separate KC gel matrix within the MCC system. In the formation of this matrix, KC absorbs water from the casein pockets, effectively compacts the protein phase closer. The increase in casein density assists in gel strength by allowing casein structures to interact as if they were a higher protein concentration, therefore increasing potential for casein-casein interactions. In addition, TSC and pH modification can lead to conformational changes in casein micelles, which can result in increased gel strength (Chapter 3.3.4, Chapter 4.3.2). Furthermore, the ability of KC to absorb moisture also offers a potential explanation for particle size data. Such competition for moisture results in less hydration of micellar structures and a reduction in overall size of casein micelles within control samples.

5.5: Conclusions

The addition of KC into diluted MCC dispersions results in significant increases in both gel strength and gelation temperature. The use of KC creates separate phases within solution that absorb water to a greater extent than casein, resulting in concentrated casein pockets within a composite network. Additional physicochemical modifications in the form of calcium chelation and alkalization improved gel strength and CGT, at the expense of a reduction in textural hardness and strain hardening properties. Interaction between KC and HC-MCC was minimal regardless of treatment, indicating that elevated gel qualities were due to the water binding effects of KC rather than the formation of a KC-casein matrix. In the future, studies with other forms of physicochemical modifications could yield additional insights into the cold gelling behavior of MCC dispersions.

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CHAPTER 6

CONCLUSION

Dispersions of HC-MCC were modified in specific manners (pH modification, TSC and KC addition) that resulted in changes (mostly favorable) in gel strength and the temperature of gelation. In addition to the changes evidenced by rheological testing, ultrastructure analysis confirms that morphological changes were occurring within the micellar structure of casein in response to these treatments. The addition of TSC to HC-MCC, regardless of protein content was found to raise gel strength and gelation temperature when at a native or acidified pH level. Changes in zeta potential and textural properties were also evidenced by calcium chelation, with TSC additions increasing strain hardening qualities, increasing the net negative charge of particles. In high concentrations (50 mM), TSC resulted in reductions in gel strength compared to lower concentrations (25 mM), with TEM micrographs depicting the formation of large casein aggregates. These newly formed structures are likely to be the cause for the reduction in storage modulus (G').

Adjustment of the sample pH also resulted in various structural changes to caseins. It was observed that storage modulus values followed an exponential positive relationship in the pH range of 6.2-6.8, with samples alkalized to 6.8 yielding the strongest gels. As expected, net charge on the casein micelles and particle size increased in response to increasing pH levels. Further alkalization to pH 7.0 continued to increase

gel strength, creating a potential pathway for further testing. TEM conducted on alkalized samples shows evidence of micellar disintegration in response to increasing pH levels with concurrent gains in gel strength. Potential increases in protein-protein and protein-water interactions as a result of micellar collapse is the current hypothesis behind the increases in gel qualities.

Tests conducted in chapter 3 led to the conclusion that protein content of the gels was the main determinant of gel strength. The addition of KC to diluted gels was found to raise the gelation point to a similar temperature level as observed in previous chapters, but gel strength and strain hardening qualities were considerably weaker compared to samples previously tested. Additional physicochemical modifications to KC added gels affected particle size, gel strength, and textural qualities, but KC content was the main determinant of gel strength and temperature of gelation. TEM micrographs depicted two separate phases within solution; one of casein and one of KC. the water binding capacity of the polysaccharide reduces the moisture available to casein, resulting in pockets of concentrated protein responsible for encouraging gelation.

Future testing of HC-MCC could explore its potential applications within the food industry. The addition of HC-MCC to products such as yogurt to observe its potential ability to thicken and prevent syneresis is a potential pathway for a future project. In addition, there is still much to learn about the gelation properties of the sample, especially when considering the physicochemical modifications applied. Another pathway to consider is how different industrial methods of manufacture could affect the gelling qualities of the product, and the effect of other milk components (higher mineral content, whey protein and lactose fraction, etc.) remaining in solution.

APPENDIX

Table A-1: Confidence Intervals of 18.5% Protein Sample Gelation Temperatures

	pH			
TSC (mM)	6.2	6.4	6.6	6.8
0	8.48 (7.50, 9.45)	10.52 (9.56, 11.48)	10.74 (9.79, 11.71)	30.19 (25.67, 34.72)
10	7.32 (4.74, 9.90)	22.87 (17.94, 27.80)	25.16 (19.66, 30.66)	32.03 (29.09, 36.96)
25	25.84 (20.35, 31.33)	27.22 (22.93, 31.50)	29.05 (25.49, 32.60)	31.33 (30.34, 32.32)
50	21.03 (14.16, 27.90)	25.16 (22.55, 27.76)	24.01 (21.06, 26.95)	26.07 (19.93, 32.22)

Table A-2: Confidence Intervals of 15% Protein Sample Gelation Temperatures

	pH			
TSC (mM)	6.2	6.4	6.6	6.8
10	No Gel	No Gel	6.41 (5.43, 7.40)	25.39 (23.67, 27.10)
25	5.06 (4.81, 5.30)	18.06 (12.84, 23.29)	25.15 (19.94, 30.36)	25.62 (22.06, 29.17)
50	14.87 (10.94, 18.80)	24.47 (17.59, 31.35)	16.24 (11.96, 20.51)	26.07 (19.91, 32.23)

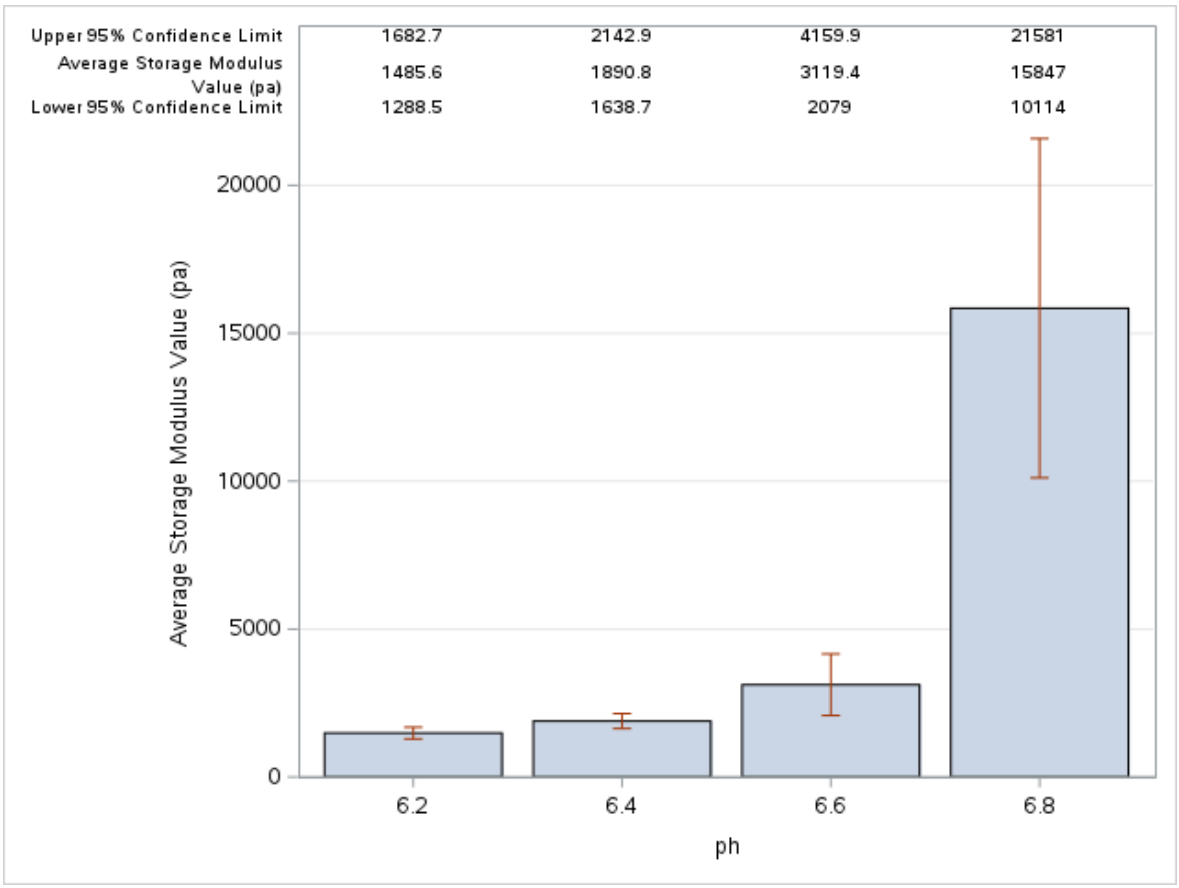


Figure A-1: 95% Confidence Intervals of G' Values – 18.5% Protein and 0mM TSC. G' values are recorded after 10 hours of holding at 5°C

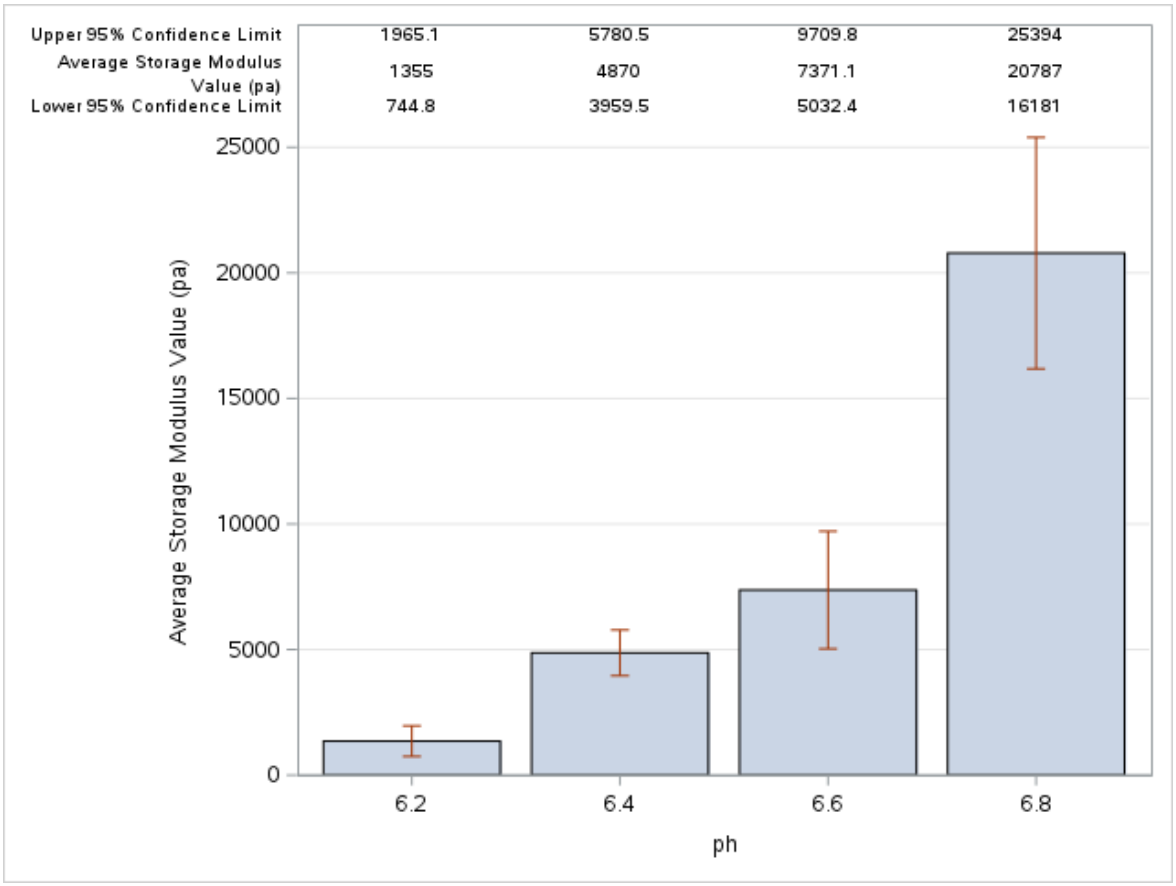


Figure A-2: 95% Confidence Intercals of G' Values - 18.5% Protein and 10mM TSC. G' values are recorded after 10 hours of holding at 5°C

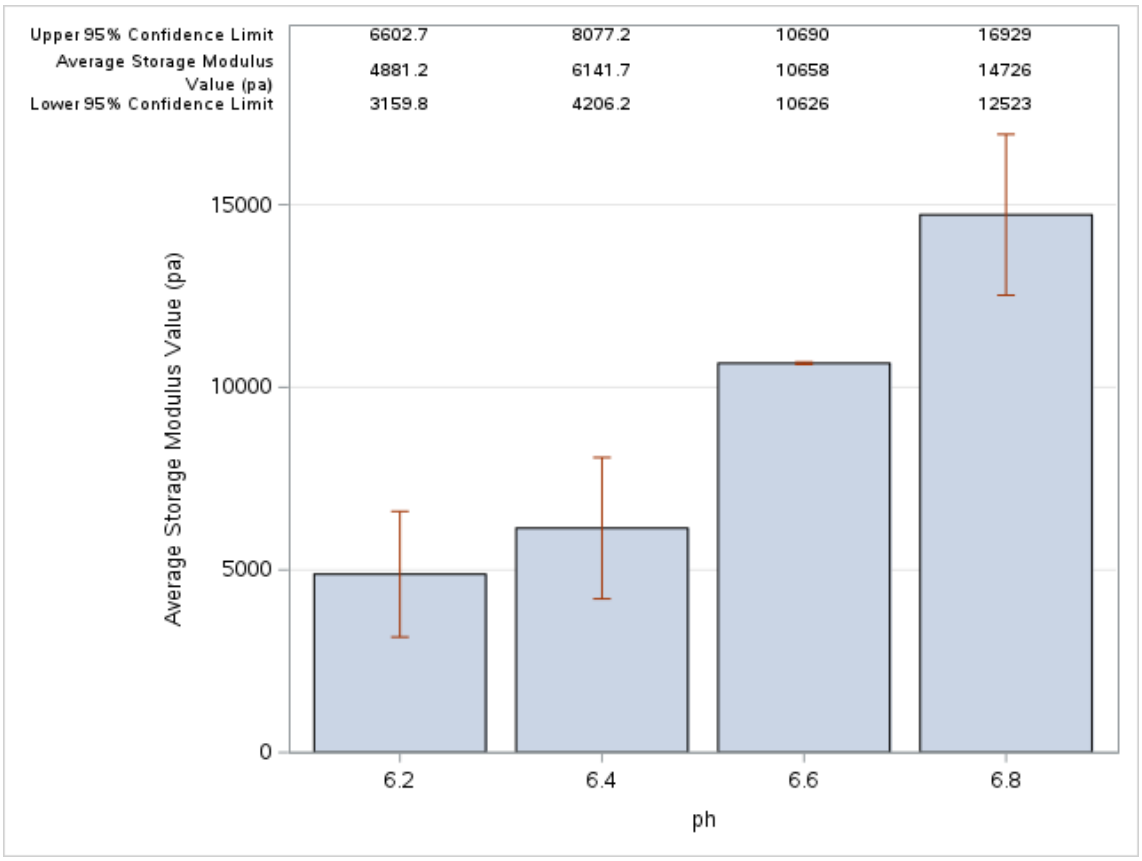
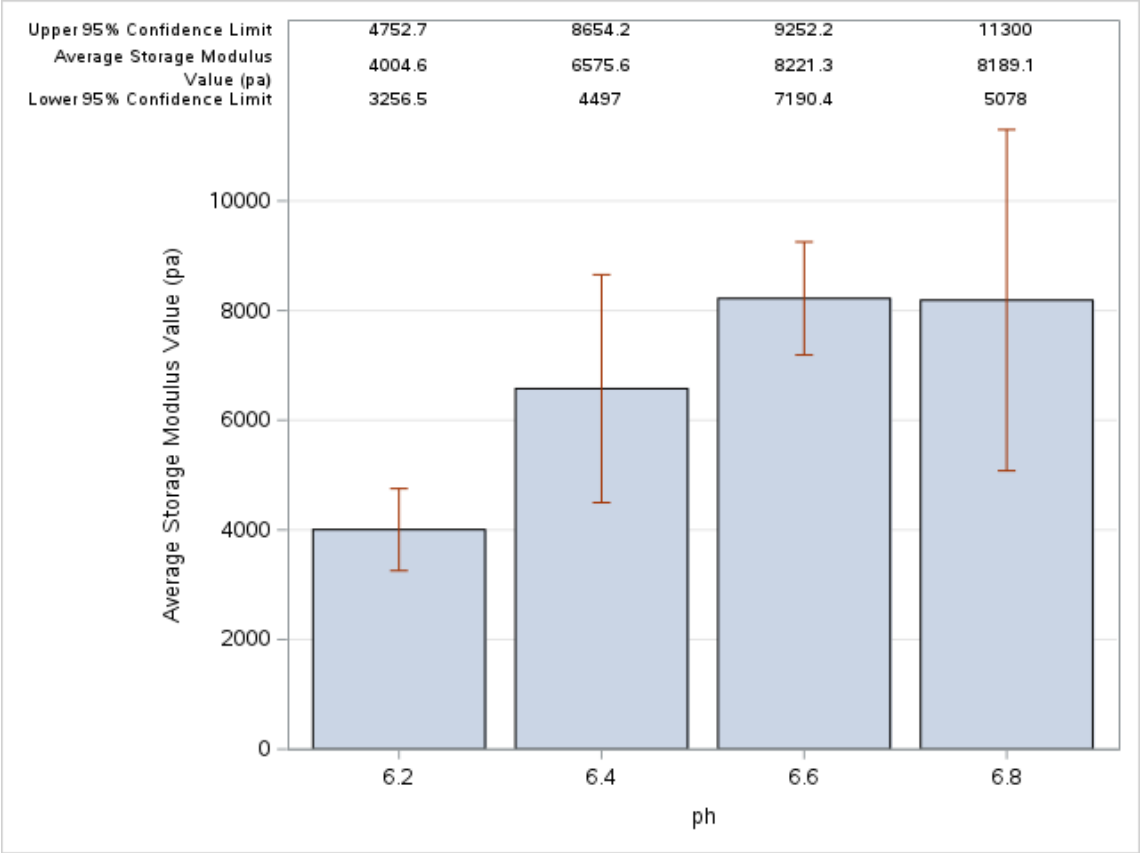


Figure A-3: 95% Confidence Intervals of G' Values – 18.5% Protein and 25mM TSC. G' values are recorded after 10 hours of holding at 5°C



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1665 **Figure A-4:** 95% Confidence Intervals of G' Values – 18.5% Protein and 50mM TSC.

1666 G' values are recorded after 10 hours of holding at 5°C