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Influence of calcium chelation and enzymatic modification of protein on the hydration characteristics of high protein dairy powders

Thesis presented by

Orla M. Power

for the degree of

Doctor of Philosophy



University College Cork

Food Science and Technology

Head of School: Prof Mairead Kiely

Supervisor(s): Dr Noel McCarthy, Dr Mark Fenelon, Dr Seamus (James) O'Mahony,

June 2020

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Declaration by the Candidate

Title: Influence of calcium chelation and protein enzymatic modification on the hydration characteristics of high protein dairy powders

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

_____

Orla Power
2nd June 2020

Dedication

This thesis is dedicated to my parents Sandra & Gerry Power and my partner Ruairi Murnane for their unwavering support and inspiration.

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Abstract

Milk protein concentrate (MPC) powders have numerous commercial applications and are integral ingredients in high quality functional dairy products and beverages. However, such high protein dairy powders pose significant technical challenges during processing and rehydration, due to high viscosity and poor powder particle dissolution, respectively. Previous work has shown that the use of calcium chelating salts is effective in improving dissolution of MPC powders; however, the use of such salts results in considerable increases in viscosity and reduction in casein micelle integrity. The objective of this research was to investigate the impact of depleting calcium in micellar casein systems after either enzymatic dephosphorylation or enzymatic crosslinking and to examine viscosity, particle size; ζ-potential and colour of the protein solutions and subsequent rehydration properties of these spray dried systems. Calcium depletion was achieved through either addition of a chelating salt (sodium hexametaphosphate; SHMP) or through ion exchange.

MPC dispersions containing enzymatically dephosphorylated casein proteins exhibited lower viscosity than phosphorylated MPC dispersions; however, the former resulted in a loss of casein micelle integrity in the presence of SHMP. Phosphate nuclear magnetic resonance spectroscopy proved useful for quantifying the depletion of calcium phosphate from casein micelles and confirmed dephosphorylation. Enzymatic crosslinking of casein proteins using transglutaminase helped maintain a lower solution viscosity after calcium chelation by SHMP, and the resultant dispersions retained a higher degree of casein micelle integrity compared to non-crosslinked MPC dispersions. Co-dried crosslinked casein and SHMP powders had improved rehydration properties. As an alternative to the addition of calcium chelating salts, a strong cation exchange resin was investigated for its ability to deplete calcium

to varying extents in the production of novel MPC powders with improved dissolution properties at low levels of calcium depletion. However, similar to with direct use of calcium chelating salts, concomitant increases in viscosity and decreases in micelle integrity were observed in MPC dispersions, with the effects increasing with extent of calcium removal. Solubility and dissolution properties of calcium depleted MPC powders exhibited improved rehydration ability and caused a shift in their heat stability towards lower pH values. MPC dispersions containing crosslinked proteins were observed to be more resistant to calcium depletion compared to their respective control samples. MPC dispersions containing crosslinked MPC proteins retained greater micelle integrity; however, extensive calcium reduction resulted in significantly higher viscosity than corresponding controls.

This thesis provides new insights into the functionality of novel MPC powders generated using enzymatic modification of casein and chemical modification of the calcium content and distribution between colloidal and serum phases, resulting in MPC powders with enhanced functionality. The outcomes of this research have application in the formulation of MPC powders with improved dissolution properties and reduced viscosity.

Publications

Papers published in peer-reviewed journals

Power, O. M., Fenelon, M. A., O'Mahony, J. A., & McCarthy, N. A. (2019). Dephosphorylation of caseins in milk protein concentrate alters their interactions with sodium hexametaphosphate. Food Chemistry, 271, 136-141.

Power, O. M., Fenelon, M. A., O'Mahony, J. A., & McCarthy, N. A. (2020). Influence of sodium hexametaphosphate addition on the functional properties of milk protein concentrate dispersions containing transglutaminase cross-linked proteins. International Journal of Dairy Technology, 104, 104641.

Power, O. M., Maidannyk, V., McSweeney, D. J., Fenelon, M. A., O'Mahony, J. A., & McCarthy, N. A. (2020). Water sorption and hydration properties of high protein milk powders are influenced by enzymatic crosslinking and calcium chelation. Powder Technology, 364, 680-688.

*McCarthy, N. A., Power, O., Wijayanti, H. B., Kelly, P. M., Mao, L., & Fenelon, M. A. (2017). Effects of calcium chelating agents on the solubility of milk protein concentrate. International Journal of Dairy Technology, 70(3), 415-423.

**Not under examination in the Thesis.*

Presentations

Oral presentations

- International Dairy Federation (IDF) Conference, Teagasc Ashtown, Co. Dublin (2018)
- Society of Dairy Technology (SDT) Conference, University College Cork, Co. Cork (2018)
- *Marie Curie* Cork Discovers Culture Night, Co. Cork (2019)
- 46th Annual Walsh Fellow Seminar Food Chemistry heat, Teagasc, Ashtown, Co. Dublin (2019)- awarded Best Presentation
- 46th Annual Walsh Fellow Seminar Final, Teagasc Johnstown Castle, Co. Wexford (2019)- awarded Institute of Food Science and Technology of Irelands (IFSTI) award for best overall food science and technology presentation
- International dairy Federation (IDF) conference, University College Cork, Co. Cork (2019)

Poster presentations

- International Dairy Federation (IDF) World Dairy Summit in Rotterdam (2016)
- International Dairy Federation (IDF) World Dairy Summit in Belfast (2017)
- Society of Dairy Technology (SDT) Conference, in University College Cork, Co. Cork (2018)

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Fig. 6.5. Heat coagulation time-pH profiles of rehydrated milk protein concentrate powders (1.5%, w/w) at 0 (■), 30 (▲), 52 (●) and 70% (♦) levels of calcium reduction.

Abbreviation

a_w	Water activity
ANOVA	Analysis of variance
BAP	Bovine alkaline phosphatase
BSA	Bovine serum albumin
CCP	Colloidal calcium phosphate
CNP	Casein-bound phosphate region
DF	Diafiltration
DP	Dephosphorylated
$D_{[3,2]}$	Sauter mean diameter
$D_{[4,3]}$	Volume mean diameter
G′	Storage modulus
G″	Elastic modulus
GAB	Guggenheim-Anderson-de Boer
HCT	Heat coagulation time
ICPMS	Inductively coupled plasma mass spectrometry
Ig	Immunoglobulins
IMF	Infant milk formula
ISE	Ion selective electrode
MF	Microfiltration
MPC	Milk protein concentrate
MPI	Milk protein isolate
N/A	Not applicable
NMR	Nuclear magnetic resonance
P^{31} NMR	Phosphate nucelar magnetic resonance
PAGE	Polyacrylamide gel electrophoresis

PAP	Potato acid phosphatase
PCP	Primary casein particle
PdI	Polydispersibility index
P*i*	Inorganic phosphate
ppm	Parts per million
RH	Relative humidity
RI	Dispersant refractive index
rpm	Revolutions per minute
RO	Reverse osmosis
SDS	Sodium dodecyl sulphate
SHMP	Sodium hexametaphosphate
SMP	Skim milk protein
TG	Transglutaminase
WPC	Whey protein concentrate
WPI	Whey protein isolate
w/w	Weight/ Weight
v/v	Volume/ Volume
UL	Ultrafiltration
a*	Red/green colour spectrum
b*	Yellow/blue colour spectrum
L*	Whiteness colour spectrum
ΔE	Colour change
Ca	Calcium
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
D_2O	Deuterium oxide
HCL	Hydrochloric acid

NaOH	Sodium hydroxide
P	Phosphorous
KCl	Potassium chloride
KOH	Potassium hydroxide

CHAPTER ONE

Literature review: Milk protein concentrate powders and modifications to improve their rehydration properties

Declaration:

This chapter was researched and written by author Orla M. Power (OMP) and reviewed by all co-authors Dr. Noel A. McCarthy (NAMC), Dr. James A. O'Mahony (JAOM) and Dr. Mark Fenelon (MK).

1.1 Introduction to high protein milk powders

The global dairy market is growing, with an estimated compounded annual rate of 5%, estimated to be worth $703.5 Billion USD by 2024 (Cobbe, 2019). Key trends emerging from the dairy sector are the popularity of high protein dairy powders as ingredients in foods and beverages to improve their functionality and nutritional content. Milk protein concentrates (MPC) and milk protein isolates (MPI) are generally marketed and transported worldwide as spray dried powders and have applications in both food and beverage products (Mulvihill and Ennis 2003). Milk protein concentrates contain both casein and whey proteins, with protein contents ranging from 42 to 85%, and provide a clean dairy taste with a reduced lactose content and hence, are popular as value-added dairy protein ingredients (Innova, 2015). In addition to protein, MPC powders also contain high levels of minerals, of nutritional significance, such as, calcium, magnesium and phosphorus. Therefore, high protein milk powders are generally utilised in the production of dairy based beverages, high protein bars, paediatric products for performance nutrition and sports recovery. MPC powders are often used to modify the functionality of nutritional products such as increasing water holding capacity, gelling, foaming, emulsification and heat stability (Ye, 2011; Huppertz and Patel, 2012). Generally, MPC powders are included in yogurt and cheese products to increase the solids content and improve gel strength and texture.

MPC has been utilised in numerous cheese manufacturing processes including Camembert, Feta, and soft cheeses including Ricotta; both liquid and powder MPC addition have also been used in the production of reduced-fat Cheddar cheeses (Pederson and Ottosen, 1992; Shakeel-Ur- Rehman *et al*, 2003a and 2003b). In a study

conducted by Shakeel-Ur-Rehman *et al* (2003), cheese milk was enriched with MPC for the production of pizza cheese and it was observed that the addition of MPC improved the yields achieved, regardless of whether cheese was made by culture or direct acidification. MPC addition was observed to alter the functional properties, including melting, calcium content and browning depending on whether the cheese was made by either culture or direct acidification. MPC powders can also be utilised in the manufacture of lactose reduced ice-cream mixes; a study by Alvarez *et al* (2005) reported that replacing skim milk with MPC did not adversely impact the functional qualities of the ice cream mix.

The market for dairy protein-based beverages for performance nutrition has grown exponentially in recent years. Mintel (2018) reported that in 2017, the US nutrition and performance drinks market was worth $14 billion, an increase of 26% compared with data for 2012. To meet Food and Drug Administration standards, protein beverages must contain a minimum of 10 g of protein per serving (Wagoner and Foegeding, 2017) and so MPC and MPI ingredients are important in the manufacture of dairy-based nutritional snack bars, which contain a minimum of 15 g of protein per serving (Loveday *et al*, 2009; Agarwal *et al*, 2015). High protein milk powders can also be incorporated during the manufacture of infant milk formula (IMF). Irish dairy products and ingredient exports exceeded €4 billion in 2018, with the top five global markets for Irish dairy products being the UK (26%), EU destinations (41%) and destinations outside the EU (mainly China and the United States, 34%), with China now accounting for 10% of total Irish dairy exports (Teagasc, 2019). However, one of the main issues with the export of high protein milk powder ingredients is their challenges with ease and completeness of rehydration. Therefore, depending on the end use, modifications may be required to existing unit operations

or formulations in order to ensure complete powder dissolution. This review will focus on the manufacture of high protein dairy powders, with an emphasis on protein and mineral modifications to improve solubility properties.

1.2. Milk proteins

Milk provides a nutritious food source containing essential nutrients for new-born mammals; however, these nutrients can be mined and separated for use in specialised ingredients for numerous dairy based foods and beverages (Haug *et al*, 2007). Milk proteins represent ~3.0–3.5% of the total macro-constituents in milk and can be divided into two subcategories; casein and whey proteins, with a ratio of approximately 80:20, respectively, in bovine mature milk (Fox and McSweeney, 2013; Table. 1).

Table. 1.1. *Protein profile of bovine milk (Fox and McSweeney, 2013)*

Protein Profile	**Protein**
	g / 100 mL
Total protein	3.3
Total casein protein	**2.6**
α_{s1}- casein	1.04
α_{s2} - casein	0.21
β- casein	0.99
κ-casein	0.31
Total whey protein	**0.7**
β-lactoglobulin (β-Lac)	0.3
α-lactalbumin (α-Lac)	0.12
Bovine Serum Albumin (BSA)	0.03
Lactoferrin	0.015
Lysozyme	Trace
IgA	0.003
IgG	0.06

1.2.1. Casein protein fractions and casein micelle

Casein proteins exist as self-assembled spherical colloids (micelles), stabilised by colloidal calcium phosphate (CCP) clusters suspended in the aqueous phase of milk (Huppertz *et al*, 2017). The casein fraction in milk is composed of four main variants, namely, α_{s1}-, α_{s2}-, β-, and κ-casein, which represent approximately 38, 10, 36, and 12% of the total casein content present in milk, and contain 199, 207, 209, and 169 amino acids, respectively. The high proportion of proline residues in casein proteins results in little secondary or tertiary structure, consequently casein proteins self-assemble and exist as a porous network of non-spherical primary casein particles (PCP) linked by CCP nanoclusters (Huppertz *et al*, 2017). Casein proteins also differ in their degree of phosphorylation, varying from one to thirteen phosphoserine residues, and so have differing extents of calcium sensitivity. κ-Casein is resistant to calcium-mediated precipitation, due its low level of phosphorylation and provides steric and electrostatic repulsion between micelles. κ-Casein is a glycosylated protein which also aids in providing micelle stability (Dalgleish, 2011; Dalgleish and Goff, 2004), with κ-Casein being the only glycosylated casein (Dziuba and Minkiewicz, 1996). Glycoproteins in bovine milk are integral to the functional properties of milk proteins and the nutritional quality of the resulting milk. The main glycoproteins found in bovine milk are lactoferrin, which is found in the whey protein fraction, immunoglobulins, milk fat globule membrane associated glycoproteins and glycomacropeptide, which is derived from κ-casein (O'Riordan *et al*, 2013).

The structure of the casein micelle has been researched and debated for many years but the composition is quite well defined, composed of ~63% water, with protein and CCP accounting for ~94 and 6% of the dry matter, respectively (Dalgleish, 2011). CCP is an important structural component of casein micelles, holding caseins together via interactions between negatively charged phosphoserine residues, which also aids

in reducing the electrostatic repulsion between the casein proteins within the micelle (Holt, 1997; Walstra, 1990). Colloidal calcium phosphate nanoclusters contain approximately 7 g of minerals per 100 g of dry casein and are comprised of calcium, phosphate, magnesium, sodium and citrate, but with the exact composition of CCP dependent on the ionic environment. The average total calcium content of bovine milk is approximately 30 mM, with ~66% associated with CCP in the casein micelles. The balance exists as either free ionic calcium (<10% of total calcium) or bound with citrate/phosphate in the serum phase of milk (Gaucheron, 2005; Lewis, 2011; Akkerman *et al*, 2019). The combination of polar and apolar amino acids and low levels of secondary and tertiary structure help aid the formation of colloidal micelles, with both hydrophobic and hydrophilic regions present at the surface. Casein micelles consist of approximately 5000 casein proteins. However, the mechanism of casein micelle self-assembly has been a topic of debate with numerous models proposed by researchers. However, it is known that casein micelles have a spherical morphology as shown in the field emission scanning electron micrographs in Fig. 1.1.

The initial micelle model proposed that the casein micelle was comprised of sub-micelles, held together by CCP nanoclusters and stabilised by a surface layer of predominately κ-casein (Fig. 1.2; McMahon and Brown, 1984). The exposed κ-casein layer orientates with the hydrophilic c-terminal protruding into the aqueous phase, creating a “hairy layer” around the micelle.

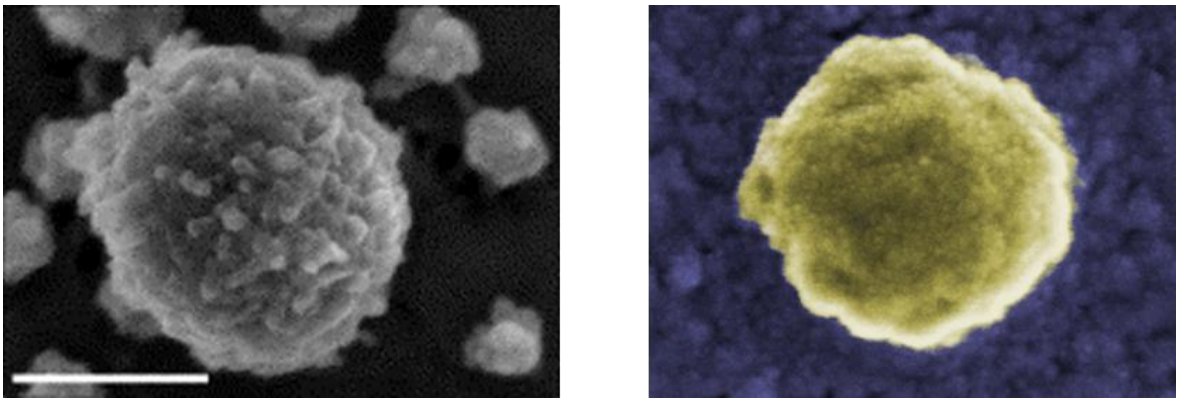


Fig. 1.1. *Field emission scanning electron micrographs of casein micelles, the scale bar is 200 nm (taken from Spagnuolo and Morris, 2004).*

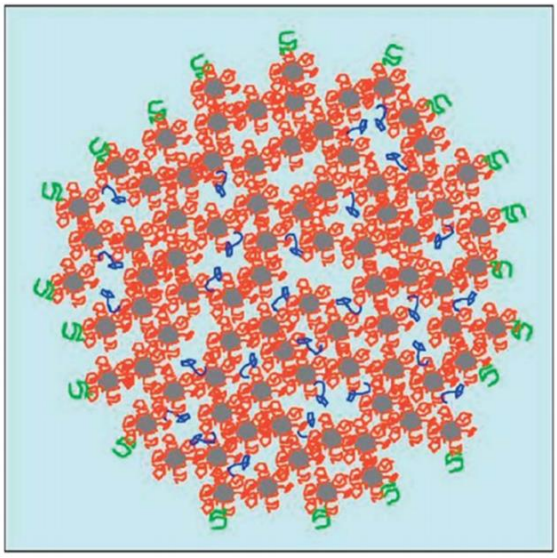


Fig. 1.2. *Schematic representation of a casein micelle showing colloidal calcium phosphate nanoclusters (shown in grey), α-caseins (shown in red) and surface-located κ-casein (shown in green). Hydrophobically bound β-casein (shown in blue) is located within the water channels inside the micelle (taken from Dalgleish, 2011).*

Models other than the sub-unit model have also been presented and described (McMahon and Brown, 1984); Dalgleish, 2011, de Kruif *et al*, 2012: Lucey and Horne,

2018). For instance, Holt (1992) suggested that α_{s1}-, α_{s2}- and β-casein, which are sensitive to calcium-induced precipitation, are connected by micro-crystals of CCP and stabilised by a surrounding layer of κ-casein (Fig. 1.3a). Horne proposed a "dual binding model" in which individual casein proteins interact with each other via hydrophobic regions while leaving the hydrophilic regions of the protein free (Fig. 1.2; Horne, 2006). The proteins are surrounded by a layer of κ-casein, orientated in the manner as described previously by Holt (1992). Huppertz *et al* (2017), proposed for caseinate particles, the main contributor to intrinsic viscosity was the presence of the non-spherical particles, these non-spherical particles in caseinate are potentially naturally present as primary casein particles (PCP) in casein micelles. PCP could be used to build casein micelles by controlled introduction of micellar salts. Hence, casein micelles could be described as a porous network of non-spherical PCP linked by calcium phosphate nanoclusters (Fig. 1.3b.). The key structural features retained in most models are the importance of CCP nanoclusters and the stabilising κ-casein layer, with the hydrophilic C-terminal protruding into the aqueous phase, creating a "hairy layer" around the micelle. More information describing casein micelle models can be found in the literature (Dalgleish, 1998; Dalgleish, 2011; de Kruif *et al*, 2012; Holt, 1992; Horne, 1998; Huppertz *et al*, 2017; Lucey and Horne, 2018; Walstra, 1990). In addition to the protein structure, casein micelles have a high degree of micellar hydration and are composed of approximately ~3.3 g of water g^{-1} protein, with water distributed in the micelle as water bound by the protein (~0.5 g water g^{-1} protein), water associated with the κ-casein brush (~1.0 g water g^{-1} of protein) and water entrapped in the casein micelles (~1.8 g water g^{-1} protein), respectively (Morris *et al*, 2000; Dalgleish, 2011; Huppertz *et al*, 2017).

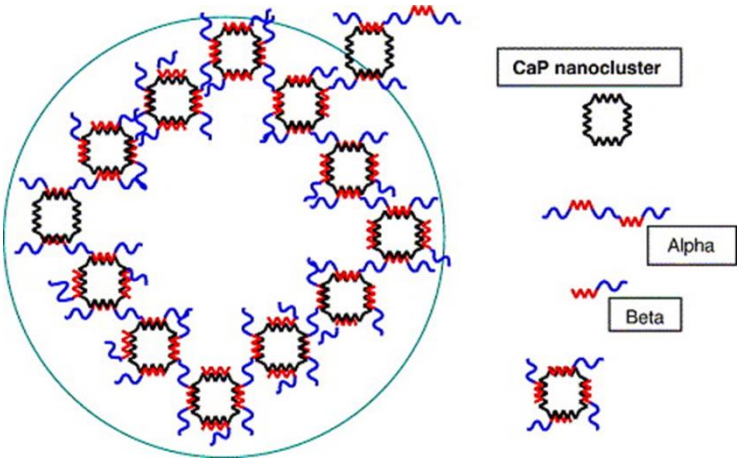


***Fig. 1.3a**. Schematic representation of the network formation in the Holt casein micelle model (Horne; 2006). α_{s1}- and α_2-Caseins are shown to have two interaction points, while β-casein has one interaction point. α_{s1}, α_{s2}-Casein binds to neighbouring nanoclusters, acting as a bridge creating a chain of linked proteins.*

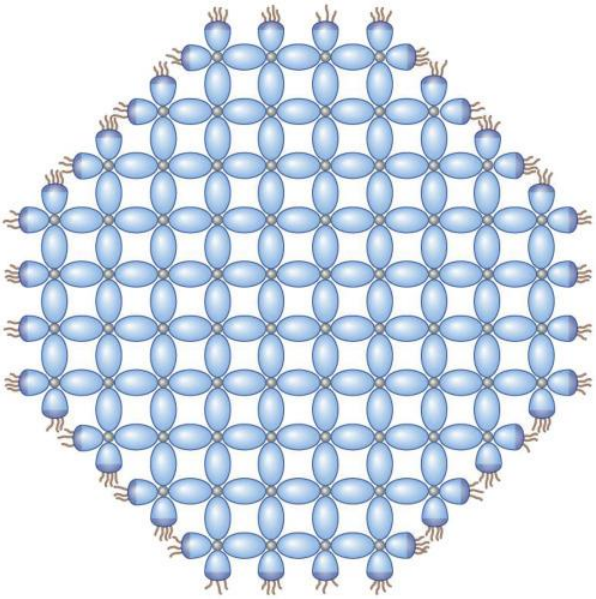


***Fig.1.3b.** Illustration of transversal section through a casein micelle: Grey – colloidal calcium phosphate nanoclusters; blue: primary casein particle, with areas consisting of α_{S1}-, α_{S2}- and β-casein in light blue and κ-casein in dark blue/brown (Huppertz et al, 2017).*

1.2.2. Whey proteins

Whey proteins in bovine milk make up ~20% of the total protein content and comprise five main globular variations, namely, β-lactoglobulin (β-lg), α-lactalbumin

(α-la), bovine serum albumin (BSA), lactoferrin and immunoglobulins (Igs) (Raikos, 2010; Wijayanti *et al*, 2014). The individual proportions of β-lg, α-la, BSA and Igs as a % of total whey protein are ~50, 20, 10 and 10%, respectively (Fox, 2001; Robson and Dalgleish, 1987; Morr and Ha, 1993). Whey proteins have a high level of tertiary structure and exist as globular folded proteins with hydrophobic amino acids arranged within the core of folded peptide chains (Singh, 2011). As a result, whey proteins are more susceptible to thermal denaturation when compared to casein proteins. β-Lg and α-la undergo structural changes during heating, forming protein aggregates, which impact on the functional properties of processed whey products such as viscosity, turbidity, protein precipitation or gelation (Buggy *et al*, 2018). Following thermal denaturation, whey protein aggregation occurs through the interaction of exposed free sulfhydryl residues (SH) of unfolded β-lg proteins, with neighbouring β-lg monomers and dimers through disulfide interchange reactions (S-S). This creates a chain reaction of free reactive SH residues promoting further protein aggregation until the reaction is terminated by disulfide linkages between two reactive SH-groups and β-lg monomers (Roefs and de Kruif, 1994; Verheul *et al*, 1998). β-Lg is highly structured and has an atypical globular protein structure, existing in a pleated sheet barrel conformation, comprising 162 amino acid per monomer. It primarily exists as dimer within the pH range 5.2 to 7.5 (Fox, 2003) and the protein has an isoelectric point of pH 5.2 and due to its rigid structure is highly resistant to proteolysis. α-La is the second most abundant whey protein fraction (20%, w/w, of total whey protein) and can be isolated from cheese whey by ion exchange, ultrafiltration or acid precipitation (Madureira *et al*, 2007), and is often used as a supplement in infant milk formula, protein-fortified beverages and protein bars due to its high nutritional quality (Mudgil and Barak, 2019). α-La has a globular structure, stabilised by four disulphide bonds at

an optimum pH range of 5.4-and 9.0. α-La contains high levels of tryptophan, approximately 6%, w/w, which is thought to be utilised in serotonin synthesis (Madureira *et al*, 2007). The main biological role of α-la is the synthesis of lactose in the mammary glands, facilitating milk production with the volume of lactose in milk, being directly correlated with the levels of α-la (Boland *et al*, 2011; Wit, 1998; Lonnerdal and Lien, 2003).

1.3. Manufacture of milk protein concentrate powders

Milk protein concentrate ingredients are used in functional food products, such as in life-stage formulae, sports nutrition products, and as a protein standardisation medium in cheese and other dairy products. The majority of these dairy-based protein ingredients are produced and distributed in powder form, allowing for cost effective transportation and extended shelf life. The production process of high protein dairy powders commences with membrane filtration of pasteurized skim milk, followed by heat treatment, evaporation and subsequent spray drying.

1.3.1. Membrane filtration, heat treatment and evaporation

Membrane technology has been employed for numerous applications in the dairy industry, including bacterial/spore removal and desalination of dairy water. However, the principal function of membrane technology in the dairy industry is to fractionate proteins, minerals and carbohydrates from within skim milk and cheese/acid whey in order to produce novel dairy ingredients (Pouliot, 2008). Generally, membrane technology has been used to isolate milk proteins, to produce casein and whey-based protein concentrates and isolates as well as a means of concentrating dry matter content in dairy streams. These novel high value protein

products range in protein content from ~40 – 90%, w/w, dry matter, with cost of production increasing with increasing protein enrichment level. Membrane filtration operates under the principle of hydrostatic pressure driving the dispersion against a semi-permeable membrane, proteins and macromolecules with high molecular weight are retained, while water and low molecular weight solutes pass through the membrane

Current pressure-driven membrane processes include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) (Fig. 1.4). Currently, the dairy industry use a combination of these membrane filtration techniques to produce MPC from skim milk using MF, UF with integrated DF, followed by heat treatment, evaporation and spray drying (Fig. 1.5). MF processes are most commonly used in the dairy industry to remove bacteria/spores and residual fat from skim milk, and have a size range of 0.1-10.0 μm. However, they are also used for the separation of micellar casein from whey proteins to produce micellar casein concentrates (Beckman *et al*, 2010). UF membranes have a Like MF membranes, UF membranes have a wide size range (pore size 1-100 nm; Van Reis *et al*, 2007; Kumar *et al*, 2013); however, the most commonly used UF membranes have a molecular weight cut-off pore size of 10 kDa which facilitates the concentration/retention of all the main milk proteins (see section 1.2). DF involves the addition of water to the retentate stream followed by further UF. This allows for further removal of lactose, minerals and other soluble components (e.g., NPN) through the permeate stream and for further concentration of the milk proteins (Mistry, 2002; Sikand, *et al*, 2011). In a continuous membrane filtration process the addition of DF water usually occurs in loops towards the end of the UF process.

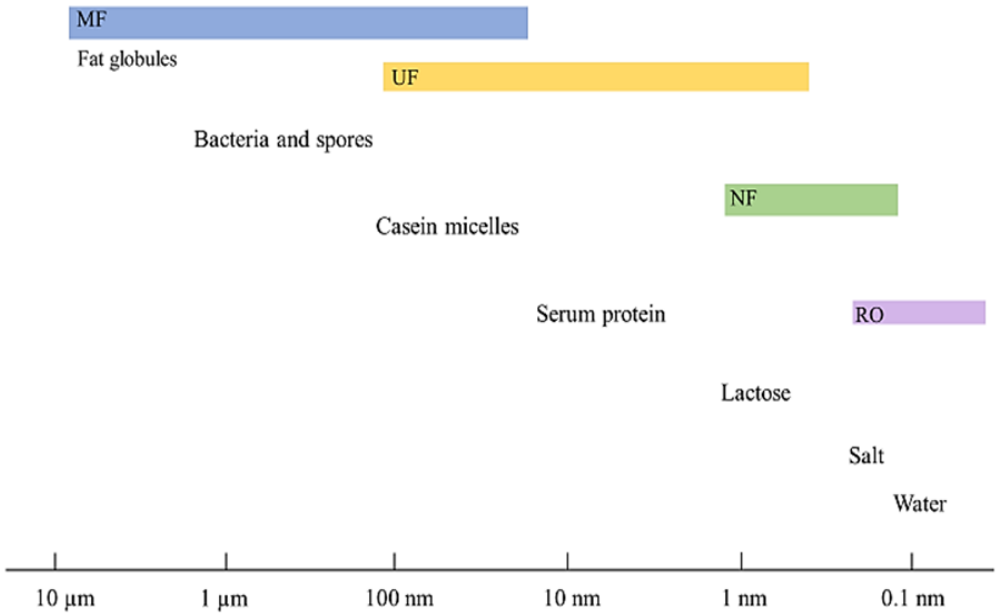


Fig. 1.4. *Milk components and membrane pore sizes for microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO (Tetra Pak, 2015).*

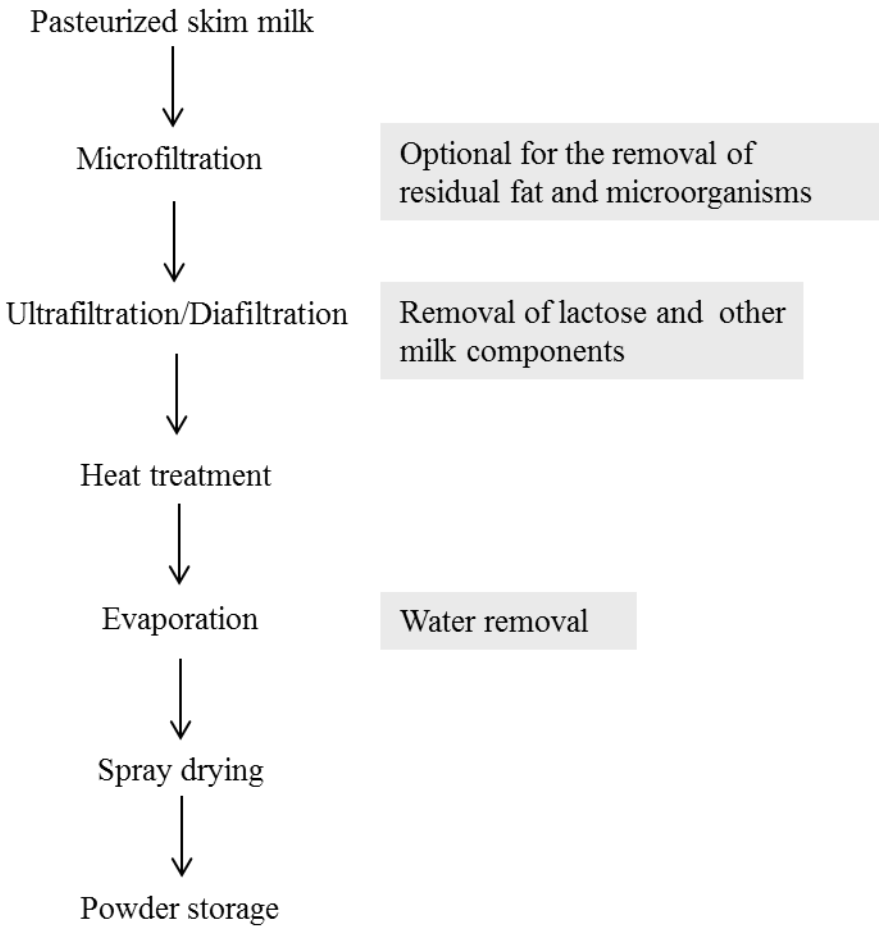


Fig. 1.5. *Modified schematic flow diagram of the steps involved in the production of high protein milk powders (Mistry, 2002).*

Following membrane filtration, high protein UF retentates are generally heat treated at high temperatures for short durations to inactivate microorganisms and to impart specific functionality to the finished ingredient, through protein denaturation/aggregation. Heat treatments can be carried out under direct or indirect heating methods. Indirect heating involves the transfer of heat from one fluid to another across a thermally conducting, but impermeable, interface. Indirect heating methods include plate, tubular and scraped-surface heat exchangers (Tetra Pak, 2020). Unlike indirect heating, direct heating processes involve the product and heating element, generally steam, to come into direct controlled contact, and the excess liquid is subsequently removed through flash cooling. Direct heating is a more thermally efficient method of heating as the latent heat of vaporisation, generally lost during conventional indirect treatments, is given up to the product as the steam condenses (Lewis, 2000; Britz and Robinson, 2008). However, heat treatment can result in increased viscosity, previous studies by Anema *et al*, (2004), Anema and McKenna, (1996), Buggy *et al*, (2017) and Kehoe *et al*, (2011) stated that protein denaturation and aggregation can be attributed to the observed increase in viscosity.

In the manufacture of protein ingredients high intrinsic viscosity is usually measured as a function of protein to solids content, with viscosity often the reason for fouling and blockages in membrane filtration plants, evaporator calandria and spray drying atomization systems (i.e., high-pressure nozzles). Fouling causes significant down time, increased cleaning costs and environmental wastewater management, with associated financial implications (O'Callaghan & Cunningham, 2005). In a study by Murphy *et al.* (2013), it was observed that infant milk formulations subjected to direct steam injection had significantly lower viscosity compared to control formulations

which were heated using indirect heat treatment (i.e., tubular heat exchanger) at an equivalent solids content. Improved viscosity in direct heating systems was attributed to lower levels of whey protein denaturation when compared to control formulations subjected to indirect heat treatment. This may be due to the lower thermal load imparted on the formulation, compared to indirect heating, resulting in a reduced residence time. In a study by Ho *et al.* (2019), it was reported that liquid MPC viscosity significantly increased after high temperature short time heat treatment; and applied an Arrhenius plot to viscosity measured as a function of temperature to predict the shift from protein unfolding to protein aggregation. These authors also showed that the protein/solids content during heat treatment significantly affected viscosity, and that where MPC liquid streams were subjected to heat treatment at lower solids, the resulting concentrate had lower viscosity. Dumpler *et al*, (2017) showed the effect of direct steam injection on skim milk concentrates whereby κ-casein/whey protein aggregates dissociated from casein micelles, making them more susceptible to calcium induced coagulation, with the higher the solids content during heat treatment the more rapid the destabilisation. After heat treatment, evaporation of UF retentate streams (~20%, w/w, dry matter) usually occurs, and because of the associated high viscosity, protein liquid streams are evaporated to relatively to low solid contents (~30% TS) when compared to skim milk concentrates (~50% TS) (Bienvenue *et al*, 2003; Park *et al*, 2016; Vélez-Ruiz and Barbosa-Cánovas, 1998).

Evaporators work on the basis that the boiling point of water is reduced as a result of a reduction in the atmospheric pressure, achieved using a vacuum pump (Liu *et al*, 2012). A number of different evaporators/configurations exist, but the most common used in the dairy industry is the multiple-effect falling-film evaporator. Briefly, falling-film evaporators work on the principle that the product enters the top

of the first effect of the evaporator where it is evenly distributed through a porous plate, guiding the product down a number of evenly distributed tubes (i.e., calandria). Water evaporation takes place while the product flows downwards forming a thin film on the inside of the calandria. This allows for increased heat transfer. The concentrate is then separated from the vapour before being pumped to the second effect and so on until the desired solids content is achieved. To increase energy efficiency the vapour from the product can be compressed (using a steam injection system) and re-used as the heating medium, known as thermal vapour recompression (TVR). Unlike TVR, mechanical vapour recompression (MVR) combines all the vapour from the evaporator and compresses it before returning it to the heating side of the evaporator. Bespoke evaporators, such as scraped surface, agitated thin-film and rising-film evaporators are designed to cope with high viscosity liquid concentrates and can be more suitable for MPC ingredients compared to typical falling film evaporators (Glover, 2004). While, evaporation occurs at relatively low temperatures the concentration of proteins and milk minerals can play a significant effect on subsequent MPC functional properties, particularly viscosity/gelation, heat stability and powder solubility. Crowley *et al*. (2014) showed how calcium ion activty, pH and lactose content significantly influence the heat stability of rehydrated MPC powders, while Cao *et al.* (2015) showed how increasing the dry matter of MPC using NF compared to evaporation caused an improvement in powder solubility.

1.3.2. Spray drying high protein milk streams

Milk protein concentrates are extremely perishable, and while they have relatively low amounts of lactose, their high protein and mineral content make them susceptible to microbial spoilage (Meena *et al*, 2017). Therefore, for preservation and

economical transport reasons, liquid protein concentrates are commonly spray dried after evaporation. Spray dryers used in the dairy industry, share a common principle, the liquid product is converted to fine droplets using spray nozzles or a disk wheel. This process is known as atomization (Schuck, 2002) and converts the liquid concentrate into droplets of approximately 10-200 μm. Spray dryers can consist of one, two or three stages, with the number of stages increasing the drying efficiency (Filkova and Mujumdar, 1995). In the first drying stage, the concentrate is converted to fine droplets using an atomization nozzle or wheel into hot circulating air in the main drying chamber (Fig. 1.6). As moisture is evaporated from the droplets, the dried powder particles fall towards the bottom of the drying chamber. The highly humidified air is removed through an outlet duct and small powder particles, known as fines, which may be entrapped in the air stream, are removed using either a cyclone or bag filter system. Fine particles can be either pneumatically conveyed to the second fluid bed in certain dryers allowing for non-agglomerated powder particles to be produced or introduced back into the atomization cloud at the top of the dryer, causing the forced agglomeration of particles (Gianfrancesco *et al*, 2008). Agglomerated milk powders have improved dispersibility, flowability and increased bulk density (Sharma, 2012).

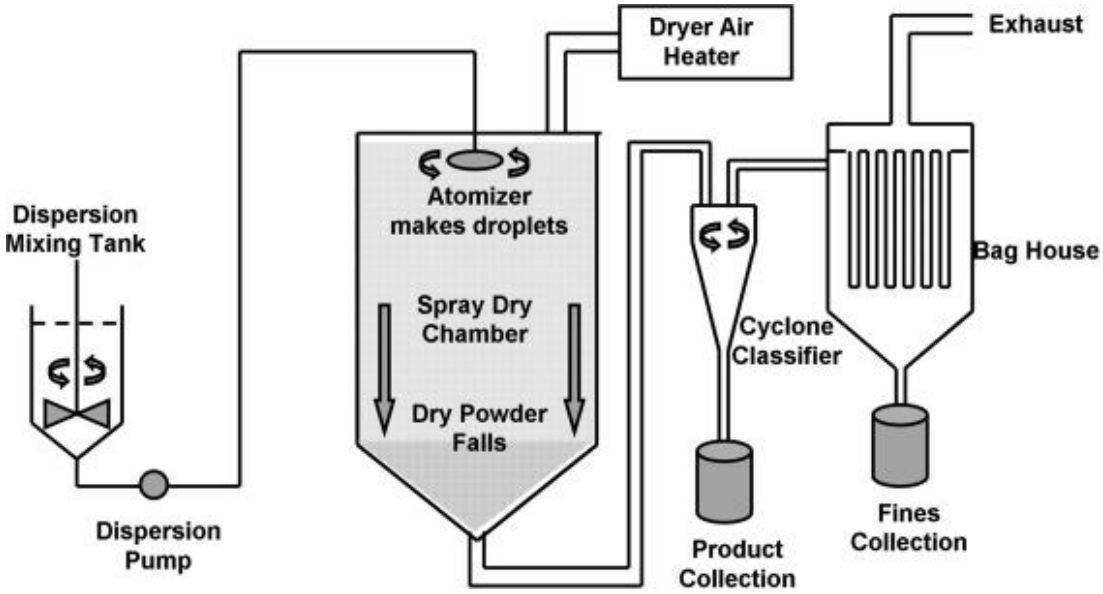


Fig. 1.6. *Schematic of a three-stage spray dryer (McKeen, 2016)*

The final stages of drying stage take place in the fluid bed of the spray dryer; at this point, product transformations can take place, such as lactose crystallisation, agglomeration (through fines returns) and lecithination (Tetra Pak. 2015), when they can be transferred to the packaging system or the next drying stage in the case of a two or three stage dryer. Aside from functionality, two and three stage dryers are more economical that single stage dryers (Masters, 1991).

The functional properties of dairy powders are significantly impacted by drying conditions, i.e., dryer type, nozzle type, inlet and outlet temperatures, agglomeration, instantization (Sharma, 2012). The use of two and three stage dryers allows for the production of agglomerated powders, which improves powder flowability and dispersibility/solubility. Another consideration is the type of nozzle used to atomise the liquid droplets. Liquid milk products dried using a rotary atomiser

have improved reconstitution characteristics, due to the formation of finer droplets which are more rapidly dried (Sharma, 2012). The inlet and outlet temperatures of the dryer also play a consequential role in powder particle properties (O'Sullivan *et al*, 2019; Schuck *et al*, 2016). An increase in inlet temperature is generally preferred as it increases the energy efficiency of the dryer and can be beneficial in producing powders with higher bulk density values; however, there is a risk of causing surface hardening of powder particles which can affect their reconstitution properties (Pisecky, 1978; Sharma, 2012). Conversely, increasing the outlet temperature can result in cracks on the surface of a powder particle; this can result in further moisture removal and a subsequent reduction in powder bulk density (Nijdam and Langrish, 2006). Therefore, low outlet temperatures are preferable and lead to the production of more uniform droplets with controlled shrinkage and improved powder bulk density (De Vilder *et al*, 1976; Kelly *et al*, 2002; O'Sullivan *et al*, 2019).

1.3.3 Powder surface composition

The impact of spray drying conditions, such as solids content, dryer inlet/outlet temperature, homogenisation, nozzle type and viscosity of the liquid stream, on the surface composition of spray dried high protein milk powder particles has been investigated by numerous researchers in order to ascertain its impact on powder functionality. Studies conducted by Fäldt *et al* (1996) demonstrated that the crust was mainly composed of protein which accumulated at the air-droplet interface even in low protein environments. Fang *et al* (2012) found that the drying temperature played a consequential role in surface composition of the particles, which dictates the later solubility of such powders. The study employed the use of mono-dispersed MPC particles manufactured using a microfluidic spray dryer. The spray drying temperature

correlated with the dissolution characteristics of the resulting powder, whereby at low temperatures, particles were more spherical and had better solubility, whilst high temperatures produced particles which had a deflated appearance and impaired solubility. SDS-PAGE analysis established that the main insoluble material in the high temperature particles was casein and not whey proteins. Research by Kim *et al* (2009) found that for skim milk powder the surface composition was largely determined by the spray drying conditions. At high solids content, or at increased drying temperatures, the content of fat and protein was lower at the powder surface, this was attributed to rapid crust formation due to increased dryer temperature and concurrently, increased droplet viscosity accredited to the increased solids content. This research suggests that a balanced combination of dryer conditions is required to control the surface composition of milk powders.

Casein's lack of tertiary structure means it has good molecular flexibility and hence has greater adsorption at the air-water interface than whey proteins which are compact globular proteins and hence, is the dominant protein component of the powder crust (Gaiani *et al*, 2011). Landström *et al* (2003) conducted a study to further define the particle surface examining the competition between β-casein and β-lactoglobulin during spray drying, with an emphasis on calcium association. The study was achieved using fluorescent quenching of labelled proteins on the powder surface; β-casein adsorbed with higher affinity at the surface with increasing inlet temperature while β-Lg had a much lower surface affinity. This absorption process is believed to be three-step in nature, with each step being dependant on the previous. The foremost being movement of proteins to air-droplet interface, followed by attachment and finally conformational changes once absorbed. The transportation of proteins to the air-droplet interface is believed to be diffusion controlled with the surface

concentration dictating the final composition. Indicating, that β-casein has a high affinity for surface migration in comparison to β-lg during crust formation.

1.4 Rehydration of milk protein concentrate powders

The hydration and solubility of high protein powders has received much attention and been the subject of extensive research for a number of years, mainly due to the significant challenges experienced with slowly-dissolving powder particles, contributing to flecking in dairy based beverages, poor emulsification properties and poor curd formation during gastrointestinal digestion (McCarthy *et al*, 2013; Toikkanen *et al*, 2018; Wang *et al*, 2018). McSweeney *et al* (2020) showed that milk protein concentrate powders with a protein content >65% (w/w) had significantly lower solubility compared to low protein milk powders (i.e., 35%, w/w, protein). The following section of the review provides an overview of some of the causes of protein powder insolubility, methods of quantifying solubility and means of improving rehydration properties and solubility of such powders.

Spray dried milk powders are considered relatively stable, particularly from a microbiological perspective, due to their low water activity; however, significant changes can still occur in powders during storage, particularly if stored under inappropriate environmental conditions. Dairy based products, such as whole milk powders, fat filled milk powders and infant milk formulas are susceptible to lipid oxidation and lactose crystallization, which are detrimental to powder physical properties and subsequent functional and nutritional quality. Conversely, MPC powders contain relatively low levels of lactose and so Maillard compounds, which are produced as a result of chemical interactions between lysyl amino acid side chains, require lactose as a co-reductant and therefore, occur more extensively in whole milk

and skim milk powders. In high protein powders, which contain low levels of fat and lactose, oxidation and crystallization can be negligible (McSweeney *et al*, 2020; Thomas *et al*, 2004); however, such powders are susceptible to issues associated with powder particle insolubility. While a number of factors play a role in high protein powder solubility two of the main elements are powder storage temperature and time, with the fraction of insoluble powder increasing with increasing temperature and duration (Bhaskar *et al*, 2001). The mechanism of MPC powder rehydration in water involves an initial wetting step, in which solvent penetrates into the powder particles by capillary forces, resulting in dissolution of cross-bridges between the primary casein particles and eventual dispersion into the surrounding solvent (Forny *et al*, 2011; Crowley *et al*, 2018).

Previously, McKenna (2000) showed that the insoluble fraction of MPC powders stored for 6 months at 20°C had impaired rehydration characteristics. Casein micelles were observed to have fused together *via* protein–protein interactions to form large particles in dispersion (average size ~100 μm), and these fused particles remained intact (45°C) for 30 min after mixing. Havea (2006) stated that the casein micelles were fused together by weak hydrophobic protein–protein interactions, the proportion of which increased with storage time at elevated temperatures. Concurrently, disulphide-linked protein aggregates were also observed in heat treated MPC powders, consisting primarily of κ-casein, β-lactoglobulin and α-lactoglobulin ; however, these aggregates were found not to play a significant role in the formation of the insoluble fraction. Boland *et al,* (2014) observed that during processing and subsequent storage of MPC powders, isopeptide bond formation can occur. Bond formation occurs between exposed reactive phosphate residues which form crosslinks with neighbouring lysyl reactive sites. Amelia *et al* (2013) observed that all major

casein fractions, particularly α_{s1}-casein, and β-lg to a lesser extent, in MPC powders were involved in protein isopeptide bond formation resulting in decreased rehydration capability. Mimouni *et al* (2010) reported that during storage, inter- and intra-molecular interactions cause casein micelles to become more closely packed, forming a monolayer "skin", while hydration of the monolayer results in the formation of a porous, gel-like structure which slows the dispersion of individual casein micelles into the surrounding liquid phase.

It must also be noted that casein and whey-based protein powders differ significantly in their rehydration properties. Casein-dominant powders have rapid initial wetting; however, once wetted, the powders swell and are slow to disperse into a homogeneous dispersion (Gaiani *et al*, 2006 and 2007). In comparison, whey-based powders, such as whey protein isolate (WPI), have poor wettability, but disperse quickly once wetted. As whey proteins are globular and hence bind less water, they do not exhibit the same level of swelling observed in casein proteins during the rehydration process. Gaiani *et al* (2007) observed that casein powders with added whey proteins had faster rehydration times, while whey protein powders with added caseins exhibited delayed rehydration compared to native WPI powders.

1.4.1. Measuring the rehydration properties of milk protein concentrate powders

Over the years a number of methods have been employed to assess the wettability and rehydration rate of high protein dairy powders including; measuring sediment height of centrifuged dispersions, gel electrophoresis (SDS-PAGE) for protein identification of supernatant, total solid analysis (Crowley *et al*, 2015; McCarthy *et al*, 2014), washburn capillary rise wettability tests (Ji *et al*, 2015; Ji *et al*, 2016) and particle size measurements (McCarthy *et al*, 2014; Mimouni *et al*, 2009).

Optical tensiometry has also been used to determine water diffusivity into a compacted sample of dry powder, a drop of known volume is deposited onto the powder surface and the contact angle is measured over the test duration. Powder wettability can be determined by the speed of water uptake and compared using the final drop angle (Crowley *et al*, 2018; Crowley *et al*, 2015; Forny *et al*, 2011; Gianfrancesco *et al*, 2008). For a more comprehensive review of analytical methodology for monitoring protein powder hydration techniques see da Silva *et al*. (2018).

Microscopy techniques have also seen major advances in the development of specific methodology for visualising and quantifying powder rehydration rates. Scanning electron microscopy (SEM) has usually been used to characterize the surface morphology of anhydrous and humidified (dairy powders, such as lactose, whole milk powder, skim milk powder (SMP) and high protein powders but due to their need to be operated under vacuum can only visualize dry powder particles, and so this has led to the use of "Environmental" SEM's which can operate at normal atmospheric pressure and allows direct real-time inspection of hydration in powders. Lately a study by Cenini *et al* (2020) showed high resolution *in situ* surface hydration of milk protein concentrate powders using an environmental scanning electron microscope. Confocal laser scanning microscopy has also been utilised to observe and determine kinetics of rehydration for dairy powders using real time visualisation of fluorescent dye penetration into individual milk protein powder particles, from which local diffusion coefficients can be measured (Maidannyk *et al*, 2019). Another recently established microscopy technique is the use of a high speed camera fitted to an optical microscope to monitor the hydration process in real time, allowing for comprehensive image analysis of powders during wetting, swelling and dispersion.

1.5 Methods to improve milk protein powder hydration

There are a number of widely used methods employed to improve the rehydration and functional properties of high protein milk powders, with many based on the partial or complete dissociation of the casein micelle, usually through chelation of calcium by salt addition or through the use of ion exchange.

1.5.1 Mechanical rehydration methods

Numerous previous studies have aimed to improve the solubility of high protein dairy powders using mechanical methods such as ultrasonication, cavitation and homogenization (McCarthy *et al*, 2014; Pathania *et al*, 2018). In a study conducted by McCarthy *et al*, 2014, it was observed that high intensity ultrasound (20 kHz; power 70.2 W) resulted in an increased rate of powder dissolution compared to stirring. Ultrasonication involves the propagation of sound waves in solution, resulting in alternating high-pressure and low-pressure cycles. Small vacuum bubbles or voids are formed during the low-pressure cycle which then collapse during the high-pressure cycle resulting in high localised turbulence, pressures and temperatures (Ashokkumar et al., 2009; Raviyan et al., 2005). McCarthy *et al*, 2014 observed that sonication effectively broke down intermolecular hydrophobic interactions in MPC powders. However, the authors noted that temperature control is crucial during sonication to prevent protein aggregation.

Hydrodynamic cavitation has been shown to improve high protein powder dissolution. Cavitation involves the formation, expansion and collapse of air bubbles within a dispersion causing localised increases in temperature and pressure. The cavitation bubbles collapse at or near the powder surface and cause disruption of

powder particles, hence providing sufficient energy for powder particle erosion and fragmentation, which promotes solubilisation through efficient mixing and enhanced mass transfer (Iskalieva *et al*, 2012; Pathania *et al*, 2018). Pathania *et al*, 2018, utilised hydrodynamic cavitation to improve the rehydration properties of MPC80 20% (w/w). Cavitation was observed to significantly reduce the dispersion particle size distribution and viscosity. High shear greatly accelerates solubilisation of otherwise insoluble dairy powders. High shear agitation has been observed to disrupt powder agglomerates and assist in the release of micellar components into dispersion. Chandrapala *et al*, 2014, investigated the impact of high shear on the dissolution of high protein dairy powders. They found that high shear techniques were able to greatly accelerate the dissolution of these powders by physically disrupting the powder agglomerates and hence, facilitating the release of individual casein micelles into solution.

More recently, novel methods for high protein powder rehydration include broadband acoustic resonance dissolution spectroscopy (BARDS), a technique that can monitor the release of air from powder particles during hydration. BARDS measures changes in acoustic resonance, which occurs during rehydration; as water migrates into the powder particle, air is released, which changes dispersion compressibility and hence, changes the resonated frequency (Vos *et al*, 2016). These methods show promising improvements to powder dissolution during the rehydration process with the primary target for dissolution being the disruption and physical break up of powder particles in solution.

1.5.2 Calcium chelating salts

As described in Section 1.2 the casein micelle model (Horne, 1998) is based on a balance between attractive hydrophobic interactions and electrostatic repulsion,

with CCP complexing with calcium phosphoserine residues of casein reducing the electrostatic repulsion and enabling hydrophobic bonds. The CCP associated with casein micelles exists in equilibrium with salts in the serum phase of milk (Gaucheron, 2005) and the calcium and phosphate distribution between soluble and insoluble phases effects the physical properties of micellar protein systems. A number of factors influence the amount of CCP associated with casein micelles such as temperature (Holt, 1995), pH (Ozcan *et al*, 2011) or the addition of calcium chelating salts (Odagiri and Nickerson, 1964; Udabage *et al*, 2000).

The calcium content of MPC powders has been shown to influence protein-protein interactions during both processing and storage, causing reduced powder solubility (Pandalaneni *et al*, 2018). In order to reduce the levels of calcium-protein interactions, calcium chelating salts, such as sodium phosphate (SP), trisodium citate (TSC), potassium citrate and sodium hexametaphosphate (SHMP) can be utilised. Calcium chelators alter the protein-mineral equilibria by binding free calcium ions in the serum phase and subsequently depleting calcium from CCP nanoclusters. Calcium chelation results in an increase in net repulsive forces between negatively charged phosphoserine residues, resulting in an increase in hydration and voluminosity of casein micelles, which can lead to an increase in dispersion viscosity (De Kort *et al*, 2009; Mekmene, *et al*, 2009; Upreti *et al*, 2006).

The mechanism by which chelating salts interact with calcium differs depending on their binding capacity, chelating either free calcium from the aqueous phase or chelating both free and CCP-associated calcium simultaneously. Strong polyphosphate chelators, like SHMP and sodium phytate, can simultaneously bind free calcium and calcium associated with CCP (de Kort *et al*, 2011). SHMP has six homogeneously distributed negative charges and can crosslink casein micelles via its

interaction with bound calcium and, if added at high concentrations, protein gelation may occur (de Kort *et al*, 2011; Guo *et al,* 2003; Mizuno and Lucey, 2007). Sodium phytate has 12 negative charges which are clustered in pairs and its relatively large molecular size and charge distribution cause steric hindrance, preventing sodium phytate from interacting with the casein micelle, unlike SHMP, despite being highly negatively charged. The addition of disodium hydrogen phosphate (Na_2HPO_4) or TSC to micellar casein systems causes the chelation of calcium to form insoluble calcium phosphate or relatively soluble calcium citrate, respectively (Guo *et al*, 2003; Mizuno and Lucey, 2007; De Kort *et al* 2011). The degree of calcium chelation is dependent on the stability of the casein micelle structure and the ionic environment, with factors such as acidification, temperature and pH strongly influencing the viscosity, turbidity and stability of such systems (De Kort *et al*, 2011; Holt *et al*, 1981; Lucey *et al,* 1997; Van Hooijdonk *et al,* 1986).

The chelation of calcium has significant knock-on effects on the physical properties of MPC dispersions, impacting casein micelle stability and resulting in either partial or full micelle dissociation. Therefore, chelating salts influence dispersion turbidity and reduces micelle light-scattering capability (Pitkowski *et al*, 2008). In a study by De Kort *et al*, (2011) it was shown that the extent of dissociation and subsequent turbidity, is dependent on the type and concentration of calcium chelator and proposed that micelles were dissociated to a greater degree in the order of SHMP $\geq$ SP > TSC > Na_2HPO_4 > Na_2UMP (Disodium uridine monophosphate). While, Mizuno *et al*, (2005) showed that different trisodium citrate (TSC) chelated calcium from CCP, causing significant casein micelle dissociation. Whereas at relatively low levels of SHMP (~0.1%, w/w) addition, complexes of SHMP with soluble calcium and casein micelles began to form, and when the SHMP concentration

was increased ≥0.5%, w/w, calcium and phosphate were released from the CCP complex, causing dissociation of the casein micelle. This was compared to weak calcium chelators, such as disodium orthophosphate (DSP), which has the ability to only chelate free ionic calcium from the aqueous phase, resulting in a gradual loss of turbidity over time.

One of the main applications for chelating salts in dairy products is in processed cheese, with often several chelating salts being added to manipulate pH, colour, texture and emulsification properties (Carić and Kalab, 1987). Kaliappan and Lucey (2011) stated that "the *difficulty in understanding the exact nature of the interaction between chelating salts and micellar casein systems lies in the fact that it is difficult to separate the effects of calcium chelation, possible chelating salt association with the casein micelles, and the concomitant shift in pH caused by addition of the salts*". Therefore, the addition of chelating salts to milk protein systems are multivariable with many different effects occurring at once. Another application for adding citrates and phosphates to dairy-based systems is to increase heat stability (De Kruif *et al*, 2012), particularly in products designed for infant and medical nutrition. The heat stability of milk protein dispersions can be improved by the addition of chelating salts to bind free divalent ions, mainly calcium, from the aqueous phase and therefore preventing calcium-induced protein bridging and aggregation during thermal treatment. In a study performed by De Kort *et al* (2012), the impact of different calcium chelators on the heat coagulation and aggregation of concentrated micellar casein dispersions was investigated. Na_2UMP was observed to increase the heat coagulation time (HCT) of concentrated micellar casein dispersions, while disodium hydrogen phosphate, trisodium citrate and sodium phytate resulted in lower HCT. The authors hypothesised that the observed differences in HCT could be

attributed to the calcium-ion activity and state of the micellar structure before and during heating. Interestingly De Kort *et al* (2012), showed SHMP was the least effective of the tested calcium chelators, resulting in the smallest increase in HCT. De Kort *et al* (2011) proposed that low impact of SHMP addition on HCT was potentially due to the high initial viscosity (Fig. 1.7). SHMP is a strong polyphosphate chelator, hence critical levels of calcium depletion from the CCP and induced high net negative charge between proteins would result in rapid dissociation of micelles during heating and therefore the dissociated casein fractions would be more susceptible to calcium-induced protein aggregation.

Aside, from their use in cheese, processed cheese and as aids in achieving greater heat stability during dairy processing, the use of calcium chelators have been shown to improve the solubility of MPC powders. In a study by McCarthy *et al* (2017) it was observed that MPC powders with added sodium phosphate, trisodium citrate (TSC) or sodium hexametaphosphate (SHMP) had increased powder dissolution rates and solubility. However, as a consequence of calcium chelation, there can be a subsequent increase in dispersion viscosity, attributed to casein micelle swelling (Mizuno and Lucey, 2007).

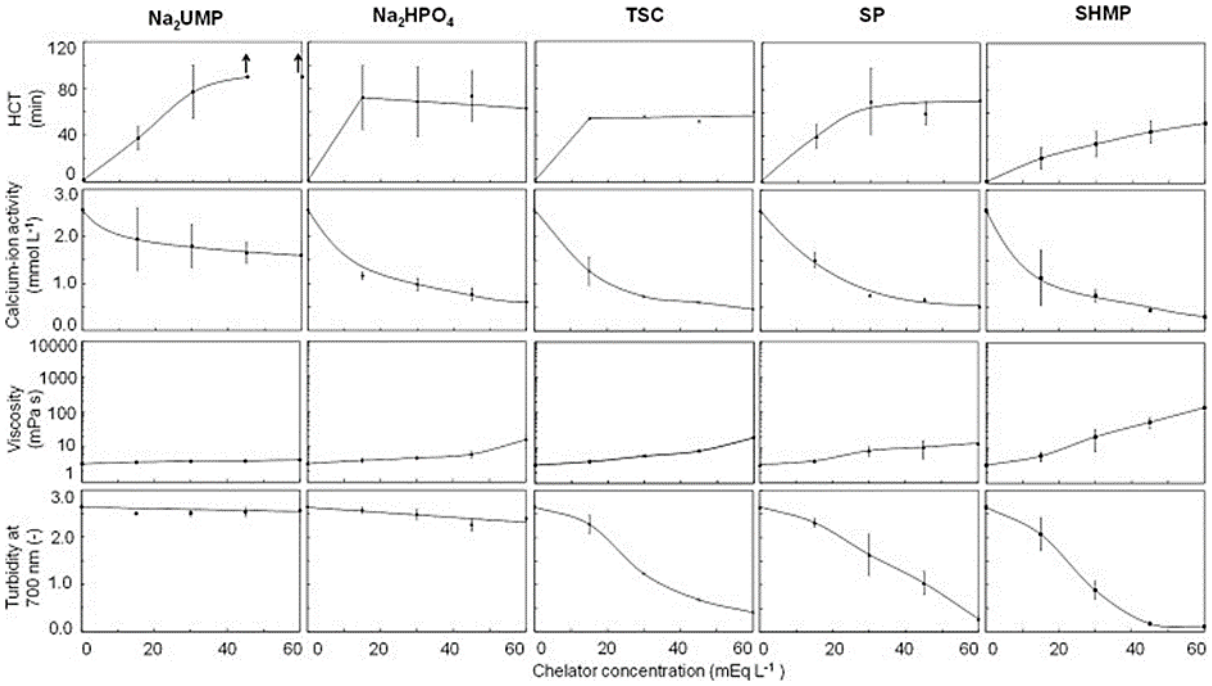


Fig. 1.7. *Heat coagulation time (HCT), calcium-ion activity, viscosity, and turbidity of a micellar casein isolate (MCI) dispersion at pH 6.7 as a function of calcium chelator concentration (Na_2UMP, disodium uridine monophosphate; Na_2HPO_4, disodium hydrogen phosphate; TSC, trisodium citrate; SP, sodium phytate; SHMP, sodium hexametaphosphate) (taken from* ***De Kort et al, 2012).***

1.5.3 Calcium depletion of milk protein using ion exchange

Chelation and depletion of calcium from milk through ion-exchange has previously been implemented to improve high protein powder hydration (Bhaskar, 2007). Ion exchange resins are insoluble matrices, normally in the form of small beads (radius = 0.25–0.50 mm) and contain an active functional group which facilitates the chelation of ions and release of a counter ion from the bead. The majority of functional groups in commercial resins are derived from polystyrene, generally from variations of polystyrene sulfonate $(CH_2CHC_6H_4SO_3H)_n$, see Fig. 1.8. During the ion exchange process the counter ion; usually sodium or potassium, is exchanged for divalent ions, such as calcium and magnesium (Moubark, 2015). Previously, the injection of CO_2 into milk to reduce the pH prior to rennet coagulation, caused an increase in the

solubility of micellar calcium phosphate, which modifies the mineral profile of cheese produced from concentrated milk (Nelson *et al*, 2004). While a study by Marella *et al* (2015) reported that the injection of CO_2 into skim milk led to a reduction in pH and subsequent increase in the solubilisation of colloidal calcium phosphate. This resulted in a reduction in the mineral content of the UF retentate stream, with the subsequent spray dried powders having significantly improved cold-water solubility (20°C).

The effective depletion of calcium from milk and milk protein systems using ion exchange has already been demonstrated. Studies by Ranjith *et al* (1999) and Xu *et al* (2016) used ion exchange to deplete calcium from milk protein concentrate in a reaction vessel under continuous agitation at ambient temperatures. While, a study by Bhaskar *et al* (2007) performed calcium ion exchange by loading resin into a stainless steel vessel and re-circulating UF protein retentate through the vessel over a period of 1-1.5 h, before the retentate was then evaporated and dried. Oftentimes a sub-sample of the protein system is depleted in calcium before being re-added to a non-calcium depleted aliquot to obtain an ingredient/product with a precise calcium content (industry know-how). Pandalaneni *et al* (2018) showed that MPC with 20 and 30% reductions in calcium content had increased heat stability, increased apparent viscosity and a decrease in particle size when compared to control dispersions, attributed to casein micelle dissociation. Bhaskar *et al* (2007) showed that calcium depleted MPC powders created through ion exchange, had improved powder solubility and could be utilised as a cheese milk extender, allowing increased yield and reduced insoluble matter.

Previous work by Xu *et al* (2016), showed using transmission electron microscopy and small angle X-ray scattering that the casein micelle and associated CCP nanoclusters were gradually dissociated with increasing levels of calcium

depletion, with the majority of casein micelles dissociating following the removal of 38.7% of the total calcium present in the system. However, ion exchange causes a concomitant increase viscosity and promotes gel formation hence, hindering the processing of demineralised MPC such as in unit operations like membrane filtration, evaporation and spray drying (Bhaskar *et al*, 2007). Increases in viscosity could be attributed to the re-aggregation of primary casein particles due to hydrophobic interactions (Huppertz *et al*, 2017). Despite the concomitant increases in protein viscosity calcium reduction can be utilised to produce a number of value added novel products. Work carried out by Dybing *et al* (2002) observed that by modifying the calcium content via ion exchange produced a range of novel protein gels, cheeses, and cheese-like products with varying calcium levels without the use of coagulating enzymes. Bhaskar *et al* (2003) showed that calcium reduced MPC retentates created through ion exchange and dried to form a protein rich ingredient which can be utilised in the preparation of novel emulsified or protein stabilised food products.

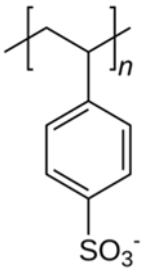


Fig. 1.8. *An example of polystyrene sulfonates structure in ion exchange resins* $(CH_2CHC_6H_4SO_3H)_n$

It is also important to note that during the ion exchange process the release of either a sodium or potassium counter ion in exchange for calcium results in an increase in monovalent cation concentration. Sodium content of micellar casein dispersions has been observed to have an impact on the heat stability and solubility of casein micelles.

A previous study by Sikand *et al* (2013) has shown that sodium addition in the form of sodium chloride (NaCl) can improve MPC hydration. Another study by Mao *et al*. (2012) showed the effect of NaCl addition during DF on the solubility, hydrophobicity and disulfide bonds of MPC powder and that MPC containing added NaCl were significantly more soluble compared to the control MPC powder.

1.6 Enzymatic modification of milk protein

The use of enzymes in dairy chemistry typically involves the hydrolysis of intact casein and whey proteins to produce peptide fractions, aimed at providing nutritional and functional benefits. Banach *et al* (2013) examined the effects of using chymotrypsin, trypsin, pepsin and cysteine protease-papain to produce MPC hydrolysates with better solubility. However, aside from the use of typical digestive enzymes to improve solubility through the generation of peptides, alternative enzymatic modification can lead to protein molecular stability. Two enzyme groups that can modify protein structure without leading to proteolysis are transferase (e.g., transglutaminase) and phosphatase (alkaline/acid phospahtases). These enzymes can be utilised in the modification of dairy proteins for use in milk protein beverages, yogurt, and cheese products.

1.6.1. Transglutaminase

Transglutaminase is an enzyme available as a food-grade preparation and has been utilised as an ingredient in a number of food manufacturing processes such as in the dairy, meat and bakery sectors (Guyot and Kulozik 2011; Faergemand and Qvist 1997; Jaros *et al*, 2007; Schorsch *et al*, 2000). Transglutaminase belongs to the

transferase family of enzymes and forms covalent crosslinks between protein molecules, resulting in significant changes in protein functionality. The enzyme consists of 331 amino acid residues with a chemical molecular weight of 37,863. The enzyme uses an acyl-transferase mechanism to catalyse the transfer of a gamma-carboxyamide group on a glutamine residue to the gamma-amine of an adjacent lysine or glutamine residue (Mahmoud and Savello, 1992; Fig.1.9). The efficacy of transglutaminase to crosslink proteins much depends on the physical structure and electrostatic charge of the substrate. When incubated with milk protein, transglutaminase can form covalent intra-micellar crosslinks between caseins. Casein protein molecules lack a secondary structure and therefore make it susceptible for transglutaminase induced crosslinking. These crosslinked caseins have altered functional properties including enhanced casein micelle stability, gelation capability, thermal stability and water-holding capacity (Sharma *et al*, 2001). Research carried out by Lorenzen *et al* (2002) observed that the degree of crosslinking in casein proteins increased with decreasing protein order: sodium caseinate > ultrafiltered skim milk powder > skim milk powder.

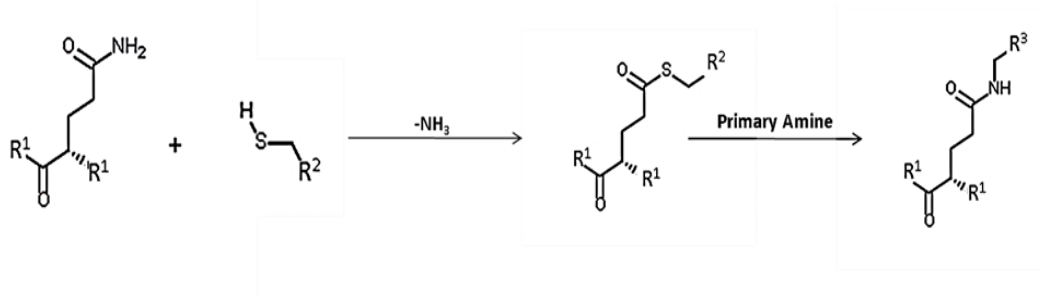


Fig. 1.9. *Reaction pathway for transglutaminase crosslinking of glutamine and lysine residues on casein proteins. R^1: Casein amino acid chain; R^2: Transglutaminase enzyme complex; R^3: Neighbouring lysine or glutamine residue*

Further work carried out by Bönisch *et al* (2004) found that sodium caseinate had a higher degree of enzymatic crosslinking due to the accessibility of glutamine and lysine residues when compared to micellised casein which has a more ordered structure.

Conversely, whey proteins have a globular structure and hence transglutaminase has little effect on their structure due to steric hindrance. As mentioned previously the tertiary structure of globular whey protein hinders accessibility of transglutaminase. However, a number of chemical methods can be applied to alter the structure of β-lg and α-la to allow enzymatic crosslinking to occur. Dithiothreitol (DTT) can be utilised to cleave disulphide bonds in whey proteins, causing the protein to unfold and allowing transglutaminase to access glutamine and lysine residues (de Jong and Koppelman, 2002). Other methods such as high pressure treatment, increases in pH (pH 8.5–9.0), high heat treatment and application of high hydrostatic pressure can be used to denature whey proteins and hence allow crosslinking to occur (Faergemand and Qvist, 1998; O'Sullivan *et al*, 2002b; Lauber *et al*, 2000)

Transglutaminase has been shown to improve casein micelle structural stability. A study carried out by Huppertz *et al* (2006) observed that crosslinked casein proteins retained a high degree of light scattering properties while the light scattering ability of native casein proteins were reduced by approximately 95% upon full disruption of micellar hydrophobic interactions or through the depletion of micellar calcium. It was observed that the extent of enzymatic crosslinking, which determines stability against dissociative agents, can be increased with increasing incubation time. After a 24 h incubation period crosslinked casein proteins were found to have an increased tolerance against dissociation when compared to shorter incubation periods.

Furthermore, O'Sullivan *et al* (2002b) showed that crosslinking of casein proteins retained casein micelle stability under conditions which would generally dissociate the casein micelle. During calcium chelation with both sodium citrate and urea it was observed that crosslinked milk did not dissociate to the same extent as observed in control milks. O'Sullivan *et al* (2002b) also studied the degree of dissociation incurred by both preheated and unheated crosslinked and non-crosslinked milk samples during high pressure treatments. Casein micelles treated with transglutaminase where shown to have increased resistance against high pressure treatment regardless of heat treatment, compared to non-crosslinked casein micelles which incurred a large reduction in size following high pressure treatment. Conversely, the casein micelle size of preheated crosslinked milk was shown to increase during high pressure treatment. O'Sullivan *et al* (2002b) and Needs *et al* (2000) theorised that this increase in size could be attributed to whey-casein complexes created through sulphydryl-disulphide reactions during high pressure treatment, causing a concurrent increase in micelle size.

Numerous studies have shown that transglutaminase can be utilised to increase heat stability milks (Moon *et al*, 2009; O'Sullivan *et al*, 2002; Smiddy *et al*, 2006). At temperatures of ~90°C, κ-casein begins to dissociate from the casein micelle (O'Connell and Fox, 2002). The stability of casein micelles are inherently affected by pH, at pH values below 6.7, β-lg forms a complex with κ-casein hence stabilising the protein and preventing dissociation. Conversely, κ-casein dissociation increases as a factor of decreasing pH, leading to the formation of β-lg and κ-casein complexes in serum and the creation of unstable κ-casein depleted micelles. At pH values ≥ 6.9, the heat stability of milk increases linearly due to a reduction in calcium ion activity and heightened protein hydration which leads to an increase in repulsion between casein

micelles. A study carried out by O'Sullivan *et al* (2002) determined that the heat treatment of milks prior to incubation with transglutaminase altered the mechanism of micelle stabilisation during heat stability analysis. In unheated milks, crosslinking reactions only occur in casein proteins. Crosslinked caseins prevented dissociation of κ-casein from the casein micelles at reduced pH values hence the micelle was more resistant to heat induced denaturation than control milks. Crosslinked preheated milks exhibited improved heat stability when compared to raw crosslinked milks. The authors theorised that this increase in stability could be attributed to crosslinking between denatured whey proteins and casein proteins (Lorenzen, 2000b).

In other applications transglutaminase has been used to enhance gelation properties in set yogurts and acid gels. Numerous studies have shown that transglutaminase incubation produces yogurts with improved gel strength and increased viscosity due to improved water-holding capacity in the gel network created by crosslinking (Færgemand and Qvist, 1997; Jaros *et al*, 2006). Research carried out by Lauber *et al* (2000) showed that there was an incremental increase in the gel strength of transglutaminase treated yoghurt, depending on the degree of crosslinking. Furthermore, Lorenzen *et al* (2002) observed by scanning electron microscopy (SEM) that enzymatic crosslinking created a tighter protein network and improved protein distribution within the yoghurt gel matrix. This accumulated to a yogurt with increased gel strength and reduced syneresis. This denser microstructure observed by Lauber *et al* (2000) was also observed in work carried out by Lorenzen *et al* (2002) observed by scanning electron microscopy (SEM) that enzymatic crosslinking created a tighter protein network and improved protein distribution within the yoghurt gel matrix. This accumulated to a yogurt with increased gel strength and reduced syneresis. This denser microstructure observed by Lauber *et al* (2000), was also observed in work carried out

by Færgemand and Qvist (1997) and confirmed that it was possible to reduce the fat and protein content with transglutaminase crosslinking. Sensory properties of set style yogurts were impacted by enzyme incubation; crosslinked set yogurts exhibited a smother, whiter yogurt surface. Yogurts found to be creamier and had a milder taste when compared to standard milk yogurts suggesting that enzymatic crosslinking could act as a replacement for fat addition (Lorenzen *et al*, 2002). Milk gels manufactured through acidification or protease action are stabilised by weak non-covalent interactions. They have application in yogurts and are generally acidified using glucono-δ-lactone (GDL). Experimental work carried out by Færgemand and Qvist, (1997) showed that crosslinked milk which underwent acidification with GDL formed a stiffer gel with a finer protein network when compared to control yogurts. Myllarinen *et al* (2007) also observed that in application with acidified sodium casein gels incubation with TGase resulted in firmer gels and improved homogeneity in comparison to gels formed using GDL.

Another application of transglutaminase in dairy-based formulations is its impact on the emulsification behaviour of milks. Merete *et al* (1998) on oil-in-water emulsions stabilised by sodium caseinate or β-lactoglobulin found that the degree of transglutaminase crosslinking affected coalescence stability, with extensive crosslinking resulting in an increase in coalescence. However, the creaming stability was stabilised by extensive crosslinking, this functional change was attributed to changes in the adsorbed layer and or increased viscosity of the continuous phase. Further work by Hinz *et al*, (2007) showed that crosslinked milk proteins had altered emulsification properties, with increased stability of milk fat globules against coalescence.

1.6.2 Enzymatic dephosphorylation

Casein phosphoserine residues in bovine milk are essential to micelle integrity, aside from its structural role; phosphoserine residues bind miceller calcium phosphate nanoclusters, which reduce the net negative charge on casein proteins, promoting attractive interactions between the hydrophobic regions of caseins. A study by Holt *et al* (1998) showed that calcium phosphate nanoclusters could be prepared *in vitro* and stabilised by casein phosphopeptides to prevent precipitation. Therefore, the removal of phosphate groups from casein has a significant impact on the structural stability of micelles. Dalgleish and Law (1989) showed the dissociation of casein micelles caused by the neutralization of the negative charge on phosphoserine residues during the acidification process. Removal of phosphate groups from dairy proteins is through enzymatic hydrolysis using phosphatase enzymes. In milk protein chemistry phosphatase enzymes hydrolyse phosphoserine residues from intact casein proteins to produce a free phosphate ion and an exposed hydroxyl group (Fig. 1.10). Numerous phosphatase enzymes can be employed to remove phosphate residues; however, few are suitable to work under the pH range of bovine milk casein. Spleen phosphoprotein phosphatase has an optimum range of pH 5.0 to 5.5, which poses a challenge as casein solubility begins to decrease due to a reduction in electrostatic charge (Bingham, 1976). Two phosphatase enzymes frequently used to catalyse dephosphorylation reactions in milk systems are bovine alkaline phosphatase (BAP) and potato acid phosphatase (PAP). Both phosphatase enzymes were selected due to relatively neutral optimum pH range (pH 6.5 to 8.5). Due to the non-globular confirmation of casein proteins full dephosphorylation is possible using phosphatase enzymes although, steric factors and neighbouring amino acid sequences can cause some hindrance to the rate of dephosphorylation (Bingham, 1976). The rate of dephosphorylation and extent can

however vary with casein conformation, with caseinates achieving a higher degree of dephosphorylation, compared with micellar casein (Bingham, 1976; Li-Chan and Nakai, 1989). Dephosphorylation of proteins can be observed using urea–PAGE, Urea PAGE or denaturing urea polyacrylamide gel electrophoresis utalises 6-8 M urea, which denatures secondary structures and hence separates proteins in a polyacrylamide gel matrix based on the molecular weight (Fig. 1.11). The following authors can be referenced in the assembly of a Urea Page; Ornstein and Davis (1964) and Summer *et al*, (2009). The dephosphorylation of casein proteins results in electrophoretic bands of lower mobility with the appearance of multiple band patterns indicative of proteins with lower charge.

Dephosphorylated casein has unique physical properties and functionality; in a study carried out by McCarthy *et al*, (2014) it was observed that the dephosphorylation of β-casein reduced its emulsification properties but also reduced its sensitivity to calcium induced aggregation. Previous work (Bingham *et al* 1972; Bingham *et al*, 1976) has also shown that α-casein incubated with potato acid phosphatase had a higher isoelectric point, reduced calcium binding and decreased electrophoretic mobility at alkaline pH values. However, dephosphorylated casein has an identical amino acid composition to phosphorylated native casein and therefore, the removal of phosphate residues with phosphatase enzymes does not alter the casein primary structure.

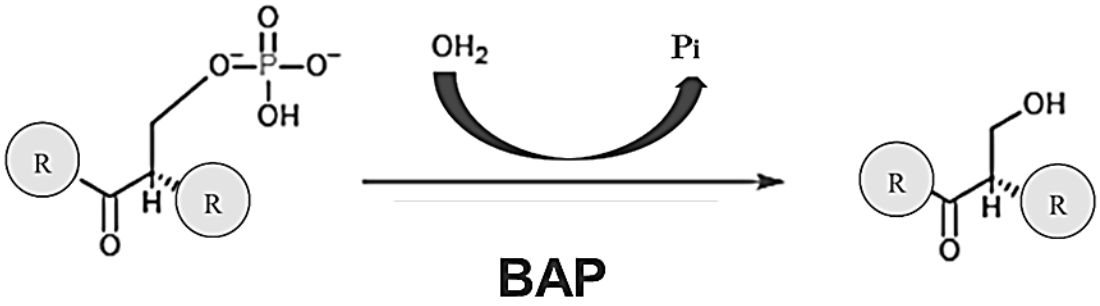


Fig. 1.10. *Reaction pathway of dephosphorylation at phosphoserine sites on casein proteins using the enzyme bovine alkaline phosphatase (BAP).*

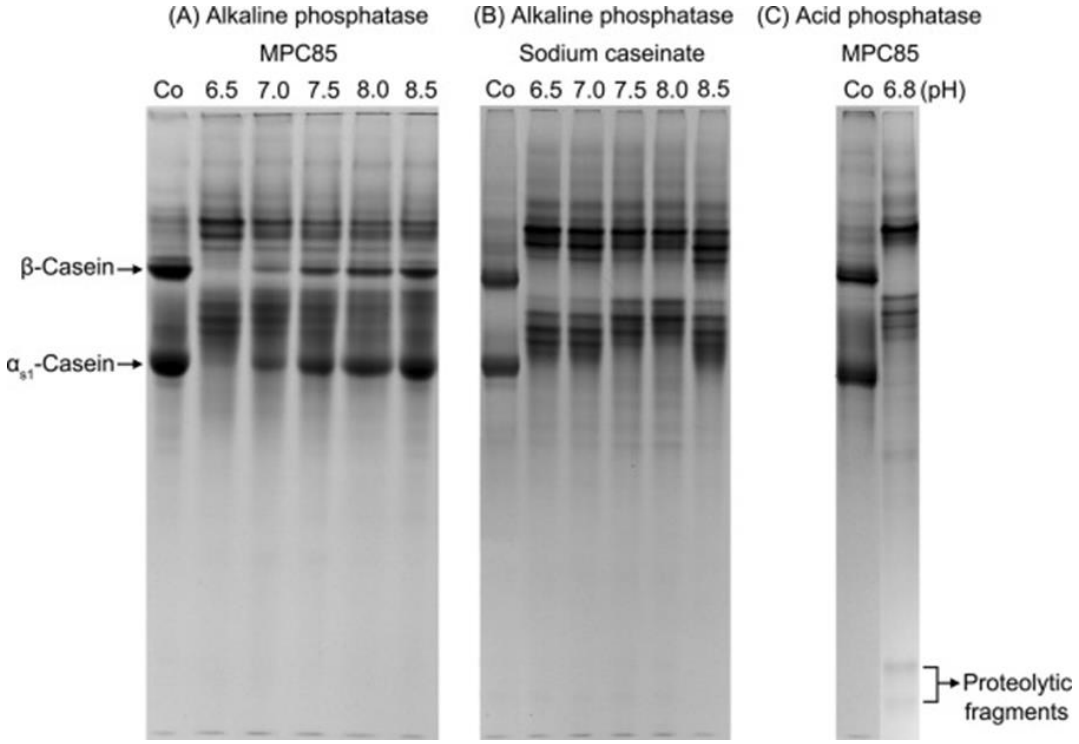


Fig. 1.11. *Urea–polyacrylamide gel electrophoresis patterns of milk protein concentrate (A) and sodium caseinate (B) dephosphorylated with calf intestinal alkaline phosphatase for 4 h at pH 6.5–8.5, and milk protein concentrate (C) dephosphorylated with potato acid phosphatase for 4 h at pH 6.8, obtained from Liu et al, (2016)*

1.6.3 Phosphorylation (Kinase) Enzymes

Modifying the number of phosphate sites on protein molecules can cause significant changes in protein functionality. As casein is the primary source of

phosphoserine groups in bovine milk, it is the primary substrate for enzymatic phosphorylation. Casein protein fractions vary in their level of phosphorylation, with α_{s1}-, α_{s2}-, β- and κ-casein containing 8, 11/12, 5 and 1 phosphate residues, respectively (De Kruif and Holt, 2003; Horne, 2006). Increasing the level of phosphorylation causes an increase in the net negative charge of proteins and therefore the isoelectric point of super-phosphorylated casein is lower than that of native casein (Van Hekken and Strange, 1997). However, the folding and distribution of the protein secondary structure remains unaffected by the modification. When compared to native casein micelles, super-phosphorylated caseins have improved water binding capacity (Matheis *et al*, 1983), lower surface hydrophobicity due to increased polarity (Van Hekken and Strange, 1997), have more flexible casein side chains (Medina *et al*, 1992) and improved foaming ability (Van Hekken *et al*, 1996). However, emulsification properties are adversely affected by super-phosphorylation, resulting in poor emulsion stability and increased dispersion viscosity (Matheis *et al*, 1983; Medina *et al*, 1992). Consequently, super-phosphorylated caseins have improved Ca^{2+} binding capacity due to the increase in negatively charged phosphate sites, while increased phosphorylation on κ-casein causes an increase in calcium mediated precipitation, resulting in reduced protein stability and impaired micelle formation (Yoshikawa *et al*, 1981).

Super-phosphorylation can be achieved using chemical modification with phosphoryl chloride ($POCl_3$) however, the pH requirements and duration of the incubation can result in dissociation of casein micelles (Van Hekken and Strange, 1997). Although enzymatic super-phosphorylation is preferable for modification of casein with intent for the food market due to its ability to alter the functionality of casein proteins, high costs have meant that chemical modification of casein with

$POCl_3$ is more common place in super-phosphorylation research (Van Hekken and Strange, 1997; Van Hekken and Dudley 1997; Van Hekken *et al*, 1996).

1.7 Conclusion

Milk protein concentrate (MPC) powders have numerous commercial applications and are integral ingredients in high quality functional dairy products and beverages. This review focused on the manufacture of high protein dairy powders, with an emphasis on protein and mineral modifications to improve solubility properties. Numerous reviews have explored the application of post spray drying mechanical and physical methodologies to improve high protein powder dissolution such as cavitation, ultrasonication and high shear mixing. However, while a number of factors play a role in high protein powder solubility two of the main elements are powder storage temperature and time, with the fraction of insoluble powder increasing with increasing temperature and duration. During storage, inter- and intra-molecular interactions cause casein micelles to become more closely packed, forming a monolayer “skin”, while hydration of the monolayer results in the formation of a porous, gel-like structure which slows the dispersion of individual casein micelles into the surrounding liquid phase. Therefore, chemically or enzymatically altering the casein micelle structure could provide a more effective dissolution aid by altering the bonding or interactions of casein proteins in solution. Hence, this review has explored the application of chemical modifications including mineral chelation and enzymatic

modification of proteins pre and post drying as a potential method to improve the dissolution properties of high protein milk powders.

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Objectives

The overall objective of the research reported in this thesis was to investigate the impact of enzymatic modification of casein and the chelation-mediated depletion of calcium on functionality of milk protein concentrate ingredients in powder and liquid format. Relatively few studies have focused on a combined approach of altering both the casein micelle structure and the mineral profile of MPC powders. The outcomes of this research will have applications in the formulation of MPC powders with improved dissolution properties, reduced viscosity and in the production of dairy products with altered mineral contents.

The specific aims of the research are as follows:

- To investigate the effects of dephosphorylation and sodium hexametaphosphate addition on the viscosity of milk protein concentrate dispersions.
- To investigate the factors responsible for increased viscosity during calcium chelation and investigate if enzymatic crosslinking with transglutaminase is effective in controlling viscosity development.
- To investigate if the hydration of milk protein concentrates can be improved by mineral chelation and if enzymatic crosslinking can be implemented to off-set issues with viscosity during processing.
- To investigate the impact of calcium depletion on enzymatically crosslinked milk proteins using calcium ion exchange on casein micelle stability and viscosity development.
- To examine the rehydration, viscosity, and thermal stability of calcium-reduced (using a strong cationic ion exchange resin) milk protein concentrates.

CHAPTER TWO

Dephosphorylation of caseins in milk protein concentrate alters their interactions with sodium hexametaphosphate

Orla M. Power[1, 2], Mark A. Fenelon[1], James A. O'Mahony[2], Noel A. McCarthy[1]

[1]Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Declaration:

This chapter was written by author Orla M. Power (OMP) and reviewed by co-authors Dr. Noel A. McCarthy (NAMC), Dr. James A. O'Mahony (JAOM) and Dr Mark Fenelon (MK). OMP co-designed the study with NAMC and performed all of the experimental work. Dr. John O'Brien and Dr. Manuel Ruether of the NMR Spectroscopy Facility at Trinity College Dublin, Ireland assisted with interpretation of ^{31}Phosphate nuclear magnetic resonance spectra data.

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2.1 Abstract

This study investigated the effects of dephosphorylation and sodium hexametaphosphate (SHMP) addition on the viscosity of milk protein concentrate (MPC) dispersions. Dephosphorylation (DP) of casein was performed using bovine alkaline phosphatase. Nuclear magnetic resonance (NMR) spectra showed that dephosphorylation depleted the casein-bound phosphate region (CNP). SHMP addition (5 mM) had no impact on the ^{31}P NMR spectra of DP-MPC; addition of 5 mM SHMP to control MPC (C-MPC) resulted in a shift in peaks associated with the CNP region, possibly caused by SHMP sequestering calcium, leading to swelling of micelles. DP-MPC exhibited a lower viscosity compared to C-MPC, with SHMP addition at 12.5 and 25 mM causing gelation of C-MPC and DP-MPC dispersions. This work confirmed the role that phosphate residues have in maintaining micelle structural stability and provides new insights into control of viscosity of MPC dispersions.

2.2. Introduction

Milk protein concentrate (MPC) powders are produced using separation processes such as ultrafiltration (UF) and diafiltration (DF) of pasteurised skim milk. They have protein contents ranging from 40 to 80% (w/w) (Havea 2006; Sikand *et al*; 2011) and a similar casein to whey protein ratio as the original skim milk (i.e., ~80:20). After membrane filtration, the liquid MPC may be heat treated and evaporated before spray drying, and while the subsequent powders are used in a wide range of applications (including infant milk formula and sports nutritional beverages and foods), their reconstitution properties can be challenging (Crowley *et al* 2016). Several factors contribute to their insolubility, such as the heat treatment of the skim milk, protein and mineral content, UF and DF conditions (e.g., volume concentration factor), spray drying temperatures, storage conditions of the subsequent powder (e.g., time and temperature) and the rehydration process (e.g., temperature of reconstitution medium and mixing conditions) (Gaiani *et al* 2010; Mimouni *et al*, 2010; Richard *et al* 2012).

There are a number of novel methods available for increasing the solubility of high protein MPC powders. Previous work (Bhaskar *et al* 2007) has shown that depleting calcium from MPC, using ion-exchange technology, can lead to significant increases in protein solubility. McCarthy *et al* (2017) showed that the addition of calcium chelating salts to MPC dispersions can increase protein solubility, but that there is a significant concomitant increase in viscosity. The structural stability of casein micelles is dependent on a delicate balance of attractive (e.g., hydrophobic interactions) and repulsive (e.g., electrostatic repulsion) forces between the constituent proteins. The colloidal calcium phosphate (CCP) associated with the proteins in casein micelles *via* phosphoserine residues can be depleted using approaches such as ion-exchange and mineral chelation, thereby reducing micelle integrity (Dalgleish and

Corredig, 2012; de Kruif *et al*, 2012; McMahon and Oommen, 2008; Horne, 2006; Wong *et al*, 1996).

Chelating salts act by sequestering free calcium ions in milk-based systems and can also potentially interact with calcium associated with the CCP located within the casein micelles, to an extent dependent on the electrostatic strength and ability of the chelator to bind calcium. The binding of calcium through the addition of chelating salts, alters the distribution of calcium between the colloidal and serum phases, which consequently influences electrostatic-mediated protein-protein interactions. This results in a greater net negative charge on the caseins, resulting in increased electrostatic repulsion, decreased protein-protein interaction and increased viscosity (De Kruif and Holt, 2003; Lin *et al*, 1972; Odagiri and Nickerson, 1964). Depending on calcium chelating salt type and concentration used, casein micelles can dissociate into single or multiple protein clusters (De Kort *et al* 2009, 2011; Panouillé *et al,* 2008)

The individual casein proteins (i.e., α_{s1}-, α_{s2}-, β- and κ-casein) have different degrees of phosphorylation, with α_{s1}-, α_{s2}-, β- and κ-casein having 8, 11/12, 5 and 1 phosphate residues, respectively (De Kruif and Holt, 2003; Horne, 2006). A number of previous studies (Yun *et al*, 1982; Liu *et al*, 2016; McCarthy *et al*, 2013) have studied the effects of dephosphorylation of casein on charge, mineral sensitivity, protein aggregation, curd forming properties and re-micellization of casein proteins.

The current study aimed to develop a mechanistic understanding of the role of phosphate groups, through dephosphorylation, on casein proteins in MPC dispersions using the advanced analytical capability of phosphate nuclear magnetic resonance (^{31}P NMR) and to control viscosity of MPC suspensions in which calcium ion activity had been altered by the addition of calcium-chelating salt. Minimising viscosity of concentrated casein systems, such as milk protein concentrate, is of academic and

industrial interest in the development of protein-dense nutritional powders and beverages as high viscosity can negatively affect unit operations (e.g., ultrafiltration, spray dryer atomisation) and finished product quality (e.g., beverage mouthfeel). Calcium chelating salts are commonly added to concentrated casein protein dispersions to enhance powder solubilisation and heat stability; however, the concurrent swelling and viscosity increases limit their use and effectiveness in such applications.

2.3 Materials and methods

2.3.1. Materials

Milk protein concentrate (MPC) powder was obtained from a local dairy ingredient manufacturer. The protein, moisture, fat and ash content of the MPC were 81.4% (w/w), 4.30% (w/w), 1.40% (w/w) and 7.80% (w/w), respectively, as provided by the manufacturer. The lactose content was determined by difference as 5.10% (w/w). Bovine alkaline phosphatase (BAP) enzyme was obtained from Sigma Aldrich (Vale Rd, Ballyraine Lower, Arklow, Co. Wicklow, Ireland).

2.3.2. Reconstitution of milk protein concentrate

MPC powders (250 g, 10%, w/w) were dissolved in preheated (50°C) distilled water aided by an overhead stirrer. Ultrasonication was carried out using an ultrasound device (Hielscher UIP1000hd, Hielscher Ultrasonics Gmbh, Warthestraβe 21 D-14513, Berlin, Germany) using the method previously described by McCarthy *et al*, (2014) at an amplitude of 50% for 10 min to promote rehydration of the MPC. Sodium azide (0.02% w/w) was added to the resulting dispersions to prevent microbial growth.

Dispersions were stirred for 4 h at 22°C prior to storage overnight at 4°C with low speed magnetic stirring.

2.3.3 Dephosphorylation of casein and addition of sodium hexametaphosphate

Reconstituted MPC dispersions (10%, w/w, protein) were incubated with BAP (1:20 w/w) at pH 6.5 and 37°C for 3 h. A control MPC (C-MPC) dispersion was prepared under the same conditions but without the addition of the enzyme. After 3 h the reaction was terminated by heating at 70°C for 5 min to inactivate the enzyme. SHMP was dissolved in 1 mL of water as a stock solution and the pH adjusted to 6.5 prior to addition to the C-MPC and DP-MPC dispersions to give a final concentration of 5, 12.5 or 25 mM.

2.3.4. Protein profile analysis by electrophoresis

Urea-PAGE was used to separate casein proteins using the method of Ornstein and Davis (1964), with a separating gel composed of 7.5% acrylamide, 0.375 M Tris–HCl, pH 8.8 and 4 M urea. Protein samples were prepared at a concentration of 5 mg/mL in sample buffer, and 20 µL of sample was loaded onto the gel. After electrophoresis, the gels were stained overnight using 0.05% (w/v) Coomassie Brilliant Blue R-250 in 25% (v/v) isopropanol and 10% (v/v) acetic acid. After staining, the gels were de-stained using a 10% (v/v) isopropanol and 10% (v/v) acetic acid solution until a clear background was achieved.

2.3.5. Nuclear magnetic resonance

^{31}Phosphate nuclear magnetic resonance (^{31}P NMR) was conducted at 25°C using a Bruker Advance III 400 NMR (Bruker UK Ltd., Coventry, UK) located at Trinity College (Dublin, Ireland) operating at 400.2 MHz for proton, 162.0 MHz for phosphorous-31 and 100.6 MHz for carbon-13 resonance. MPC powders (10-20 mg) were prepared in deuterium oxide (D_2O) solvent (1 ml) and NMR data was analysed using TopSpin 3.5 software (Bruker UK Ltd).

2.3.6. Rheological measurements of milk protein concentrate dispersions

Rheological measurements of MPC dispersions (10%, w/w, protein) were carried out using a controlled-stress rheometer (AR2000ex rheometer, TA Instruments, Crawley, UK), equipped with a concentric cylinder geometry. SHMP was prepared as described in Section 2.3 and added to 17 mL of MPC dispersions (10%, w/w, protein). Samples were inverted ten times prior to testing to ensure a homogenous mixture. Viscosity measurements were carried out at 20°C with pre-shearing at 100 s^{-1} for 10 s, followed by a peak hold step at a shear rate of 100 s^{-1} for 2 h. Low-amplitude oscillatory shear rheological measurements of the C-MPC and DP-MPC dispersions containing 12.5 and 25 mM SHMP were also determined using a 60 mm parallel plate geometry. SHMP was added to the MPC dispersion to give 12.5 or 25 mM final concentration and stirred for 5 min to allow adequate mixing prior to analysis. The sample was then pre-sheared at 50 s^{-1} for 10 s at 20°C to eliminate any shear history, followed by a time sweep at 0.25% strain and 1 Hz frequency over 6 h, during which the rheological parameters elastic (G') and viscous (G'') moduli were recorded.

2.3.7. Dynamic light scattering and particle size distribution analysis

Particle size measurements were performed on C-MPC and DP-MPC dispersions with added SHMP concentrations of 0, 5, 12.5 and 25 mM using a Zetasizer nano (Malvern Instruments, Worcestershire, England). Measurements were conducted 1 h after SHMP addition to ensure equilibration. Dispersions were diluted (1:50) in deionised water with a dispersant refractive index (RI) of 1.33 and viscosity of 0.89 mPa.s used. The refractive index used for the protein particles in the sample was 1.45 in conjunction with an absorption value of 0.001. Experiments were conducted in triplicate at 25°C at a backscattering angle of 173°. Data was displayed as the z-average (nm), correlating to the intensity-weighted mean size of all particles present in the dispersion.

2.3.8. Statistical analysis

Rheological measurements were analysed using a Paired T-test with a 95% confidence interval. Particle size data was statistically analysed using one-way analysis of variance (ANOVA), with posthoc Tukey analysis. The level of significance was considered as $P < 0.05$. All statistical analysis was carried out using Minitab 17 (Minitab Inc, Coventry, United Kingdom).

2.4 Results

2.4.1. Protein profile of non-dephosphorylated and dephosphorylated casein

Incubation of MPC with BAP for 3.5 h at 37°C resulted in dephosphorylation of casein, as shown by urea-PAGE profiles in Fig. 2.1. Urea-PAGE separates proteins based on their charge-to-mass ratio and so dephosphorylated protein bands were observed to have lower electrophoretic mobility on the urea-PAGE gel (Fig. 2.1; lane 4) due to a reduction in their charge-to-mass ratio. These bands corresponded to α_{s1}-

and β-casein with varying degrees of dephosphorylation. Urea-PAGE profiles showed a greater reduction in the original β-casein band intensity, compared to α_{s1}-casein suggesting a greater extent of dephosphorylation of the former casein. In the current study, BAP was chosen as the dephosphorylating enzyme over potato acid phosphatase (PAP) due to a proteolytic side reaction during incubation of PAP with MPC as evident on urea-PAGE gels from preliminary trials (data not shown) and confirmed by previous studies conducted by Liu *et al,* (2016) and McCarthy *et al*, (2013).

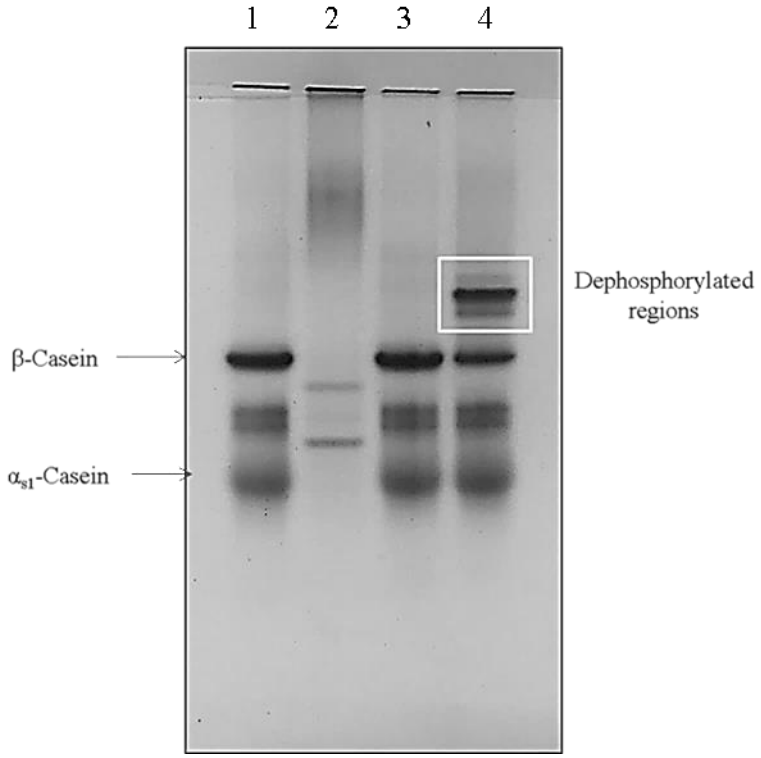


Fig 2.1. *Urea-PAGE protein profiles of MPC (lane 1), bovine alkaline phosphatase enzyme (lane 2), C-MPC dispersion (lane 3) and DP-MPC dispersion (lane 4).*

2.4.2. Nuclear magnetic resonance spectroscopy of milk protein concentrate dispersions

Fig. 2.2 shows ^{31}P NMR profiles of both C-MPC and DP-MPC dispersions with and without SHMP addition. Profiles of control MPC without SHMP addition (Fig. 2.2A) showed the calcium phosphate nano-cluster regions (CPN) associated with the casein micelle as a broad multiplet between 1.89-3.37 ppm and a further broad singlet at 0.93 ppm, attributed to non-serine bound inorganic phosphate (P_i) DP-MPC dispersions (0 mM SHMP) showed a significant change in the NMR spectra from that of C-MPC, with the absence of a CPN region (Fig. 2.2C). Following addition of SHMP to MPC dispersions, peaks were observed at ~-21 and -24 ppm (Fig. 2.2B and D). Addition of SHMP (5 mM) to the C-MPC dispersion caused a shift in the ^{31}P spectra in the CPN region from 1.76-3.36 ppm to 1.93-2.66 ppm, with larger and sharper peaks being observed also (Fig. 2.2B). In contrast, addition of the same level of SHMP to DP-MPC created only a single large P_i peak at 0-2.0 ppm (Fig. 2.2D), with no evidence of CPN peaks.

2.4.3. Rheological measurements of milk protein concentrate dispersions

Viscosity profiles of C-MPC and DP-MPC dispersions without and with added SHMP (5 mM) are shown in Fig. 2.3. C-MPC and DP-MPC dispersions exhibited similar viscosity in the absence of SHMP, with not significantly different final viscosity values of 10 and 9.0 mPa.s ($P > 0.05$), respectively. The addition of 5 mM SHMP resulted in an increase in viscosity for both C-MPC and DP-MPC, with the viscosity of DP samples being significantly ($P < 0.05$) lower than those of the control MPC sample with added SHMP (C-MPC; Fig. 2.3). The viscosity of DP-MPC increased slowly from an initial value of 13 mPa.s to a final viscosity of 40 mPa.s over a 2 h period, compared to the viscosity for C-MPC which increased progressively from 13 mPa.s to a final viscosity of 125 mPa.s over the same time frame (Fig. 2.3). Gelation

properties of MPC dispersions containing 12.5 and 25 mM SHMP are shown in Fig. 2.4. The C-MPC sample with 12.5 mM SHMP showed an elastic (G′) and viscous modulus (G″) crossover at 12.8 min (G′ and G″ = 5.89 Pa), with final G′ and G″ values of 557 and 219 Pa, respectively, after 2.5 h (Fig. 2.4A). The C-MPC sample with 25 mM SHMP had a G′-G″ moduli crossover time of 18.6 min (G′ and G″ = 20.0 Pa), with final G′ and G″ values of 419 and 200 Pa after 2.5 h, respectively.

In contrast, DP-MPC with 12.5 mM SHMP formed a gel after an extended timeframe of 20.4 min (G′ and G″ = 6.17 Pa), with final G′ and G″ modulii of 245 and 124 Pa, respectively (Fig. 2.4B). DP-MPC dispersions with 25 mM added SHMP formed a gel network with an elastic and viscous moduli crossover at 31.8 min (G′ and G″ = 30.5 Pa), with final G′ and G″ values of 86.2 and 69.9 Pa, respectively (Fig. 2.4).

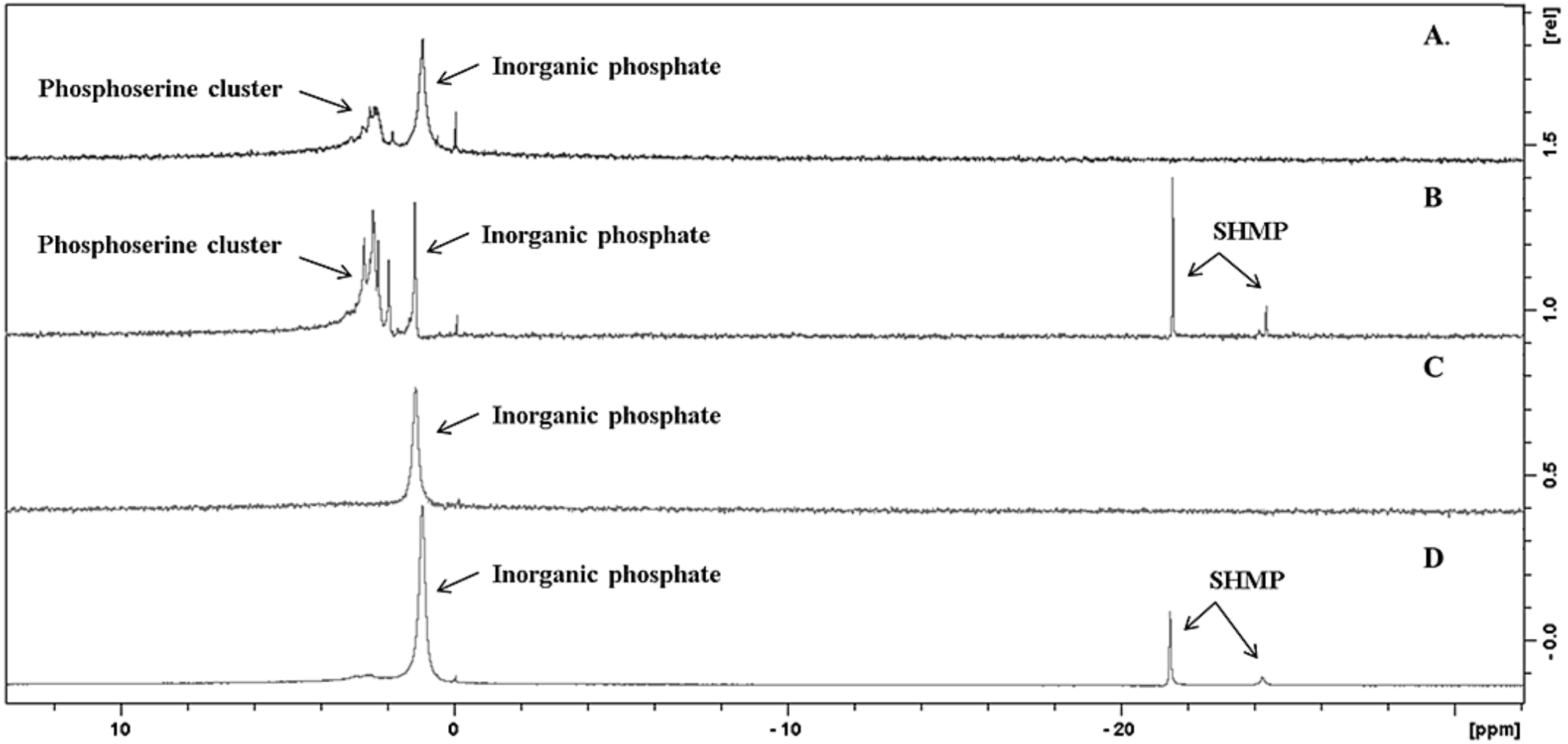


Fig 2.2. *^{31}P NMR profiles of C-MPC at SHMP concentrations of 0 mM (A) and 5 mM (B) and DP-MPC at SHMP concentrations of 0 mM (C) and 5 mM (D).*

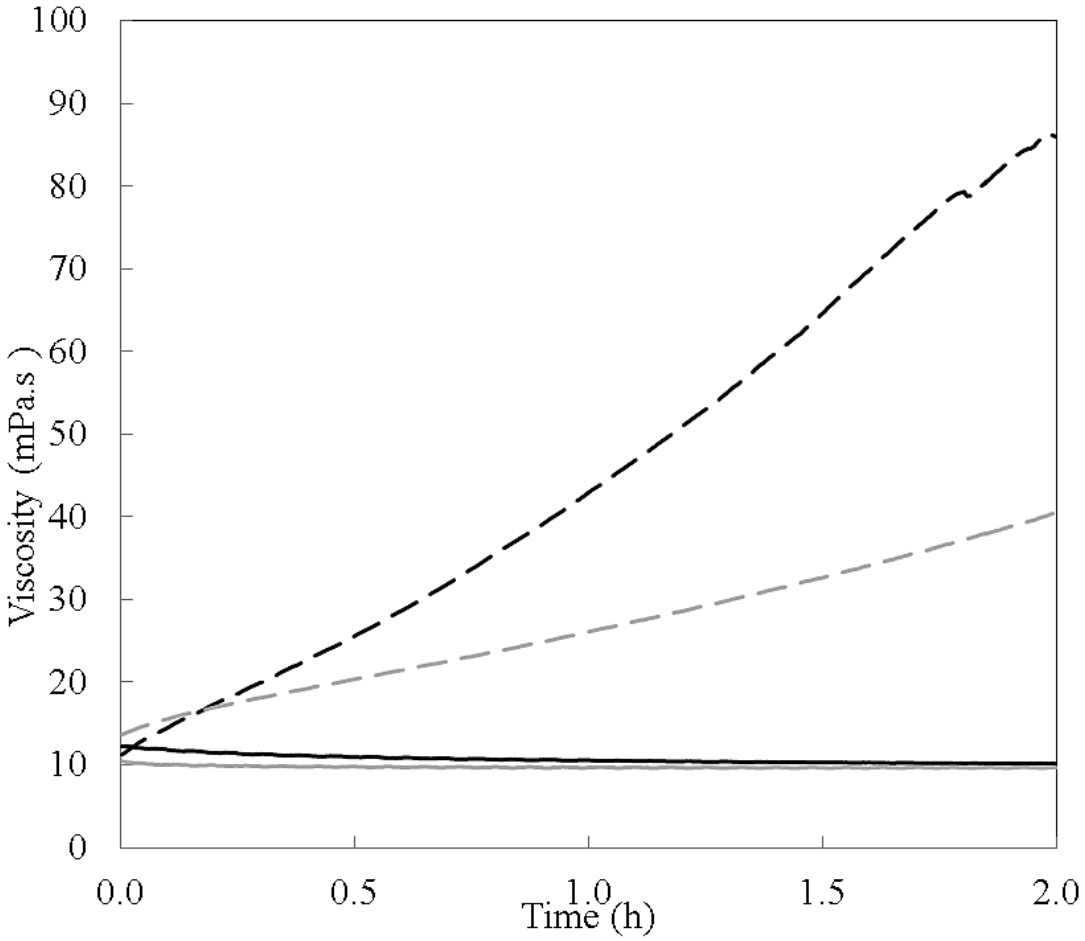


Fig 2.3. *Viscosity profiles of C-MPC (black lines) and DP-MPC (grey lines) dispersions (10%, w/w, protein) containing 0 (—) or 5 (- - -) mM sodium hexametaphosphate, measured at a shear rate of 100 s-1 at 20 °C.*

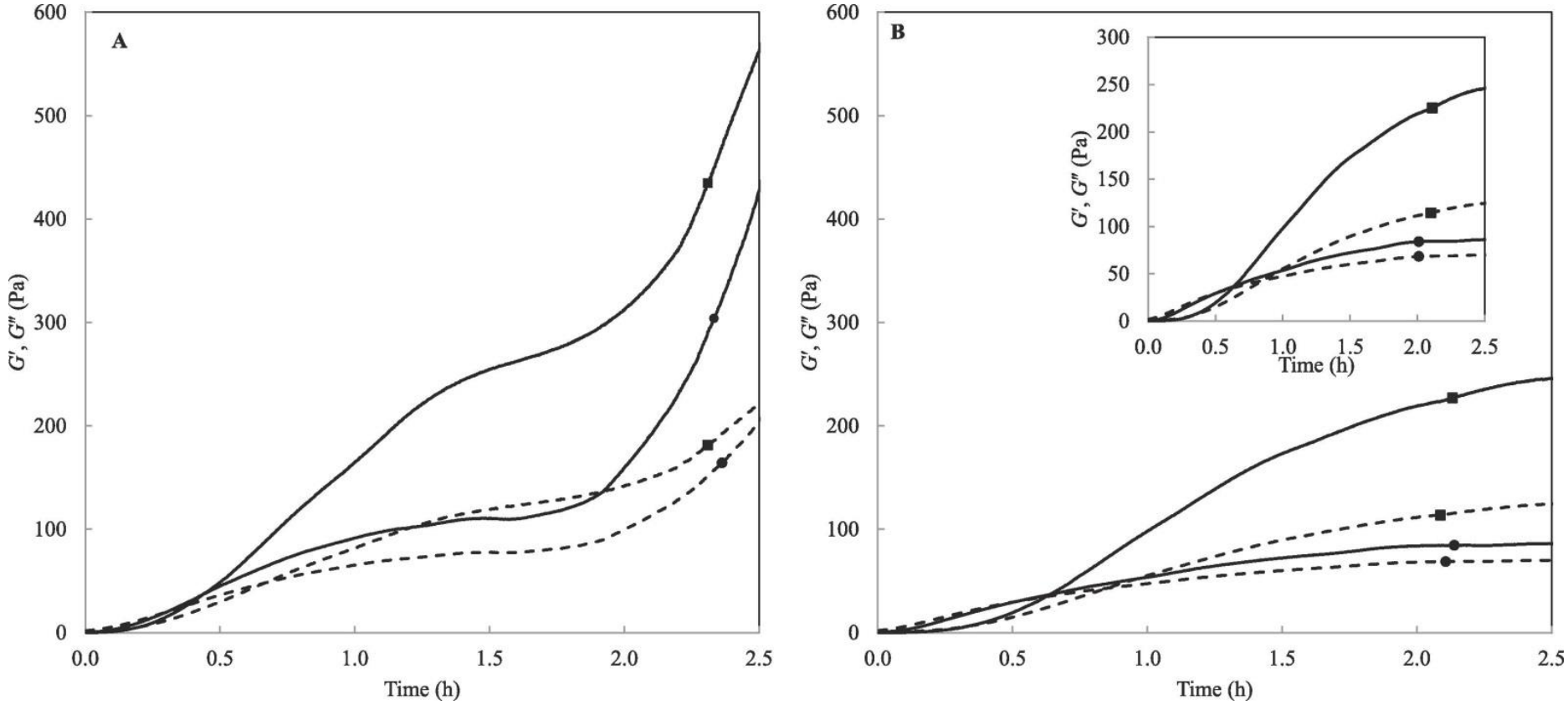


Fig 2.4. *Elastic, G' (—; Pa) and viscous G'' (- - -; Pa) moduli, of C-MPC (A) and DP-MPC (B) dispersions (10%, w/w, protein) containing 12.5 (■) and 25 (●) mM sodium hexametaphosphate, measured at a frequency of 1 Hz and strain of 0.25%.*

2.4.4. Particle size measurements of milk protein concentrate

Data for particle size analysis of MPC dispersions is shown in Fig. 2.5. The z-average particle size values for C-MPC and DP-MPC dispersions containing no SHMP were 168 and 183 nm ($P > 0.05$), respectively (Fig. 2.5A). Size distribution profiles showed large monomodal peaks for MPC dispersions with no SHMP addition (Fig. 2.5B and C). C-MPC and DP-MPC dispersions containing 5 mM SHMP had z-average particle size values of 205 and 206 nm, respectively, indicating that both samples displayed similar swelling behaviour upon SHMP addition ($P > 0.05$), as evident from a shift in monomodal size distribution peaks towards larger particle size (Fig. 2.5B and C). However, C-MPC particle size values were significantly ($P < 0.05$) lower at 12.5 mM SHMP addition (107.3 nm), indicating partial dissociation of casein micelles. In contrast, the particle size of DP-MPC dispersions with 12.5 mM SHMP decreased to 143 nm, also indicative of micelle disintegration, albeit not to the same extent as observed in the C-MPC dispersion. Addition of 25 mM SHMP resulted in even greater micelle dissociation, as evidenced by particle size values for C-MPC and DP-MPC dispersions of 108 and 100 nm ($P > 0.05$), respectively (Fig. 2.5A, B and C).

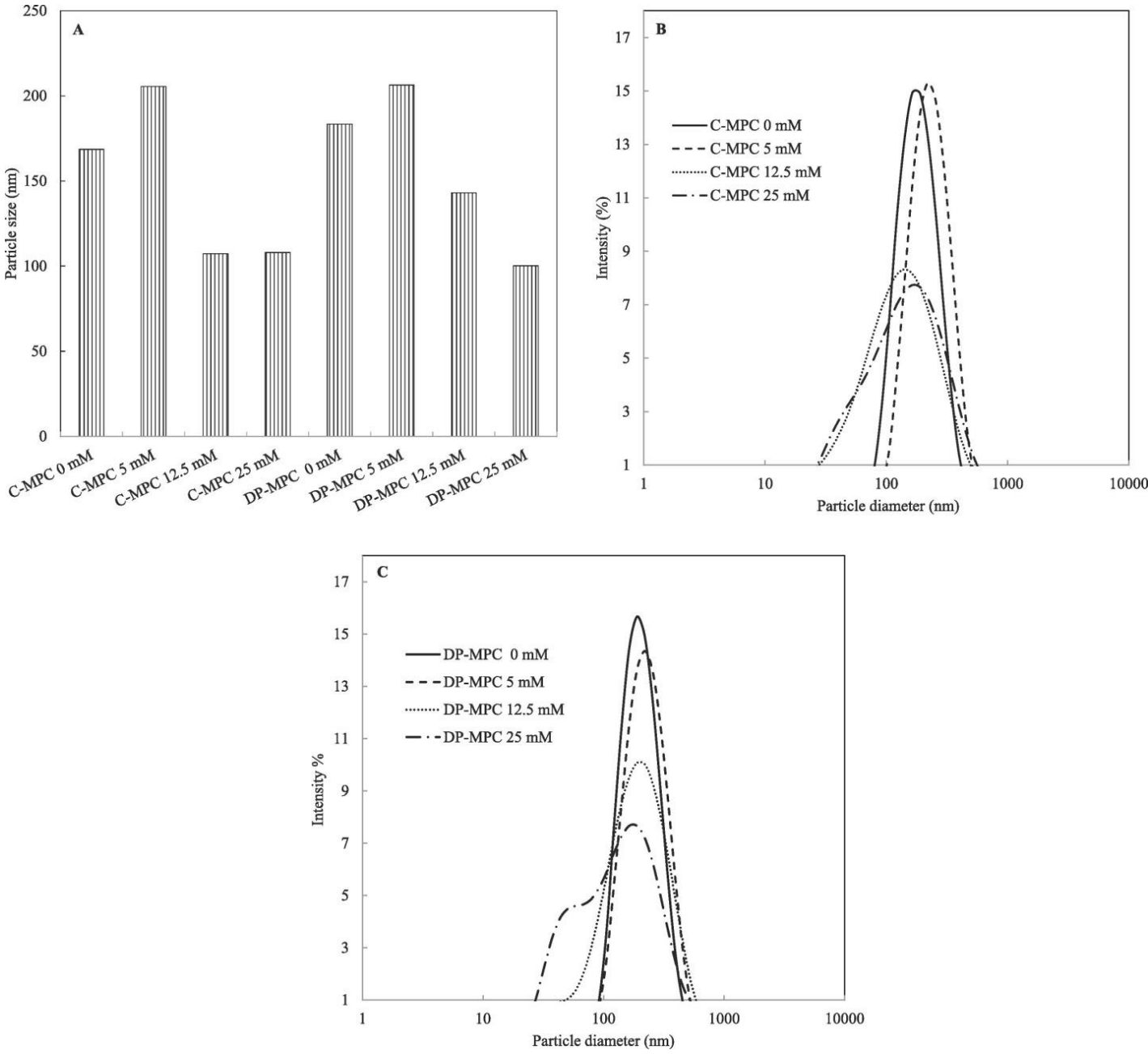


Fig 2.5. *Z-average particle size values for C-MPC and DP-MPC dispersions (A) and particle size distribution profiles for C-MPC (B) and DP-MPC (C) dispersions containing (0, 5, 12.5 and 25 mM) sodium hexametaphosphate. Data shown is the mean value derived from triplicate analysis in each case.*

2.5. Discussion

Previous studies (De Kruif and Holt, 2003; Lin *et al*, 1972; Odagiri and Nickerson, 1964) have shown the benefits of adding SHMP to concentrated milk protein dispersions prior to drying, aiding subsequent powder dissolution and protein hydration. However, the benefits achieved in powder dissolution can be undermined by the high viscosity of the protein concentrate stream prior to spray drying; often requiring such MPC concentrates to be dried at lower solids content. The aim of this study was to understand in more detail the fundamental chemistry underlying the increased viscosity of MPC concentrates on addition of a strong polyphosphate chelating salt such as SHMP. This new understanding will allow better prediction and control of viscosity of MPC concentrate dispersions.

SHMP is a hexadentate ligand, and hence, in milk protein systems, can simultaneously chelate calcium in the aqueous phase and chelate calcium from CCP (De Kort *et al*, 2011). This chelation of cations changes the ionic balance of the aqueous phase, thereby affecting electrostatic casein-casein interactions and the structural integrity of the micelle, leading to swelling (Fig. 2.5). This increase in casein micelle swelling is caused mainly by increased electrostatic repulsion between proteins created by newly-exposed serine phosphate groups (De Kort *et al*, 2009 and 2011; McCarthy *et al*, 2017; Mizuno and Lucey, 2007; O'Connell *et al*, 2003; Panouille *et al*, 2005).

NMR data showed that dephosphorylation and addition of SHMP significantly influenced the NMR phosphate signals in MPC dispersions (Fig. 2.2). Boiani *et al* (2017) identified the effects of diafiltration water containing citrate on miceller casein-based milk proteins with an emphasis on the phosphate signals using ^{31}P NMR. The authors highlighted a peak located at ~1.00 ppm to be that of P_i ($H_2PO_4^-$ and HPO_4^{2-})

along with broad, low-intensity peaks ranging from 2.0 to 3.0 ppm, identified as casein-associated phosphate as well as a smaller peak located at ~0.5 ppm, identified as a phosphate ester. The addition of 5 mM SHMP to C-MPC (Fig. 2.2B) resulted in larger and sharper peaks for NMR analysis compared to C-MPC samples without SHMP addition (Fig. 2.2A). This may be due to the action of the SHMP chelating calcium leading to micelle swelling, and possible partial dissociation (Fig. 2.5) which would lead to an increase in the exposure of serine phosphate residues, and therefore, an increase in their peak signal from NMR analysis. This experimental data demonstrates that SHMP did impact the casein micelle structure, due to the change in the distinct peak pattern of the phosphoserine residues located at 2-3 ppm which resolved into a multiplet feature upon addition of 5 mM SHMP (Fig. 2.2B). Belton *et al* (1985) examined the impact of EDTA on whole cows' milk using ^{31}P NMR and observed that upon EDTA addition the P_i peak increased in intensity and decreased in width. This was likened to the chelation of free P_i in solution by EDTA and subsequent dissociation of calcium bound by phosphoserine residues. Furthermore, the addition of EDTA caused the phosphoserine peak located at 3.2 ppm to resolve into three distinctive peaks indicating that chelating action impacts on micelle integrity. This can also account for the changes observed in P_i peak intensity in the current study. Boiani *et al*, (2017) showed similar results when MPC manufactured using diafiltration water containing citrate was shown to have better resolution and increased signal peaks for phosphoserine residues. When DP-MPC dispersions were analysed in the absence of SHMP it was observed that the multiplet feature attributed to the phosphoserine region was absent, confirming that dephosphorylation had indeed occurred. The intensity of the P_i peak in Fig. 2.2C is larger and narrower than the peak observed in Fig. 2.2A, suggesting that CCP associated with casein proteins *via* phosphoserine residues had

been, at least partially, disintegrated. The addition of 5 mM SHMP to DP-MPC (Fig. 2.2D) showed no change at the phosphoserine site, indicating that no interaction occurred between SHMP and phosphoserine-bound calcium.

The significantly ($P < 0.05$) higher gel strength observed for C-MPC compared to DP-MPC at 12.5 and 25 mM SHMP addition levels (Fig. 2.4A and B) could indicate that gelation brought about by SHMP interactions with calcium bound to phosphoserine residues was hindered in DP-MPC. Similarly, C-MPC also had higher viscosity values compared to DP-MPC dispersions (Fig. 2.3). The lower viscosity values for DP-MPC could be attributed to a number of possible factors. CCP has a complex composition and structure, containing high proportions of calcium, magnesium and sodium, with the exact composition of CCP dependent on the ionic environment. CCP is an important structural component of casein micelles, holding caseins together *via* interactions between negatively charged phosphoserine residues, reducing electrostatic repulsion (Holt, 1997; Walstra, 1990). During dephosphorylation, negatively charged phosphate groups are enzymatically removed from serine phosphate residues, greatly limiting casein-casein interactions *via* CCP, thus reducing the rigidity of the micelle network. Interestingly, gel strength (as measured by G') increased with SHMP addition level only up to 12.5 mM after which a decrease in gel strength was observed on increasing SHMP concentration to 25 mM. This may be due to partial dissociation of casein micelles through reduction of CCP content as a result of increasing SHMP addition level. At sufficiently high SHMP concentrations (i.e., >12.5 mM), the reduction in viscosity caused by disintegration of casein micelles (as evidenced from particle size distribution analysis) may have been greater than the increase in viscosity caused by electrostatic interaction strength-mediated micelle swelling.

In a somewhat analogous manner, Ennis *et al* (1998) showed that during the hydration of rennet casein (consisting of a *para*-caseinate curd as opposed to native casein micelles in MPC) there was a reduction in the maximum viscosity index observed at high concentrations of chelating salts due to disruption of calcium-mediated cross-bridging of casein proteins. In another study, Panouille *et al,* (2005) reported that the aggregation and gelation of casein sub-micelles is dependent on casein and polyphosphate salt concentration, pH, and ionic strength. These authors stated that the aggregation and gelation process may involve a restructuring of submicelles through interactions with calcium and phosphate, leading to the formation of branched chains of small spheres. These spheres are composed of complexes involving ionic calcium, phosphates, and casein molecules. However, in the present study, at sufficiently high levels of addition of SHMP the level of free calcium ions in the aqueous phase is diminished, resulting in decreased calcium-mediated cross-bridging of casein particles. Therefore, the current study has shown that phosphoserine residues are involved, through their association with calcium, in both the stability and swelling of casein micelles and that through dephosphorylation the viscosity of concentrated casein systems can be controlled in the presence of SHMP.

2.6. Conclusion

NMR analysis proved effective in providing new information on the influence of polyphosphate salt addition to MPC, while having the ability to simultaneously show changes in both phosphoserine and P_i signals in control and dephosphorylated samples. DP-MPC samples had lower viscosity and gel strength in the presence of SHMP when compared to C-MPC. This demonstrates that phosphoserine groups play an important role in viscosity development, and even more so, gel formation, in MPC

dispersions on SHMP-mediated chelation of calcium. This novel study clearly demonstrates that dephosphorylation is effective in the control of viscosity development in MPC dispersions in which calcium-chelating salts are added for technological reasons such as increasing protein dispersion heat stability and enhancing powder rehydration properties.

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CHAPTER THREE

Influence of sodium hexametaphosphate addition on the functional properties of milk protein concentrate dispersions containing transglutaminase cross-linked proteins

Orla M. Power[1, 2], Mark A. Fenelon[1], James A. O'Mahony[2], Noel A. McCarthy[1]

[1]Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[2]School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Declaration:

This chapter was written by author Orla M. Power (OMP) and reviewed by co-authors Dr. Noel A. McCarthy (NAMC), Dr. James A. O'Mahony (JAOM) and Dr. Mark Fenelon (MK). OMP co-designed the study with NAMC and performed all of the experimental work.

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3.1 Abstract

The functional properties of milk protein concentrate (MPC) powders are often hindered by their poor solubility. Calcium chelating salts have been shown to improve powder solubility but generally their action contributes to higher viscosity due to disintegration of casein micelles and higher levels of serum-phase calcium. To help mitigate increases in viscosity associated with calcium chelation, transglutaminase (TGase), an enzyme which covalently crosslinks protein, was employed in an effort to stabilise the casein micelle structure. Sodium hexametaphosphate (SHMP) was added to control (C-MPC) and TGase crosslinked MPC (TG-MPC) dispersions at concentrations of 5, 12.5 and 25 mM prior to analysis. TG-MPC dispersions had lower viscosity than C-MPC dispersions across all SHMP concentrations studied. Crosslinking limited micelle dissociation on SHMP addition and led to greater retention of the white colour of the protein dispersions, while the turbidity of C-MPC dispersions decreased with increasing SHMP addition.

3.2. Introduction

High protein dairy based powders, such as milk protein concentrate (MPC), milk protein isolate, micellar casein isolate and whey protein isolate are increasingly being used as ingredients in value-added dairy products such as beverages, yogurts and infant formulae. MPC powders are manufactured from skim milk using ultrafiltration, followed by diafiltration and possible evaporation, prior to spray drying to create high protein powders which are transported globally (Mistry, 2002; Sikand *et al*, 2011). One of the greatest challenges encountered during the processing of milk protein concentrates is the high viscosity after ultrafiltration and evaporation. This high viscosity can be caused by the concentration of proteins and an increase in bound moisture due to denaturation/aggregation of protein during heat treatment (Henriques *et al*, 2017). In a study by Ho *et al*, (2018), it was shown that high heat treatment temperatures resulted in a significant ($P < 0.05$) increase in viscosity from 36.3 to 74.8 mPa.s. Consequently, such high protein systems are typically evaporated to relatively low total solids content, compared to skim milk, prior to drying (Bienvenue *et al*, 2003; Rupp *et al*, 2018; Vélez-Ruiz and Barbosa-Cánovas, 1998).

A common challenge in using MPC powders is their poor rehydration properties, which is primarily due to the close proximity of protein molecules and the hydrophobic nature of the casein protein constituent (Crowley *et al*, 2015; de Kruif *et al*, 2012; Holt *et al*, 2013; Horne, 2006; Mimouni *et al*, 2010). Several different formulation and technological approaches have been investigated to improve rehydration of MPCs, such as ultrasonication of liquid MPC, calcium depletion using ion exchange and addition of calcium chelating agents (Bhaskar *et al*, 2007; McCarthy *et al*, 2014; McCarthy *et al*, 2017).

The use of calcium chelating salts has been shown to improve the dissolution of MPC when added during the rehydration process (McCarthy *et al*, 2017); however, their addition to MPC systems can further contribute to viscosity, resulting in processing challenges during evaporation, spray drying or in end use applications. Calcium chelators work by sequestering calcium from the aqueous phase, causing a change in the electrostatic environment and depletion of calcium from within the casein micelle through alteration of the calcium equilibrium. Calcium depletion causes increased hydration and subsequent swelling of casein micelles due to reduced structural rigidity (de Kort *et al*, 2011; McCarthy *et al*, 2017; Omoarukhe *et al*, 2010; Power *et al*, 2019). Polyphosphate-based chelators, such as sodium hexametaphosphate (SHMP), have multiple calcium-binding sites, and therefore can simultaneously bind calcium in the serum phase and associated calcium from colloidal calcium phosphate (CCP) nano clusters within casein micelles. Through interactions with colloidal calcium, SHMP can also crosslink caseins *via* calcium phosphate complexes, thereby further increasing viscosity of MPC dispersions (De Kort *et al*, 2009, 2011; Lucey and Horne, 2018; Mizuno and Lucey, 2007).

One particular means of increasing casein micelle stability may be through the use of the enzyme transglutaminase (TGase) to create covalent linkages between casein proteins. Previous work by O'Sullivan *et al*, (2002) showed that TGase alters the heat stability of milk by crosslinking individual casein proteins and preventing dissociation of κ-casein from the micelles. Further research by Smiddy *et al.* (2006) and Moon *et al*, (2009) showed that casein micelles incubated with TGase had increased stability against micellar disruption by urea, sodium dodecyl sulphate or heating in the presence of ethanol, with stability increasing progressively with incubation time. Therefore, enzymatic crosslinking by TGase could be used to restrict

increases in viscosity caused by greater hydration and micelle swelling in MPC samples treated with calcium chelators and prevent destabilisation of the casein micelle. Thus, this study aimed to control micelle stability and maintain a lower viscosity of MPC dispersions in the presence calcium chelating agents by prior enzymatic crosslinking of casein proteins.

3.3 Materials and methods

3.3.1. Materials

Milk protein concentrate (MPC) powder (casein:whey protein ratio of 81:19) was obtained from a local dairy ingredient manufacturer and had protein, moisture, fat, lactose and ash content of 81.4% (w/w), 4.30% (w/w), 1.40% (w/w), 5.1% (w/w) and 7.8% (w/w), respectively. Transglutaminase enzyme preparations (Activa MP, Ajinomoto enzyme preparations, Ajinomoto foods Europe) were sourced from Healy Group (Cookstown Industrial Estate, Tallaght, Co. Dublin, Ireland). Sodium hexametaphosphate (CAS number: 68915-31-1) was obtained from Sigma Aldrich (Vale Rd, Ballyraine Lower, Arklow, Co. Wicklow, Ireland).

3.3.2. Rehydration of milk protein concentrate powder and crosslinking of casein proteins

MPC powder was rehydrated (250 g sample at 10%, w/w, protein) as per the method outlined by Power *et al*, (2019). Sodium azide (0.02%, w/w) was added to MPC dispersions to prevent microbial growth. Proteins were covalently cross-linked using the enzyme transglutaminase (TGase) as described previously by Huppertz and de Kruif, (2008). MPC dispersions (10%, w/w) were preheated to 30°C and incubated with Activia TGase (0.5 g L^{-1}) for 24 h at pH 6.5. Following incubation, the enzyme

was inactivated by heating at 80°C for 5 min. A control dispersion was prepared and treated using the same procedure without the addition of TGase enzyme. Control MPC and enzymatically crosslinked MPC dispersions are abbreviated to C-MPC and TG-MPC, respectively.

3.3.3 Rheological analysis

3.3.3a Viscosity of milk protein concentrate dispersions as a function of temperature

C-MPC and TG-MPC were divided into 50 mL aliquots and stored at 4, 20, 30 and 50°C for 3 h prior to rheological analysis. Dispersions were analysed using a controlled-stress rheometer (AR-G2 Rheometer, TA Instruments, Crawley, UK) equipped with a concentric cylinder geometry. Sample aliquots were initially conditioned at a temperature of 4, 20, 30 or 50°C and pre-sheared at 100 s^{-1} for 10 s, followed by a peak hold step at a shear rate of 300s^{-1} for 5 min.

3.3.3b Viscosity of milk protein concentrate dispersions with sodium hexametaphosphate addition

Sodium hexametaphosphate (SHMP) was dissolved in 1 mL of water and the pH adjusted to 6.5 prior to addition to C-MPC and TG-MPC dispersions (17 mL; 10%, w/w, protein) to give final SHMP concentrations of 5, 12.5 or 25 mM. Following SHMP addition, samples were inverted ten times to ensure homogeneity prior to analysis using a controlled stress rheometer (AR-G2 Rheometer, TA Instruments, Crawley, UK) equipped with a concentric cylinder geometry. Rheological analysis consisted of a conditioning step performed at 20°C and a pre-shear at 100 s^{-1} for 10 s, followed by a peak hold step at a shear rate of 100 s^{-1} for 2 h.

3.3.4 Particle size distribution of milk protein concentrate dispersions as a function of temperature

Particle size measurements were carried out using a Zetasizer nano (Malvern Instruments, Worcestershire, UK) on both TG-MPC and C-MPC dispersions as a function of temperature (i.e., 4, 20, 30 or 50°C) after a 3 h storage period. Dispersions were diluted 1:50 with tempered deionised water prior to analysis. Sample analysis parameters were set at a dispersant refractive index (RI) of 1.330 and viscosity of 0.8872 cp. The viscosity of dispersions was measured in disposable cuvettes, and the samples were characterised as protein using an RI of 1.45 and absorption value of 0.001. Experiments were carried out in triplicate with a backscattering angle of 173°.

Size measurements were also carried out on C-MPC and TG-MPC dispersions with added SHMP at concentrations of 0, 5, 12.5 and 25 mM using the Zetasizer nano as described above. All measurements were carried out 1 h after SHMP addition at 20°C.

3.3.5. Zeta-potential analysis of milk protein concentrate dispersions

The ζ-potential of C-MPC and TG-MPC dispersions was measured as a function of pH using a Zetasizer (Malvern Instruments, Worcestershire, UK). Samples were diluted 1:10 using deionised water at 22°C prior to pH adjustment using concentrated hydrochloric acid or sodium hydroxide. Zeta potential analysis was performed using water as the dispersant, with an RI of 1.330 and viscosity of 0.8872 cp. Dispersions were measured using disposable folding capillary cells (DTSI060/DTSI061). The protein was characterised using an RI of 1.45 and absorption value of 0.001. Measurements were performed in triplicate at 25°C using the Smoluchowski model (Smoluchowski and Marian, 1927).

3.3.6. Colour analysis of control and cross-linked protein concentrate dispersions as a function of sodium hexametaphosphate concentration

The colour of C-MPC and TG-MPC dispersions containing 0, 5, 12.5 and 25 mM SHMP were measured using a Minolta Chroma Meter CR-400 colorimeter (Minolta Ltd., Milton Keynes, UK). Data was calculated using three parameters L^*, a^* and b^* to describe the colour spectrum of a sample within the three-dimensional visible colour range. The colorimeter was calibrated against a white standard prior to analysis. Measurements were taken three times and the mean calculated. Measurement data was displayed as L^*, which represents a scale from black (0) to white (100), a^* which represents the green-red spectrum with a range from -60 (green) to +60 (red) and b^* which represents the blue-yellow spectrum, ranging from -60 (blue) to +60 (yellow), respectively (Mohammadi *et al*, 2008).

Total colour difference (ΔE) was calculated using Equation 1 where L_o, a_o and b_o refer to the colour of C-MPC and TG-MPC dispersions without SHMP addition, while *L*, *a* and *b* denote the respective colour parameters of samples containing SHMP.

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \qquad \text{(Eq.1)}$$

3.3.7. Statistical data analysis

All trials and measurements were carried out in triplicate. Rheological measurements were analysed using a paired T-test with a 95% confidence interval. Particle size and colour data was statistically analysed using one-way analysis of variance (ANOVA), with posthoc Tukey analysis. The level of significance was

considered as P < 0.05. All statistical analysis was carried out using Minitab 17 (Minitab Inc, Coventry, United Kingdom).

3.4. **Results and discussion**

3.4.1. Rheological and particle size analysis of milk protein concentrate dispersions

The viscosity of C-MPC and TG-MPC dispersions decreased with increasing temperature. For example, the viscosity of C-MPC was significantly ($P < 0.05$) lower at 20°C (11 mPa.s) than at 4°C (37 mPa.s) (Fig. 3.1). The viscosity of TG-MPC dispersions showed a similar trend (i.e., 15 mPa.s at 4°C and 8 mPa.s at 20°C; Fig. 3.1), with the viscosity of C-MPC and TG-MPC dispersions decreasing progressively with increasing temperature; however, there was no significant ($P > 0.05$) difference in viscosity between non-crosslinked and crosslinked protein dispersions at 30 or 50°C. A previous study by Ho *et al* (2018) showed similar results to the present study where the viscosity of MPC increased with decreasing temperature from 55 to 25°C. This decrease in viscosity could be attributed to a higher degree of flexibility within the casein micelles and reduced interactions between casein micelles, due to a decrease in intra- and intermolecular hydrophobic interactions, respectively. This is in agreement with particle size distribution profiles shown in Fig. 3.2, whereby the z-average diameter for C-MPC samples decreased with increasing temperature from 163 nm at 4°C to 156 nm at 50°C. Weakening of hydrophobic interactions within the casein micelle, results in the release of β-casein from the casein micelle into the serum phase at low temperature (<20°C), leading to increased micelle hydration and size (Zhao and Corredig, 2016).

Conversely, particle size values were lower for TG-MPC dispersions at 4°C (158 nm) than at 50°C (170 nm; Fig. 3.2). The contrasting trend observed for TG-MPC

dispersions compared to C-MPC dispersions could potentially be due to the covalent bond network created by TGase-induced crosslinking of casein proteins restricting free movement of proteins (i.e., β-casein) within and out of the casein micelle at 4°C. Therefore, while hydrophobic bonds are weaker at 4°C, swelling is prevented due to the more rigid cross-linked protein structure in the TGase treated samples. Casein proteins, both in micellar and non-micellar form, are the primary substrate for enzymatic crosslinking, which is due to the abundance of glutamic acid within casein. TGase catalyses cross-linking of peptide chains through the formation of an isopeptide bond between the γ-carboxyamide group of glutamine side chains and an amine donor of neighbouring lysine or glutamine residues depending on steric location (Jaros *et al* 2006; Moon *et al* 2009; O'Sullivan *et al* 2002). Ercili-Cura *et al* (2013) reported that gels produced from TGase modified milk had a significantly higher water holding capacity because of restricted particle movement due to crosslinking. Prior incubation of milk with TGase resulted in gels consisting of a higher order network of small aggregates with defined pore sizes; hence preventing network contraction/rearrangement and subsequent syneresis of water. Potentially the high water holding capacity of TG-MPC dispersions could result in larger particle size values as a consequence of casein micelle swelling at increased temperatures (i.e. 50°C: Fig. 3.2D).

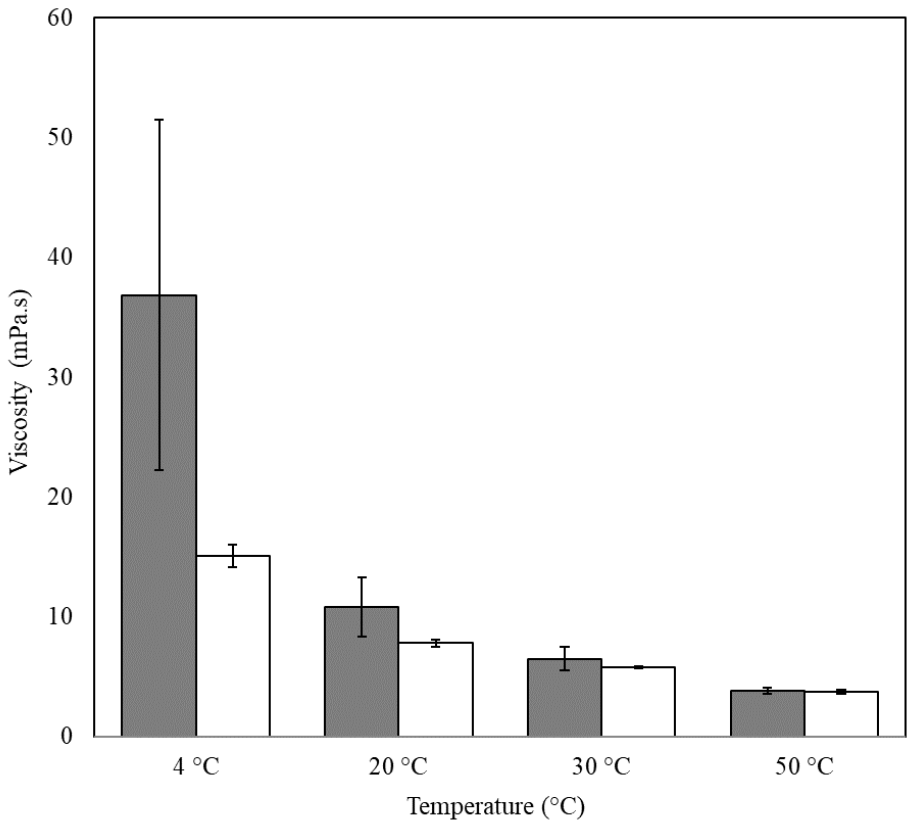


Fig. 3.1 *Apparent viscosity of control (■) and transglutaminase cross-linked (□) milk protein concentrate dispersions (10%, w/w, protein) at 4, 20, 30 or 50°C, measured at a shear rate of 100 s-1. Values presented are the mean values ± SD.*

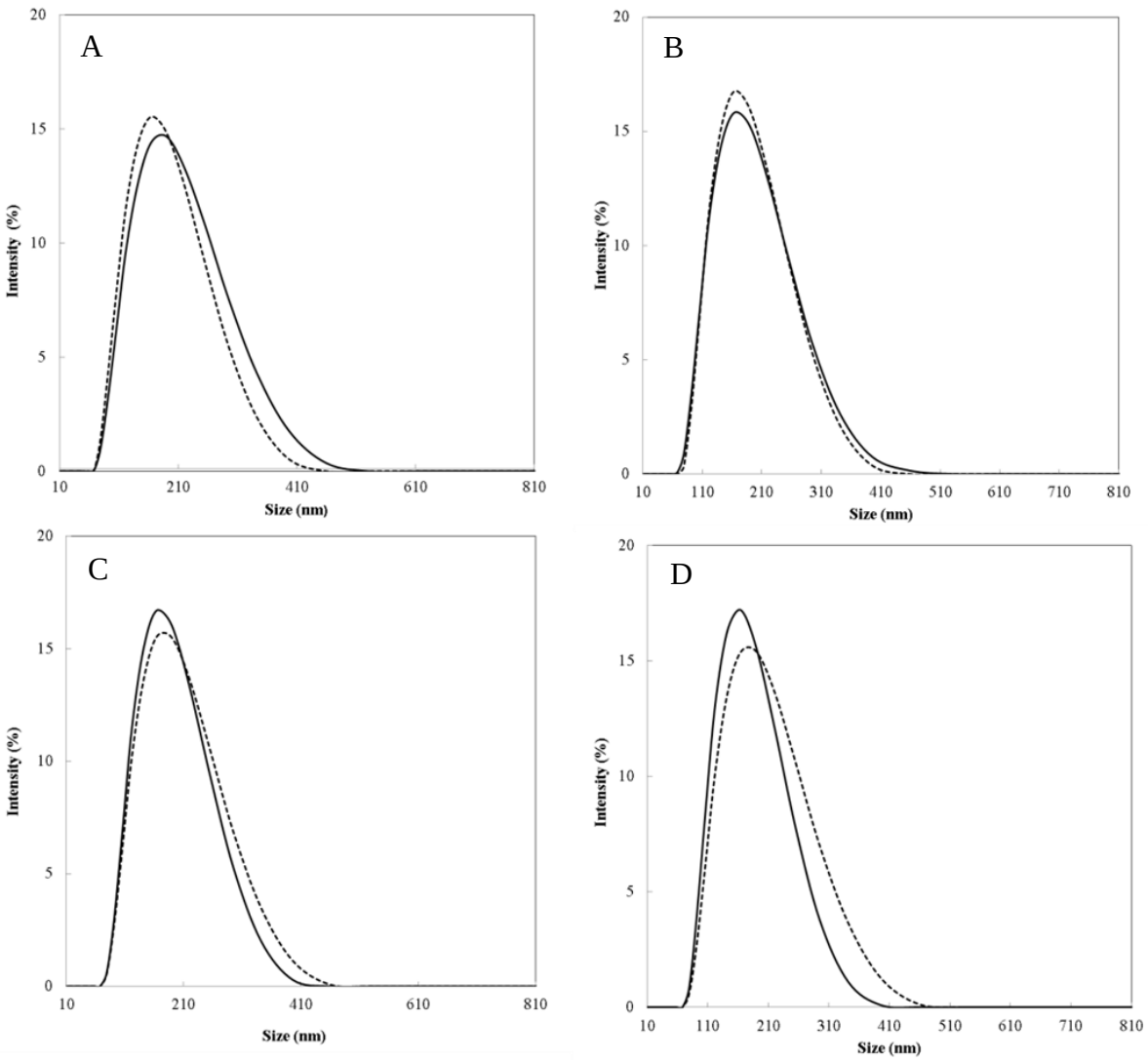


Fig. 3.2. *Particle size distribution profiles of control (—) and transglutaminase cross-linked (--) milk protein concentrate dispersions (10%, w/w, protein) measured at temperatures of 4 (A), 20 (B), 30 (C) or 50°C (D).*

3.4.2. Zeta-potential of milk protein concentrate dispersions

The ζ-potential of C-MPC and TG-MPC dispersions as a function of pH are shown in Fig.3.3. At an initial pH of 6.7, C-MPC had a ζ-potential of -18.8 mV while TG-MPC had a ζ-potential of -20.7 mV (Fig. 3.3). These values are in line with previous work carried out on skim milk at pH 6.7, which had a ζ-potential of −18 mV (Wade *et al*, 2009). As expected, the ζ-potential of both C-MPC and TG-MPC dispersions became less negative with decreasing pH. Nogueira *et al* (2019) reported similar results with ζ-potential values of ~ -20 mV at pH 6.0 for a cross-linked micellar

casein system. However, in the present study, the ζ-potential at pH 5.5 for C-MPC and TG-MPC was -14.3 and -13.9 mV, respectively, compared to Nogueira *et al*, (2018) who reported a ζ-potential of -18 mV at pH 5, while at pH less than 5.5 in the present study, the ζ-potential was not measured due to extensive precipitation of casein. The fact that there were no significant differences in ζ-potential between C-MPC and TG-MPC dispersions showed that crosslinking did not alter the surface charge of the casein micelles. This is in line with previous work carried out by de Kruif *et al*, (2002) who showed, using small-angle neutron scattering (SANS), that crosslinking of casein micelles caused little or no restructuring of the casein micelle other than the formation of covalent linkages.

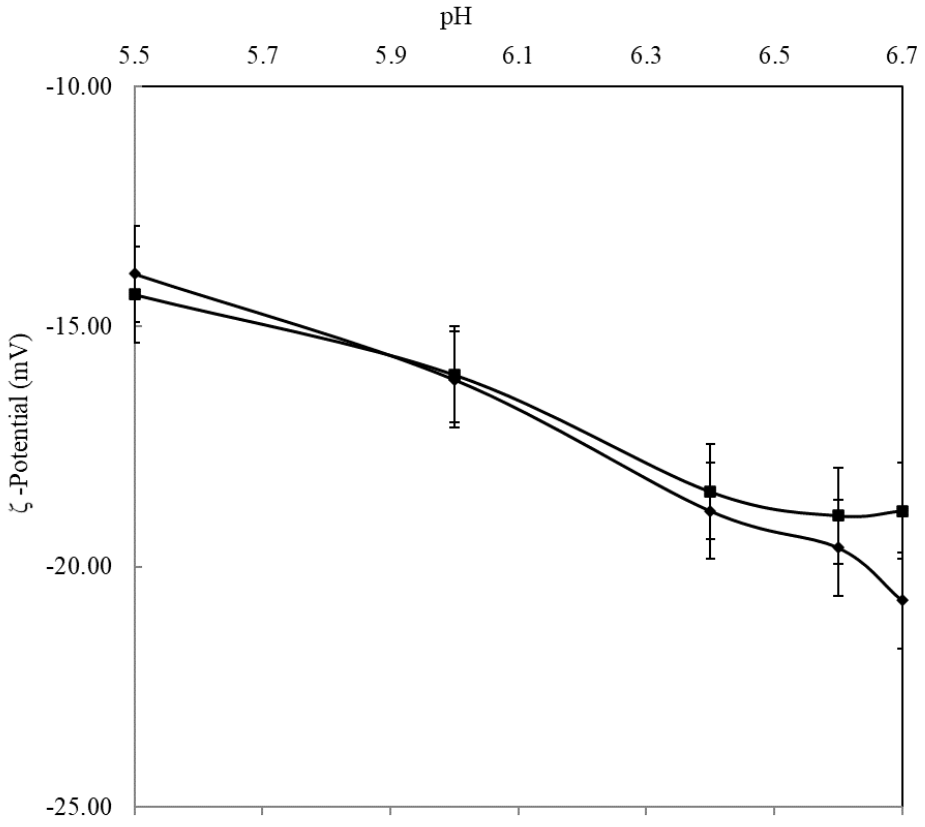


Fig. 3.3*. Zeta-potential of control (■) and transglutaminase cross-linked (♦) milk protein concentrate dispersions (10%, w/w, protein), as a function of pH. Values presented are the mean values ± SD.*

3.4.3. Particle size and colour analysis of milk protein concentrate dispersions containing sodium hexametaphosphate

C-MPC dispersions without (0 mM) and with 5 mM SHMP addition displayed narrow monomodal size distribution profiles, with particle size ranging from 68.1 to 459 nm (Fig. 3.4A). However, C-MPC dispersions containing 12.5 and 25 mM SHMP were significantly different, with a shift in profile towards a broader particle size distribution (Fig. 3.4A). The effect of SHMP addition on the size distribution profiles of MPC dispersions was also highlighted by the significant ($P < 0.05$) changes in polydispersity index (PdI) (Table 3.1), with PdI values of C-MPC dispersions increasing (0.09-0.41) with increasing SHMP content (Table 3.1). Particle size results for C-MPC dispersions correlated well with colour analysis (Table 3.2), with C-MPC dispersions containing 0 and 5 mM being relatively similar in terms of L^*-values (82.7 and 80.2, respectively) but with some differences observed in b^*-values, in conjunction with a ΔE value (i.e., 3.10; Table 3.2) denoting a visible change. A significantly ($P < 0.05$) lower L^*-value was observed for C-MPC dispersions containing 12.5 and 25 mM SHMP (48.3 and 46.0, respectively), with respective ΔE values of 34.5 and 37.0 (Table 3.2; Fig. 3.4A inset). Considered collectively, these data provide evidence for the dissociation of casein micelles into primary casein particles (De Kort *et al*, 2009, 2011; Panouillé *et al*, 2005; Pitkowski *et al*, 2008). Strong polyphosphate-based calcium chelators, such as SHMP, cause partial disintegration of casein micelles by the depletion of calcium from the casein micelle, resulting in partial collapse of the micelle, releasing individual casein proteins/particles into the serum phase.

No differences were observed in particle size distribution profiles for TG-MPC dispersions containing 5 and 12.5 mM SHMP compared with the control. Previous studies (Myllärinen *et al*, 2007; O'Sullivan *et al*, 2002) showed similar results to those

found in the current study, with TGase-treated casein micelles having increased resistance to dissociation during calcium depletion. Only when 25 mM SHMP was added to TG-MPC dispersions was a broadening of particle size distribution profile observed, with a primary peak between 78.8 and 825 nm, indicating increased casein micelle swelling and hydration, with a secondary smaller peak between 28.2 and 78.8 nm, indicating the presence of smaller casein micelle fragments (Fig. 3.4B). These primary casein particles have been shown to have a diameter of approximately 20 nm (De Kort *et al*, 2009 and 2011; Panouillé *et al*, 2005; Pitkowski *et al*, 2008, Huppertz *et al*, 2017). This was also observed in PdI values for TG-MPC samples, with no significant ($P > 0.05$) difference in dispersions between 0 and 12.5 mM (0.13 to 0.14), while at 25 mM, the PdI value was significantly ($P < 0.05$) higher at 0.24 (Table 3.1). Similarly, the L^*-value for TG-MPC dispersions also decreased with increasing SHMP addition level (L^*-value decreased from 84.0 to 59.9; Table 3.2), albeit, to a significantly ($P < 0.05$) lesser extent than observed for C-MPC dispersions. The resistance of TG-MPC dispersions to disintegration, and retention of whiteness, can be related to the strong isopeptide bond formed between amino acids during TGase incubation process. Therefore, casein micelles treated with TGase had improved micelle stability and as a result retained more light scattering ability in the presence of high concentrations of SHMP (see Fig. 3.4B inset).

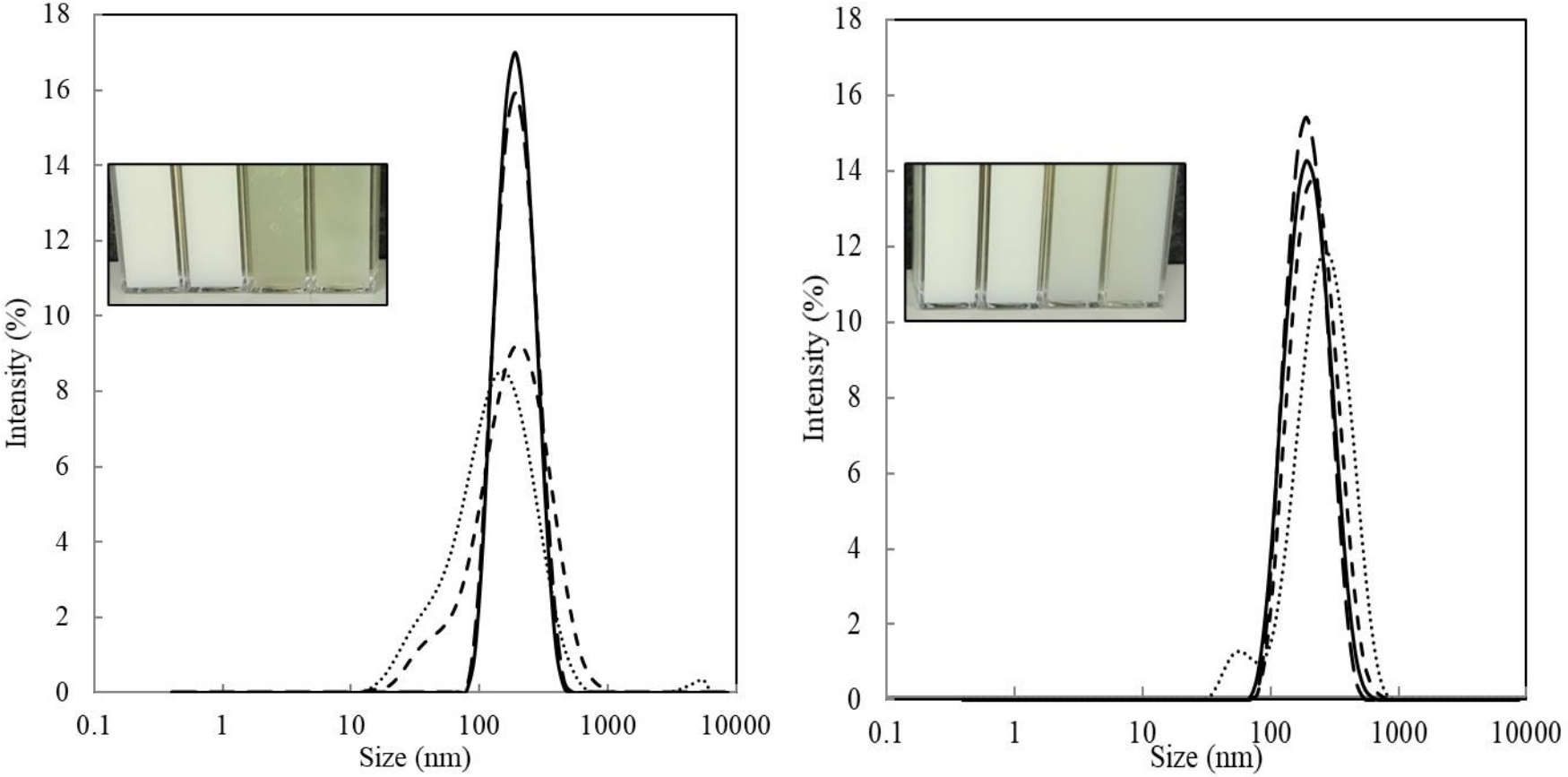


Fig. 3.4. *Particle size distribution profiles of control (a) and transglutaminase cross-linked (b) milk protein concentrate dispersions (10%, w/w, protein) containing 0 (—), 5 (— —), 12.5 (---) and 25 (•••) mM sodium hexametaphosphate. Inset: Photographic image of control and transglutaminase cross-linked milk protein concentrate dispersions containing 0 (i), 5 (ii), 12.5 (iii) or 25 (iv) mM sodium hexametaphosphate*

Table 3.1. *Particle size distribution parameters for control (C-MPC) and transglutaminase cross-linked (TG-MPC) milk protein concentrate dispersions with added sodium hexametaphosphate (SHMP).*

SHMP concentration	C-MPC		TG-MPC	
	Z-Average	PdI	Z-Average	PdI
(mM)	(nm)	(-)	(nm)	(-)
0	180[a]	0.09[a]	182[a]	0.13[a]
5	180[a]	0.10[a]	182[a]	0.12[a]
12.5	138[b]	0.34[b]	200[b]	0.14[a]
25	988[c]	0.41[c]	211[c]	0.24[b]

Z-Average – Intensity weighted mean hydrodynamic size of particles measured by dynamic light scattering (DLS)
PDI- polydispersibility Index
Values within a column not sharing a common superscript letter differ significantly ($P < 0.05$)

Table 3.2. *Colour chromaticity co-ordinates of control (C-MPC) and transglutaminase cross-linked (TG-MPC) milk protein concentrate dispersions with added sodium hexametaphosphate (SHMP).*

SHMP concentration	C-MPC				TG-MPC			
(mM)	L^*	a^*	b^*	ΔE	L^*	a^*	b^*	ΔE
0	82.7[a]	-4.20[c]	2.06[b]	-	84.0[a]	-4.14[a]	1.79[a]	-
5	80.2[a]	-4.67[c]	0.29[d]	3.10[a]	82.5[a]	-4.63[a]	1.04[b]	1.76[a]
12.5	48.3[b]	-1.65[b]	1.93[c]	34.5[b]	66.3[b]	-5.08[a]	-4.33[c]	18.8[b]
25	46.0[c]	-0.67[a]	3.84[a]	37.0[c]	59.9[c]	-4.26[a]	-4.45[c]	24.9[c]

L^* − Whiteness
a^*− Green-red spectrum with a range from -60 (green) to +60 (red)
b^* − Blue-yellow spectrum with a range from -60 (Blue) to +60 (yellow)
ΔE - Total colour difference
Values within a column not sharing a common superscript letter differ significantly ($P < 0.05$).

3.4.4. Viscosity measurements of milk protein concentrate dispersions containing sodium hexametaphosphate

Viscosity profiles of C-MPC and TG-MPC dispersions with added SHMP at concentrations of 0, 5, 12.5 and 25 mM are shown in Fig. 3.5. At 5 mM SHMP addition both C-MPC and TG-MPC dispersions had higher viscosity with final values of 25 and 29 mPa.s, respectively, compared to the respective samples without SHMP addition. Significantly ($P < 0.05$) higher viscosity was observed in C-MPC dispersions containing 12.5 and 25 mM SHMP, with final viscosities of 48 to 3217 mPa.s and from 72.7 to 3838 mPa.s, respectively (Fig. 3.5A). This higher viscosity can be attributed to chelation of calcium, which causes diffusion of calcium from the micelle into the serum phase and a reduction in the proportion of micellar CCP. The reduction in CCP causes increased electrostatic repulsion between casein proteins within the micelle, which together with the loss of the CCP-mediated casein protein cross-links, causes reduced structural integrity and increased swelling of the casein micelles (De Kort *et al*, 2009 and 2011; Holt, 1992).

TG-MPC dispersions displayed considerably less change in viscosity with addition of SHMP (Fig. 3.5B). At higher addition levels of SHMP (i.e., 12.5 and 25 mM), TG-MPC dispersions had significantly ($P < 0.05$) lower final viscosity values (162 and 991 mPa.s, respectively), compared to the corresponding C-MPC dispersions (3212 and 3838 mPa.s, respectively). The resistance of TG-MPC to increases in viscosity on the addition of SHMP can be attributed to the impact of enzymatic crosslinking, which creates a secondary structural organisation within the casein micelles, conferring increased resistance to micellar disintegration. TGase-mediated crosslinking could also potentially hinder the access of SHMP to CCP within the casein micelle (Moon, *et al* 2009).

Previous work carried out by Power *et al* (2019) showed that a change in ^{31}p nuclear magnetic resonance signal occurred upon the addition of SHMP to reconstituted MPC dispersions, indicative of a change in the structure of phosphate prompted by an interaction between SHMP and phosphate-bound calcium associated with the casein micelle. Chelation increases exposure of negatively-charged phosphate residues through depletion of bound calcium, hence changing the charge on the phosphate residues and its associated resonance signal (Holt, 1997; Walstra, 1990). Therefore, TGase-mediated crosslinking may restrict the ability of SHMP to chelate calcium which is bound to phosphate residues on the casein micelle due to steric hindrance.

3.5. Conclusion

The chelation of calcium in milk protein concentrate dispersions significantly modifies the structural integrity of casein micelles, leading to increased viscosity. In this study, the use of transglutaminase to cross-link casein micelles prior to addition of sodium hexametaphosphate greatly reduced this viscosity development, with the effect being most pronounced at low temperature. Enzymatically crosslinking casein micelles also helped maintain the natural white colour of MPC even after SHMP addition. This may also be useful in other studies which use strategies such as ion-exchange to reduce the calcium content of micellar casein ingredients. Overall, this study provided new knowledge relating to the factors responsible for increased viscosity during calcium chelation, mainly casein micelle swelling and micelle dissociation, and demonstrated that enzymatic crosslinking is effective in controlling viscosity development in MPC systems with added calcium chelating salts.

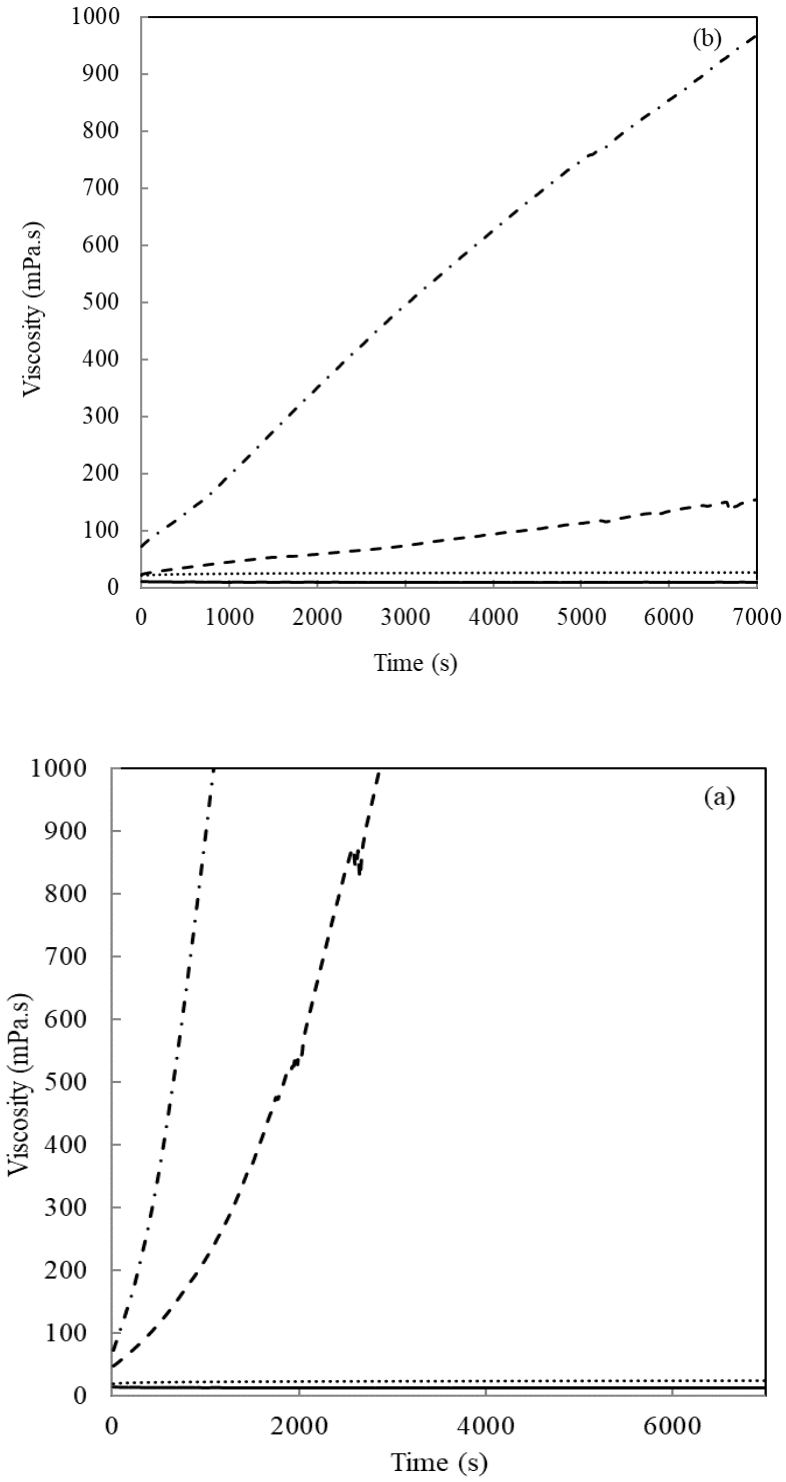


Fig. 3.5. *Viscosity profiles of control (A) and transglutaminase cross-linked (B) milk protein concentrate dispersions (10%, w/w, protein) containing 0 (—), 5 (···), 12.5 (- - -) and 25 (-·-·) mM sodium hexametaphosphate, measured at a shear rate of 100 s-1 at 20°C.*

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CHAPTER FOUR

Water sorption and hydration properties of high protein milk powders are influenced by enzymatic crosslinking and calcium chelation

Orla M. Power[1, 2], Valentyn Maidannyk [2], David J. McSweeney [1,2], Mark A. Fenelon[1], James A. O'Mahony[2], Noel A. McCarthy[1]

[1]Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[2]School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Declaration:

This chapter was written by author Orla M. Power (OMP) and reviewed by co-authors Dr. Noel A. McCarthy (NAMC), Dr. James A. O'Mahony (JAOM) and Dr. Mark Fenelon (MK). OMP co-designed the study with NAMC and performed all of the experimental work, with the exception that David J McSweeney (DJMS) assisted in temperature dependent dissolution and capillary rise measurements. Valentyn Maidannyk (VM) provided microscopy images and water sorption isotherms.

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4.1 Abstract

Calcium chelating agents, such as sodium hexametaphosphate (SHMP), can be added to milk protein (MPC) dispersions to aid in rehydration; however, this leads to a concomitant increase in dispersion viscosity due to micelle swelling/dissociation. Crosslinking casein proteins in MPC using transglutaminase (TGase) can help retain casein micelle structure and maintain low viscosity. This study aimed to determine the water sorption and hydration properties of MPC powders, as influenced by the crosslinking of milk proteins and the addition of SHMP. Crosslinked casein protein (TG-MPC) powders without SHMP addition had improved wettability, water sorption and water diffusion compared to the non-crosslinked control (C-MPC) powder. All powders containing SHMP were observed to have increased water sorption compared to control powders without SHMP addition. Powder dissolution data showed that increasing SHMP content increased powder particle size, compared to powders without SHMP addition, indicating increased powder particle swelling due to increased water uptake.

4.2. Introduction

Milk protein concentrate (MPC) powders have numerous commercial applications and are used as ingredients in value added dairy products, such as in high protein yogurts, medical nutrition, infant milk formula, dairy-based beverages and muscle building products (Crowley *et al*, 2016; de Kort *et al*, 2011; Havea, 2006; Sikand *et al*, 2011). MPC is manufactured from skim milk through ultrafiltration using membranes with a molecular weight cut-off of 5 to 10 kDa. Additional water is often added during filtration (i.e., diafiltration) to further remove lactose and minerals, allowing for even higher protein to solids ratios to be achieved. Liquid MPC is usually then heat-treated, evaporated and spray dried. Spray dried MPC powders (i.e., >60%, w/w, protein) have been shown to have challenges with rehydration with poor wetting and hydration properties making them difficult to incorporate in to finished products (Toikkanen *et al*, 2018; Wang *et al*, 2018).

Insolubility of freshly produced MPC powders can be mainly attributed to non-covalent hydrophobic interactions between proteins, due to the exposure of hydrophobic amino acids during the drying process (Baldwin and Truong, 2007). Casein proteins are highly hydrophobic and are able to assemble through the formation of weak hydrophobic forces and calcium binding, with a number of models proposed for the structure of the casein micelle (Dalgleish *et al* 2004; Holt *et al*, 2013; Lucey and Horne, 2018; McMahon *et al*, 2009). Several methods have been employed to improve solubility of MPC powders, with a number of these methods targeting casein micelle integrity through the depletion of calcium from the serum, and consequently, colloidal phases (Bhaskar *et al*, 2007; de Kort *et al*, 2011).

Casein micelle integrity is dependent on the equilibrium between serum and colloidal minerals, as well as attractive (e.g., hydrophobic interactions) and repulsive

(e.g., electrostatic repulsion and attraction) forces between proteins resulting in the dissociation of casein micelles and allowing water uptake and subsequent hydration (Horne, 2006; McMahon and Oommen, 2008; Kruif *et al*, 2012; Wong *et al*, 1996). The addition of calcium chelating salts to milk protein systems changes this electrostatic balance by binding calcium from the serum phase and depletion of calcium from colloidal calcium phosphate (CCP) nanoclusters, depending on the electrostatic strength of the calcium chelator (De Kort *et al*, 2009; McCarthy *et al*, 2010; Mizuno and Lucey, 2007; Panouillé *et al*, 2005)

Aside from powder insolubility, one of the major challenges associated with MPC production is the high viscosity incurred at relatively low solids content (i.e., 20-30%, w/w), limiting the achievable dry matter prior to spray drying, compared to skim milk concentrates which are dried at solids contents of ~50%, w/w, (Bienvenue *et al*, 2003; Park *et al*, 2016; Vélez-Ruiz *et al*, 1998). Increases in MPC viscosity can be attributed to increases in protein-protein interactions during heat treatment and the innate high water holding capacity of casein micelles (~3.3 g g^{-1} of protein; Huppertz *et al*, 2017). In addition, calcium chelation can cause further increases in viscosity depending on the strength of the chelator, resulting in dissociation of the casein micelle into multiple protein clusters and reduced turbidity (De Kort *et al*, 2009 and 2011; Panouillé *et al*, 2005; Pitkowski *et al*, 2008). Therefore, to combat high viscosity in MPC systems a previous study Power *et al* (2020) crosslinked milk proteins using the enzyme transglutaminase in an attempt to control viscosity during calcium chelation. Transglutaminase creates isopeptide bonds *via* an acyl transfer reaction between the carboxyl group of glutamine residues and the α-amino group of lysine residues, forming intra-micellar crosslinks (Hinz *et al*, 2007; Lorenzen *et al*, 2002; O'Sullivan *et al*, 2002; Sharma *et al*, 2001). However, the combined effects of crosslinking milk

proteins and the addition of SHMP on the subsequent water sorption and hydration properties of MPC powders are unknown. Thus, the objective of this study was to determine if the addition of SHMP could improve powder rehydration properties and if protein crosslinking (to control viscosity) would affect these attributes.

4.3. Materials and methods

4.3.1. Materials

Milk protein concentrate (MPC) powder was obtained from a local dairy ingredient manufacturer. The protein, moisture, fat and ash content of this MPC was 81.4% (w/w), 4.30% (w/w), 1.40% (w/w) and 7.8% (w/w), respectively. The lactose content was determined by difference as 5.1% (w/w). Transglutaminase (Activa MP, Ajinomoto enzyme, Germany) was obtained from Healy Group (Tallaght, Co. Dublin). Sodium hexametaphosphate (SHMP) was obtained from Sigma Aldrich ltd (Vale Rd, Ballyraine Lower, Arklow, Co. Wicklow).

4.3.2. Pilot-scale production of milk protein concentrate powders with enzymatic crosslinking and calcium chelator addition

Milk protein concentrate powder was rehydrated (10%, w/w, total solids) at 50°C using a Y-Tron ZC-1 high shear mixer (YTRON, Process Technology GmbH & Co. KG, Handwerkerpark, Germany). Following rehydration, the MPC dispersion was cooled to 30°C and divided into a control batch and enzyme modified batch (batch size 16 kg). Protein crosslinking was carried out by incubating the MPC dispersion with the TGase enzyme (0.5 g/L) at 30°C and stored for 24 h under gentle agitation to allow sufficient crosslinking of casein proteins to occur (TG-MPC; Smiddy *et al*, 2006). The control batch (C-MPC) was also treated under the same incubation

conditions but without the addition of TGase. Sodium azide (0.02% w/w) was added to prevent microbial growth in both batches. Following incubation, each batch was sub-divided and combined with SHMP to give final chelator concentrations of 0, 2.5, 5, 10 or 25 mM. All MPC dispersions were then stirred for 30 min before being heated to 50°C prior to drying using a single-stage spray dryer equipped with a two fluid nozzle. Inlet and outlet air temperatures were set at 187 and 83°C, respectively. All powders were stored for analysis in aluminium foil bags.

4.3.3. Particle size measurement of milk protein concentrate powders

The particle size distribution (volume in %) of C-MPC and TG-MPC powders were determined using a Malvern Morphologi G3 (Malvern Instruments, Malvern, UK) image analysis-based particle characterisation system (McCarthy *et al*, 2014)

4.3.4 Scanning electron microscopy of milk protein concentrate powders

C-MPC and TG-MPC powder samples were attached to double-sided adhesive carbon tabs and mounted on scanning electron microscope (SEM) stubs, after which tabs were coated with chromium as per the method used by Maidannyk, *et al* 2019 (K550X, Emitech, Ashford, UK:). Scanning electron microscopy images were collected using a Zeiss Supra 40P field emission SEM (Carl Zeiss SMT Ltd., Cambridge, UK) at 2.00 kV. Representative micrographs were taken at 200×, 500×, 1000×, 5000× and 10000× magnification.

4.3.5 Capillary rise of water in milk protein concentrate powders

Wettability was determined for C-MPC and TG-MPC powders using a modified version of the Washburn capillary rise method, as described previously by Ji *et al* (2015) and Ji *et al* (2016). Powders were loaded into a glass cylindrical tube with the bottom covered by a filter. The tube was then held at the surface of a body of distilled water at 20°C and wettability was calculated based on the increase in mass of the powder when weighed after 10 min of absorption. All measurements were repeated five times to insure repeatability.

4.3.6. Determination of the initial water content of milk protein concentrate powders

The water content of spray dried powders was determined by weighing ~1.0 g of each powder and placing them in a Jeio Tech vacuum oven (Jeio Tech®, Seoul, Korea) at 70°C and at an absolute pressure of <10 mBar for 24 h. The difference in mass of samples before and after drying (g/100g of dry solids) was defined as the initial water content (IWC).

4.3.7 Water sorption analysis of milk protein concentrate powders

Water sorption analysis was carried out on vacuum dried C-MPC and TG-MPC powders after storage in desiccators over phosphorous pentoxide (P_2O_5). Each powder was stored in an evacuated desiccator (21°C) for 120 h over saturated solutions of lithium chloride (LiCl), potassium acetate (CH_3COOK), magnesium chloride ($MgCl_2$), potassium carbonate (K_2CO_3), magnesium nitrate ($Mg(NO_3)_2$), sodium nitrite ($NaNO_2$), sodium chloride (NaCl) and potassium chloride (KCl) (Sigma Chemical Co., St. Louis, MO. U.S.A.), which at equilibrium provided water activities (a_w) of 0.11, 0.23, 0.33, 0.44, 0.545, 0.66, 0.76 and 0.85, respectively. Water activity

of each powder was measured after storage using a Novasina water activity meter (Labmaster.a_w, Novatron, London, UK). Powders were weighed at intervals of 0, 2, 4, 6, 8, 10, 24, 48, 72 h and then every 24 h up to 240 h (Potes *et al*, 2014). The water content in each system was plotted as a function of time, and the Guggenheim-Anderson-de Boer (GAB) relationship was fitted to data to relate water activity and water content of anhydrous powders (Eq. 1):

$$\frac{m}{m_0} = \frac{Cka_w}{(1- ka_w)(1 - ka_w + Cka_w)} \quad (1)$$

Where m is the water content (g of water/100 g of dry solids), m_0 - the monolayer value of water content, C, k - constants related to energy constant, which can be calculated from m_0.

4.3.8. Confocal laser scanning microscopy of milk protein concentrate powders

A Leica TCS SP5 confocal laser scanning microscope (CLSM; Leica Microsystems CMS GmbH, Wetzlar, Germany) was employed for powder particle visualisation. The DPSS 561 nm laser was engaged. C-MPC and TG-MPC powder particles were placed onto a glass slide and labelled using rhodamine dye (0.1%, Rhodamine B; Sigma-Aldrich Co., St. Louis, MO, USA.) in ultrapure water. The confocal images of each system were taken using 20x air and 63x oil immersion objective with numerical aperture of 1.4. The areas of particles were measured using Image Pro Premier 3D software and z-Stacks were obtained in order to generate a 3-D structure of selected particles. Green pseudo-coloured pictures (8-bit), 512x512

pixels in size, were acquired using a zoom factor of 1-3 (Maher *et al*, 2015; Maidannyk *et al*, 2019).

4.3.9 Real time visualisation of liquid phase water diffusion in milk protein concentrate powders

Real time visualisation of liquid phase water diffusion in C-MPC and TG-MPC powders was carried out in accordance with the method described by Maidannyk *et al* (2019). Rhodamine B and PEG 200 were added to anhydrous C-MPC and TG-MPC powders. Images were obtained at fixed time intervals using a Leica TCS SP5 CLSM and particle diameters were detected using the Leica TCS SP5 software in the size range 3-200 μm. The areas of individual powder particles were measured using spherical approximation which allowed for the local effective diffusivity of the liquid phase in individual powder particles to be calculated.

4.3.10. Bulk diffusion coefficients for milk protein concentrate powders

The effective bulk diffusivity of C-MPC and TG-MPC powders was determined experimentally from water kinetic profiles as per the method outlined by Maidannyk *et al* (2019). It was assumed that water sorption kinetics are limited only by diffusion. The solution of Fick's second law equation for one-dimensional slab can be obtained as shown in Eq. 2 (Murrieta-Pazos *et al*, 2011)

$$\Gamma = \frac{(\mathrm{M}-\mathrm{Me})}{(\mathrm{Mo}-\mathrm{Me})} = \frac{8}{(\Pi)^2}\sum_{n=0}^{\infty}(2n+1)^{-2}\exp\frac{Deff(2n+1)^2\,(\Pi)^2 t}{4L^2} \quad (2)$$

Where M_e is equilibrium moisture content, M_o is initial moisture content, M is moisture content at time t and L is thickness of the slab. This equation assumes that the initial moisture content is uniform and that surface moisture and density are constant. The thickness of the sample volume (L) was measured using a numeric micrometer (5 times) and was assumed to be constant for each powder. Eq. 3 has been used to determine the effective or apparent diffusion (Lomauro *et al*, 1985)

$$Deff = -\left(\frac{4L^2}{\Pi^2}\right) * Slope \quad (3)$$

The slope was determined from a plot of LnΓ versus time. When a break in this plot was observed, diffusion coefficients were determined for each part of the curve (Crank, 1979; Rizvi, 1986)

4.3.11. Temperature dependant dissolution of milk protein concentrate powders

Solubility characteristics of C-MPC and TG-MPC powders were analysed using a laser-light diffraction unit (Malvern Mastersizer, Malvern Instruments Ltd, Worcestershire, UK). Dispersions were prepared by adding 7 g of MPC powder to 100 mL of tempered deionised water at 20 or 40°C along with 3 drops of silicon antifoam emulsion. Dispersions were high shear mixed for 30 s using a solubility index mixer (Labinco l295, Labinco B.V, Netherlands). Following mixing, dispersions were transferred to beakers and allowed to rest for 30 s after which aliquots were added to distilled water re-circulating at 20°C in the dispersion unit (Hydro MV; Malvern Mastersizer) at 1760 rpm. Measurements were performed using particle and dispersant refractive indices of 0.001 and 1.33, respectively. All particle size distribution profiles and mean diameters ($D_{(4,3)}$) presented were carried out in triplicate.

4.3.12. Data analysis

Water sorption and morphology analyses were performed in triplicate. Confocal microscopy data was obtained using five replicates. Statistical analysis was carried out using one-way analysis of variance (ANOVA), with posthoc Tukey analysis using SPSS software (SPSS V.18, IBM, New York, US). The level of significance was determined at $P < 0.05$.

4.4. Results and discussion

4.4.1. Powder particle size analysis

The powder particle size distribution for C-MPC and TG-MPC are given in Fig. 4.1. The majority of C-MPC powder particles were in the size range 0-200 µm (Fig.4. 1A), while the addition of SHMP caused a shift in size distribution towards larger particles with the extent of this shift increasing with increasing SHMP concentration (i.e., 0 to 10 mM)., It is noted that C-MPC dispersions containing 25 mM SHMP developed such high viscosity prior to drying that atomization was not possible and so powder was not produced (Avvaru *et al*, 2006). TG-MPC without SHMP had a particle size distribution ranging from 0-100 µm (Fig. 4.1B). TG-MPC powders containing SHMP had a maximum particle size of ~300 µm (Fig. 4.1B), compared to C-MPC powders which had a maximum particle size well in excess of 300 µm (Fig. 4.1A). The increase in powder particle size with increasing SHMP addition level in both C-MPC and TG-MPC may be explained by the increase in viscosity of the MPC feed prior to spray drying.

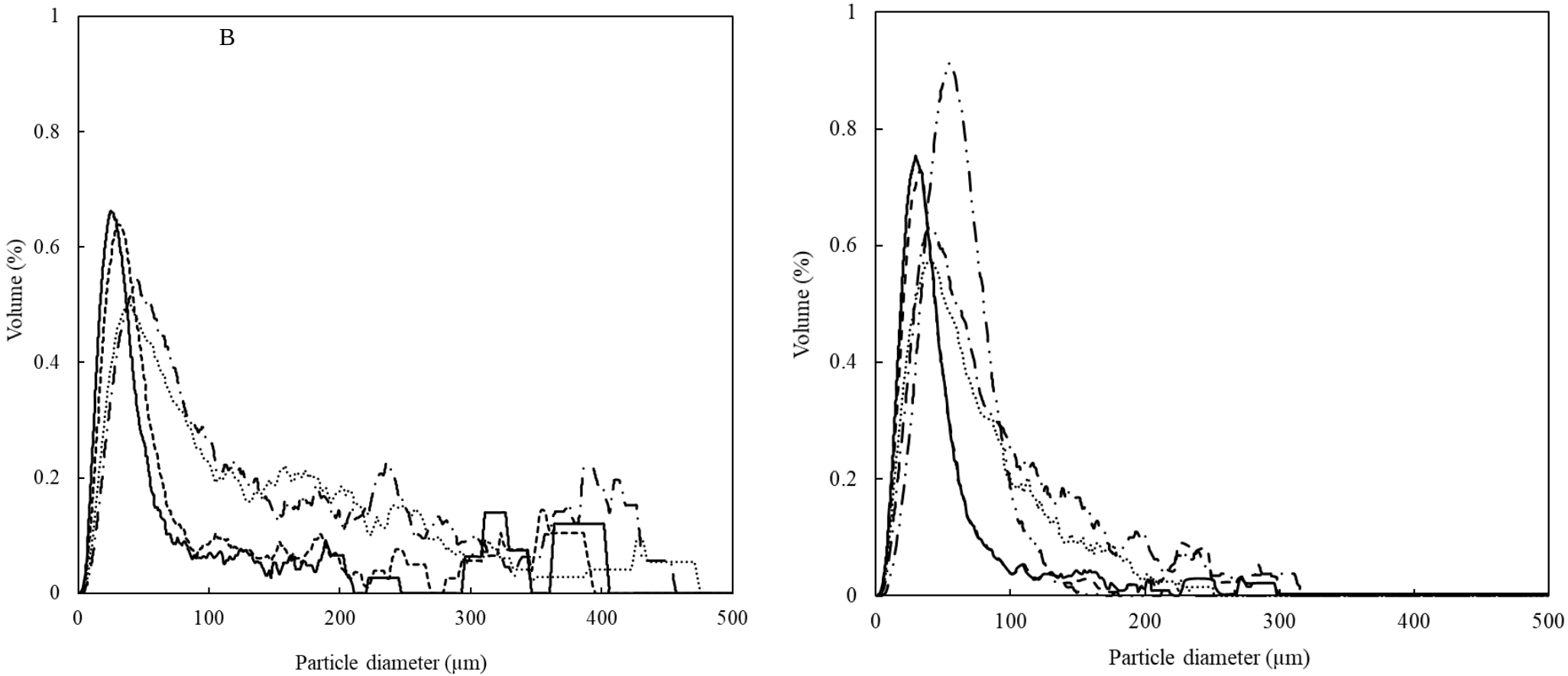


Fig. 4.1. *Powder particle size distribution for anhydrous control (A) and crosslinked (B) casein protein powders, containing 0 (—), 2.5 (---), 5 (···), 10 (−·−·) and 25 mM (−··−) sodium hexametaphosphate.*

Power *et al* (2020), showed that the addition of 5 mM SHMP to MPC systems caused a significant increase in viscosity from 13 to 125 mPa.s. Murphy *et al* (2013) found similar results to those presented in the current study, with a significantly larger particle size for infant formula powders produced from a high viscosity concentrate compared to the same system with a low viscosity. Therefore, if the liquid feed viscosity increases, but the energy supplied to the atomization nozzle remains constant then the atomized droplet size will be greater. The feed viscosity also affects spray pattern with a more viscous liquid typically producing a narrower spray angle. Increased viscosity can also sometimes increase the product flow rate and contribute to the formation of even larger droplets. Power *et al* (2019) described the mechanism behind the increased viscosity with increasing SHMP concentration through micelle swelling and dissociation, *via* chelation of calcium in the aqueous phase and potential solubilisation of CCP. Mineral chelation also changes the electrostatic balance in such high protein systems, and therefore impacts protein-protein interactions (Power *et al*, 2020; De Kort *et al*, 2011). SEM images of C-MPC powders showed that increasing the SHMP concentration caused the surface morphology of particles to progressively change from having a shrivelled, collapsed shape to a smoother, spherical particle surface (Supplementary data: Fig. S41A-D). In contrast, TG-MPC powder morphology remained relatively unchanged with increasing SHMP concentration (Supplementary data: Fig. S41E-H).

4.4.2. Capillary rise and water sorption kinetics

Capillary rise data for C-MPC and TG-MPC powders is shown in Fig. 2. Water absorption in general increased with increasing levels of SHMP in both C-MPC and TG-MPC powders. TG-MPC powders, with and without SHMP, had higher levels of

absorbed water than the corresponding C-MPC powders. Water sorbance was almost two-fold higher in C-MPC and TG-MPC powders containing 10 mM SHMP (0.07 and 0.10 g, respectively) compared to C-MPC and TG-MPC powders with no SHMP added (0.04 and 0.05 g, respectively). Ji *et al* (2015) showed similar results to the current study for non-agglomerated and agglomerated whey protein isolate powders with water absorption levels of 0.05 and 0.07 g, respectively. Water sorption profiles of C-MPC and TG-MPC powders as a function of relative humidity are shown in Fig. 4.3A and B, respectively. All powders showed an increase in water content with increasing relative humidity, with no evidence of lactose crystallization occurring, similar to results shown by Hogan and O'Callaghan (2010) for MPC powders. Similar to the trend observed for the capillary rise data, all TG-MPC powders were found to contain higher water content than the corresponding control powders at the same relative humidity. At a relative humidity of 85% C-MPC powders containing 10 mM SHMP had a final water content of 21.2 g/100 g compared to 17.3 g/100 g for C-MPC powders without SHMP. While water sorption values for TG-MPC were significantly ($P < 0.05$) higher than C-MPC powders, water content did not significantly increase with increasing SHMP content (Fig. 4.3B). Water sorption data was modelled using the GAB sorption isotherm model and is shown in Fig. 4.4. The data showed the increase in water content for TG-MPC powders compared to C-MPC powders, especially at water activity values in the range of 0.1 to 0.5 (Fig. 4.4A and B). All powders exhibited typical sorption isotherm profiles for non-crystalline high protein powders, with the GAB model having a good fit with the experimental data over the water activity range 0.1 to 0.9.

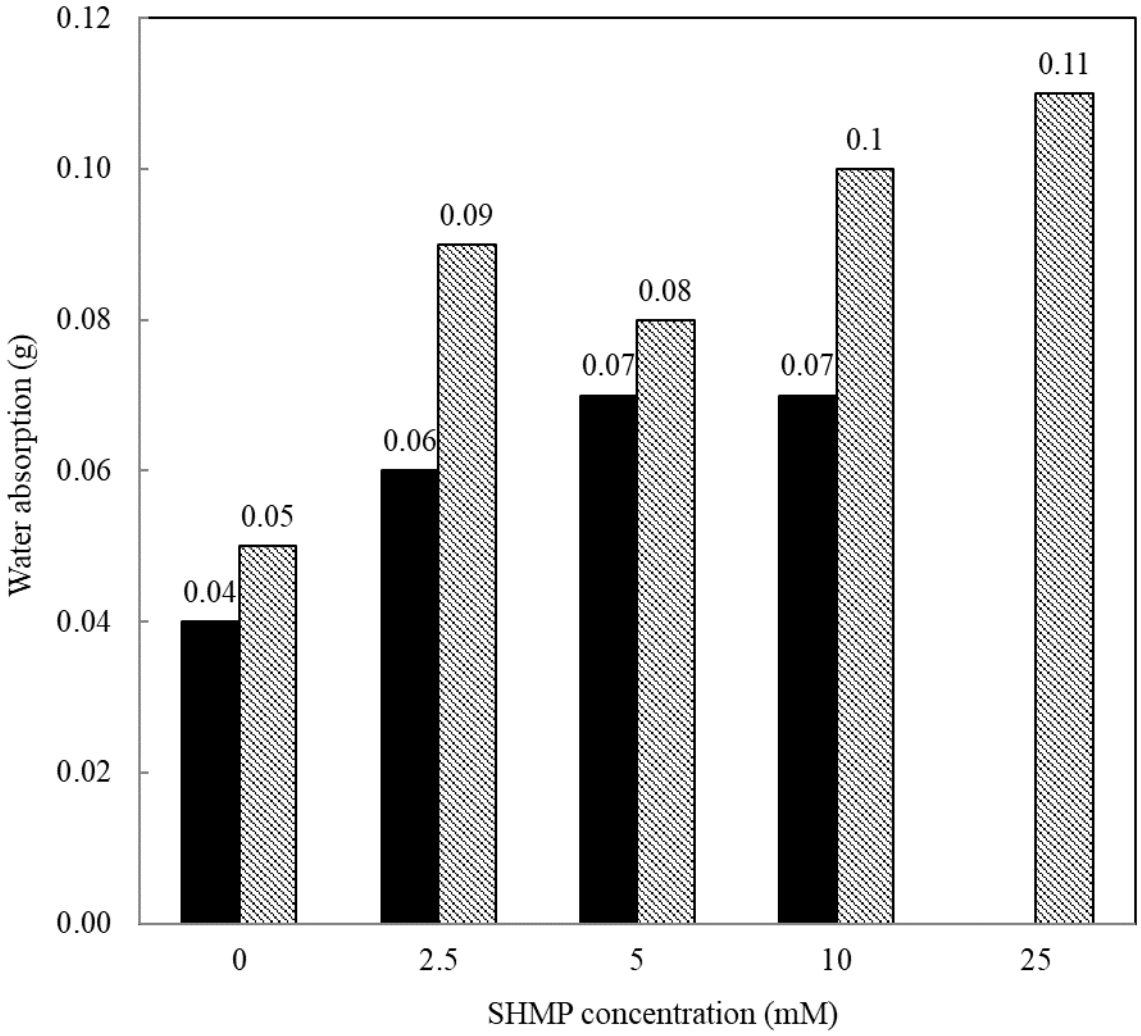


Fig. 4.2. *Water absorbance through capillary rise for control (■) and crosslinked (□) milk protein powders containing 0, 2.5, 5, 10 and 25 mM sodium hexametaphosphate.*

A

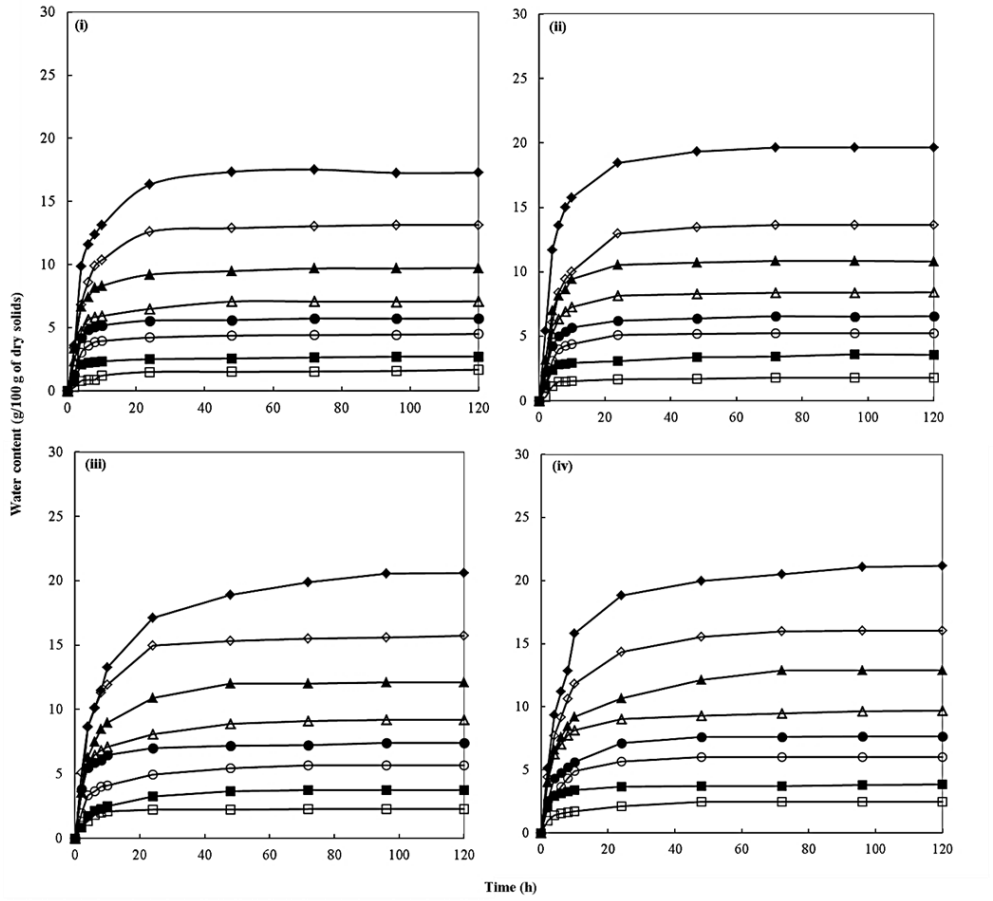


Fig. 4.3a. *Water sorption kinetics for control milk protein concentrate powders at 0 (i), 2.5 (ii), 5 (iii) and 10 mM (iv) sodium hexametaphosphate (SHMP) addition, measured at relative humidity values of 11 (□), 23 (■), 33 (○), 44 (●), 55.5 (Δ), 65 (▲), 76 (◊) and 85% (♦) over 120 h at 21 ±2°C*

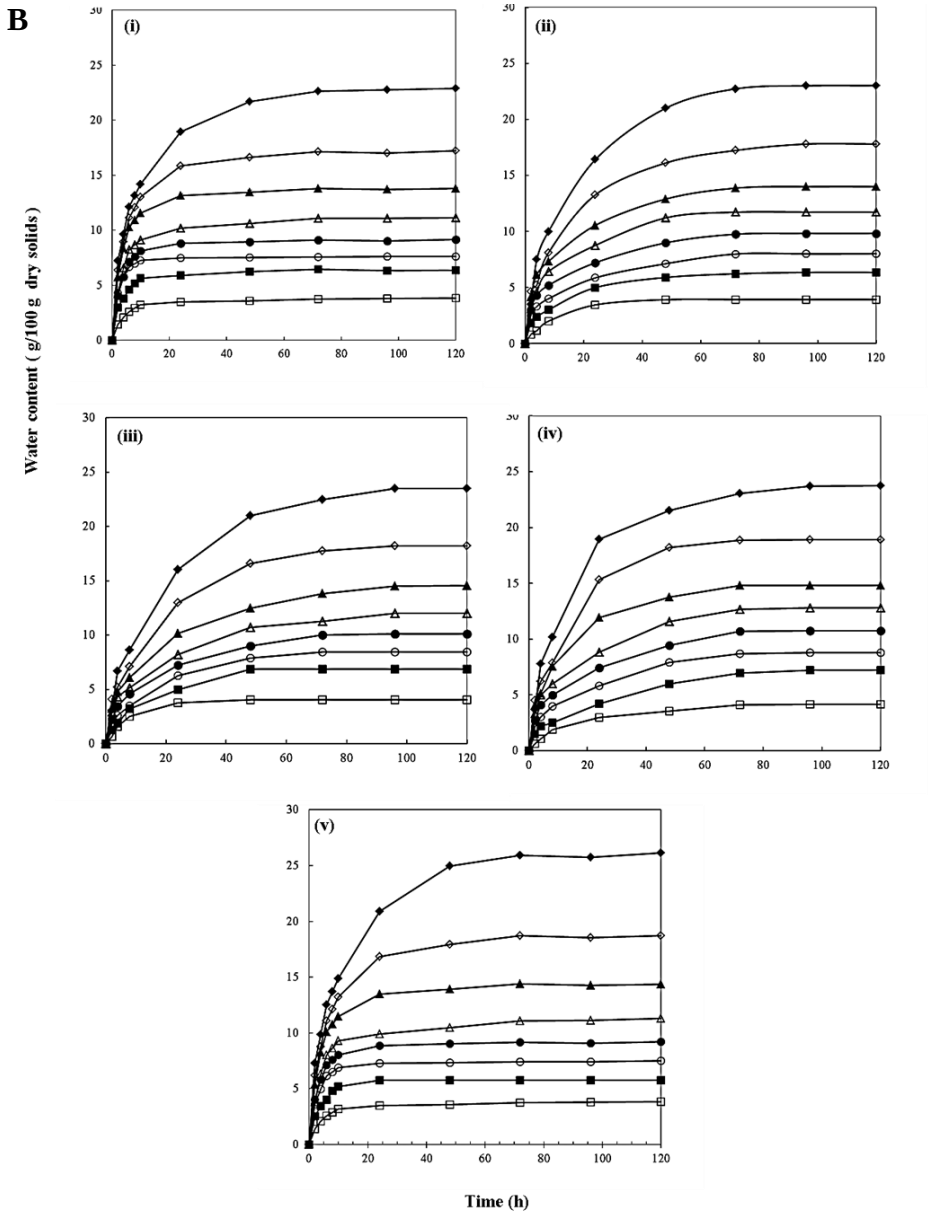


Fig. 4.3b. *Water sorption kinetics for crosslinked milk protein concentrate powders at 0 (i), 2.5 (ii), 5 (iii), 10 (iv) and 25 mM (v) sodium hexametaphosphate (SHMP) addition, measured at relative humidity values of 11 (□), 23 (■), 33 (○), 44 (●), 55.5 (Δ), 65 (▲), 76 (◊) and 85% (♦) over 120 h at 21 ±2°C*

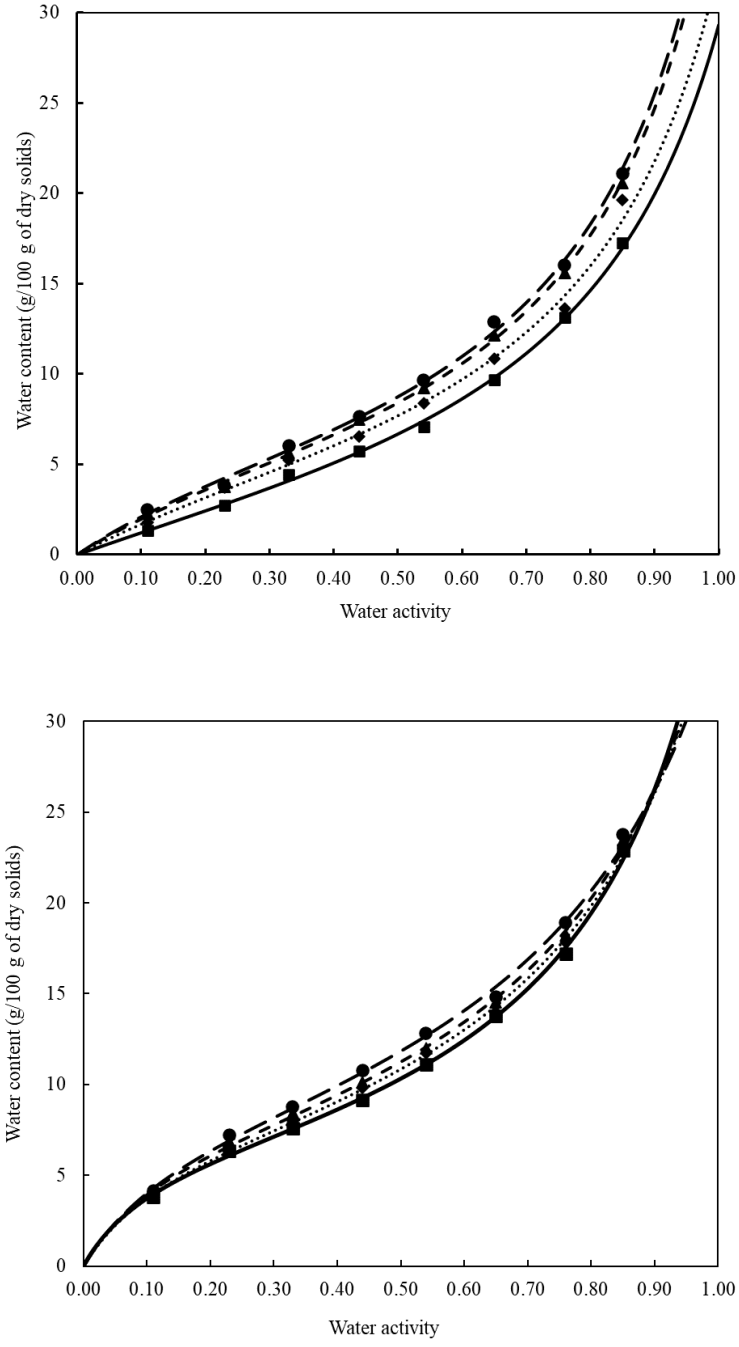


Fig. 4.4. *Guggenheim-Anderson-de Boer (GAB) water sorption isotherms for control (A) and crosslinked (B) casein protein concentrate 0 (−), 2.5 (····), 5 (---) and 10 mM (−−) sodium hexametaphosphate addition and experimental data points at 0 (■), 2.5 (♦), 5 (▲) and 10 mM (●), measured at 21 ± 2 °C .*

4.4.3. Real time visualization of liquid phase water diffusion and effective diffusivity behaviour in milk protein concentrate powders

Diffusion of Rhodamine B dye molecules into the anhydrous powder particles allowed for the visualization of dye penetration into the particle centre, indicative of powder hydration, while preventing particle solubilisation and any major disruption to powder morphology (Supplementary data: Fig. 4.S2). The sorption of water containing fluorescent Rhodamine B dye into, and through, dry powder particles occurred more quickly in TG-MPC powders containing 10 mM SHMP than the corresponding C-MPC powder. Complete dye penetration to the centre of the powder particle occurred after ~65 min for TG-MPC powders but took ~80 min for C-MPC powders (results not shown). From the penetration of dye into the powder particles of known size (i.e., data from Fig. 4.1A and B) at set time intervals the effective diffusivity of liquid water into individual powder particles could be calculated. The dependence of local diffusion coefficients on particle size is shown in Fig. S4.3. For both C-MPC (Fig. S4.3A) and TG-MPC (Supplementary data: Fig. S4.3B) powders the effective diffusion coefficients increased linearly with increasing particle size. The relationship between dye diffusion and SHMP concentration in the powders is shown in Fig. 4.5, with a linear relationship established between dye diffusion and SHMP concentration (Fig. 4.5; R^2 0.89 and 0.98 for C-MPC and TG-MPC powders, respectively). TG-MPC powders had higher effective diffusivity compared to C-MPC powders across all SHMP concentrations (Fig. 4.6). The increase in water sorption and hydration observed in TG-MPC may be due to a restructuring of the casein molecules in the MPC. Agyare and Damodaran (2010) showed that the surface hydrophobicity of whey protein isolate solutions decreased significantly after 30 h of incubation with

microbial TGase and reported that the decrease in surface hydrophobicity was due to partial burial or occlusion of hydrophobic cavities or clefts on the protein surface.

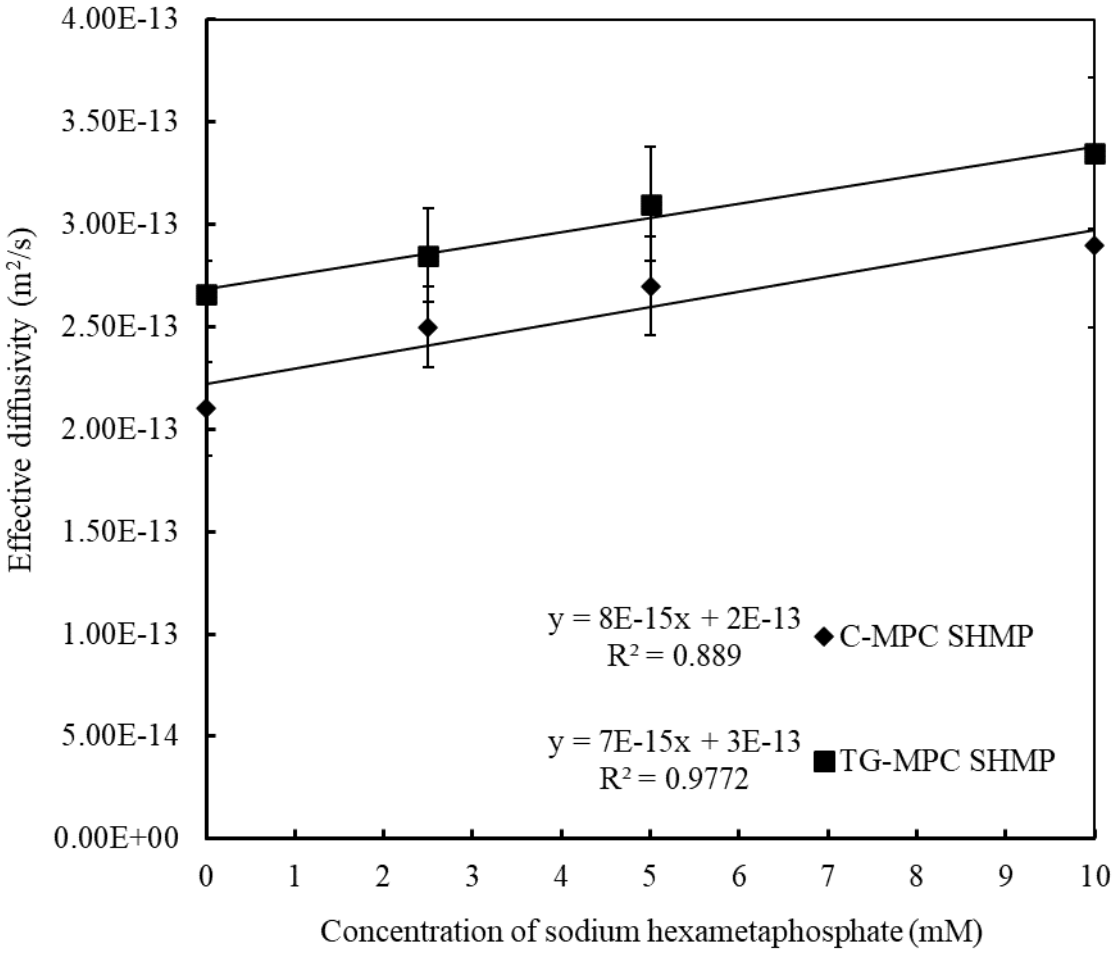


Fig. 4.5. *Effective diffusivity of control (♦) and crosslinked (■) casein protein concentrate powders measured as a function of sodium hexametaphosphate concentration. Symbols are experimental data obtained by confocal laser scanning microscopy (CLSM).*

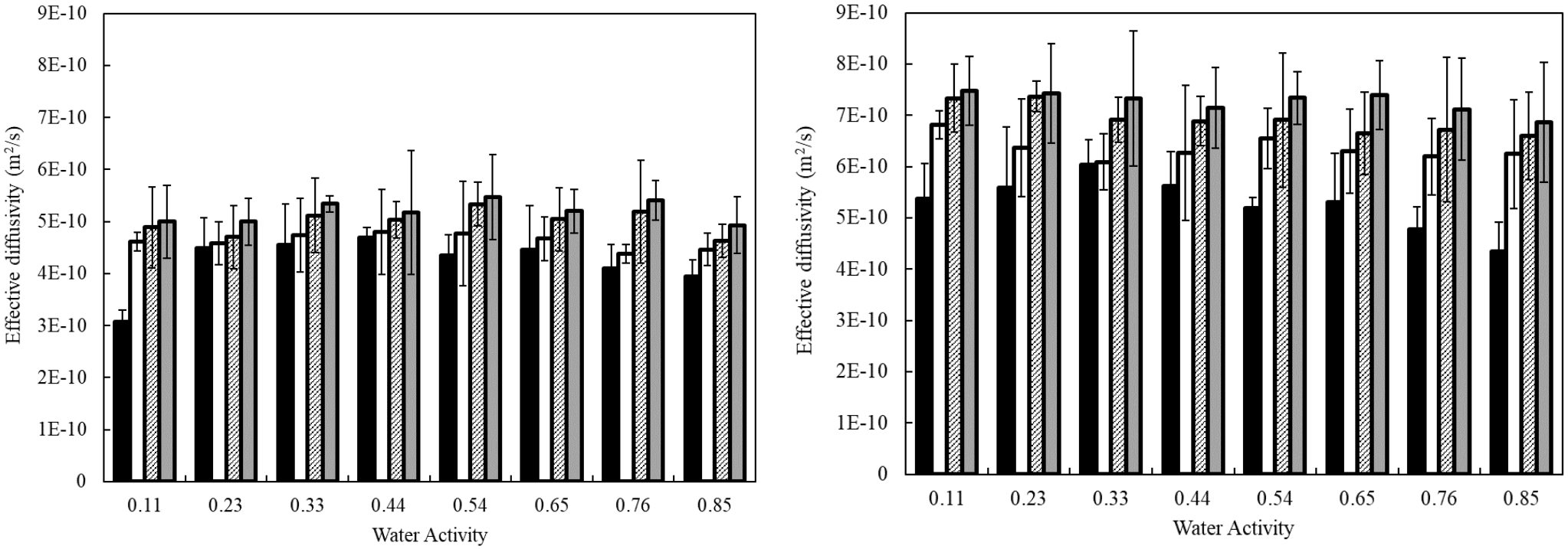


Fig. 4.6. *Effective diffusivity of control (A) and crosslinked (B) milk protein concentrate powders measured as a function of water activity at concentrations of 0 (■), 2.5 (□), 5(■) and 10 (■) mM sodium hexametaphosphate.*

4.4.4. Vapour phase diffusion in milk protein concentrate powders

The effective moisture (vapour phase) diffusivity of powder was calculated using the method of slopes and the raw data from Fig. 4.6B (Lomauro *et al*, 1985) Fig. 4.6 shows the results of effective diffusivity for C-MPC (Fig. 4.6A) and TG-MPC (Fig. 4.6B) powders. All powders showed a trend towards increasing effective diffusivity with increasing SHMP concentration. The effective diffusivity of C-MPC powders containing SHMP were observed to remain relatively constant over the entire a_w range studied (Fig. 4.6A). TG-MPC powders showed significantly ($P < 0.05$) higher effective diffusivity compared to C-MPC over the entire water activity range studied (Fig. 4.6). The increased levels of vapour sorption in TG-MPC powders may be due to lower surface hydrophobicity of the casein proteins following TGase treatment. However, studies have shown that the hydrophobicity of proteins after crosslinking using TGase vary depending on the length of time allowed for crosslinking. Liu *et al* (2010) monitored the surface hydrophobicity of wheat gluten during incubation with TGase and showed that the surface hydrophobicity initially increased and then decreased with incubation time. The authors stated that the increase in hydrophobicity may be attributed to the exposure of hydrophobic residues induced by cross-linking and the latter decreases due to the deamidation of gluten mediated by TGase, which led to hydrophilic and ionised carboxylic acid side chains. A previous study by Jaros and Rohm (2006) stated that in the absence of primary amines, water molecules can substitute as acyl acceptors causing deamination of glutamine residues and the formation of glutamic acid and ammonia in solution.

4.4.5. Temperature dependant hydration properties of milk protein concentrate powders

Particle size distribution profiles of C-MPC and TG-MPC powders re-dispersed in water at 20 and 40°C are shown in Fig. 4.7. Monomodal size distribution profiles were observed for all C-MPC dispersions, with and without SHMP. The size distribution profiles of C-MPC dispersions at 20°C (Fig. 4.7A) exhibited increasing particle size with increasing SHMP addition level, with $D_{(4,3)}$ values increasing from 25.1 to 53.8 μm. A similar trend was observed for C-MPC powders dispersed at 40°C with an increase in particles size with increasing SHMP content; however, the $D_{(4,3)}$ values increased from 25.1 to 64.0 μm. This increase in particle size with increasing SHMP concentration could be due to a change in electrostatic interactions within the casein micelle caused by calcium chelation, resulting in micellar swelling and a subsequent increase in powder particle size. McCarthy *et al*, (2017) found that even after 2 h of agitation, MPC dispersions containing SHMP had a bimodal particle size distribution. This is one of the principal characteristics of casein dominant powders which are highly hydrophobic and disperse slowly during the rehydration process, resulting in the irregular dissolution of powder particles.

In Fig. 4.7C, a bimodal size distribution was observed for TG-MPC powders containing no SHMP dispersed at 20°C, with a primary peak ranging from 4.23 to 119 μm and a smaller secondary peak ranging from 134 to 484 μm. Interestingly, TG-MPC powders containing 5 mM SHMP showed a bimodal size distribution similar to the profile for TG-MPC without SHMP but, with a significantly larger secondary peak (Fig. 4.7C). However, a monomodal size distribution profile was observed for TG-MPC powders containing 2.5, 10 and 25 mM SHMP, all of which had a larger primary peak than TG-MPC without SHMP. All TG-MPC dispersed at 40°C were monomodal

with the exception of the powder containing 5 mM SHMP which remained bimodal but with a small peak ranging from 0.05 to 0.64 μm and a larger peak ranging from 4.89 to 105 μm (Fig. 4.7D). Particle size $D_{(4,3)}$ values ranged from 29 to 67 μm for TG-MPC dispersions with 0 to 25 mM SHMP (results not shown). In general, the addition of SHMP to liquid MPC prior to drying caused significant changes to water sorption and hydration properties even after only 30 s of high shear mixing.

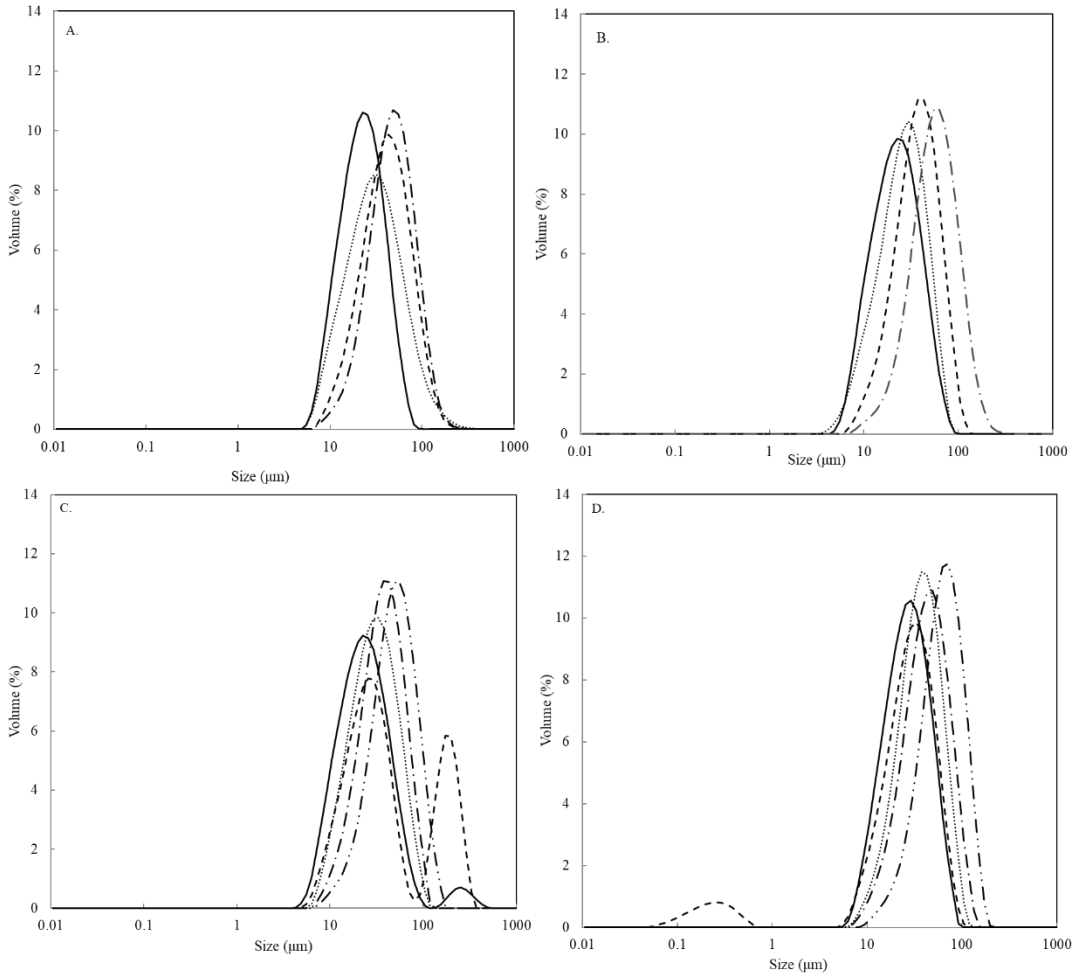


Fig. 4.7. *Particle size distribution profiles of control protein powders dispersed at 24 (A) and 40°C (B) and crosslinked milk protein concentrate powders dispersed at 24 (C) and 40°C (D) at sodium hexametaphosphate concentrations of 0 (—), 2.5 (···), 5 (---)10 (−·−·) and 25 mM (−···−).*

4.5. Conclusion

This study has demonstrated that the hydration of milk protein concentrates can be improved by mineral chelation and that enzymatic crosslinking of casein proteins can be implemented to off-set increases in viscosity during processing, primarily due to the network of covalent bonds helping to retain casein micelle structure. The addition of SHMP improved water sorption and hydration properties of MPC powders. SEM images of control crosslinked MPC powders demonstrated that increasing the SHMP concentration caused the surface morphology of powder particles to progressively change from having a shrivelled, collapsed appearance to a smoother, spherical particle surface. In contrast, TG-MPC powder morphology remained relatively unchanged with increasing SHMP concentration. Crosslinking improved powder hydration and water sorption, even without addition of SHMP, when compared to the corresponding non-crosslinked control. Overall, crosslinking casein proteins in MPC powders had positive effects on water sorption and hydration in addition to decreasing concentrate viscosity during the drying process.

4.6. Supplementary data

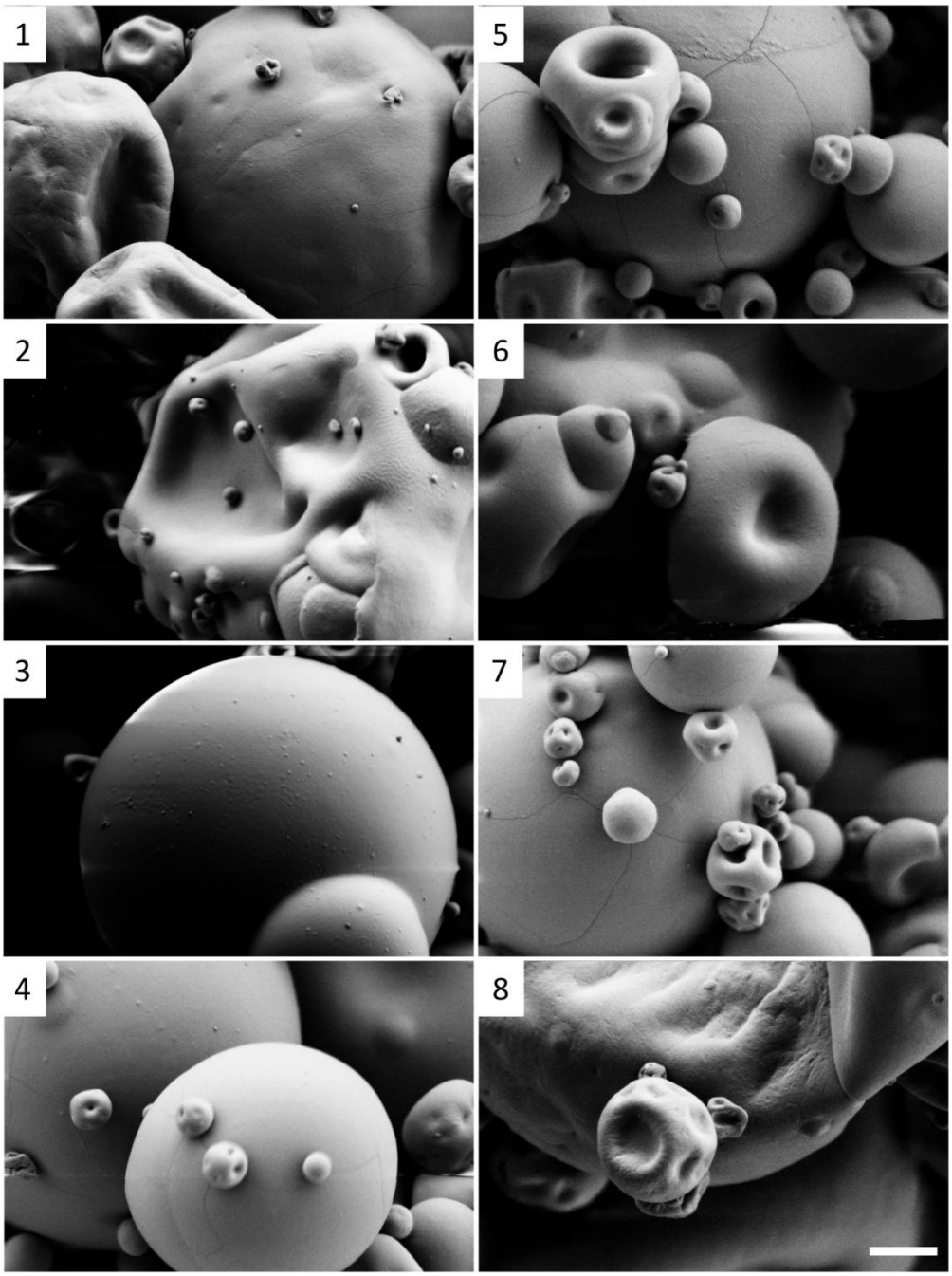
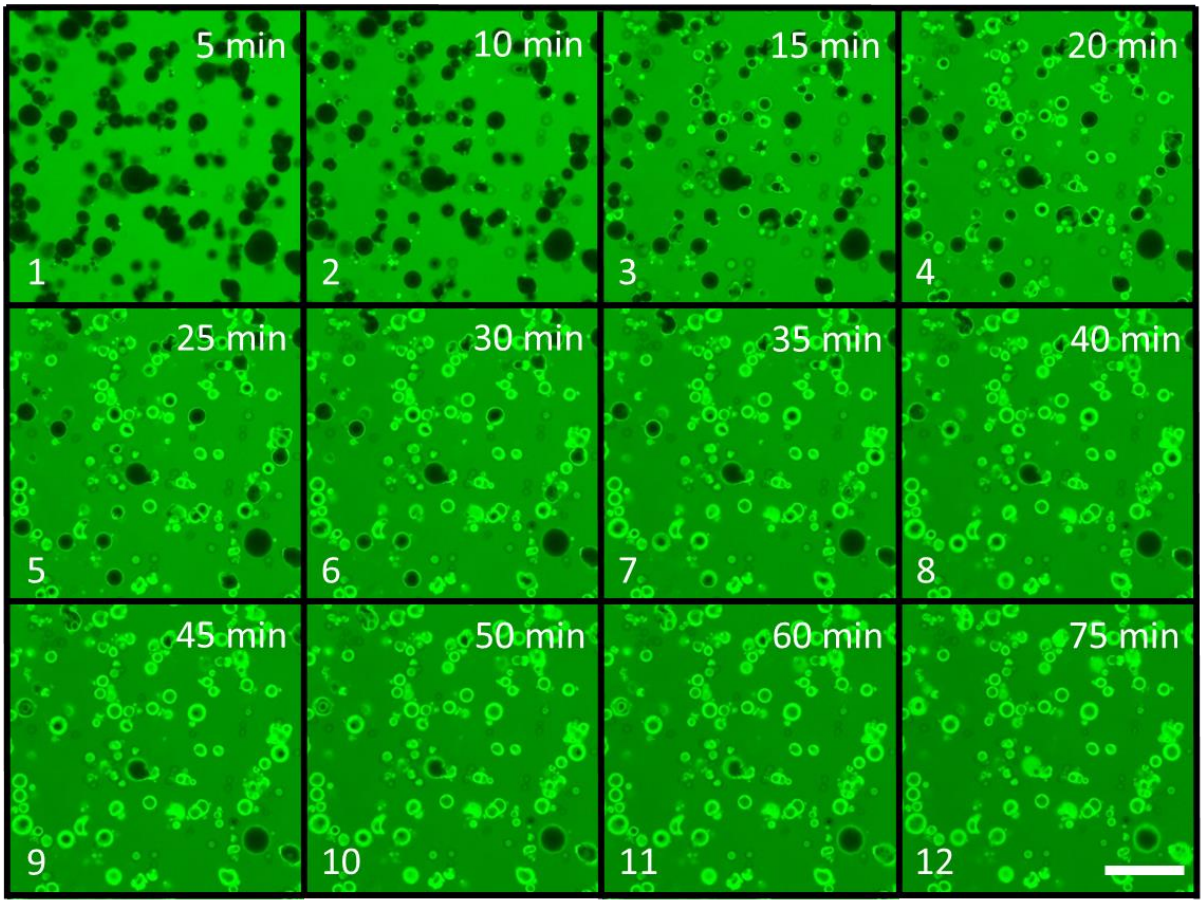
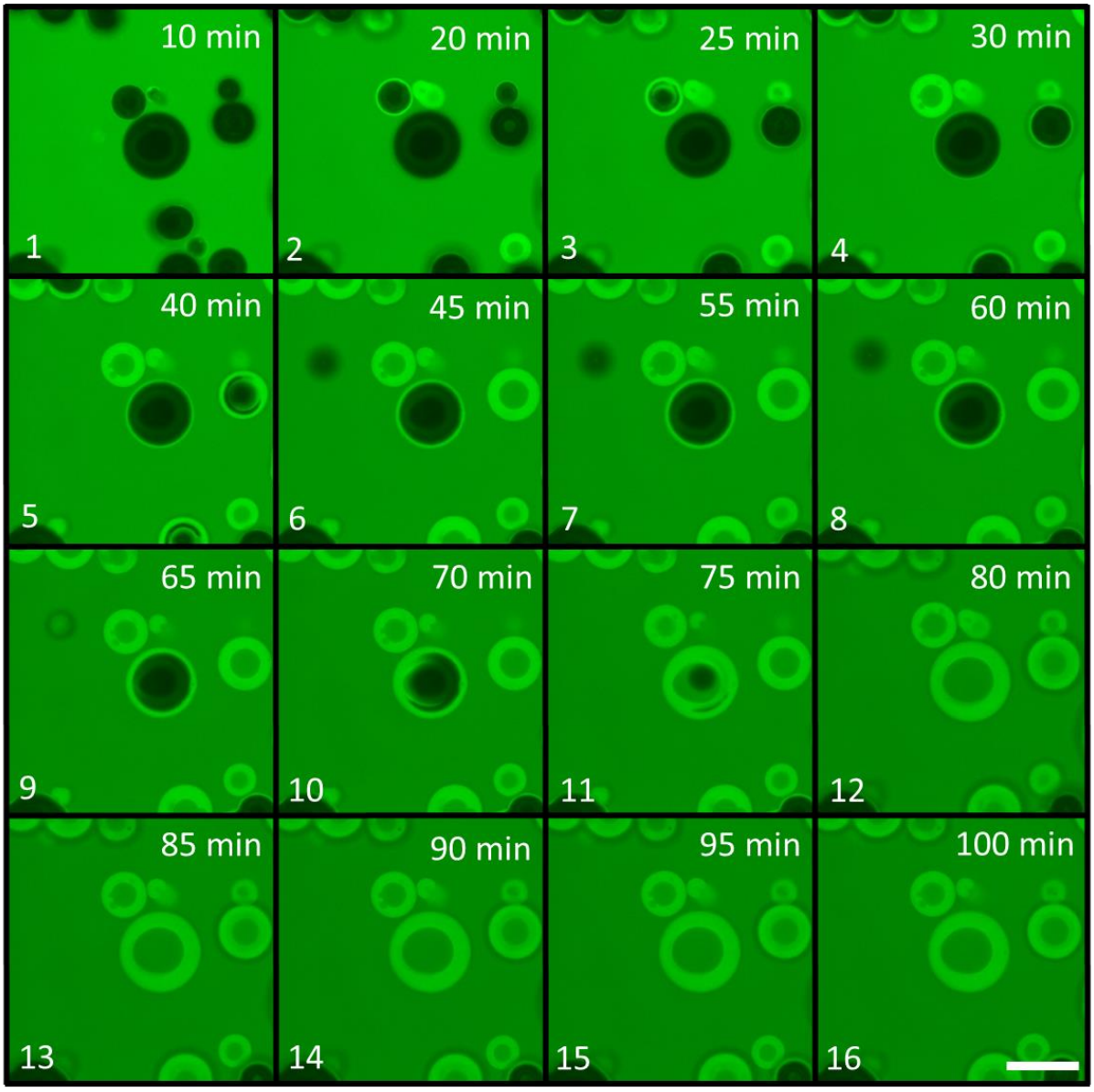


Fig. S4.1. *Scanning electron microscopy (SEM) images of control milk protein concentrate powder containing 0 (1), 2.5 (2), 5 (3) or 10 mM (4) sodium hexametaphosphate and crosslinked milk protein concentrate powder containing 0 (5), 2.5 (6), 5 (7) or 10 mM (8) sodium hexametaphosphate.*



S2.1. Confocal scanning laser micrographs of anhydrous control milk protein concentrate powder 0 mM at 10 (1), 20 (2), 25 (3), 30 (4), 40 (5), 45 (6), 55 (7), 60 (8), 65 (9), 70 (10), 75 (11), 80 (12), 85 (13), 90 (14), 95 (15) and 100 min (16) after addition of Rhodamine B dye. Scale bar = 25 µm.



S2.2. Confocal scanning laser micrographs of anhydrous crosslinked milk concentrate protein 0 mM powder at 5 (1), 10 (2), 15 (3), 20 (4), 25 (5), 30 (6), 35 (7), 40 (8), 45 (9), 50 (10), 60 (11) and 75 (12) min after addition of Rhodamine B dye. Scale bar = 250 µm.

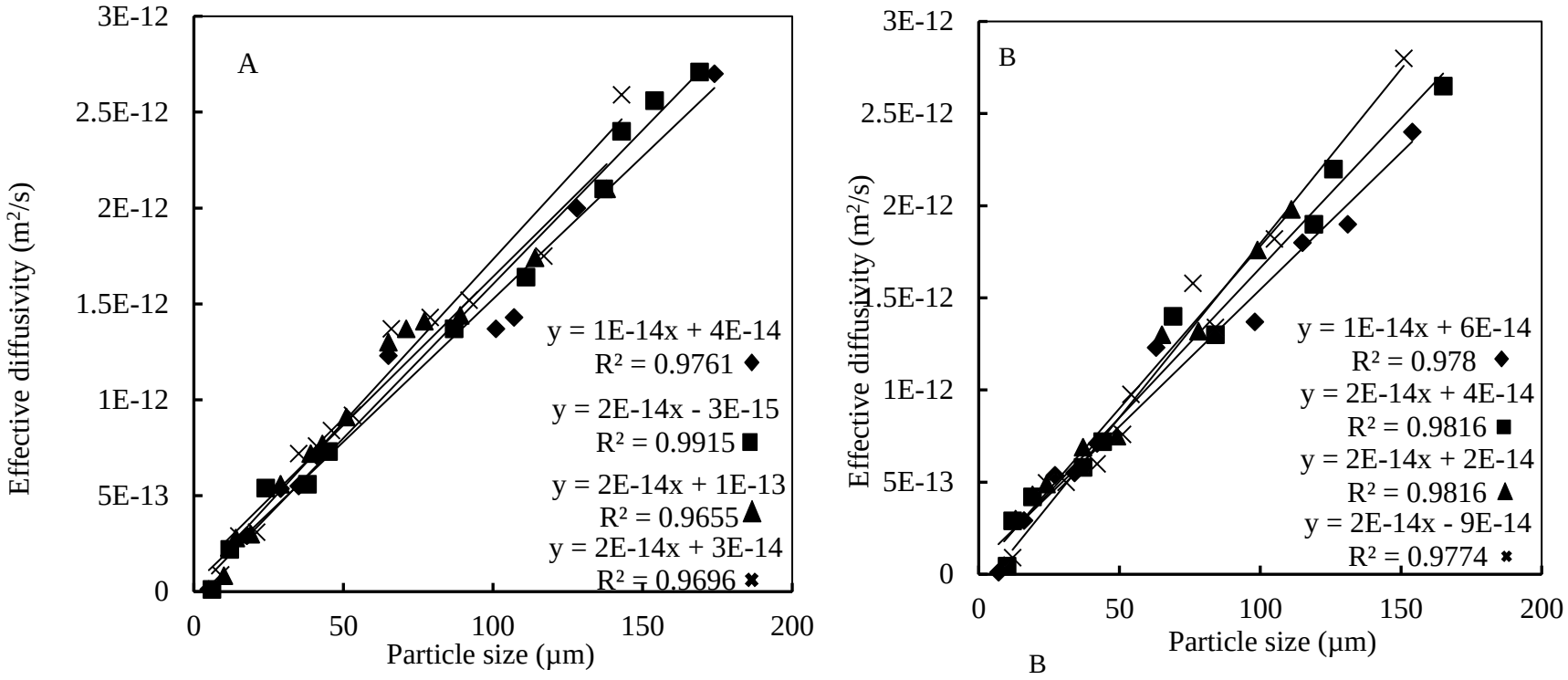


Fig. S4.3. *Effective diffusivity of Rhodamine B (0.1% in water) measured as a function of particle size of control (A) and crosslinked milk protein concentrate powder (B) with 0 (◊), 2.5 (■), 5 (▲) and 10 mM (×) additions of sodium hexametaphosphate. Data were compiled and calculated from confocal laser scanning microscopy images.*

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CHAPTER FIVE

Calcium depletion using ion-exchange influences the physical properties of milk protein concentrates containing enzymatically crosslinked caseins

Orla M. Power[1, 2], Mark A. Fenelon[1], James A. O'Mahony[2], Noel A. McCarthy[1]

[1]Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[2]School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Declaration:

This chapter was written by author Orla M. Power (OMP) and reviewed by co-authors Dr. Noel A. McCarthy (NAMC), Dr. James A. O'Mahony (JAOM) and Dr. Mark Fenelon (MK). OMP co-designed the study with NAMC and performed all of the experimental work.

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5.1 Abstract

The aim of this study was to determine the effect of calcium depletion using ion-exchange on the physical properties of non-crosslinked (C) and transglutaminase crosslinked (TG) milk protein concentrate (MPC) dispersions. Addition of ion-exchange resin to C-MPC dispersions caused reductions in total calcium content and calcium ion activity. TG-MPC dispersions were more resistant to calcium chelation compared to C-MPC, due to the installation of covalent bonds, which increased the structural stability of the casein micelles; however, ionic calcium was depleted to a similar extent with increasing resin addition level. Depletion of calcium resulted in increased viscosity, reduced turbidity and increased levels of serum casein in C-MPC. TG-MPC dispersions retained a higher degree of turbidity and had lower polydispersity index values compared to C-MPC samples, mainly due to a greater ability to resist dissociation of casein micelles. However, TG-MPC dispersions extensively depleted of calcium were observed to have a higher viscosity, compared to the corresponding C-MPC dispersions.

5.2 Introduction

The global dairy market is expanding at an estimated annual growth rate of 5%, amounting to ~$704 billion by 2024 (Cobbe, 2019). High protein dairy powders have gained popularity as ingredients in value-added nutritional products (e.g., beverages) to improve both functional and nutritional properties. However, the poor dissolution properties of MPC powders mean that modifications are often required during their manufacture to facilitate more rapid, and complete powder rehydration. Poor dissolution properties of high protein dairy powders can be attributed to the slow penetration of water through the surface of the powder particles due to the presence of hydrophobic casein proteins and hence retards the rate at which constituents of the powder particles are released into the aqueous phase (Anema *et al*, 2006; Crowley *et al*, 2015; Fang *et al*, 2011; Mimouni *et al*, 2009; Gaiani *et al*, 2007).

A number of previous studies aimed at improving solubility of high protein dairy powders used mechanical unit operations such as ultrasonication, cavitation and homogenization (McCarthy *et al*, 2014; Pathania *et al*, 2018) and chemical modifications such as calcium chelation and ion-exchange (Bhaskar *et al*, 2007; de Kort *et al*, 2011; McCarthy *et al* 2017; Xu *et al*, 2016). These chemical modifications have generally been shown to be more successful in increasing powder solubility, while the mechanical unit operations are usually effective only in improving dispersion properties of such powders, but do little to increase powder solubility, particularly if applied to the concentrate prior to spray dying. Calcium chelating salts bind positively charged calcium ions using negatively charged counter ions such as citrate, phosphate or ethylenediaminetetraacetic acid (i.e., EDTA). Depending on the type and concentration of chelator used, both serum phase and bound calcium may be chelated, resulting in disruption of casein micelles. Alternatively, calcium can be

depleted from milk protein solutions using strong cationic ion exchange resins, as the resins simultaneously bind calcium and release monovalent counter ions, usually potassium or sodium. Bhaskar *et al* (2007) observed that MPC powders with a reduced calcium content achieved using ion exchange, had improved solubility, while Marella *et al* (2015) reported that MPC powders with reduced calcium content, achieved using carbon dioxide injection into skim milk during ultrafiltration, had improved solubility in cold water due to the solubilisation of colloidal calcium phosphate (CCP).

However, ion exchange has been shown to contribute to concomitantly higher viscosity and can result in gel formation within the ion exchange column. In a patent by Bhaskar *et al* (2003), calcium-reduced MPCs, achieved using calcium ion exchange, were utilised to manufacture dairy gels for application in cheese and dairy-based food products. Such increases in viscosity could potentially cause challenges during pumping, limit evaporation capacity and alter the physical properties of resultant spray dried powders (Bienvenue *et al*, 2003; Huppertz *et al*, 2017; Park *et al* (2016); Power *et al*, 2020; Vélez-Ruiz *et al*, 1998). Previous work by Xu *et al* (2016) demonstrated, using transmission electron microscopy and small angle X-ray scattering (SAXS), that both the casein micelle structure and CCP nanoclusters were partially dissociated at 38.7% calcium reduction, with the majority of CCP nanoclusters fully dissociated at 83.6% calcium reduction. The depletion (even partial) of calcium from CCP destabilises casein micelles and thereby causes dissociation of micelles, resulting in increased viscosity of protein dispersions (de Kort *et al*, 2011; Odagiri and Nickerson, 1964; Pitkowski *et al*, 2008; Power *et al*, 2019; Power *et al*, 2020).

Transglutaminase (TGase) has been used to improve colloidal and heat stability of caseins, as a consequence of isopeptide bond formation (O'Sullivan *et al*, 2002).

Enzymatic crosslinking results in greater mechanical stability of casein micelles and confers greater resistance to dissociation of micelles. Previous work by Smiddy *et al*, (2006) and Moon *et al* (2009) has shown that casein micelles incubated with TGase had increased stability against micellar disruption by dissociating agents such as urea, sodium dodecyl sulphate, or heating. Previously, Power *et al* (2020) showed that crosslinking caseins using TGase was effective in controlling viscosity of MPC dispersions, which otherwise would have increased significantly due to calcium chelation by sodium hexametaphosphate (SHMP). In the present study, it is aimed to examine the impact of ion exchange-based calcium depletion on cross-linked casein proteins and determine their stability to dissociation, by measuring non-sedimentable casein, particle size and viscosity.

5.3. Materials and methods

5.3.1. *Materials*

Milk protein concentrate (MPC80, Glanbia Ireland, Ballyragget, Ireland) contained 80.5% (w/w) protein, 1.5% (w/w) fat and 7.49% (w/w) ash. A strong cationic ion-exchange resin (Amberlite^{TM} IR120; $(C_{10}H_{11})A(C_8H_9O_3S)B(C_2H_6)$) was obtained from Fisher Scientific (Fisher Scientific, Dublin 15, Ireland), with a bead size range of 0.6-0.8 mm. Transglutaminase enzyme (Activa MP, Ajinomoto Enzyme Preparations) was purchased from Healy Group (Tallaght, Co. Dublin).

5.3.2. Rehydration and enzymatic crosslinking of milk protein concentrate

MPC powder was rehydrated (250 g sample at 10%, w/w, protein) as per the method outlined by Power *et al* (2019), with the addition of sodium azide (0.02%, w/w) to prevent microbial growth. TGase was used to covalently crosslink rehydrated

MPC (TG-MPC), using the approach described previously by Huppertz and De Kruif (2008). In brief, MPC dispersions were preheated (30°C) prior to incubation with TGase (0.5 g L^{-1}) for 24 h at pH 6.5 ± 0.1, with minor pH adjustments being made, as required, using 0.1 M NaOH or HCl. TGase was inactivated by heating at 80°C for 5 min and a control MPC (C-MPC) dispersion was prepared using the same procedure as outlined above, but without enzyme addition.

5.3.3. Calcium ion-exchange of milk protein concentrate dispersions

Calcium-depleted MPC dispersions were generated using an approach similar to that described by Xu *et al*. (2016) and was carried out by the addition of a strong cationic ion exchange resin (Amberlite^{TM} IR120) to 250 g of control (C-MPC) and crosslinked (TG-MPC) milk protein dispersions. Prior to addition of ion exchange resin, the pH of dispersions was adjusted to pH 5.9 to compensate for the pre-determined increase in pH arising from addition of the ion-exchange resin. Dispersions were gently agitated for 1.5 h, followed by filtration using cheese cloth to remove the resin beads. Following removal of the resin, the pH of dispersions were adjusted to pH 6.7. The ion-exchange resin was added at four different levels to deplete MPC of calcium with protein:resin ratios of 100:0, 76:24, 56:44 or 38:62.

5.3.4 Mineral composition and calcium ion activity of milk protein concentrate dispersions

Total calcium, sodium, magnesium and potassium content of MPC samples were determined using inductively coupled plasma mass spectrometry (ICPMS) as per the method of Pacquette and Thompson (2017). Calcium ion activity of C-MPC and

TG-MPC dispersions was analysed in triplicate using a Sension^{+} bench-top laboratory ion meter (Sension^{+} MM374 GLP 2 channel, HACH, Germany) equipped with a calcium ion-selective electrode (Sension^{+} 9660C calcium combination ion-selective electrode (ISE), HACH, Berlin, Germany) as per the method previously described by Gulati *et al,* (2019). Prior to analysis, the electrode was prepared by immersing in a calcium chloride ($CaCl_2$; 10 mg L^{-1}) solution for 30 min. An ISE calibration curve was then generated using 10 mL of the following $CaCl_2$ solutions (0.5, 1.0, 2.5 and 5 mM) and 0.1 mL of 3 M potassium chloride (KCl). Potassium chloride (KCl, 3 M) was added to all samples and to the standard solutions at a level of 1% (v/v) to attain a similar ionic strength in all samples prior to analysis.

5.3.5 Protein profile analysis

Protein profiles of C-MPC and TG-MPC dispersions were determined using sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) before and after centrifugation (produced by centrifugation at 100,000 × g using an Eppendorf centrifuge 5417R, Hamburg, Germany). The samples were dissolved under reducing and non-reducing conditions in an SDS buffer; 10 μL of each dispersion were added to wells in a 12% Bis-Tris Nu-PAGE gel and electrophoretic analysis was performed using an X-Cell Surelock electrophoresis unit (Novex Technologies, Fischer Scientific, Blanchardstown, Dublin, Ireland) at a constant voltage of 180 V for 55 min. Samples for SDS-PAGE analysis contained 1 μg protein per μL of sample buffer solution. Gels were then stained overnight using 0.05% (w/v) Coomassie brilliant blue R-250 in 25% (v/v) isopropanol and 10% (v/v) acetic acid. After staining, the gels were de-stained using a 10% (v/v) isopropanol and 10% (v/v) acetic acid solution until a clear background was achieved.

5.3.6. Particle size and zeta-potential analysis of milk protein concentrate dispersions

Particle size measurements were performed using a Zetasizer nano (Malvern Instruments, Malvern, Worcestershire, UK) on both C-MPC and TG-MPC dispersions. Dispersions were diluted 1:50 with tempered deionised water. The refractive index of the dispersant (water) was 1.330 with an assigned viscosity of 0.8872 cP. C-MPC and TG-MPC dispersions were measured in disposable cuvettes and the samples were characterised as protein using a refractive index of 1.45 and absorption value of 0.001. Experiments were carried out in triplicate with a backscattering angle of 173°.

The zeta (ζ)-potential of C-MPC and TG-MPC dispersions were measured using a Zetasizer (Malvern Instruments, Worcestershire, UK). Samples were diluted 1:10 with deionised water and ζ-potential analysis was performed using water as the dispersant; the dispersant was assigned a refractive index of 1.330 and viscosity of 0.8872 cp. Measurements were carried out using disposable folding capillary cells (DTSI060/DTSI061). The milk proteins were characterised using a refractive index of 1.45 and absorption value of 0.001. All measurements were performed in triplicate at 25°C.

5.3.7 Colour analysis of milk protein concentrates dispersions

The impact of calcium depletion on the colour of C-MPC and TG-MPC dispersions was assessed using a Minolta Chroma Meter CR-400 colorimeter (Minolta Ltd, Milton Keynes, UK) as per the method previously described by Magan *et al*

(2019). The L*a*b* values were determined using the CIE method, with lightness (L*), red/green colour (a*), and yellow/blue colour (b*). Dispersions were placed into plastic cuvettes and measured in triplicate. The total colour difference (ΔE) was calculated using Equation 1 where L_o, a_o and b_o refer to the colour of C-MPC and TG-control samples (100:0), while L, a and b denote the respective colour parameters of dispersions with resin addition.

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \qquad \text{(Eq.1)}$$

5.3.8. Viscosity analysis of milk protein concentrate dispersions

Viscosity analysis of C-MPC and TG-MPC dispersions was performed using a controlled-stress rheometer (AR-G2, TA Instruments, Crawley, UK) equipped with a parallel plate geometry. Dispersions were pre-sheared at 200 s^{-1} for 1 min, followed by a continuous shear rate ramp from 0.1 to 250 s^{-1} over 5 min at 25°C. All measurements were performed in triplicate. Temperature-dependant rheological measurements of C-MPC and TG-MPC dispersions were performed using a controlled-stress rheometer (AR-G2 Rheometer, TA Instruments, Crawley, UK) equipped with concentric cylinder geometry to prevent evaporation from the dispersions. The analysis method consisted of a conditioning step at 20°C and pre shear at a shear rate of 100 s^{-1} for 10 s, succeeded by a temperature ramp from 20 to 55°C for 10 min at a shear rate of 100 s^{-1}. Analysis was carried out in triplicate.

5.3.9. Statistical data analysis

One-way analysis of variance (ANOVA), with posthoc Tukey analysis was used to determine the statistical significance. The level of significance was considered as $P<0.05$. A paired T-test was used to ascertain the confidence interval of rheological

data. All statistical analyses were carried out using Minitab 17 (Minitab Inc, Coventry, United Kingdom).

5.4. Results and discussion

5.4.1. Mineral composition and calcium ion activity of milk protein dispersions

The mineral composition and calcium ion activity of milk protein concentrate (MPC) dispersions is shown in Table 5.1 and Fig. 5.1, respectively. Mineral analysis showed significant differences in calcium concentration with C-MPC samples significantly more depleted in calcium compared to TG-MPC (Table 5.1) on addition of ion-exchange resin, particularly at a protein:resin ratio of 56:44. However, calcium concentrations were similar for C-MPC and TG-MPC after ion exchange at a protein:resin ratio of 38:62. As the calcium concentration decreased with increasing resin addition level, a concomitant increase in sodium content was observed, as sodium acts as the counter-ion during the ion exchange process. Calcium ion activity decreased progressively in both C-MPC and TG-MPC dispersions with increasing resin addition (Fig. 5.1), with ~50% decrease in ion activity at a protein:resin ratio of 76:24, compared to 100:0 samples. However, the total calcium content of TG-MPC dispersions remained relatively unchanged until a protein:resin ratio of 38:62 was applied (Table 5.1).

Table 5.1. *Mineral content (g 100 g^{-1}, dry matter) of control (C-MPC) and crosslinked (TG-MPC) protein dispersions measured using inductively coupled plasma mass spectrometry (ICPMS), as a function of protein to resin ratio.*

Protein:resin	Calcium		Sodium		Magnesium		Potassium	
	C-MPC	TG-MPC	C-MPC	TG-MPC	C-MPC	TG-MPC	C-MPC	TG-MPC
100:0	2.12^{aA}	2.15^{aA}	0.7^{dA}	1.72^{bB}	0.09^{aA}	0.09^{aA}	0.93^{aA}	0.93^{aA}
76:24	2.05^{aA}	1.94^{aA}	1.85^{cA}	1.82^{bA}	0.08^{abA}	0.08^{aA}	0.54^{bA}	0.57^{bA}
56:44	1.49^{bB}	2.19^{aA}	2.58^{bA}	3.67^{aB}	0.06^{bA}	0.09^{aA}	0.31^{cA}	0.43^{cA}
38:62	1.36^{bA}	1.21^{bB}	4.08^{aA}	3.61^{aA}	$0.06b^{A}$	0.06^{bA}	0.26^{dA}	0.21^{dA}

$^{a-d}$Values within a column not sharing a common superscript differ significantly ($P < 0.05$).

$^{A-B}$Values within a row for each mineral not sharing a common superscript differ significantly ($P < 0.05$).

The depletion of calcium in C-MPC dispersions can be attributed to their susceptibility to micellar dissociation, with the reduction of calcium from the serum phase resulting in the diffusion of micellar calcium and subsequent casein micelle dissociation (Dalgleish and Corredig, 2012, de Kruif *et al*, 2012, McMahon and Oommen, 2008, Horne, 2006, Wong *et al*, 1996). While the reduced level of ionic calcium in TG-MPC dispersions observed after resin addition could be due to preferential binding of free ionic calcium in the serum phase during the ion-exchange process, TGase crosslinked casein proteins within micelles are highly resistant to dissociation and may have hindered the chelation of calcium from CCP within these micelles (O'Sullivan *et al*, 2002). The discrepancy between the calcium ion activity decreasing and total calcium remaining unchanged in cross-linked samples at protein:resin ratios of up to, and including, 56:44 may be due to the relatively low amount of ionic calcium in MPC compared to the total calcium content, with approximately 3-5% of the total calcium in MPC in the ionic form. Previously, Lin *et al* (2006) showed that <10% of the total calcium present in milk was ionic. The depletion of magnesium followed a similar trend to calcium, with levels in C-MPC decreasing incrementally with increasing addition level of resin. However, magnesium levels in TG-MPC samples once again did not decrease significantly ($P < 0.05$) until a protein:resin ratio of 38:62 was used. Potassium levels in C-MPC and TG-MPC both decreased with increasing protein:resin ratio.

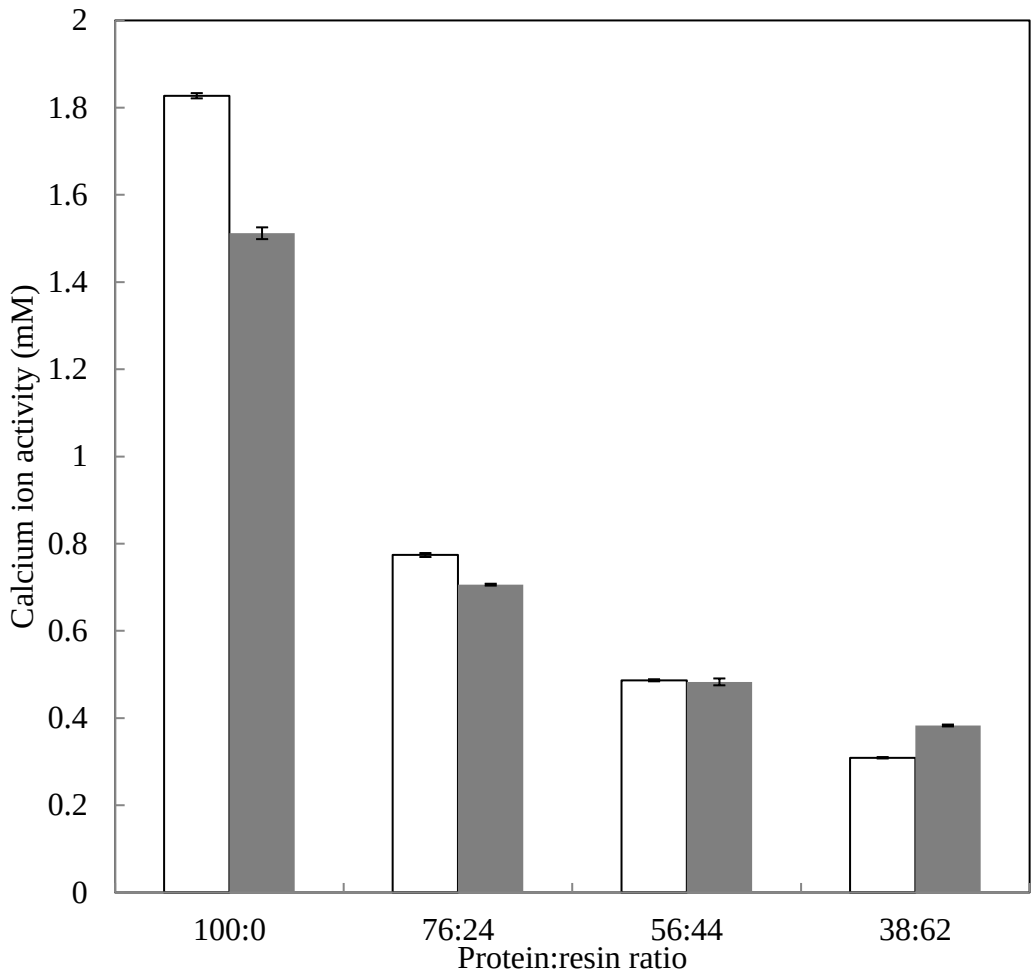


Fig. 5.1. *Calcium ion activity (mM) for control (□) and crosslinked (■) casein protein dispersions at protein to resin ratios of 100:0, 76:24, 56:44 and 38:62.*

5.4.2. Protein profile analysis

SDS-PAGE protein profiles of C-MPC samples, performed under reducing conditions, showed all the major casein and whey protein bands (Fig. 5.2A; lanes 5-8), with a lower band intensity for κ-casein and α-lactalbumin, and particularly β-lactoglobulin, in non-reducing than in reducing gels, indicative of the presence of disulphide linkages due to heat treatment during manufacture of the MPC powder (Fig. 5.2A; lanes 1-4). Lower intensities of bands corresponding to α_{s1}-, β- and κ-casein were observed in TG-MPC dispersions, compared with C-MPC dispersions, under reducing conditions (Fig. 5.2B; lanes 5-8), however; whey proteins were relatively

unaffected. The reduction in monomeric casein protein in TG-MPC dispersions can be attributed to enzymatic crosslinking of the casein proteins (Moon *et al*, 2009).

Incubation with TGase results in an acyl-transferase mechanism, which catalyses the transfer of a gamma-carboxyamide group of a glutamine residue to the gamma-amine of an adjacent lysine or glutamine residue, resulting in covalent linking of casein proteins (Jaros *et al*, 2006). SDS-PAGE protein profiles of the serum phase of ultracentrifuged C-MPC dispersions showed the significant effect of calcium depletion on casein micelle structure. It was clear that calcium depletion resulted in an increase in soluble casein (Fig. 5.2C). At a protein:resin ratio of 76:24, higher levels of soluble casein (Fig. 5.2C; lane 6) were observed compared to non-calcium depleted (i.e., protein:resin ratio 100:0) MPC dispersions (Fig. 5.2; lane 5). The serum phase of TG-MPC dispersions showed a similar trend in protein profile to C-MPC, but again, band intensities were significantly lower for the casein proteins, with the whey proteins relatively unaffected, particularly α-lactalbumin (Fig. 5.2D).

The lower band intensity for crosslinked caseins in the serum phase indicated significant casein micelle sedimentation during ultracentrifugation and that the micelle was not reliant solely on calcium to maintain structural integrity. Previous research by Sharma *et al* (2001) and Smiddy *et al* (2006) showed the order of casein susceptibility to enzymatic crosslinking to decrease in the order κ- > β- > α_{s1}-casein and for that individual casein susceptibility to be related to the preferential location of individual caseins in casein micelles. Monomeric κ-casein and β-casein were more readily crosslinked by TGase due to their respective locations in casein micelles, while α_{S1}-casein is primarily located within casein micelles, as previously described by De Kruif and Holt (2003). Previous work by Xu *et al* (2016) showed, using transmission electron microscopy and small angle X-ray scattering, that the casein micelles, and

associated CCP nanoclusters, were gradually dissociated with increasing levels of calcium depletion, with the majority of casein micelles dissociating following the removal of 38.7% of the overall calcium present in the system.

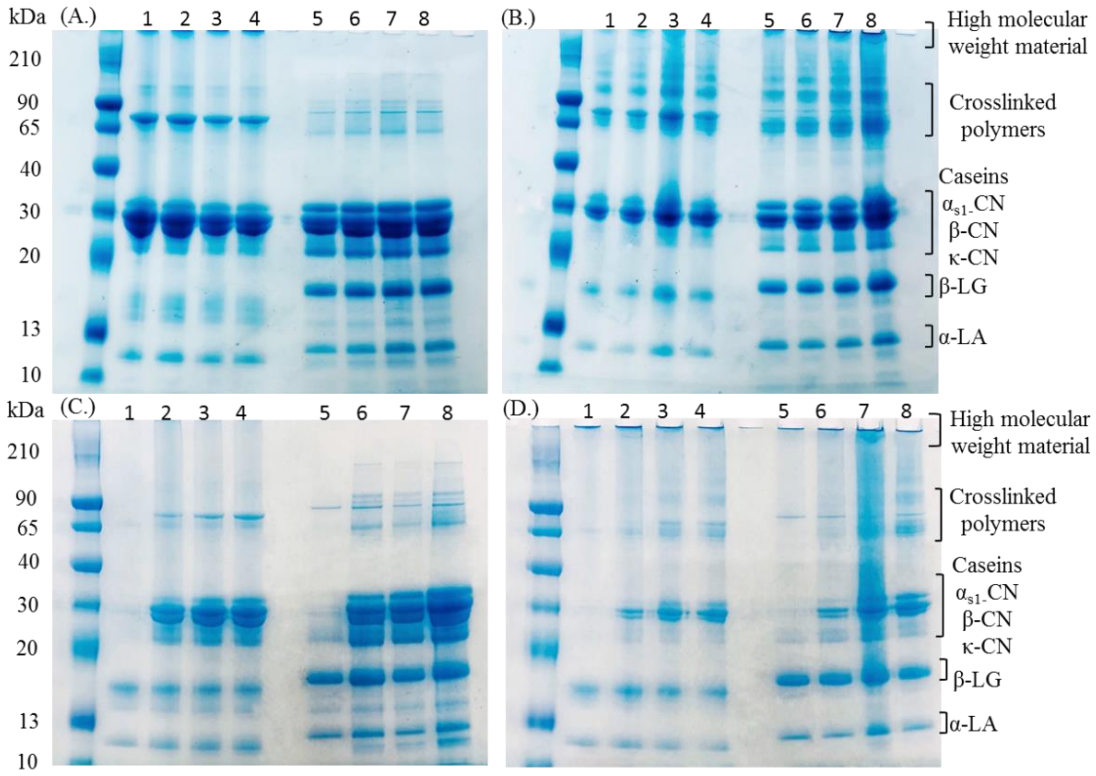


Fig. 5.2. *Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles of control (A) and crosslinked (B) milk protein dispersions and their respective non-sedimentable protein fractions (C and D), under non-reducing (lanes 1-4) and reducing (lanes 5-8) conditions, with protein:resin ratios of 100:0 (lanes 1 and 5), 76:24 (lanes 2 and 6), 56:44 (lanes 3 and 7) and 38:62 (lanes 4 and 8).*

5.4.3. Particle size, zeta-potential and colour analysis

C-MPC and TG-MPC dispersions with protein:resin ratios of 100:0 and 76:24 had similar monomodal particle size distribution (PSD) profiles (Fig. 5.3) and polydispersity index (PdI) values (0.14 and 0.11 for C-MPC and 0.13 and 0.12 for TG-MPC dispersions, respectively; Table 5.2). However, increasing the protein:resin ratio to 56:44 resulted in the C-MPC dispersion having a broader PSD profile (ranging from 28 to 459 nm) than C-MPC dispersions with protein:resin ratios of 100:0, indicative of partial dissociation of the casein micelles, caused by the significant depletion of calcium (Table 5.1).

Further depletion of calcium in C-MPC dispersions with a protein:resin ratio of 38:62 resulted in a substantial alteration to the PSD profile as shown in Fig. 5.3A. This is also shown by the significant ($P < 0.05$) increase in PdI values from 0.14 to 0.43 (Table 5.2), and correlates to the high level of soluble casein as shown in SDS-PAGE profiles (Fig. 5.2). The depletion of calcium resulted in a gradual dissociation of the casein micelle into primary casein particles, hence the diverse particle size population observed in C-MPC dispersions. The broadening of the size distribution, particularly with regard to the larger particles up to 1000 nm (Fig. 5.3) was seen previously by Power *et al*, (2020) whereby a calcium chelator (sodium hexametaphosphate) was used to bind calcium from both the aqueous and micellar phase in MPC dispersions, and even though the micelle structure was disrupted the particle size of the protein solutions ranged from <100 nm to ~1000 nm. Interestingly, Panouillé *et al* (2004) stated that primary casein particles have the ability to re-aggregate and that the rate of aggregation strongly depends on the casein concentration, temperature and pH; and depending on these factors extensive aggregation of casein particles can eventually lead to gelation. Panouillé *et al* (2004)

also found particles of up to 1000 nm in size in phosphocasein systems, whereby the casein micelles were dissociated using calcium chelating salts and suggested that it may be due to the presence of residual fat droplets; however it is more likely that it is a result of primary casein particle re-aggregation. The presence of residual fat droplets causing the large particle size in Fig. 5.3 in the current study is unlikely, as the presence of particles up to 1000 nm are not present in the C-MPC with protein:resin ratio of 100:0, and therefore is more likely a result of re-aggregation of primary casein particles in the calcium depleted samples.

Table 5.2. *Zeta potential (mV) and polydispersability index (PdI) values for control (C-MPC) and crosslinked (TG-MPC) casein protein dispersions measured as a function of protein to resin ratio.*

Protein:Resin ratio	Control		Crosslinked	
	ζ-Potential (mV)	PdI (-)	ζ- Potential (mV)	PdI (-)
100:0	-21.93	0.14	-21.63	0.13
76:24	-23.43	0.11	-23.97	0.12
56:44	-25.27	0.23	-25.50	0.18
38:62	-26.87	0.43	n.a.	0.28

n.a. Data not available as sample gelled

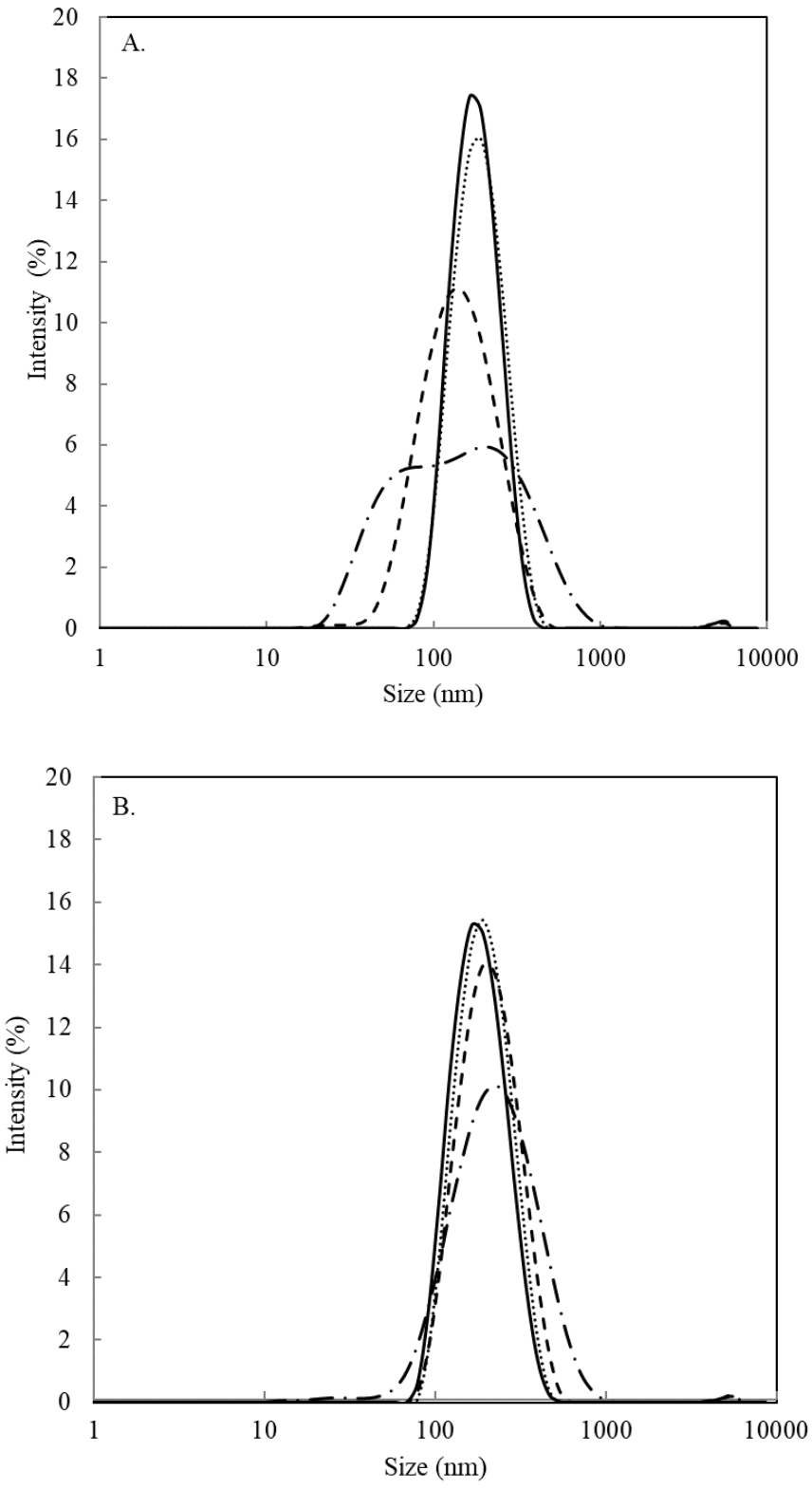


Fig. 5.3. *Particle size distribution profiles for control (a) and crosslinked (b) casein protein dispersions at protein:resin ratios of 100:0 (—), 76:24 (••••) (−•−), 56:44 (- - -) and 38:62(−•−).*

Aside from the loss of micelle structural stability due to the depletion of positively charged calcium ions, a simultaneous change was the increase in ζ-potential of the system from -21.9 to -26.8 mV (Table 5.2). This shift towards more negative net charge of calcium depleted C-MPC dispersions may be attributed to the exposure of negatively charged phosphate residues associated with casein proteins and the decrease in ionic calcium (Fig. 5.1) from the aqueous phase. The consequent increase in electrostatic repulsion between protein molecules may have added to the loss in micelle integrity.

Unlike the broad size distribution profiles observed for calcium depleted C-MPC dispersions, TG-MPC dispersions with protein:resin ratios of 76:24 and 56:44 had monomodal PSD profiles, similar to the non-calcium depleted crosslinked sample (Fig. 5.3). TGase has been shown to stabilise casein micelles against the action of dissociating agents. Previous studies performed by O'Sullivan *et al* (2002), Smiddy *et al* (2006) and Moon *et al* (2009) showing that TGase alters the heat stability of milk by crosslinking individual caseins, prevented dissociation and concurrently increased stability against micellar dissociation by urea and SDS, with casein micelle stability increasing with enzyme incubation time. Although it must be noted that the TG-MPC samples that were subjected to the same protein:resin ratios of 76:24 and 56:44 as C-MPC samples had significantly lower levels of depletion (Section 5.4.1). Therefore, it may be expected that the PSD profiles of TG-MPC dispersions would have been affected to a lesser extent. However, a monomodal PSD profile was still observed for the TG-MPC sample with a protein:resin ratio of 38:62, while it experienced a similar level of calcium depletion as its corresponding C-MPC sample (Table 5.1). This was also supported by the correspondingly lower PdI values, compared to C-MPC samples (PdI values of 0.13 and 0.28, respectively; Table 5.2). Interestingly, the observed

increase in ζ-potential for calcium-depleted TG-MPC samples was comparable to C-MPC dispersions and may be a result of the similar reduction in ionic calcium as shown in Fig. 5.1. Particle size data correlated well with colour analysis for all samples (Table 5.3), with an initial decrease in *L**-value for C-MPC dispersions with protein:resin ratio of 76:24 but with no such decrease observed for the crosslinked sample. The C-MPC dispersion with a protein:resin ratio 38:62 was the most distinctly different from the dispersion with a protein:resin ratio of 100:0, with a total colour difference (ΔE value) of 34.6, compared to 22.5 for TG-MPC, owing to the differences in micelle dissociation.

Table 5.3. *Colorimetric coordinates for control (C-MPC) and crosslinked (TG-MPC) milk protein dispersions*

Resin ratio	Control				Crosslinked			
	L^*	a^*	b^*	ΔE	L^*	a^*	b^*	ΔE
100:0	77.1	-4.15	0.47	-	77.1	-4.38	-0.41	-
76:24	70.6	-4.86	-1.91	6.9	74.8	-4.73	-1.74	2.72
56:44	55.5	-3.50	-3.22	21.9	65.7	-4.75	-4.53	11.4
38:62	42.8	-0.49	3.47	34.6	54.8	-3.20	-2.93	22.5

L^* − Whiteness
a^* − Green-red spectrum with a range from -60 (green) to +60 (red)
b^* − Blue-yellow spectrum, ranging from -60 (Blue) to +60 (yellow)
ΔE − Total colour difference

5.4.4. Rheological measurements of milk protein dispersions

Viscosity profiles of C-MPC and TG-MPC dispersions, measured as a function of shear rate and temperature are shown in Fig. 5.4 and 5.5, respectively. C-MPC without calcium depletion showed a distinctive decrease in viscosity with increasing shear rate; however, interestingly calcium-depleted samples did not show this shear-thinning behaviour, with the increase in calcium depletion resulting in an increase in viscosity (Fig. 5.4A). This is due to extensive calcium depletion resulting in the weakening and dissociation of the casein micelle structure into primary casein particles (PCP). A study by Huppertz *et al* (2017) showed that the non-spherical particle shape derived from PCP in caseinate dispersions was found to be the main contributor to intrinsic viscosity as opposed to exclusively protein hydration.

The measured viscosity for C-MPC in the present study is much lower than values shown previously by Power *et al* (2020), for calcium-depleted MPC systems using sodium hexametaphosphate (SHMP). However, unlike the use of SHMP, which chelates calcium, and would have remained as an SHMP-calcium complex within the system, the ion-exchange process employed in the current study removed the chelated calcium from the protein dispersion, replacing it with sodium ions. Although it must be noted that performing calcium depletion in these MPC systems with a protein concentration of ≥15% (w/w) using ion-exchange was not possible under the conditions used in this study due to extremely high viscosity (data not shown). During the ion exchange process, changes in the electrostatic balance of minerals in the serum causes the diffusion of micellar calcium into the aqueous phase, resulting in increased miceller hydration and swelling (De Kort *et al*, 2009 and 2011; Holt, 1997, Power *et al*, 2020).

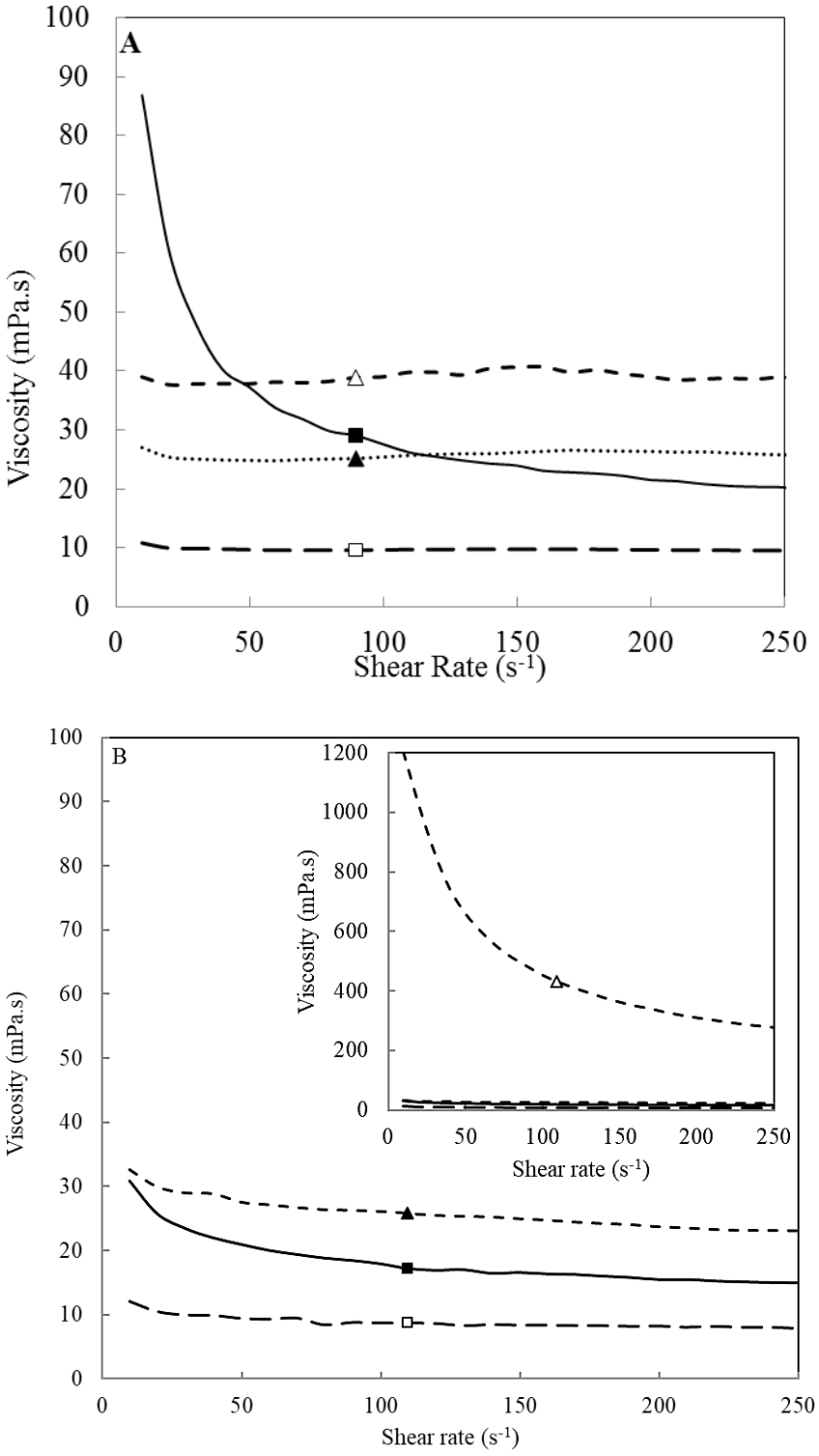


Fig. 5.4. *Apparent viscosity as a function of shear rate for control (a) and cross-linked (b) casein protein dispersions at protein:resin ratios of 100:0 (−■−), 76:24 (−□−), 56:44 (-▲-) and 38:62 (•Δ•). Fig. 5.4B inset shows the viscosity of crosslinked protein dispersion at a protein to ion-exchange resin ratio of 38:62 (•Δ•).*

TG-MPC dispersions with protein:resin ratios of 100:0, 76:24 and 56:44 all had lower viscosity compared to the corresponding C-MPC dispersions (Fig. 5.4B); however, the TG-MPC dispersion with a protein:resin ratio of 38:62 was significantly higher in viscosity than all other samples with an initial viscosity of ~1200 mPa.s, (shear rate of 0.1 s^{-1}) which decreased to 276 mPa.s at a shear rate of 250 s^{-1}, as a consequence of shear thinning (Fig. 5.4B inset). The extremely high viscosity observed at low shear rates, with samples displaying a weak gel-like behaviour, may be due to the capability of crosslinked primary casein particles to bind high levels of water. Huppertz and De Kruif (2008) stated that crosslinked casein micelles could be considered a nanogel-type system, with extreme stability against dissociation agents. In addition, Huppertz *et al* (2007) stated that covalently-linked casein micelles, from which micellar calcium phosphate has been solubilised, displayed increased swelling behaviour upon changes in solvent environment. It may be hypothesized that at extensive calcium depletion, such as the TG-MPC sample at a protein:resin ratio of 38:62, that the aqueous phase has significantly changed to this casein nanogel-type network and so crosslinked caseins are capable of increased hydration.

The influence of temperature on viscosity indicated that all dispersions had lower viscosity with increasing temperature (Fig. 5.5), with the greatest decrease in viscosity observed for the TG-MPC dispersion with protein:resin ratio of 38:62 (viscosity decreasing from 190 mPa.s at 20°C to 50 mPa.s at 55°C). Furthermore, it may be anticipated that gelation could occur in extensively calcium-reduced MPC systems, as seen in previous studies by Bhaskar *et al* (2007) where the authors showed that calcium reduction of 20-85% in the temperature range 35-95°C can result in the formation of milk gels. However, maintaining temperature in a range that minimises

viscosity issues is crucial for efficient calcium depletion using ion exchange technology.

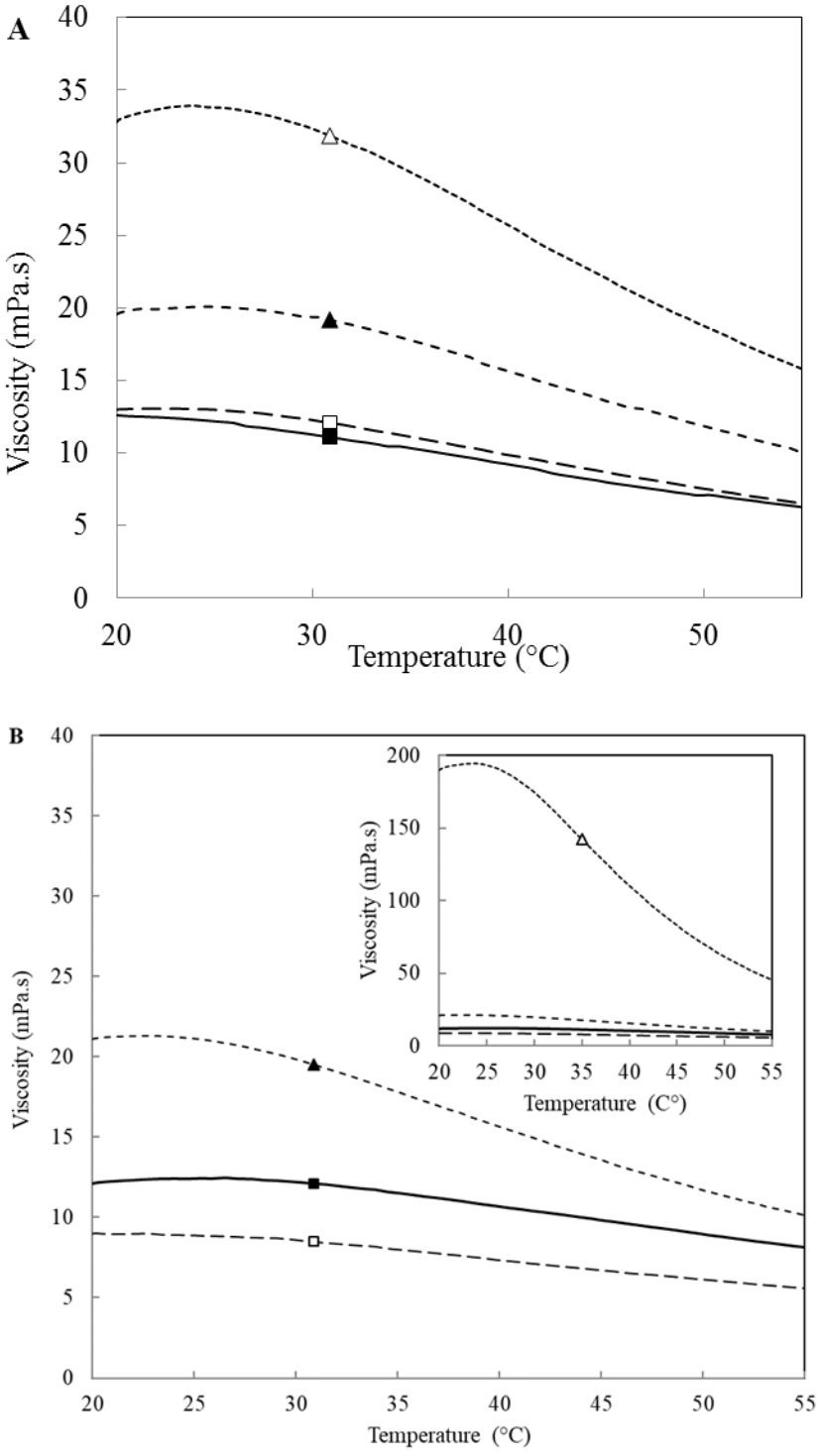


Fig. 5.5. *Apparent viscosity at shear rate of 100 s^{-1} for control (A) and crosslinked (B) protein dispersions at protein:resin ratios of 100:0 (−■−), 76:24 (−□−), 56:44 (-▲-) and 38:62 (•Δ•), measured as a function of temperature. Fig. 5.5B inset shows the viscosity of crosslinked protein dispersion at a protein:resin ratio of 38:62 (•Δ•).*

5.5. Conclusion

The depletion of calcium from milk protein concentrate dispersions using ion-exchange resin technology resulted in significant physicochemical changes in the protein dispersions, with an observed reduction in whiteness and increase in viscosity. The use of ion-exchange to deplete calcium in transglutaminase crosslinked casein protein dispersions was not as effective at low levels of resin addition, due to the covalent linkages in the structure of the micelles, hindering micellar calcium from chelation. However, micelles were progressively dissociated and weakened with increasing resin addition level, with dispersion viscosity increasing significantly as a consequence. This may be due to increased water binding capability owing to the structure of covalently linked primary casein particles. From a nutritional perspective, one must also take into account the significant increase in the counter ion, sodium, as a result of this approach to calcium depletion, which may need to be considered when formulating nutritional products. This work has provided an insight into the impact of calcium depletion through ion exchange and provides evidence for improved casein micelle stability, but that extensive calcium depletion in crosslinked casein systems results in highly viscous protein concentrates.

5.6. References

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CHAPTER SIX

Calcium reduction using ion-exchange alters the rehydration and thermal stability properties of milk protein concentrate powders

Orla M. Power[1, 2], Mark A. Fenelon[1], James A. O'Mahony[2], Noel A. McCarthy[1]

[1]Food Chemistry and Technology Department, Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[2]School of Food and Nutritional Sciences, University College Cork, Cork, Ireland

Declaration:

This chapter was written by author Orla M. Power (OMP) and reviewed by co-authors Dr. Noel A. McCarthy (NAMC), Dr. James A. O'Mahony (JAOM) and Dr. Mark Fenelon (MK). OMP co-designed the study with NAMC and performed all of the experimental work.

6.1. Abstract

The aim of this study was to examine the rehydration, viscosity and thermal stability of calcium-reduced milk protein concentrates (MPC; achieved using a strong cationic ion exchange resin). The extent of calcium reduction was 0 (MPC-0; i.e., control), 30 (MPC-30), 52 (MPC-52) and 70% (MPC-70). Calcium-reduced MPC powders had altered rehydration, turbidity and heat coagulation properties. Powder rehydration characteristics, as determined via particle size analysis, was significantly more rapid and extensive in calcium-reduced samples, compared with the control sample. Viscosity was measured at 10%, w/w, protein, and increased with increasing calcium depletion. The heat stability of MPC suspensions (1.5%, w/w) was measured at 140°C in the pH range 6.2–7.2 and at pH values <6.8, the heat stability of MPC-0 was <2 min, while at pH values ≥6.8, the HCT was significantly higher. The reduction of calcium resulted in the heat stability of MPC-50 and MPC-70 having highest HCT at pH 6.2.

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6.2. Introduction

Proteins are valuable major components of milk and can be utilised in the formulation of numerous food products, in either dried powder or liquid concentrate form. Milk protein concentrate (MPC) powders, with protein content of 47-90%, can be produced from skim milk using ultrafiltration/diafiltration, evaporation, heat treatment and spray-drying (Havea, 2006). MPC powders often have poor dissolution properties, primarily due to the close proximity of protein molecules and the hydrophobic nature of the casein protein constituents, with previous studies identifying dispersion of the casein fraction as the rate limiting step during rehydration of MPC powders containing ⩾80% protein (Crowley *et al* 2014; de Kruif *et al*, 2012; Holt *et al*, 2013; Horne, 2006; Mimouni *et al*, 2010). During powder particle dissolution, the slow penetration of water into primary particles contributes to the poor rehydration characteristics of casein-dominant powders by impeding the subsequent release of colloidal material (Anema *et al*, 2006; Arnaud *et al* 2009; Bastiaan *et al*, 2016; Fang *et al*,, 2012; Gaiani *et al*, 2007; Arnaud *et al*, 2009).

Several different formulation and technological approaches have been investigated to improve the dissolution properties of MPC powders, including ultrasonication, extrusion porosification, calcium-depletion, mineral addition and enzymatic modification (Bhaskar *et al*, 2007; de Kort *et al*, 2011; McCarthy *al* 2014; McCarthy *et al*, 2017; Power *et al*, 2020b; Xu*et al*, 2016). Bouvier *et al*, (2013) reported enhanced powder rehydration properties in MPC powders processed using extrusion porosification, a technique which increases the size and number of pores in MPC powder particles. In a study by McCarthy *et al*, (2014), the application of high intensity ultrasound (i.e., ultrasonication), significantly accelerated the dissolution of MPC powders.

Modification of the calcium content of high protein dairy powders, using both calcium chelating salts and ion exchange chromatography have proven to be effective in enhancing the solubility of such powders. Sikand *et al* (2011) reported that the solubility of commercial MPC (40 and 80%, protein) and MPI (90%, protein) was strongly influenced by mineral composition (e.g., calcium, magnesium, phosphorus and sodium) of the powders. The calcium content of MPC liquid concentrates can be reduced using different approaches; calcium chelating salts, which bind positively-charged calcium ions using negatively-charged counter ions (e.g., citrate, phosphate or EDTA), while calcium ion exchange resins exchange sodium counter ions for calcium ions. Depending on the strength of the calcium chelator or quantity of exchange resin used, both serum and colloidal (i.e., protein bound and colloidal calcium phosphate) can be removed, resulting in disruption of casein micelles and consequent loss of turbidity (de Kort *et al*, 2011; Pandalaneni *et al* 2018; Power *et al*, 2020a; Xu *et al*, 2016). Calcium chelating salts have been shown to improve dissolution properties (McCarthy *et al*, 2017), to increase thermal stability (Pandalaneni *et al*, 2018), to improve solubility and reduce insoluble matter in milk solutions (Bhaskar *et al*, 2007) and improve cold water solubility (Marella, *et al*, 2015) of MPC powders. However, the use of calcium chelating salts often contributes to higher viscosity of MPC dispersions, resulting in processing challenges during evaporation, pumping, spray drying or in end use applications (Power *et al*, 2018). These increases in viscosity can be attributed to hydration and swelling of casein micelles, due to reduced structural rigidity following the reduction of colloidal calcium phosphate (de Kort *et al*, 2011; McCarthy *et al*, 2017; Omoarukhe *et al*, 2010; Power, *et al*, 2020a). With our group having previously shown that the addition of calcium chelating salts improves dissolution characteristics of MPC powders (Power *et al*,

2018 and 2020b), this study aims to investigate the impact of reducing calcium content, using ion exchange, on the dissolution, heat stability and viscosity characteristics of spray dried MPCs and builds on the previous work in Chapter five which examined the impact of calcium ion exchange on transglutaminase modified MPC dispersions.

6.3. Materials and methods

6.3.1. Materials

Milk protein concentrate (MPC) powder, with a casein:whey protein ratio of 81:19 was purchased from a local dairy ingredient company and had protein, moisture, fat, lactose and ash content of 81.4% (w/w), 4.30% (w/w), 1.40% (w/w), 5.1% (w/w) and 7.8% (w/w), respectively. Cationic ion-exchange resin (Amberlite™, IR120; $(C_{10}H_{11})_A(C_8H_9O_3S)_B(C_2H_6)$, bead particle size 0.6 to 0.8 mm) was obtained from Fisher Scientific (Dublin 15, Ireland). Sodium azide was obtained from Sigma Aldrich (Arklow, Co. Wicklow, Ireland).

6.3.2. Rehydration, calcium depletion and spray drying of milk protein concentrate

Milk protein concentrate powder was rehydrated (10%, w/w, protein; 100 kg batch size) in reverse osmosis (RO) water at 50°C using a YTron high shear mixer (YTRON Process Technology GmbH & Co. KG, Germany). MPC powder was added slowly to the YTron high shear mixer and re-circulated through an agitated jacketed tank prior to gentle overnight agitation at 4°C.

Four aliquots of MPC (15 kg, 10%, w/w, protein) were heated to 30°C and the pH adjusted to pH 5.9 using hydrochloric acid (2 M HCl) to compensate for the increase in pH during the ion-exchange process, as determined from preliminary trials.

A control sample was prepared under the same conditions but without the addition of resin (MPC control). Calcium reduction was performed using a strong cationic calcium ion exchange resin (Amberlite^{TM} IR120) at 25°C. Resin beads were added to the MPC dispersion at protein:resin ratio of 100:0 (Control), 78:22, 59:41, 42:58, necessary to achieve progressive calcium reduction levels of 0 (i.e., Control), 30, 52 and 70% of the original calcium content. After resin addition, MPC dispersions were gently agitated for 1.5 h, before filtering through cheese cloth to remove the resin beads, and after resin removal, the pH of the MPC dispersions were readjusted to pH 6.7 using 2 M HCl (2M) or sodium hydroxide (2 M) prior to spray drying. Liquid MPC dispersions were then heated to 50°C prior to drying at inlet and outlet temperatures of 184 and 85°C using a two fluid nozzle atomization system, as described by Power *et al* (2020b). The resulting powders were packaged in sealed bags and stored in the dark at 4°C. All spray drying trials were performed in duplicate.

6.3.3. Mineral profile and calcium ion activity of milk protein concentrates

Total calcium, sodium, magnesium and potassium content of MPC samples were determined using inductively coupled plasma mass spectrometry (ICP-MS) as per the method of Lawrence *et al* (2018). The calcium ion activity (mmol/L) of rehydrated dispersions was analysed in triplicate using a Sension^{+} bench-top laboratory ion meter (Sension^{+} MM374 GLP 2 channel, HACH, Germany) equipped with a calcium ion selective electrode (Sension^{+} 9660C, HACH, Germany) calcium combination ion-selective electrode (ISE), at pH 6.5 as per the method of Power *et al* (2020a).

6.3.4. Temperature-dependant dissolution of milk protein concentrate powders

The dissolution properties of MPC powders were analysed using a laser-light diffraction unit (Malvern Mastersizer, Malvern Instruments Ltd, Malvern Worcestershire, UK) as per the method previously described by Power *et al*, (2020b). All particle size analyses were performed in triplicate.

6.3.5. Protein particle size distribution of milk protein concentrates

Particle size distributions of MPC dispersions (10% protein, w/w) were assessed in disposable cuvettes using a Zetasizer nano (Malvern Instruments, Malvern, Worcestershire, UK). Dispersions were diluted 1:50 with tempered deionised water. The refractive index (RI) of water, used as dispersant, was 1.330, with an assigned viscosity of 0.8872 cP. Samples were characterised as protein using RI of 1.45 and absorption value of 0.001. Experiments were carried out in triplicate with a backscattering angle of 173°.

6.3.6. Colour analysis of milk protein concentrates

The colour parameters of dispersions and powders were analysed using a Minolta Chroma Meter CR-400 colorimeter (Minolta Ltd., Milton Keynes, UK) as per the method of Magan *et al* (2019). Parameters included L^*, denoting whiteness (0-100), a^* indicating green-red spectrum (green (-60) to red (+60)) and b^* indicating blue-yellow spectrum (blue (-60) to yellow (+60)). Dispersions were measured in plastic cuvettes in triplicate and from the L^*, a^* and b^*, the total colour difference (ΔE) was calculated using Equation 1 (Mohammadi *et al*, 2008). In the equation L_o, a_o and b_o refer to control samples without calcium reduction while L, a and b denote the colour of calcium reduced samples.

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \quad \text{(Eq.1)}$$

6.3.7. Viscosity analysis of milk protein concentrate dispersions

Viscosity analysis of MPC dispersions of all calcium reduced samples were performed using a controlled-stress rheometer (AR-G2 Rheometer, TA Instruments, Crawley, UK) equipped with a parallel plate geometry. Dispersions were pre-sheared at 200 s^{-1} for 1 min, followed by a continuous shear rate ramp from 0.1 to 300 s^{-1} over 5 min at 25°C. All measurements were carried out in triplicate.

Temperature-dependent viscosity analysis of MPC dispersions was performed using a controlled-stress rheometer (AR-G2 Rheometer, TA Instruments, Crawley, UK) equipped with a concentric cylinder geometry. Dispersions were conditioned using a shear rate of 100 s^{-1} for 10 s at a constant temperature of 20°C. Temperature-dependent viscosity was analysed using a temperature ramp step from 20-55°C for 10 min, at a constant shear rate of 100 s^{-1}. All analysis was carried out in triplicate.

6.3.*8. Thermal stability of milk protein concentrate dispersions*

The heat coagulation time (HCT)-pH profiles of rehydrated MPC dispersions were measured using a temperature-controlled Elbanton oil bath (Hettich Benelux Laboratory Equipment, Geldermalsen, the Netherlands). Dispersions were reconstituted to 1.5% (w/w) and pH adjusted in increments of 0.2 between pH 6.2 and 7.2, using 1 M HCl or NaOH. Aliquots (3.4 mL) of dispersions were pipetted into glass tubes and stoppered prior to insertion into the rack of the oil bath. The rack was then inserted into the oil bath at 140°C and gently rocked, during which the time was recorded when visible coagulation first occurred in dispersions. All analyses were carried out in triplicate.

6.4. Results and discussion

6.4.1. Mineral analysis of milk protein concentrate

Concurrent reductions in total and ionic calcium content were measured in MPC powders with increasing resin:protein ratio (Tables 6.1 and 6.2), with the calculated extent of total calcium reduction amounting to 0 (i.e., Control), 30, 52 and 70% of the total calcium content of the original (i.e., control) sample. In a parallel manner, the concentration of ionic calcium decreased from 4.95 in the Control sample to 1.27 mM in the 70% reduced sample. Due to the ion-exchange process, the reduction of calcium was associated with increases in sodium (i.e., counter-ion) content from 0.53 to 2.18 g 100 g^{-1} of powder for the MPC-0 and MPC-70 samples, respectively (Table 6.1). The concentrations of mono- and divalent minerals, such as potassium and magnesium, both decreased significantly with increasing resin:protein ratio; however, the concentration of phosphorus remained unchanged as the majority of phosphorus in milk systems is present as negatively-charged phosphate ions as an integral component of colloidal calcium phosphate (CCP) nanoclusters. CCP has a complex composition and structure, containing high proportions of calcium, magnesium, and sodium, with the exact composition of CCP dependent on the ionic environment. CCP is an important structural component of casein micelles, holding caseins together via interactions between negatively charged phosphoserine residues, reducing electrostatic repulsion between casein molecules (Holt 1997, Walstra, 1990). Therefore, the reduction of calcium alters the ionic balance of the aqueous phase, increasing the electrostatic repulsion between proteins created by newly exposed serine phosphate groups and causing structural instability of casein micelles.

Table 6.1. *Mineral content (g 100 g^{-1}, dry matter) of milk protein dispersions measured using inductively coupled plasma mass spectrometry (ICPMS), as a function of protein to resin ratio.*

Protein:resin ratio	Calcium	Magnesium	Phosphorus	Potassium	Sodium
	g 100 g^{-1} of powder				
100:0	1.94[a]	0.09[a]	1.27[a]	0.63[a]	0.53[a]
78:22	1.35[a]	0.06[a]	1.30[a]	0.40[b]	1.28[b]
59:41	0.91[b]	0.04[b]	1.29[a]	0.23[c]	1.74[c]
42:58	0.57[c]	0.03[b]	1.38[b]	0.16[c]	2.18[d]

[a-d]Values within a column not sharing a common superscript differ significantly ($P < 0.05$).

Table 6.2. *Calcium ion activity (mM) and polydispersability index (PdI) values of milk protein concentrate (MPC), expressed as a percentage of calcium reduction.*

Calcium reduction (%)	Calcium ion activity	PdI
	mM	
MPC-0	4.95	0.27
MPC-30	2.47	0.21
MPC-52	1.57	0.20
MPC-70	1.27	0.37

Calcium ion activity was measured at pH 6.5

6.4.2. Temperature-dependant dissolution of milk protein concentrate powders

The dissolution properties of MPC powders dispersed in deionised water at 24 and 40°C are shown in Fig. 6.1A and B, respectively. The particle size distribution (PSD) of the control (i.e., MPC-0) sample was monomodal, with a broad primary peak ranging between ~4-135 μm at 24°C. Calcium reduction was observed to alter the PSD, in MPC-30 dispersions a tight monomodal peak was observed with a greater particle size range, ~5-175 μm, indicating significant particle hydration and swelling, even at only 30% calcium reduction however, the volume of larger particles was observed to be lower in MPC-30 ($D_{4,3}$ = 26.9 μm) than the control MPC-0 dispersion ($D_{4,3}$ = 38.6 μm; Fig. 6.2A). In comparison, the PSD profiles of MPC-52 and MPC-70 had the largest volume of small particles, ranging between 0.01 and 1.0 μm, indicating a substantial increase in particle dissolution. The substantially greater powder particle dissolution in calcium-reduced MPC, compared to MPC-0, has significant consequences for powder solubility, particularly at relatively low rehydration temperatures. In a previous study Bhaskar *et al* (2004), reported that calcium reduction using ion exchange improved the solubility of high protein powders at temperatures ≤20°C.

Control and calcium reduced powders were rehydrated at 40°C resulting in improved powder dissolution for all powders tested, compared to 24°C (Fig. 6.1B), with an immediate dissolution of particles, but still with the trend that MPC-0 and MPC-30 displayed the highest volume of large particles (i.e, particles between 3.0 and 100 μm). Several previous studies have demonstrated that rehydration of high protein dairy powders is most rapid and extensive at temperatures between 40 and 55°C (Baldwin, 2010). This has important implications in reducing costs associated with rehydration and the risks of microbial growth during rehydration of high protein

content dairy powders. However, the concentration of sodium ions was observed to increase with calcium removal (Table 6.1). Previous studies by Sikand *et al*, (2013), have shown that sodium addition in the form of sodium chloride (NaCl) have also improved MPC hydration but not to the same extent as calcium removal in the present study.

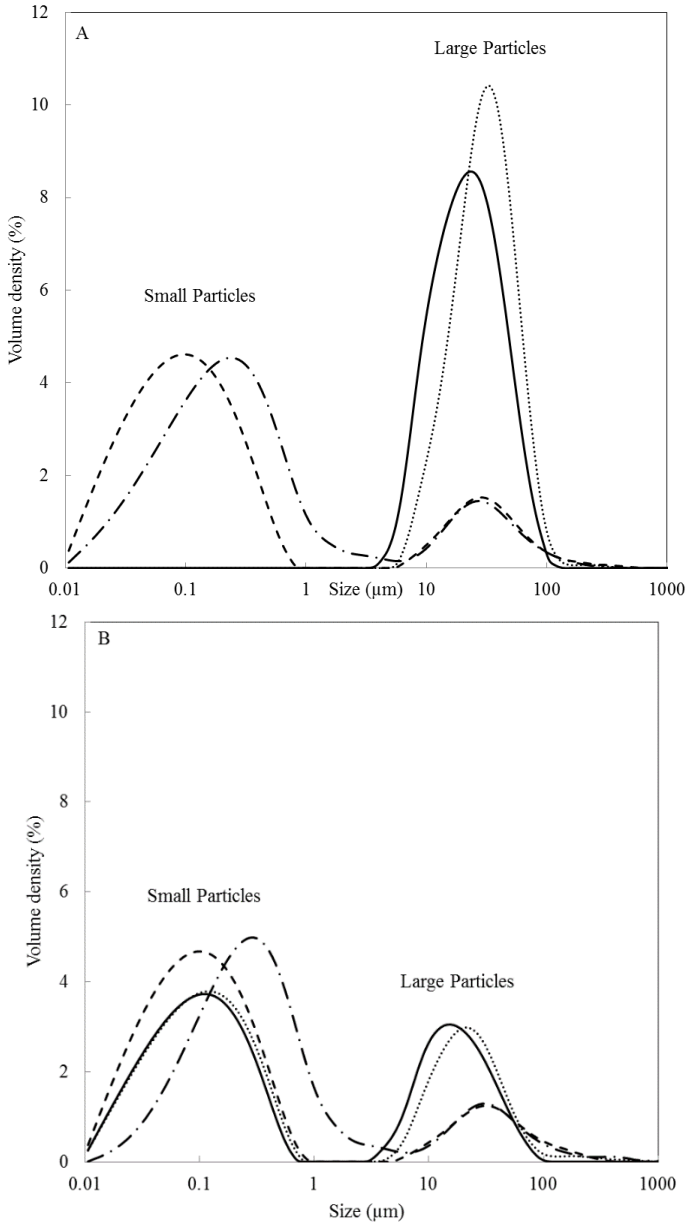


Fig. 6.1. *Particle size distribution profiles of milk protein concentrate powders rehydrated (10% protein, w/w) using high shear mixing at 24°C (A) and 40°C (B) for 30 s at calcium reduction levels of 0 (−), 30 (•••), 52 (- - -) and 70% (−•−).*

6.4.3. Particle size distribution and colour analysis of milk protein concentrate

MPC-0 was observed to have a monomodal primary peak in the 79-460 nm region, indicative of rehydrated MPC powder particles (McCarthy *et al*, 2017b). In powders with 30% calcium reduction, a slight deviation in size profile was observed when compared to MPC-0 (Fig. 6.2); however, it was observed that the progressive reduction of calcium caused a significant change to the protein particle size distribution; MPC-52 and MPC-70 had significantly broader size range from ~80 to 1000 nm, also indicated by the increase in polydispersity (PdI) values as shown in Table 6.2. The greater size range may be due to micelle swelling but also the subsequent aggregation of primary casein particles after the dissociation of casein micelles. A concomitant reduction in whiteness (L^*-value) was also observed in rehydrated MPC powders, with the L^* value decreasing with progressive calcium reduction, from 82.9 to 55.1 nm in MPC-0 and MPC-70, respectively (Table 6.3). Xu et al (2016) reported that upon 29.6% reduction of calcium in an MPC system, there was an 80% decrease in turbidity, but only a 33% decrease in mean particle size.

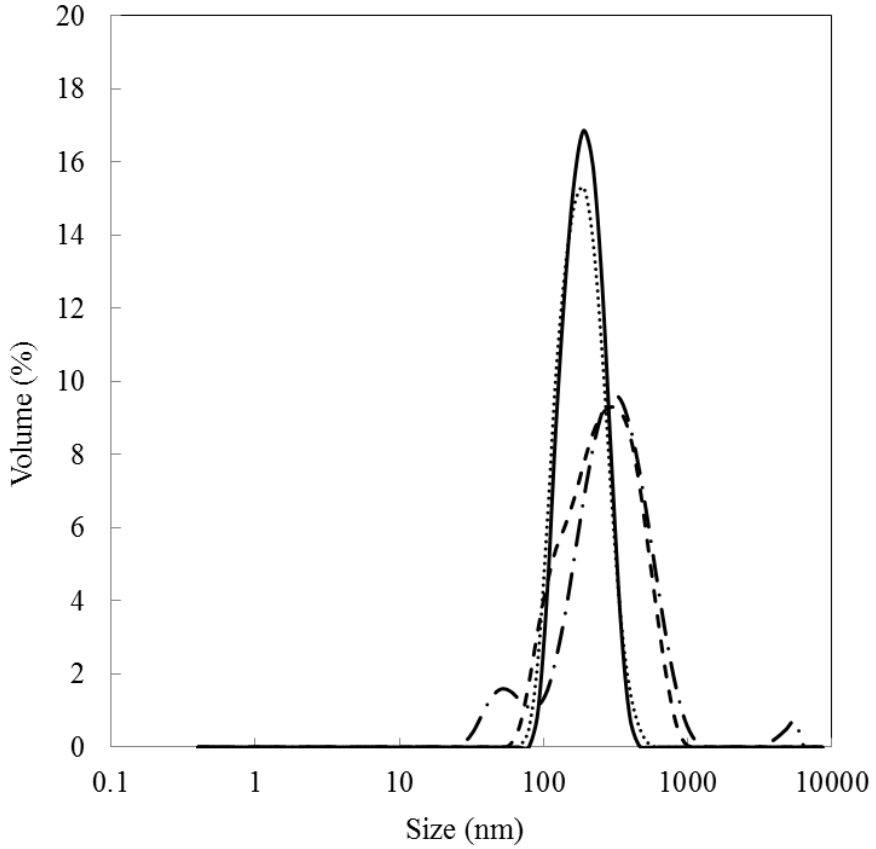


Fig. 6.2. *Particle size distribution profiles of rehydrated milk protein concentrate at 0 (−), 30 (•••), 52 (- - -) and 70% (−•−) calcium reduction.*

Table 6.3. *Colorimetric coordinates of calcium reduced milk protein concentrates and subsequent spray dried powders.*

% calcium reduction	Rehydrated protein dispersions				Powders			
	L*	a*	b*	ΔE	L*	a*	b*	ΔE
MPC-0	82.9[a]	-4.14	1.38	-	94.3	-1.49	7.06	-
MPC-30	75.5[b]	-4.71	-0.13	7.55	92.9	-1.79	8.06	1.79
MPC-52	68.9[c]	-4.30	0.20	14.0	93.2	-1.84	8.82	2.13
MPC-70	55.1[d]	-3.25	-0.65	27.9	93.4	-1.66	8.67	1.85

L* − Whiteness
a* − Green-red spectrum, ranging from -60 (green) to +60 (red)
b* − Blue-yellow spectrum, ranging from -60 (Blue) to +60 (yellow)
ΔE − Total colour difference

6.4.4. Viscosity of milk protein concentrate dispersions

The viscosity of MPC dispersions increased with increasing calcium reduction, with final viscosity of 8.1, 10.4, 19.7 and 25.0 mPa.s for MPC-0, MPC-30, MPC-52 and MPC-70, respectively (Fig. 6.3). Higher viscosity at higher levels of calcium removal from MPC has been shown previously by McKenna *et al* (2000); the authors observed during the manufacture of high protein powders using ultrafiltration and diafiltration prior to drying, dissociation of colloidal calcium phosphate, resulting in swelling of casein micelles due to a loss of structural rigidity. The effect of increasing temperature on the viscosity of MPC dispersions is shown in Fig. 6.4. The viscosity of MPC-0, MPC-30, MPC-52 and MPC-70 were observed to decrease with increasing temperature (Fig. 6.4). This could potentially be due to a higher degree of flexibility in casein micelle structure and reduced intra- and intermolecular mobility which may counteract the fact that hydrophobic interactions within casein micelles would also have increased (Ho *et al*, 2018).

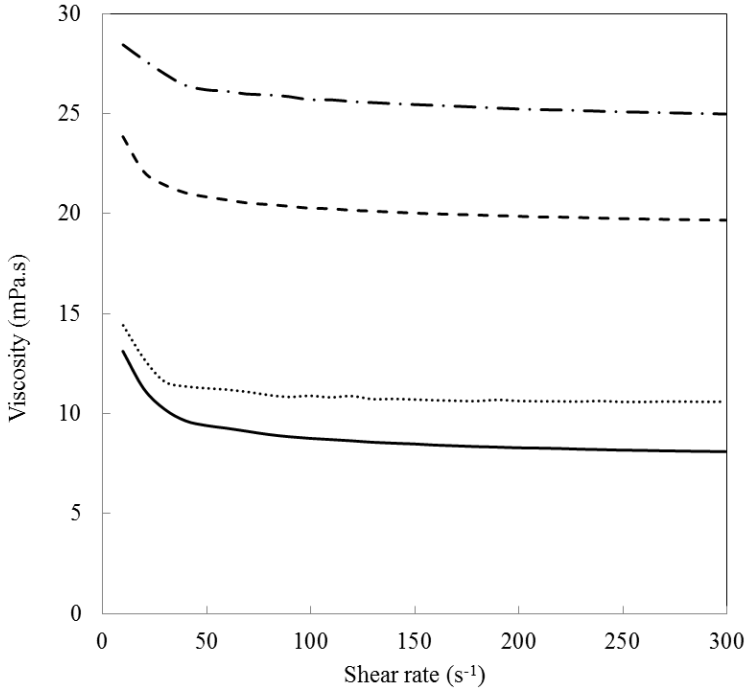


Fig. 6.3. *Viscosity measured as a function of shear rate for milk protein concentrate (10%, w/w, protein) dispersions at calcium reduction levels of 0 (−), 30 (•••), 52 (- - -) and 70% (−•−).*

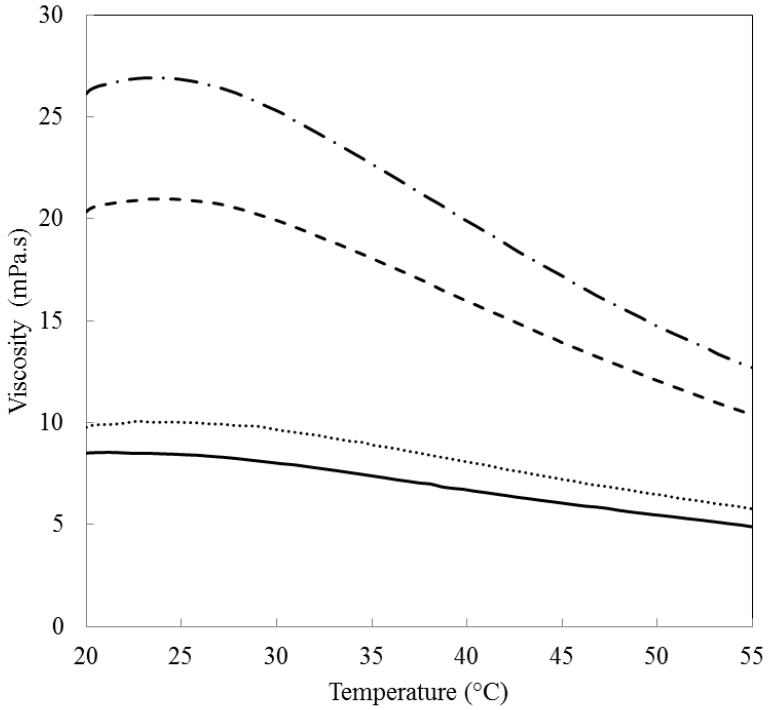


Fig. 6.4. *Viscosity (shear rate 100 s^{-1}) of milk protein concentrate dispersions measured as a function of temperature from 20 to 55°C at 0 (−), 30 (•••), 52 (- - -) and 70% (−•−) levels of calcium reduction.*

6.4.5. Thermal stability of milk protein concentrate dispersions

The HCT profiles of protein dispersions, measured as a function of pH, at 140°C are shown in Fig. 6.5. The HCT of MPC-0 was <1 min at pH values <6.8, with significantly higher HCT at pH 7.0 (13 min) and pH 7.2 (16 min). This was similar to that shown previously by Crowley *et al* (2014) who reported that MPC suspensions exhibited higher HCT with increasing pH, due to a reduction in calcium ion activity which off-set the expected destabilization by heat-induced dissociation of κ-casein. Previous studies, such as De Kort *et al* (2012), Gaucheron (2005), Philippe *et al* (2003) and Sievanen *et al* (2008) have shown that high calcium ion activity is a primary contributor to reduced heat stability in the pH range 6.3–6.7. However, a very different HCT-pH profile was observed for MPC-30, which had a minimum HCT at pH 6.2 (<1 min), maximum HCT at pH 6.4 (9.46 min) and a significantly lower HCT thereafter at pH values of 7.0 and 7.2 (2.38 and 2.27 min, respectively). HCT-pH profiles of MPC-52 and MPC-70 displayed the opposite trend in HCT-pH profile to that of MPC-0, with HCT of ~8 min at pH 6.2, which continued to decrease with increasing pH. The higher heat stability at pH values $\leq$6.8, compared to MPC-0, can be attributed to the overall lower total calcium content in the systems and so even with solubilisation of calcium phosphate with decreasing pH, its overall ionic calcium content remained lower. Interestingly, the level of phosphorus remained unaffected by the ion exchange process as shown in Table 6.1; therefore, the phosphorus:calcium ratio increased with increasing extent of calcium reduction and so may also have contributed to the higher heat stability observed at pH values <6.8.

Eshpari *et al* (2017) showed that calcium reduction of MPC (80%, w/w, protein) through acidification and ultrafiltration, caused a signifcant reduction in their heat stability, compared to the original skim milk, when measured at pH ~6.7. The

consensus that the serum phase composition of milk plays a pivotal role in influencing heat stability, and is dependent on factors such as levels of urea, lactose, ionic calcium and phosphate, is well established (Metwalli and van Boekel, 1996; Singh, 2004). Crowley *et al* (2014) found that the addition of urea to MPC systems did little to affect the heat stabilty at pH values <6.8, but significantly increased their heat-induced coaglution times at pH > 6.8. Jeurnink and de Kruif (1995) demonstrated that milk dispersions with reduced calcium content had lower heat stability, attributed to the dissociation of casein micelles into primary casein particles and hence resulting in a loss of κ-casein-induced stabilisation, resulting in lower heat stability. In the current study, as shown in Fig. 6.6, the increased heat stability of calcium reduced MPC may find potential in low pH product applications.

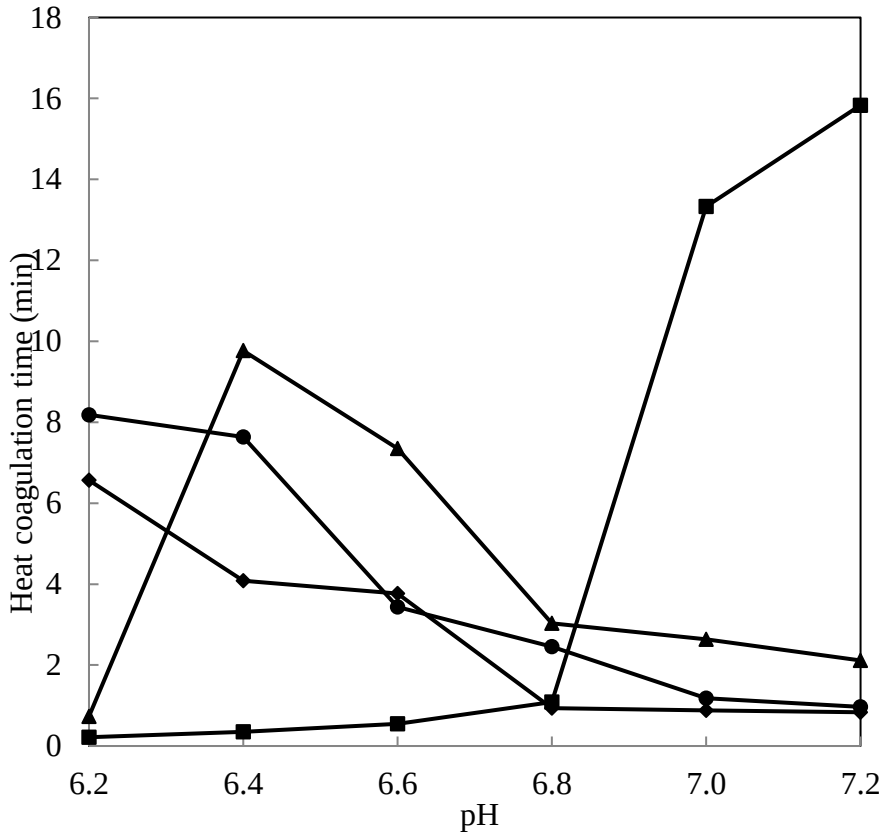


Fig. 6.5. *Heat coagulation time-pH profiles of rehydrated milk protein concentrate powders (1.5%, w/w) at 0 (■), 30 (▲), 52 (●) and 70% (♦) levels of calcium reduction.*

6.5. Conclusion

A reduction in the calcium content of MPC samples significantly reduced the structural integrity of casein micelles, due to the reduction of colloidal calcium phosphate nanoclusters. This reduction in calcium content improved the rehydration properties of MPC powders, resulting in more rapid powder dissolution, even at room temperature. Manipulation of the calcium content of MPC also significantly impacted the pH-heat stability relationship. The pH of maximum heat stability became more acidic with calcium removal and decreased with increasing pH. Improved powder dissolution characteristics at ambient temperature may prove beneficial for use of such powder, as ingredients in cold soluble nutritional powder applications. However, the mineral composition may require adjustment during product formulation due to the increased levels of sodium and reduced calcium content.

6.6. References

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CHAPTER SEVEN

General discussion and suggestions for future research

Declaration:

This chapter was written by author Orla M. Power (OMP) and reviewed by co-authors Dr. Noel A. McCarthy (NAMC), Dr. James A. O'Mahony (JAOM) and Dr. Mark Fenelon (MK).

7.1. General discussion

Milk protein concentrate (MPC; 80%, w/w, protein) powders are important ingredients in nutritional dairy products, often included in infant formula, medical nutrition, muscle building and sports recovery beverages. As ingredients, MPC powders can be used to modify the functional properties of foods, including water binding capacity, gelling, foaming, emulsification and heat stability (Ye, 2011; Huppertz and Patel, 2012). One of the greatest challenges encountered during the processing of milk protein concentrates is the high associated viscosity on ultrafiltration and evaporation. Increased viscosity can be caused by the concentration of milk proteins and an increase in bound moisture due to denaturation/aggregation of protein during heat treatment, hence high protein concentrates are typically evaporated to relatively low total solids content, compared with skim milk, prior to drying to prevent process performance issues (e.g., high pumping energy costs, blockages and fouling) related to such high viscosity (Ho *et al*, 2013; Buggy *et al*, 2017). MPC powders often exhibit poor rehydration properties, primarily due to the proximity of protein molecules and the strongly hydrophobic nature of casein proteins (McCarthy *et al*, 2017). The overall objective of the research reported in this thesis was to investigate the impact of enzymatic modification of casein and the chelation-mediated depletion of calcium on functionality of milk protein concentrate ingredients in powder and liquid format.

Calcium chelating salts have been added for technological reasons, such as increasing heat stability of protein dispersions and enhancing powder rehydration properties of MPC systems (De Kort *et al*, 2011, McCarthy *et al*, 2017, De Kruif and Holt, 2003, Lin *et al*, 1972, Odagiri and Nickerson, 1964; Ramchandran *et al*, 2017).

However, such intended benefits can be undermined by the high viscosity of the protein concentrate stream prior to spray drying, requiring such MPC concentrates to be dried at even lower solids contents. The aim of this study (**Chapter 2**) was to understand in more detail the fundamental chemistry underlying the increased viscosity of MPC concentrates on addition of the calcium chelating salt sodium hexametaphosphate (SHMP). Dephosphorylation of casein was performed using bovine alkaline phosphatase and phosphate nuclear magnetic resonance (P^{31} NMR) analysis proved effective in providing new information on the influence of polyphosphate salt addition to MPC, concurrently showing changes in both phosphoserine and inorganic phosphate (Pi) signals in control and dephosphorylated samples. Dephosphorylation was observed to fully deplete the colloidal calcium phosphate (CCP) nanoclusters, SHMP addition (5 mM) had no discernible impact on the ^{31}P NMR spectra of dephosphorylated MPC, however the addition of 5 mM SHMP to control MPC dispersions resulted in a shift in peaks associated with the CCP region, attributed to SHMP sequestering calcium, contributing to swelling of casein micelles. Dephosphorylated MPC exhibited a lower viscosity compared to control MPC dispersions; however, SHMP additions at 12.5 and 25 mM caused gelation in both control and dephosphorylated MPC dispersions. This study clearly demonstrated that dephosphorylation is effective in the control of viscosity development in MPC dispersions in which calcium-chelating salts are added for technological reasons such as increasing heat stability of MPC dispersions and enhancing powder rehydration properties.

A second study (**Chapter 3**) focused on controlling viscosity and maintaining casein micelle stability in MPC dispersions with added SHMP. As shown previously in Chapter 2, calcium chelation in MPC contributes to higher viscosity due to

dissociation of casein micelles and changes to the calcium equilibrium. In this study (**Chapter 3**) crosslinking casein proteins using transglutaminase prior to addition of SHMP significantly reduced associated viscosity development, with the effect being most pronounced at low temperature. Enzymatic crosslinking enhanced casein micelle structural stability and contributed to greater retention of the whiteness of MPC dispersions, even after SHMP addition. This chapter provided new knowledge on the factors responsible for increased viscosity during calcium chelation, mainly casein protein dissociation and micelle swelling, and demonstrated for the first time that enzymatic crosslinking is effective in controlling viscosity development in MPC systems with added calcium chelating salts.

Based on the findings of **Chapter 3**, spray dried MPC powders were produced with crosslinked casein proteins and added SHMP. This work (**Chapter 4**) demonstrated that the hydration of milk protein concentrates can be improved by mineral chelation and that enzymatic crosslinking of casein proteins can be implemented to off-set increases in viscosity during processing, primarily due to the network of covalent bonds helping to retain casein micelle structure. Crosslinking MPC samples resulted in smaller powder particle size values compared to non-crosslinked samples when dried in the presence of SHMP, potentially due to the lower viscosity during atomization. The addition of SHMP improved water sorption and hydration properties of MPC powders. The higher viscosity of control non-crosslinked MPC liquid feed prior to spray drying can be related to findings in **Chapter 2**; it was shown that the addition of 5 mM SHMP to MPC caused a significant increase in viscosity, hence if the viscosity of the liquid concentrate increases but the energy supplied to the atomization nozzle remains constant the resulting atomized droplet size will be larger. Increased feed viscosity affects spray pattern, with a more viscous liquid

typically producing a narrower spray angle, with larger atomised droplets as reported by Murphy *et al* (2013). SEM images of control crosslinked MPC powders demonstrated that increasing the SHMP concentration caused the surface morphology of powder particles to progressively change from having a shrivelled, collapsed appearance to a smoother, spherical particle surface. In contrast, TG-MPC powder morphology remained relatively unchanged with increasing SHMP concentration. Crosslinking improved powder hydration and water sorption, even without addition of SHMP, when compared to the corresponding non-crosslinked control. Overall, crosslinking casein proteins in MPC powders had positive effects on water sorption and hydration in addition to decreasing concentrate viscosity during the drying process.

Calcium ion exchange can be implemented as an alternative method for calcium depletion in MPC dispersions. Calcium ion exchange resins simultaneously deplete calcium and release monovalent positively charged counter ions. The work in **Chapter 5** examined the effects of calcium depletion using ion-exchange on the physical properties of non-crosslinked and crosslinked MPC dispersions, providing an insight into the impact of ion-exchange mediated calcium reduction and provided evidence for improved casein micelle stability, but only up to certain point of calcium depletion. The ion-exchange resin was added at four different levels to deplete MPC of calcium with protein:resin ratios of 100:0, 76:24, 56:44 or 38:62. The depletion of calcium from milk protein dispersions using ion-exchange resin caused significant changes to casein micelle structure, with a reduction in whiteness and increase in viscosity. The use of ion-exchange to deplete calcium in crosslinked casein protein dispersions was not as effective as non-crosslinked controls; covalent transglutaminase-mediated linkages hindered micellar calcium from being chelated.

However, the micelle structure was weakened and dissociated as resin concentration increased, with viscosity being significantly higher in crosslinked 38:62 dispersions than non-crosslinked protein dispersions, potentially due to increased water binding capability owing to the structure of covalently linked primary casein particles.

In **Chapter 6**, liquid MPC with varying degrees of calcium reduction, produced through calcium ion exchange, were spray dried at pilot scale to produce calcium reduced powders. The powders were then analysed for the impact of calcium reduction on rehydration, viscosity and thermal stability produced. The extent of calcium depletion was 0 (MPC-0; i.e., control), 30 (MPC-30), 52 (MPC-52) and 70% (MPC-70). The viscosity of rehydrated calcium depleted MPC powders increased with increasing calcium depletion. Powder rehydration, as determined from particle size measurements, was significantly more rapid and extensive in calcium-depleted samples at room temperature (24°C) than the control MPC powder. Removal of calcium from MPC resulted in improvements in powder solubility, but significantly altered the heat stability of the rehydrated MPC dispersions. Thermal stability measured at 140°C showed that calcium chelation caused the pH of maximum heat stability to decrease and that at pH values above 6.8 the heat stability was reduced.

Examined collectively, the studies conducted in this thesis have developed and applied novel methods, generating new insights to improve the rehydration characteristics of MPC powders, while controlling associated increases in viscosity and demonstrating how disruption of the casein micelle structure results in changes to the overall functionality of the resulting dispersion. Micellar casein systems are more difficult to disperse. Previous studies by Mimouni et al, (2010 a,b) investigated the rehydration process and the mechanisms responsible for the loss of solubility of MPC powders. They concluded that storage induced loss of solubility was due to changed

rehydration kinetics and not as a consequence of the formation of insoluble material over time. The results suggested that the release of casein micelles from powder particles was the rate-limiting step of the MPC rehydration process and compounded with storage. Water penetration into the powder particles was not considered as a rate-limiting factor as molecules larger than water (whey proteins, lactose ect) were freely released out of the powder structure in both fresh and aged MPC (Mimouni and others 2010 a,b). Further research conducted by Maidannyk et al, (2019) showed that MPC powder particles hydrated and swelled in solution but were slow to dissociate into individual casein micelles, indicating the presence of a "glue" type bond between casein micelles, or the colloidal calcium phosphate cluster (CCP).

Hence, casein phosphoserine residues in bovine milk are essential to micelle integrity, aside from its structural role in the CCP; phosphoserine residues bind miceller calcium phosphate nanoclusters which reduce the net negative charge on casein proteins, promoting attractive interactions between the hydrophobic regions of caseins. A study by Holt et al. (1998) showed that calcium phosphate nanoclusters could be prepared in-vitro and stabilised by casein phosphopeptides to prevent precipitation. Therefore, one cannot underestimate the role that calcium phosphate phosphoserine interactions play in micellar casein rehydration. The role of colloidal calcium phosphate and serine residues in structural stability and hydration were explored through the installation of covalent bonds using transglutaminase (chapters 3 and 4), the disruption of CCP using dephosphorylating enzymes to deplete phosphoserine residues (chapter 1) and through calcium reduction using calcium chelators and calcium ion exchange resins (chapter 5 and 6). Desphosphorylation depleted serine-phosphate residues and resulted in a loss of structural stability and subsequent reduction in viscosity. Therefore, poor micellar casein solubility is the

result of the combined effect of hydrophobic, hydrogen, van der Waals forces and phosphoserine-calcium phosphate interactions between casein micelles which are compacted together during the spray drying process.

The author would like to state that the experimentation was conducted using multiple bags of MPC 80 powder over a four-year period, which could be attributed to discrepancies in control viscosities, zeta potentials and particle size measurements. Spray dried dephosphorylated MPC powders where not produced at pilot scale due to the author exploring the concept of stabilising the casein micelle using covalent bonds, which was achieved using Transglutaminase (chapter 3 and 4).

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7.4. Suggestions for future work

- Based on the research in **Chapter 4,** future work on the effects of storage conditions on crosslinked protein powders may be of interest. Previous work by Mimouni *et al* (2010) stated that protein-protein interactions occur between micelles during storage, leading to the formation of a monolayer skin on the surface of powder particles composed of packed closely packed casein micelles which prevents rapid dissolution. However, crosslinking of casein proteins could hinder casein-casein surface interactions during storage, as crosslinked casein molecules may have less mobility to reorganise hydrophobic groups towards the powder particle surface.

- The inclusion of transglutaminase modified powders in the formulation of protein-based beverages. As observed in Chapter 3 transglutaminase crosslinking of casein proteins prior to addition of SHMP significantly reduced associated viscosity development but also improved subsequent rehydration properties of spray dried powders (Chapter 4). Therefore, the application of the modified powders could improve viscosity and rehydration properties of complex nutritional formulations. This can be substantiated by the higher viscosity observed in the control non-crosslinked MPC liquid samples, which prevented spray drying at high levels of SHMP addition.

- The inclusion of calcium reduced MPC (**Chapter 6**) in protein-based beverages to assess the rehydration and dispersibility behaviour. As it was determined that powder rehydration was significantly more rapid and

extensive in calcium depleted MPC samples at room temperature (24°C) than the control MPC powder. It would also be interesting to assess the impact of altering the mineral content of the calcium reduced powders, *i.e.*, reducing the sodium content (which increased with calcium reduction due to its presence as the counter ion associated with the ion exchange resin) or increasing the calcium content again on the functional properties.

- In-vitro digestion
    - Transglutaminase uses an acyl-transferase mechanism to catalyse the transfer of a gamma-carboxyamide group on a glutamine residue to the gamma-amine of an adjacent lysine or glutamine residue forming covalent bonds (Fig. 1.9). Therefore, it would be interesting to investigate the impact of the covalent bonds on curd formation and enzymatic hydrolysis under in-vitro digestion conditions.

- The extent of dissociation and subsequent turbidity loss in MPC dispersions, is dependent on the type and concentration of calcium chelator, with a greater degree of casein micelle dissociation observed in the order of SHMP ≥ SP > TSC > Na_2HPO_4 > Na_2UMP calcium chelating agents. The extent of turbidity loss is dependent on the strength of the calcium chelator to dissociate the casein micelle. Hence, it would be interesting to investigate the impact of different calcium chelators on the viscosity, turbidity and rehydration properties of crosslinked and dephosphorylated casein protein liquids and powders (based on the experimental methods of **Chapters 2/3/4**)

- A limiting factor in the use of transglutaminase in industrial processing is the duration (24 h) and temperature (30°C) required for incubation which may promote microbial growth. For experimental applications sodium azide can be employed to prevent microbial spoilage, however, a detailed examination of using transglutaminase under refrigeration temperatures to form covalent bonds is worthy of further investigation.