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**THE CASEIN MICELLE AS AN ENCAPSULATION SYSTEM FOR
TRICLOSAN: MECHANISMS OF MICELLE DISSOCIATION,
ENCAPSULATION, RELEASE, AND *IN VITRO* DELIVERY**

A Dissertation Presented for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Adrienne L. Roach
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DEDICATION

Scientific research is most purposeful if human lives are positively impacted. It was my daily prayer before entering the lab every morning that through this research process, His name is glorified and His people are edified. This dissertation is dedicated to those motivated by that statement. Whatever it is you are doing, impact the lives of those around you. Be a teacher whenever the opportunity presents itself. Operate with a purpose. To much is given much is expected. Give until there is nothing left to give. The revolution is here.

Be the change *you* want to see – Gandhi.

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ABSTRACT

This dissertation reports on the use of the casein micelle as an encapsulation and potential delivery system for a low molecular weight hydrophobic compound, triclosan. The ability of the casein micelle to serve in this capacity was tested in a series of experiments investigating: 1) micelle dissociation to expose hydrophobic binding sites of interaction, 2) encapsulation of triclosan, 3) the release of triclosan under various conditions, and 4) the intercellular delivery of triclosan to human liver cells *in vitro* for the inactivation of a malaria-causing parasite. Casein micelle dissociation and reassociation was achieved through high pressure homogenization and solvent-mediated pressure-induced dissociation. Triclosan was found to naturally associate to casein micelles in skim milk (up to 70%) and this association is enhanced by 30% at homogenization pressures reaching 300 MPa. The release of triclosan is governed by the disruption of micelle integrity, i.e., the enhancement casein-solvent interactions, where triclosan likely exists as a triclosan-protein complex as opposed to free triclosan. In the presence of milk, the release of triclosan is prevented in simulated gastric solutions but released in a simulated intestinal solution, demonstrating the ability of milk to protect triclosan from the acidic environment of the stomach and promote its release in the intestine, a measure of bioaccessibility. The intercellular delivery of triclosan was most affected by the type of milk protein. Triclosan transported within β -casein isolates where most sufficient in preventing the growth and development of *Plasmodium berghei*, the rodent malaria parasite, in human liver cells. Triclosan carried within milk digestates were also efficient in delaying parasite growth, whereas triclosan carried within milk

serum by serum proteins where completely ineffective. Triclosan in the presence of milk also provided a protective effect on host cells from the acute toxicity of triclosan in free form. These experiments introduced new functional properties of the casein micelle. The casein micelle has the ability to serve as an encapsulation system and casein proteins, as a potential delivery system. This introduces new functional properties of the casein micelle for use novel usage within the food, pharmaceutical, and/or cosmetic industry.

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INTRODUCTION

In nature, the casein micelle has a simple, yet indispensable, biological role, i.e., to transport calcium, minerals, bioactive peptides, and other nutrients to the feeding mammalian young. What is intriguing about this milk protein, which has been around since the first mammals roamed the earth, likely unchanged, is the intrinsically disordered structure of the individual casein proteins. These amphiphilic molecules have little to no secondary structure and exist in what is commonly referred to as a random coil (Holt and Sawyer 1993). However, these random coils associate through both hydrophobic and electrostatic interactions to form one of the most fascinating protein structures, the casein micelle. Commercial uses for casein proteins reflect the diversity of this supramolecular structure, ranging in use from surfactant molecules in food emulsions to industrial strength adhesives for a number of applications. The efficiency of this nanoparticle in doing so, as well as its incorporation into numerous products, is attractive to polymer scientists especially with the recent popularity of nanotechnology.

Casein Micelle Biogenesis

One of the most differentiated and metabolically active organs in the body is the lactating mammary gland, which secretes lactose, proteins, enzymes, fat globules and cellular membranes for the nourishment of mammalian young. The casein micelle is an association colloid formed within the secretory cells of the mammary glands through the biological assemblage of the four amphiphilic casein proteins, κ -, α_{s1} -, α_{s2} -, and β -casein. Post translational phosphorylation occurs on the serine residues of the calcium sensitive caseins, α_{s1} - α_{s2} - and β -casein, providing a site for the binding of calcium and the subsequent formation of calcium phosphate nanoclusters that serve to link the hydrophilic

regions of the protein molecules and aid in the overall stability of the casein micelle (Farrell and others 2006).

Protein synthesis of casein molecules occurs within the ribosomes on the rough endoplasmic reticulum (ER). Casein polypeptide chains contain cleavable amino targeting sequences called signal sequences recognized by receptors on the ER that eventually give rise to the individual casein proteins. Although not well defined, protein folding occurs prior to entering the Golgi apparatus. Small vesicles at the ER exit site export proteins from the ER to the *cis* face of the Golgi apparatus. The Golgi apparatus is the site of post-translational phosphorylation of the casein proteins and the site of “partial” casein micelle assemblage. Casein phosphorylation is carried out by casein kinase and occurs at different, possibly precise, compartments within the Golgi network. Turner and others (1993) report that phosphorylation of the α -caseins occur in the *cis* Golgi apparatus as submicellar structures have been detected there. Phosphorylation of κ - and β -casein is believed to occur further along the pathway in the *trans* Golgi prior to cell secretion. After secretion to the lumen, these newly phosphorylated proteins are exposed to Ca^{2+} , which initiate the final process of micelle formation.

The phosphorylation of the casein proteins is essential for Ca^{2+} binding and the association of the caseins to form micelles. The most popular type of phosphorylation is the formation of phosphate ester bonds with hydroxyl side chains of serine and threonine residues (Kjeldsen and others 2007). In the bovine casein micelle, phosphorylation occurs along the serine residues (SerP) of the calcium sensitive caseins within the Golgi

apparatus of mammary epithelial cells. The ratio of SerP in the caseins is approximately 1:5:8:11 for κ -, β -, α_{s1} - and α_{s2} -casein, respectively.

Micellar Stability

While the actual structure of the casein micelle has been under debate for quite some time, there are 3 undisputed integral components of the system that contribute to its stability: 1) the surface location of κ -casein, 2) the stabilizing effect of colloidal calcium phosphate through cross-bridging and 3) the presence of hydrophobic protein-protein interactions (Walstra 1990).

Kappa-casein

Kappa-casein, the calcium insensitive casein, is stable in high ionic environments and solely responsible for suspending the micelle within aqueous solutions, as it is located on the surface of the micelle (de Kruif 1999). The C-terminal region of κ -casein is hydrophilic, highly hydrated and extends into solution and often referred to as a polyelectrolyte brush. Its negative surface charge provides steric repulsions preventing aggregation with adjacent micelles. However, the collapse of this brush or the removal of caseinomacropeptide (the hydrophilic portion that extends into solution) initiates micelle flocculation, precipitation or gelling.

Colloidal calcium phosphate

The role of calcium is apparent in the stability and overall structure of the micelle based on the ability of caseins to fluidize the abundant amount of calcium present that would otherwise calcify the mammary gland or precipitate out of solution. Calcium is present in milk at an average concentration of 32 mM, where 2 mM of this exists as free

ionic Ca^{2+} . Studies using microscopy have revealed the uniform distribution of calcium phosphate nanoclusters within the casein micelle. However, whether these nanoclusters link casein submicelles or exist dispersed within a network of casein proteins connected through altering hydrophobic and hydrophilic residues, remains to be determined. Micelle dissociation can be initiated by the removal of colloidal calcium phosphate through a number of methods, which include the use of chelating agents, dialysis against Ca^{2+} , acidification, or pressure (Panouille and others 2004). Often, for complete micelle disruption, hydrophobic sites of interaction between the protein constituents also need to be disrupted or destabilized.

The Casein Micelle as a Potential Carrier for Hydrophobic Compounds

Disruption of the casein micelle to its submicellar constituents either as smaller aggregates (<100 nm), individual casein proteins, or a mixture of both, exposes not only binding sites of interaction, but also sanctions the proteins to behave as individual copolymers where their amphiphilic nature can be realized. Of particular interest is the exposure of hydrophobic sites of interaction. Nanoparticles formed from amphiphilic copolymers, both natural and synthetic, have been extensively explored as carriers for hydrophobic compounds (Tang and Du 2008; Sun and others 2001). Biopolymers can be an appealing alternative to synthetic polymers as a delivery system based on commercial availability, relatively low cost to produce, the absence of harsh chemical reagents to initiate polymerization, and biodegradability. Commonly used natural polymers include gelatin, collagen, starch, dextran, and chitosan (Sun and others 2001).

A limiting factor in realizing the use of the casein micelle as a carrier molecule for a number of compounds is that its actual quaternary structure has not been resolved. Explaining micellar dissociation and reassociation can be difficult if the native structure is ill defined. On the contrary, molecular interactions that contribute to stability are well known as well as the properties of the individual proteins. Micelle stability is based on the ability of the casein micelle to maintain its structural integrity under adverse environmental conditions. Disruption of calcium cross-bridging and hydrophobic interactions are the only two properties that can lead to micellar dissociation. Temperature, pH, salt concentration, the presence of organic solvents, and chelating agents all affect the functional stability of the casein micelle promoting both association and/or dissociation. While the effects of these components on micelles are individually well defined, the combined effects of two or more can act synergistically on the micelle and behave in a manner completely different than it would alone.

The Current State and Future Potential of Casein Proteins

As highlighted earlier, the casein proteins enjoy widespread application in a number of products for both food and non-food applications. In food, they are often utilized for the improvement of functional properties such as water binding, viscosity enhancement, emulsion stability, and texture. Non-food applications include glue, adhesives, and stabilizing components in paint, to name a few. One of the latest applications explores the use of the casein micelle for the delivery bioactive compounds and pharmaceuticals (Huppertz and de Kruif 2008). In this case, the casein proteins are treated as block copolymers that self-associate under the proper conditions. Block

copolymers are amphiphilic molecules, having a hydrophilic region and hydrophobic region, i.e., blocks that self assemble and used in a number of industries often for encapsulation purposes. Viewing casein proteins as block copolymers can allow applications that parallel it to the use synthetic polymers. For example, Bajpai (1999) investigated the release behavior of drugs from caseins cross-linked with polyacrylamide hydrogels. In a more recent study, Huppertz and de Kruif (2008) examined the structure and stability of nanogels prepared by crosslinking caseins with transglutaminase to create a stable casein network. This study was a preliminary exploration to create a structure suitable for the sustained release of pharmaceuticals or other bioactive compounds.

The use of caseins for the aforementioned purposes is beneficial for a number of reasons. Biocompatibility (aside from allergic responses) along with biodegradability of starting components and byproducts are the two top benefits of using this biopolymer for applications currently dominated by synthetic polymers. However, a brief history lesson on materials used before the petrochemical-based polymers highly produced after World War II, would reveal that the majority of the plastics before that time were protein-based and largely of casein and soy origin. The non-food use of caseins dates as far back as ancient Egyptians who used it to bind paint pigments. The first patents on casein-based plastics were granted in 1885 and later combined with other components to expand its functional properties such as durability and hardness. Interestingly, in 1926 over 50% of the worlds buttons were made of casein. Casein production increased from 9,071 metric tonnes in 1930 to 54,430 metric tonnes in 1932 worldwide. In 1938 a chemist in New York developed a method for making casein plastic without the use of formaldehyde for

hardening and secured a patent but the adoption of synthetic plastics during and after WWII halted the use of protein-based plastics. [Ralston and Osswald 2008]

OUTLINE OF DISSERTATION

This dissertation will explore the uniqueness of the casein micelle and introduce it as a biopolymer capable of polymer-like behavior such as micellar assembly, the encapsulation of hydrophobic compounds, and triggered release. **Chapter 1** will explore the use of high pressure homogenization as a means to disrupt casein micelles into submicellar components and micellar properties post treatment. **Chapter 2** reports on the association of triclosan to the casein micelle as induced by pressure and the presence of solvents to enhance casein dissociation. **Chapter 3** takes a microscopic look into the structural changes of the casein micelles subjected to dissociation and re-association via solvent, pressure, and pH induced dissociation. **Chapter 4** compares the release of triclosan from casein micelles under 3 different release conditions: 1) aqueous diffusion, 2) pH triggered release, and 3) release during simulated oral digestion. **Chapter 5** closes the dissertation with a qualitative look into the use of casein digestates and β -casein micelles as delivery vehicles to prevent the development of *Plasmodium* parasites in the liver-stage progression of malaria. The work was done at the Food Science and Technology Department at the University of Tennessee except that of delivery experiments that were conducted *in vitro* and analyzed through live cell imaging at the Bernhard-Nocht Institute for Tropical Medicine in Hamburg, Germany. Chapters 1 and 2 have been published in the *Journal of Innovative Food Science and Emerging Technologies* (2008) and in the *Journal of Food Science* (2009), respectively. Chapter 4 will be submitted as a manuscript (Journal to be determined).

REFERENCES

- Bajpai SK. 1999. Casein cross-linked polyacrylamide hydrogels: Study of swelling and drug release behavior. *Iranian Polymer Journal*, 8(4): 231-239.
- De Kruif CG. 1999. Casein micelle interactions. *International Dairy Journal*, 9(3-6): 183-188.
- Farrell HM, E Malin, E Brown, P Qi. 2006. Casein micelle structure: What can be learned from milk synthesis and structural biology. *Current Opinion in Colloid and Interface Science*, 11(2-3): 135-147.
- Holt C, L Sawyer. 1993. Caseins as rheomorphic proteins-interpretation of primary and secondary structures of the alpha-s1-caseins, beta-caseins and kappa caseins. *Journal of the Chemical Society-Faraday*, 89(15): 2683-2692.
- Huppertz T, CG de Kruif. 2008. Structure and stability of nanogel particles prepared by internal cross-linking of casein micelles. *International Dairy Journal*, 18(5): 556-565
- Kjeldsen F, M Savitski, M Mikhail, ML Nielsen, L Shi L, RA Zubarev. 2007. On studying protein phosphorylation patterns using bottom-up LC-MS/MS: the case of human alpha-casein. *Analyst*, 132(8): 768-776.
- Panouillé M, T Nicolai, D Durand. 2004. Heat induced aggregation and gelation of casein submicelles. *International Dairy Journal*, 14(4): 297-303.
- Ralston BE, TA Osswald. The history of tomorrow's materials: Protein-based biopolymers. *Plastics Engineering*, 64(2): 36-40.
- Sun Y, DC Watts, JR Johnson, AJ Shukla. 2001. Biodegradable drug delivery systems. *American Pharmaceutical Review*, 4(4):25-30.
- Tang YF, YM Du. 2008. Chitosan-based injectable thermosensitive hydrogel. *Progress in Chemistry*, 20(2):239-244.
- Turner MD, SE Handel, CJ Wilde, RD Burgoyne. 1993. Differential effect of brefeldin-a on phosphorylation of the caseins in lactating mouse mammary epithelial-cells. *Journal of Cell Science*, 106 (4): 1221-1226.
- Walstra P. 1990. On the stability of casein micelles. *Journal of Dairy Science*, 73(8): 1965-1979.

CHAPTER 1

Disruption and sedimentation of casein micelles and casein micelle isolates under high-pressure homogenization

ABSTRACT

Native casein micelles were isolated from raw skim milk by ultrafiltration (< 30 kDa) or microfiltration (< 0.2 μm) and subjected to high-pressure homogenization (HPH) at 100, 200, 250, 300, and 350 MPa. Of particular interest was the effect of HPH on casein micelle size in solutions varying in ionic strength (0, 5, 10, and 15 mM CaCl_2) and micelle size populations. Particle size distribution reflected an initial decrease in micelle diameter in all samples at 100 MPa. In samples containing 10 and 15 mM CaCl_2 , there was an abrupt increase in particle size and subsequent casein precipitation followed by sedimentation upon centrifugation at elevated pressures (300 and 350 MPa). The amount of sedimentable casein protein increased as CaCl_2 concentration (10 and 15 mM) and pressure (300 and 350 MPa) increased as determined by UV absorbance of sample supernatant. SDS-PAGE indicated extensive micellar disruption at elevated pressures (300 and 350 MPa) and confirmed that the sedimented portion of the samples contained casein proteins and minimal amounts of whey proteins. Results indicated that through HPH treatment casein micelle size can be modified based on CaCl_2 concentration and pressure applied. Based on these findings, HPH in combination with an appropriate suspending medium has the ability to modify micelles to a desired size for a number of potential applications.

* Manuscript was modified from its original published version to accommodate dissertation formatting.

INTRODUCTION

The use of high-pressure technology to improve the quality of food products dates as far back as the early 1900's with the observations of Hite (1899) on the extended shelf life and quality of milk and the sterilization of fruits (Hite and others 1914). Protein denaturation by use of high pressure was first observed when Brigman (1914) non-thermally coagulated egg whites. Until recently, the use of high pressure was limited to hydrostatic batch methods or, as with the homogenization of milk, restricted to low processing pressure (< 20 MPa). Because advances in homogenization design have greatly improved, processing pressures exceeding 350 MPa are now possible. Potential and current applications include the inactivation of enzymes in milk (Hayes and others 2005), non-thermal treatment of fluid foods, enhancement of rheological and emulsifying properties of whole milk (Kietczewska and others 2003), bacterial inactivation (Diels and others 2004; Diels and others 2003; Wuytack and others 2003; Wuytack and others 2002), molecular weight reduction of hydrocolloids (Floury and others 2000), production of fine lipid dispersions, and improved efficacy of cosmetic products and pharmaceuticals by the reduction of particle size in active ingredients (Jia 2005; Işcan and others 2005; Möschwitzer and Müller 2006).

The modification of protein structure by use of high pressure is also a growing area of interest. Protein function is determined by its three-dimensional conformation defined by its tertiary and quaternary structure. During HPH proteins are subjected to a simultaneous combination of applied stresses such as cavitation, turbulence, shear stress, friction, and heat. These forces have the ability to alter protein shape and hence, protein

functions. The proteins of the casein micelle are stabilized in an aqueous system of salts, lactose, and soluble serum proteins by steric and electrostatic interactions due to layers of κ -caseins that extend their hydrophilic C-terminal region from the surface of the casein micelle into solution (Walstra 1990). Micelle stability is based on three integral components of the micelle system: 1) κ -casein, 2) colloidal calcium phosphate, and 3) hydrophobic interactions. Altering the components that affect their stability can either result in micellar aggregation or dissociation into their submicellar components.

De Kruif (1999) describes κ -casein in functional terms as a salted brush of polyelectrolyte molecules that extend freely into the ionic environment of milk serum and serve to increase the solvency of the casein micelle by electrostatic and steric repulsions. When this surface protein is disturbed, the solvency of the casein micelle is affected. Under hydrostatic pressure, casein proteins are soluble in the order of $\beta > \kappa > \alpha s1 > \alpha s2$ -casein as pressure increases (Huppertz and others 2004) and κ -casein is solubilized up to 20% at 200 MPa (Needs and others 2000). At the same time, the “collapse” or removal of the polyelectrolyte brush can decrease micellar solubility and can induce micellar aggregation.

Micelle dissociation is induced when colloidal calcium phosphate is removed or dissolved by various processes such as chelation, dialysis, acidification (Panouillé and others 2004), or pressurization (Huppertz and others 2006a). High-pressure homogenization of casein micelles has been found to increase the amount of soluble Ca^{2+} and reduce the micelle effective diameter (Huppertz and others 2006a). The disruption of the micellar structure is also the consequence of pressure induced unfolding that alters the

tertiary and quaternary protein conformation, due to in part, to changes in secondary structure (Floury and others 2000) that can promote the liberation of Ca^{2+} into solution. However, salts in the aqueous environment of casein micelles have the ability to increase micellar solubility by the stabilization of the hydrophilic end of κ -casein through ionic bonding. Solubilized Ca^{2+} has been suggested to protect casein micelles against disruption by high pressure. Ionic Ca^{2+} can “screen” or “shield” charges on casein proteins during pressurization (Huppertz and others 2006a).

The hydrophobicity of caseins is important in the assemblage of the supramolecular structure of the casein micelle. Beta-casein is bound to κ -casein by hydrophobic interactions and characterized by its mobility and hydrophobicity serving as a link between submicelles (Gatti and others 1995). High pressure can disrupt these hydrophobic interactions and initiate micelle dissociation or aggregation, depending on the extent of applied pressure. The key determinate between micellar dissociation and aggregation is the stability of the polyelectrolyte brush of κ -casein. Aside from pressure, its functional stability is affected by other dynamics such as temperature, pH, salt concentration, and the presence organic solvents. It is suggested that only the extended brush is able to sterically stabilize the casein micelle and maintain micellar solvency (De Kruif 1999).

Current studies have demonstrated notable effects of HPH on the functional properties of skim milk (Adapa and others 1997; Hayes and others 2005). HPH has a disruptive effect on electrostatic and hydrophobic interactions within the micelle and among its protein constituents. The objective of this work was to study the effects of

high-pressure homogenization on the particle size of casein micelles and casein micelle isolates in solutions varying in calcium concentration.

MATERIALS AND METHODS

Milk supply and casein micelle separation and isolation

Raw whole milk was collected from a local dairy farm (University of Tennessee Agricultural Research Station, Knoxville, TN) and skimmed by centrifugation at 15,000 $\times g$ for 15 min. The following processes were employed for casein micelle separation and isolation: filtration of raw skim milk on 1) laboratory scale ultrafiltration cartridge (Millipore, Fisher Scientific, GA) made of regenerated cellulose with a nominal cut-off of 30 kDa and 2) laboratory scale hollow fiber microfiltration module (Spectrum Laboratories, CA) with a 0.2 μm polyethersulfone membrane cut-off. Two particle size populations of caseins were collected in an effort to account for the effects of whey proteins. Imidazole buffer solutions (20 mM) were prepared at calcium chloride (CaCl_2) concentrations: 0, 5, 10, and 15 mM. All buffer solutions were adjusted to a pH of 6.7 with 1N HCl. As permeated serum containing whey protein and other milk components was discarded, displaced volume was replaced with respective buffer solution. Retentate from each separation was collected for experimental analysis. Solution pH was re-adjusted to 6.7, if needed. Raw skim milk was also used in experiments for comparison purposes. Sodium azide (0.04%) was added to each buffer solution prior to filtration or preparation to prevent microbial growth. Milk samples were stored at 4°C prior to HPH treatment.

High-pressure homogenization

Casein micelle isolates (CMI) suspended in their respective buffer solutions were pressure treated by high-pressure homogenization (HPH) (model FPG 12500, Stanstead Fluid Power, Essex, UK) at the following pressures: 0, 100, 200, 250, 300, and 350 MPa. The operating structure consists of a bench top unit providing synchronized homogenization using two hydraulic intensifiers. Valve temperature was regulated by water bath (Isotemp 3016D, Fisher Scientific, Pittsburgh, PA) at a constant temperature of 4°C. CMI were collected in centrifuge tubes to a volume of 50 mL per pressure level. Pressure was increased until desired pressure level was reached and kept constant until total sample volume was collected. Temperature increases were noted during sample collection. After pressure treatment, samples were stored immediately at 4°C until further analysis.

Particle size distribution

Particle size distribution of homogenized samples was determined by static light scattering with a Horiba LA-910 particle size analyzer (Horiba Instruments Inc., Irvine, CA). Pressure treated samples were diluted at a ratio of 1:1000 (v/v) in either simulated milk ultrafiltrate (SMUF) or imidazole buffer. Apparent particle size measurements were made at a scattering angle of 90° using a relative refractive index of 108-000. Calibration was checked with casein micelles in buffer solutions at atmospheric pressure and a pH of 6.7. All measurements were conducted at room temperature (25°C).

Calcium quantification

Free soluble calcium was measured using a calcium combination electrode (Denver Instruments, Denver, CO) in pressure treated samples. The electrode was standardized for calcium quantification by serial dilution of a 1000 mg L⁻¹ stock solution of calcium provided by the manufacturer at a concentration range from 5 mg L⁻¹ to 300 mg L⁻¹. Sample pH was not adjusted prior to measurements.

Turbidity measurements

UV absorbance at 280 nm was measured on supernatant of pressure treated samples, 24 h after HPH treatment, using a BioMate 5 UV–Visible spectrophotometer (Thermo Spectronic, Rochester, NY). Prior to analysis, samples were centrifuged for 1.5 h at 4000 × *g*. Supernatant was carefully removed and transferred to glass vials for use in turbidity measurements. Supernatants were diluted in their respective buffer solutions as described previously. Dilutions were made accordingly, a 1:40 dilution was most sufficient for UV absorbance measurements. Quartz cuvettes were used for all UV absorbance measurements.

Protein identification by gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Bio-Rad (Hercules, CA) mini-gel electrophoresis unit. Samples were pre-heated (95°C for 4 min) in the presence of 2-mercaptoethanol. Proteins were stained using Coomassie Brilliant Blue R-250. Pressure treated samples used for analysis were subjected to centrifugation (4000 × *g* for 1.5 h) and supernatant generated was kept

frozen until analysis. SDS-PAGE was carried out on the supernatant of 30 kDa and 0.2 μ m CMI in buffer solutions containing 5 and 15 mM CaCl₂.

RESULTS

Temperature elevation during HPH

Temperature at the homogenizing valve (Fig. 1) * will increase as pressure increases due to friction and shear forces during the homogenization process. Generally, homogenization temperature increased 17°C per 100 MPa in a linear manner ($r^2 = 0.97$). An increase in temperature of approximately 12–18°C/100 MPa during high-pressure homogenization has been reported in similar studies (Sandra and Dalgleish 2005; Thiebaud and others 2003; Hayes and Kelly 2003). The circulating water bath maintained at a constant 4°C, distributed throughout the homogenizing system by tubing, aided in regulating temperature increases at elevated pressures. The degree of temperature fluctuation between experiments (Fig. 1) relates to the drift in pressure during experiments at a constant pressure. Normal pressure fluctuation is ± 20 MPa, as stated by the manufacturer. Therefore, temperature will also fluctuate accordingly, generally between 5°C and 10°C with greater fluctuation at higher pressures.

* All figures and tables are located in the appendix section found at the end of each chapter.

Particle size distribution of casein micelles: effects of HPH and calcium chloride concentration on casein dissociation and aggregation

Skim milk

Apparent casein micelle size of milk samples subject to high-pressure homogenization are shown in Fig. 2 for skim milk and Fig. 3 (a–d) for nano- and micro-filtrated samples. Dependent upon the milk sample, buffer solution, and pressure achieved, casein micelle size was either reduced or increased. As displayed in Fig. 2, casein micelle size in raw skim milk was greatly affected by pressure treatment. Average casein micelle size decreased by approximately 30% in a seemingly linear fashion ($r^2 = 0.92$) from 0 to 200 MPa with reduction in particle size from 278 nm (± 2.23) to 171 nm (± 0.035). However, at 250 MPa there is an increase in micelle size to 178 nm (± 0.036) and size continues to increase to 200 nm (± 0.771) at 350 MPa. Similar behavior was observed in casein micelle isolates but highly dependent upon the amount of CaCl_2 added to the buffer solutions.

Nano-filtrated casein suspension

In the nano-filtrated casein suspension (< 30 kDa), and in the absence of added CaCl_2 , micelle diameter decreased from 277 nm (± 0.084) to 184 nm (± 0.040) from 0 to 200 MPa and remained stable up to 250 MPa. Higher pressures, 300 and 350 MPa, sharply increased apparent diameter to 241 nm (± 0.078) and 544 nm (± 0.865), respectively. When CaCl_2 concentration was increased, micelle size remained at a constant average size of 180 nm (± 0.742) from 0 to 200 MPa in both 5 and 10 mM CaCl_2 solutions. In 5 mM, micelle size increased moderately at 250 MPa to 238 nm (± 1.41) and

remained at that size as pressure increased to 350 MPa. In 10 mM CaCl_2 , micelle size remained constant at about 188 nm (± 0.892) up to 250 MPa. At 300 MPa size slightly increased to 240 nm (± 2.48) but was followed by a drastic increase at 350 MPa to 14,200 nm (± 7.35). Similar behavior was observed in the 15 mM solution, except there was an initial decrease in micelle size from 237 nm (± 0.798) to 162 nm (± 0.034) at 200 MPa. Particle size increased slightly to 244 nm (± 3.26) at 300 MPa and increased abruptly at 350 MPa to 10,800 nm (± 5.96). This substantial size increase in micelles suspended in 10 and 15 mM CaCl_2 buffer at 300 and 350 MPa was manifested by the formation of micellar precipitation that was able to sediment upon centrifugation.

Micro-filtrated casein suspension

In the micro-filtrated casein suspension ($< 0.2 \mu\text{m}$), in the absence of CaCl_2 , casein micelle size remained at a stable 240 nm (± 0.068) from 0 to 250 MPa. Size then increased moderately to 277 nm (± 0.095) and 363 nm (± 0.190) at 300 and 350 MPa, respectively. In suspensions containing 5 mM CaCl_2 micelle size reduced initially from 237 nm (± 2.61) to 182 nm (± 0.039) from 0 to 200 MPa and increased moderately thereafter to 363 nm (± 1.07) at 350 MPa. In 10 mM CaCl_2 , micelle size remained at constant 182 nm (± 0.655) at the initial pressures of 0 and 100 MPa and increased moderately to 244 nm (± 1.67) at 250 MPa. At 300 and 350 MPa micelle size increased considerably to 12,400 nm (± 7.33) and 16,200 nm (± 8.83), respectively, (as noted previously in the nano-filtrated suspension at 350 MPa). A similar increase in size was also observed in the 15 mM CaCl_2 suspension where micelle size increased from 10,800 nm (± 7.43) at 300 MPa to 12,300 nm (± 10.0) at 350 MPa. Initially, there was a slight

decrease in size from 181 nm (± 0.313) to 161 nm (± 0.035) at 0 and 100 MPa. As observed in the nano-filtrated suspensions, casein micelles precipitated at 300 and 350 MPa, in both the 10 mM and 15 mM CaCl_2 suspensions and were able to sediment upon centrifugation.

Serum phase UV absorbance analysis

Absorbance measurements were performed on the aqueous portion of centrifuged (1.5 h, 4000 x *g*) samples (Fig. 4). A reduction in absorbance intensity reflects a higher degree of sedimentable milk components post high-pressure homogenization. A substantial decrease in turbidity was observed in samples subject to 300 and 350 MPa but based on the amount of CaCl_2 added to buffer solution. Samples containing 10 and 15 mM CaCl_2 had the most pronounced amount of sedimentable casein and a considerable effect on casein micelle size as discussed in the previous section. Turbidity remained fairly constant in skim milk throughout pressurization (Fig. 5).

DISCUSSION

The reduction of casein micelle size at pressures as low as 100 MPa in skim milk, nano- and micro-filtrations, contrast with Hayes and Kelly (2003) where casein micelle size was not affected at 150 MPa and only decreased minimally at 200 MPa. However, Hayes and Kelly (2003) used whole milk and achieved a significant reduction in fat globule size where as our focus was on skim milk and different populations of casein micelle isolates. Sandra and Dalgleish (2005), using skim milk powder, achieved a reduction in casein micelle size from 252 nm to 114 nm at 186 MPa during HPH. They concluded that this reduction was most likely due to the surface disruption of proteins

nearest the micellar surface and not from complete or partial micelle dissociation based on the presence of casein proteins, κ - and α_{s1} , in the serum portion of skim milk and absence of β -casein as determined by gel electrophoresis.

In our study, the presence of soluble casein proteins was reduced dramatically at pressures of 250 MPa and greater (with the exception of 0.2 μ m in 5 mM CaCl_2) while serum proteins, α -la and β -lg, remained at a fairly stable concentration throughout the pressure range in SDS-PAGE (Fig. 6 and Fig. 7). This suggests that the increase in casein micelle size was due to increased hydrophobic interactions of submicelles and not from the complexation of whey proteins, mainly β -lg, with surface components of the micelle, as complexed whey protein would have precipitated with the caseins. Sandra and Dalgleish (2005) noted that casein dissociation into submicelles was unlikely at pressures up to 184 MPa, but occurred rather from the detachment of surface components of the micelle, i.e., κ -casein. Extending this theory to higher pressures, it is likely that micelle disruption is possible at elevated pressures if the disruptive effects are multiplied as pressure increases. The minimal presence of soluble casein proteins in contrast to the presence of whey proteins at elevated pressures (300 and 350 MPa) indicates this possibility in our samples. It is, however, too premature to state whether complete disruption of micelles to their submicellar components was achieved due to the immediate re-association of micelles post homogenization.

HPH also resulted in the increase of particle size and aggregation of casein micelles at elevated pressures, mainly 300 and 350 MPa. Based on the findings of Huppertz and others (2004) under high hydrostatic pressure, casein micelle size can

increase due to primarily two occurrences, 1) the formation of β -lg-casein complexes and 2) casein aggregation. The denaturation of whey protein increases with rising pressure (> 250 MPa) and temperature (75–80°C) and, therefore, increases the interaction with the surface components of the casein micelle resulting in an increase in casein micelle size (Guyomarc'h and others 2003; Sandra and Dalgleish 2005; Datta and others 2005; Zobrist and others 2005). In the second theory of high-pressure induced aggregation, the collapse or removal of κ -casein reduces the steric repulsion between the micelles and causes them to cluster together. This aggregation is due to the decreased solubility of the micelle after solubilization of κ -casein. Kappa-casein is solubilized up to 20% under hydrostatic pressure at 200 MPa (Needs and others 2000). This reduces the negative charge and electrostatic repulsions between the micelles promoting micellar aggregation, thus increasing micelle diameter (Needs and others 2000).

Hydrophobic interactions of the casein proteins also play a major role in both casein dissociation and aggregation. High pressure can disrupt hydrophobic bonds within the micelle resulting in a reduction in micelle size or complete dissociation as with hydrostatic pressure at 400 MPa or greater (Huppertz and others 2004; Hayes and others 2005; Huppertz and others 2006a; Huppertz and others 2006b). Due to the novelty of high-pressure homogenization on the modification of milk proteins, there is a lack of information concerning its effect on casein micelles at pressures exceeding 250 MPa. Casein aggregation can also be the result of increased hydrophobicity once hydrophobic sites are exposed under high pressure. Submicellar casein particles are extremely hydrophobic and can result in substantial re-aggregation once pressure is released (Needs

and others 2000). Johnston and others (1992) reported that exposure of hydrophobic sites begin to increase at 200 MPa under hydrostatic pressure. Walstra (1990) distinguishes between different types of aggregation as the flocculation of micelles that fuse into larger micelles in a rough spherical shape resulting in visible particles and sedimentation. We believe that these phenomena are the most likely explanation for casein aggregation and subsequent precipitation in our samples.

The combined effects of temperature and pressure during the homogenization process affects the solubility of calcium phosphate. Solubility is reduced at elevated temperatures but enhanced at elevated pressures (Huppertz and others 2006a). This occurrence explains, at least partially, the behavior of the casein proteins during HPH in the presence of different concentrations of calcium in solution in the form of CaCl_2 . Precipitated calcium phosphate enhances the hydrophobic aggregation of the casein molecules into micelles (O'Connell and others 2001) and aids in protecting the micelle from pressure induced dissociation. Calcium not associated with colloidal calcium phosphate exists as free calcium ions and can serve to “protect” or “shield” charges on casein proteins during pressurization (Huppertz and others 2006a). With the exception of raw skim milk, Ca^{2+} concentration remained fairly stable during HPH (data not shown). In raw skim milk, Ca^{2+} concentration increases to 146 mg L^{-1} at 250 MPa from its initial value of 130 mg L^{-1} and peaks at 300 MPa with a concentration of 169 mg L^{-1} (Fig. 8). This abrupt increase in concentration accompanied with a decrease in particle size indicates that the solubilization of calcium phosphate, in combination with hydrophobic disruption, caused the reduction in casein micelle size. The role of added Ca^{2+} to the

nano- and micro-filtered casein isolates in our experiments is not clear, nonetheless, it is evident that increased amounts of Ca^{2+} at elevated pressures initiates micelle precipitation.

CONCLUSIONS

High-pressure homogenization can be used as a tool to modify the functional aspects of proteins in skim milk. Casein micelles will form large aggregates at pressures exceeding 300 MPa in the presence of excess calcium in solution. The combined effects of pressure on the exposure of hydrophobic sites, insolubility of calcium phosphate at high temperatures, and presence of additional calcium in solution led to the dissociation and immediate re-association of casein micelles. This resulted in casein aggregation and subsequent precipitation. The loss micelle stability once κ -casein is solubilized/released into solution, can also lead to precipitation. A significant reduction in particle size can be achieved when pressures ranging from 100–250 MPa are applied to casein micelles and casein micelle isolates. These results indicate that the use of HPH has the ability to appreciably alter micellar components based on micro- and macro-analysis. This technology is applicable to various areas of research and industry where the disruption of nano-sized particles is desired.

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REFERENCES

- S. Adapa, K.A. Schmidt and R. Toledo. 1997. Functional properties of skim milk processed with continuous high pressure throttling, *Journal of Dairy Science* 80 (9) pp. 1941–1948.
- P.W. Brigman, The coagulation of albumen by pressure, *Journal of Biological Chemistry* 19 (4) (1914), pp. 511–512.
- C.G. De Kruif, Casein micelle interactions, *International Dairy Journal* 9 (3–6) (1999), pp. 183–188.
- A.M. Diels, L. Callewaert, E.Y. Wuytack, B. Masschalck and C.W. Michiels, Moderate temperatures affect *Escherichia coli* inactivation by high-pressure homogenization only through fluid viscosity, *Biotechnology Progress* 20 (5) (2004), pp. 1512–1517.
- A.M. Diels, E.Y. Wuytack and C.W. Michiels, Modeling inactivation of *Staphylococcus aureus* and *Yersinia enterocolitica* by high-pressure homogenisation at different temperatures, *International Journal of Food Microbiology* 87 (1–2) (2003), pp. 55–62.
- N. Datta, M.G. Hayes, H.C. Deeth and A.L. Kelly, Significance of frictional heating for effects of high pressure homogenization on milk, *Journal of Dairy Research* 72 (4) (2005), pp. 1–7.
- J. Flourey, A. Desrumaux and J. Lardières, Effect of high-pressure homogenization on droplet size distributions and rheological properties of model oil-in-water emulsions, *Innovative Food Science and Emerging Technologies* 1 (2) (2000), pp. 127–134.
- Gatti et al., 1995 C.A. Gatti, P.H. Risso and M.S. Pires, Spectrofluorometric study on surface hydrophobicity of bovine casein micelles in suspension and during enzymatic coagulation, *Journal of Agricultural and Food Chemistry* 43 (1995), pp. 2339–2344.
- Guyomarc'h et al., 2003 F. Guyomarc'h, A.J.R. Law and D.G. Dalgleish, Formation of soluble and micelle-bound aggregates in heated milk, *Journal of Agricultural and Food Chemistry* 51 (16) (2003), pp. 4652–4660.
- Hayes and Kelly, 2003 M.G. Hayes and A.L. Kelly, High pressure homogenization of raw whole milk (a) effects on fat globule size and other properties, *Journal of Dairy Research* 70 (3) (2003), pp. 297–305.
- Hayes et al., 2005 M.G. Hayes, P.F. Fox and A.L. Kelly, Potential applications of high pressure homogenization in processing of liquid milk, *Journal of Dairy Research* 72 (1) (2005), pp. 25–33.

Hite, 1899 B.H. Hite, The effects of pressure in the preservation of milk, West Virginia Agricultural Experimental Station 58 (1899), pp. 15–35.

Hite et al., 1914 B.H. Hite, N.J. Giddings and C.E. Weakly, The effects of pressure on certain microorganisms encountered in the preservation of fruits and vegetables, West Virginia Agricultural Experimental Station 146 (1914), pp. 1–67.

Huppertz et al., 2006a T. Huppertz, P.F. Fox, K.G. DeKruif and A.L. Kelly, Disruption and reassociation of casein micelles under high pressure, *Journal of Dairy Research* 73 (3) (2006), pp. 294–298.

Huppertz et al., 2006b T. Huppertz, P.F. Fox, K.G. DeKruif and A.L. Kelly, High pressure-induced changes in bovine milk proteins: A review, *Biochimica et Biophysica Acta* 1764 (3) (2006), pp. 593–598.

Huppertz et al., 2004 T. Huppertz, P.F. Fox and A.L. Kelly, High pressure treatment of bovine milk: Effects on casein micelles and whey proteins, *Journal of Dairy Research* 71 (1) (2004), pp. 97–106.

Işcan et al., 2005 Y. Işcan, S.A. Wissing, S. Hekimoglu and R.H. Müller, Solid lipid nanoparticles (SLNTM) for topical drug delivery: Incorporation of the lipophilic drugs N,N-diethyl-m-toluamide and vitamin K, *Pharmazie* 60 (12) (2005), pp. 905–909.

Jia, 2005 L. Jia, Nanoparticle formulation increases oral bioavailability of poorly soluble drugs: Approaches, experimental evidences and theory, *Current Nanoscience* 1 (3) (2005), pp. 237–243.

Johnston et al., 1992 D.E. Johnston, B.A. Austin and R.J. Murphy, Effects of hydrostatic pressure on milk, *Milchwissenschaft* 47 (12) (1992), pp. 760–763.

Kietczewska et al., 2003 K. Kietczewska, A. Kruk, M. Czerniewicz, M. Warmińska and E. Haponiuk, The effect of high-pressure homogenization in changes in milk colloidal and emulsifying systems, *Polish Journal of Food and Nutrition Sciences* 12/53 (1) (2003), pp. 43–66.

Möschwitzer and Müller, 2006 J. Möschwitzer and R.H. Müller, Spray coated pellet as carrier system for mucoadhesive drug nanocrystals, *European Journal of Pharmaceutics and Biopharmaceutics* 62 (3) (2006), pp. 282–287.

Needs et al., 2000 E.C. Needs, R.A. Stenning, A.L. Gill, V. Ferragut and G.T. Rich, High-pressure treatment of milk: Effects on casein micelle structure and on enzyme coagulation, *Journal of Dairy Research* 67 (1) (2000), pp. 31–42.

O'Connell et al., 2001 J.E. O'Connell, A.L. Kelly, P.F. Fox and K.G. de Kruif, Mechanism for the ethanol-dependent heat-induced dissociation of casein micelles, *Journal of Agricultural and Food Chemistry* 49 (9) (2001), pp. 4424–4428.

Panouillé et al., 2004 M. Panouillé, T. Nicolai and D. Durand, Heat induced aggregation and gelation of casein submicelles, *International Dairy Journal* 14 (4) (2004), pp. 297–303.

Sandra and Dalgleish, 2005 S. Sandra and D.G. Dalgleish, Effects of ultra-high-pressure homogenization and heating on structural properties of casein micelles in reconstituted skim milk powder, *International Dairy Journal* 15 (11) (2005), pp. 1095–1104.

Thiebaud et al., 2003 M. Thiebaud, E. Dumay, L. Picart, J.P. Guiraud and J.C. Cheftel, High-pressure homogenization of raw bovine milk. Effects on fat globule size distribution and microbial activation, *International Dairy Journal* 13 (6) (2003), pp. 427–439.

Walstra, 1990 P. Walstra, On the stability of casein micelles, *Journal of Dairy Science* 73 (8) (1990), pp. 1965–1979.

Wuytack et al., 2002 E.Y. Wuytack, A.M. Diels and C.W. Michiels, Bacterial inactivation by high-pressure homogenisation and high hydrostatic pressure, *International Journal of Food Microbiology* 77 (3) (2002), pp. 205–212.

Wuytack et al., 2003 E.Y. Wuytack, L.D. Phuong, A. Aertsen, K.M. Reyns, D. Marquenie and B. De Ketelaere et al., Comparison of sublethal injury induced in *Salmonella enterica* serovar Typhimurium by heat and by different nonthermal treatments, *Journal of Food Protection* 66 (1) (2003), pp. 31–37.

Zobrist et al., 2005 M.R. Zobrist, T. Huppertz, T. Uniacke, P.F. Fox and A.L. Kelly, High-pressure-induced changes in the rennet coagulation properties of bovine milk, *International Dairy Journal* 15 (6–9) (2005), pp. 655–662.

APPENDIX I - FIGURES

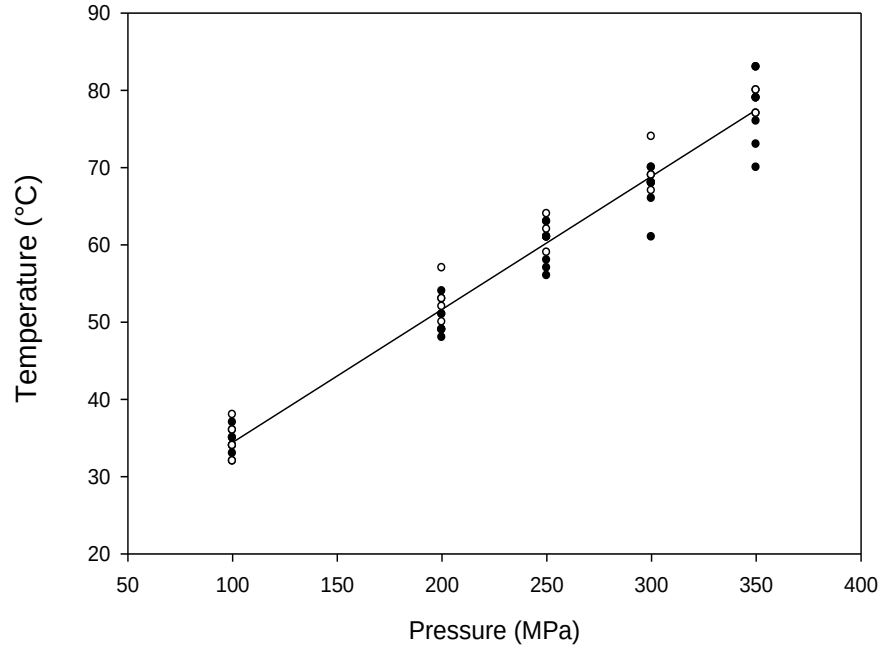


Fig 1. Temperature increases during high-pressure homogenization. Data points represent individual experiments. Pressure fluctuates ± 20 MPa at each pressure level and causes the subsequent fluctuation of temperature by approximately 10°C per pressure level. Linear regression for all points in plot ($r^2 = 0.97$).

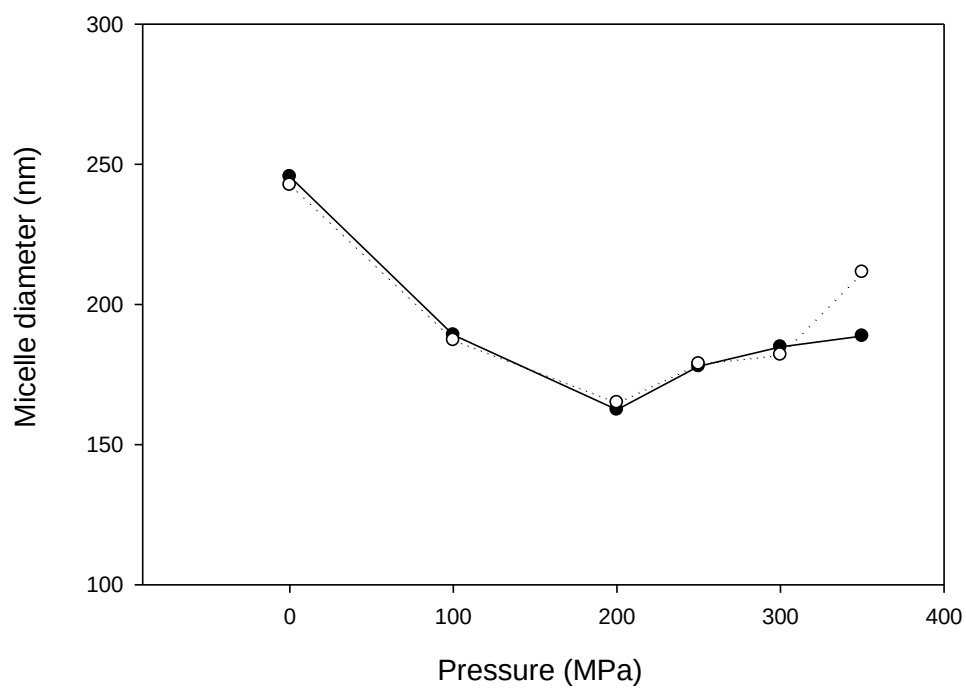


Fig 2. Micelle diameter of raw skim milk after pressurization. Particle size reduction from 0 to 200 MPa reduces in a semi-linear fashion ($r^2=0.92$). Data points (open and closed circles) represent two independent experiments.

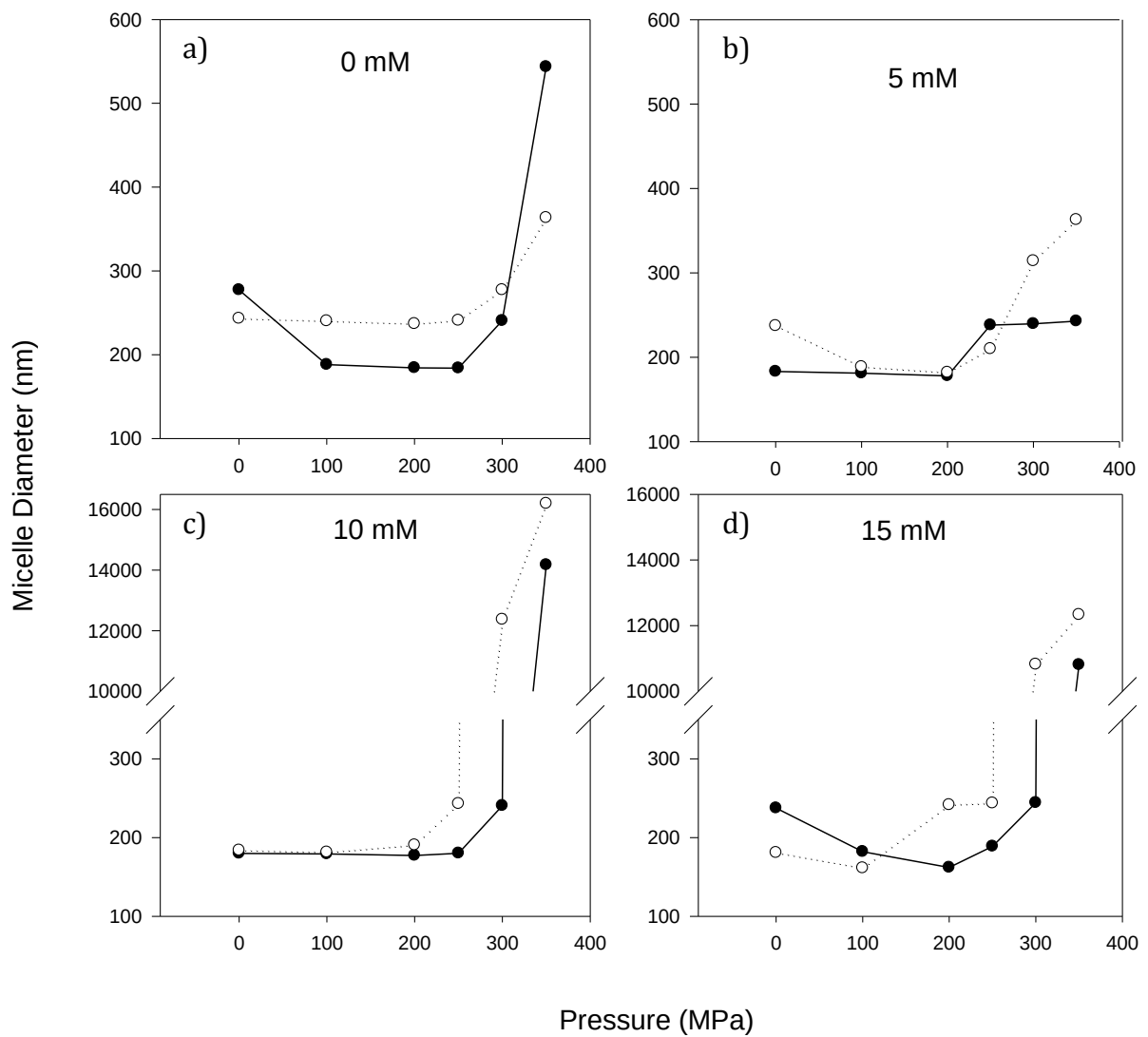


Fig 3. Micelle diameter of 30 kDa (closed circles and solid line) and 0.2 μm (open circles and dashed line) skim milk retentate subjected to high-pressure homogenization in buffer solutions containing a) 0 mM CaCl_2 , b) 5 mM CaCl_2 , c) 10 mM CaCl_2 , and d) 15 mM CaCl_2 .

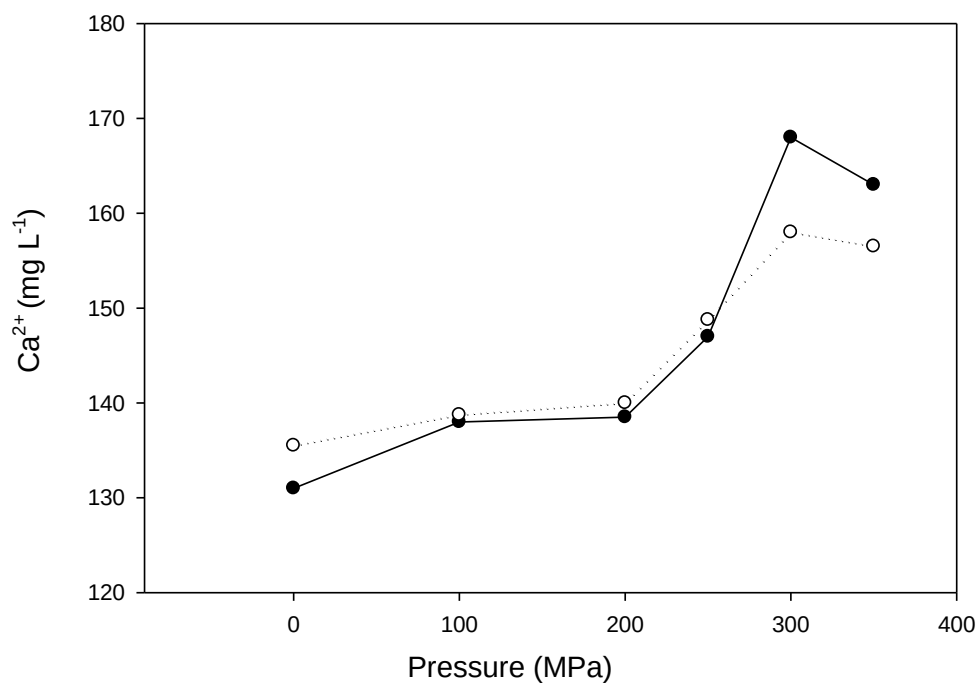


Fig 4. Calcium concentration of raw skim milk during pressurization from 0 to 350 MPa. Data points (open and closed circles) represent two independent experiments.

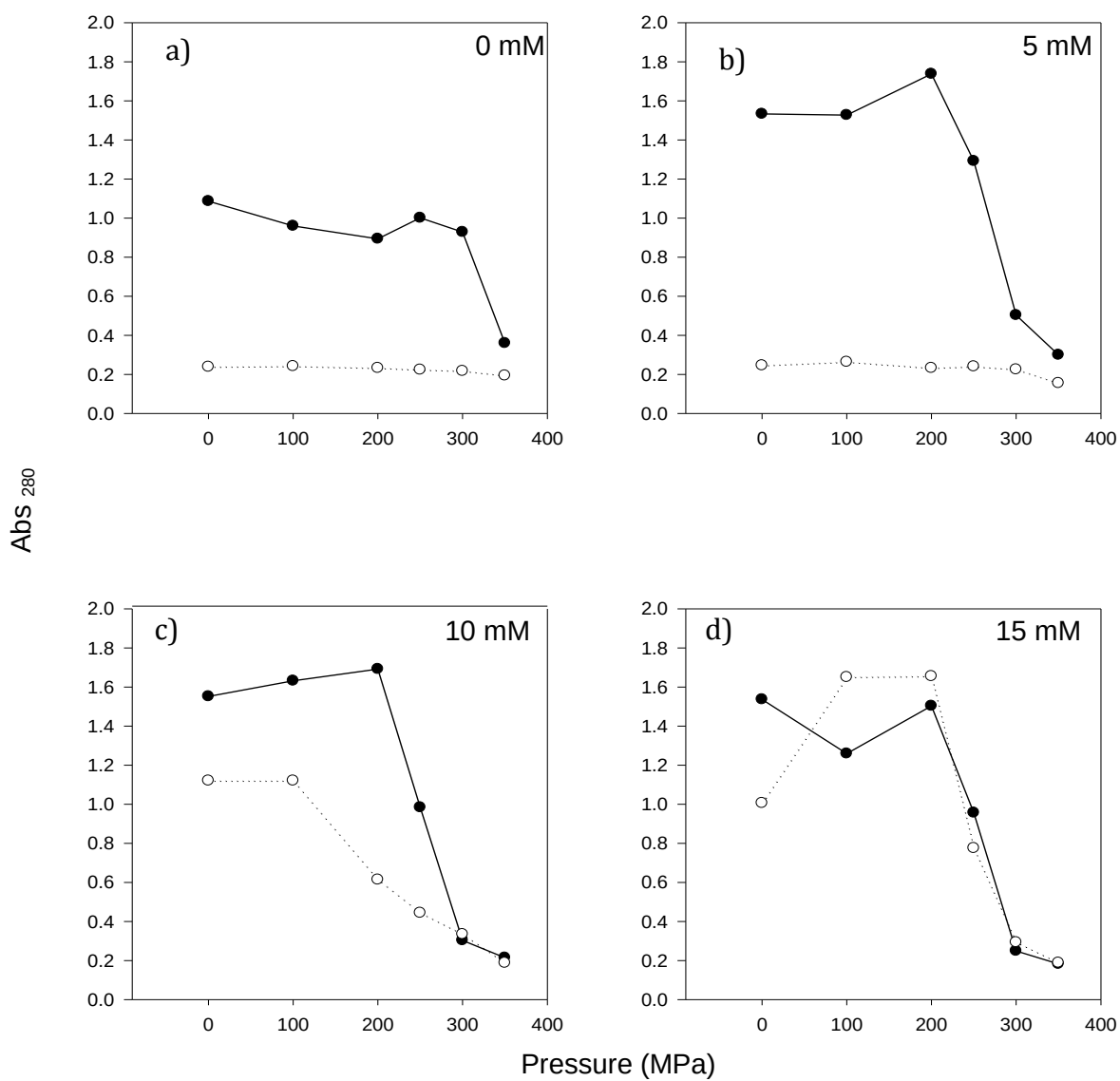


Fig 5. Absorbance (280 nm) measurements of supernatant of pressure treated samples. 30 kDa (closed circles and solid line) and 0.2 μm (open circles and dashed line) skim milk retentate in buffer solutions containing a) 0 mM CaCl₂, b) 5 mM CaCl₂, c) 10 mM CaCl₂, and d) 15 mM CaCl₂.

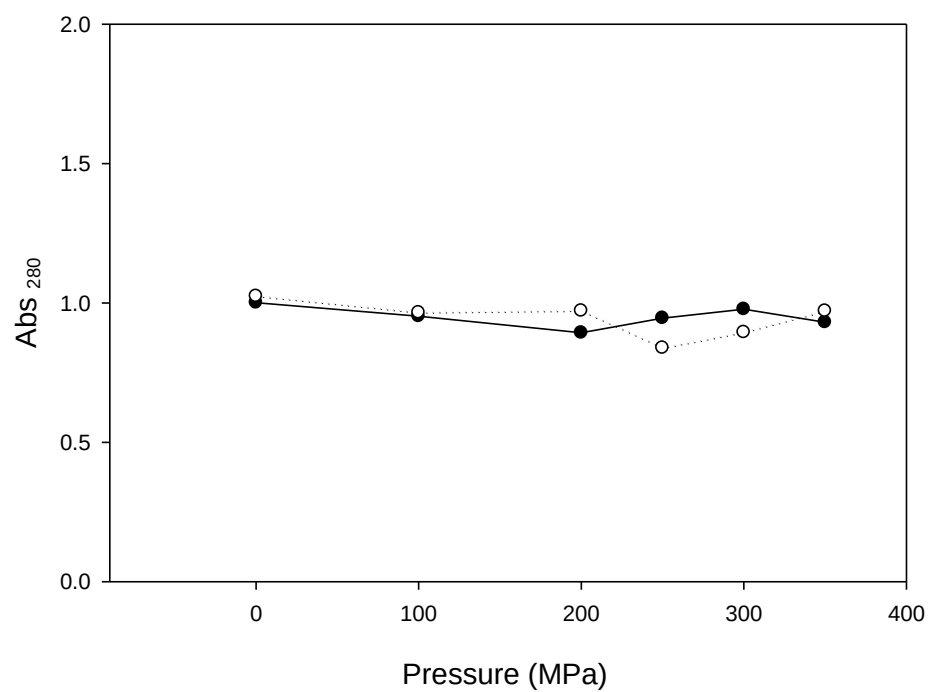


Fig 6. Absorbance (280 nm) measurements of supernatant of raw skim milk after pressurization. Data points (open and closed circles) represent two independent experiments.

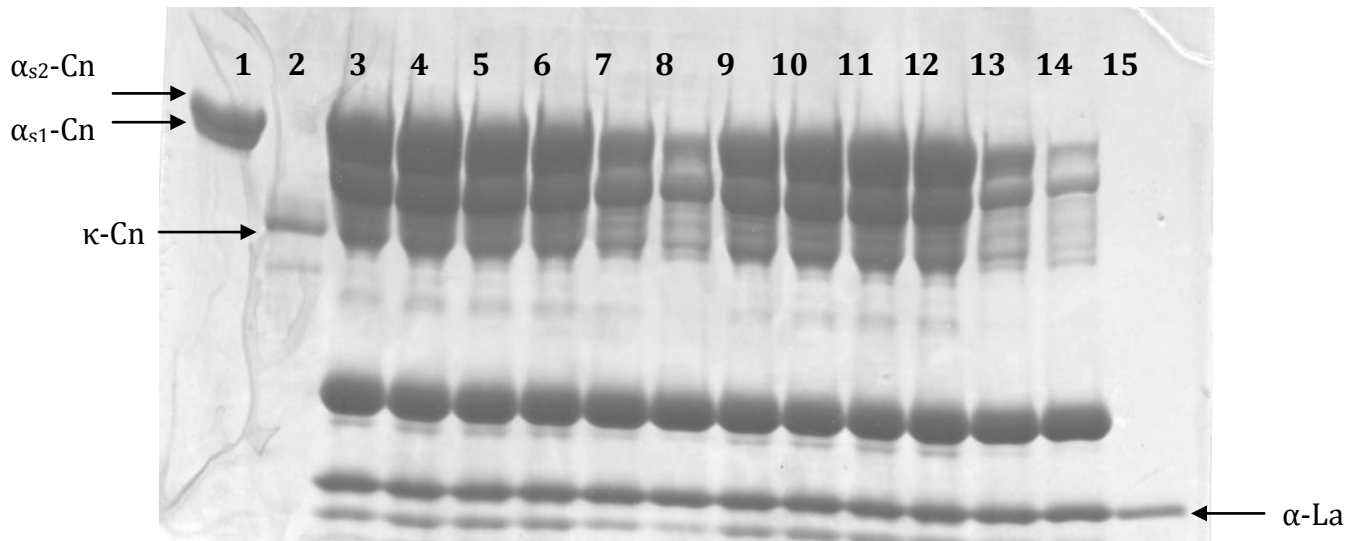


Fig 7. SDS-PAGE of 30 kDa CMI (casein micelle isolates) subjected to HPH in 5 and 15 mM CaCl_2 buffer solutions. Lanes 1, 2, and 15 contain α_{s1} -Cn, α_{s2} -Cn, κ -Cn, and α -La standards. Lanes 3-14 follow in consecutive order: CMI in 5 mM CaCl_2 at 0, 100, 200, 250, 300, and 350 MPa (lanes 3-8) and CMI in 15 mM CaCl_2 (lanes 9-14).

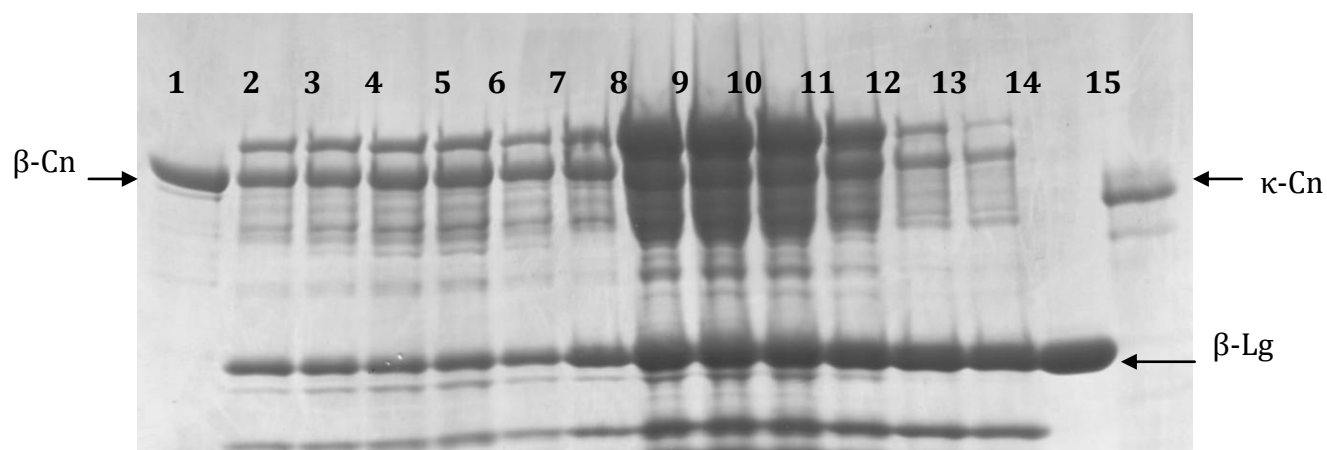


Fig 8. SDS-PAGE of 0.2 μ m CMI subjected to HPH in 5 and 15 mM CaCl_2 buffer solutions. Lanes 1, 2, and 15 contain κ -Cn, β -Lg, and β -Cn. Lanes 3-14 follow in consecutive order: CMI in 15 mM CaCl_2 at 0, 100, 200, 250, 300, and 350 MPa (lanes 3-8) and CMI in 5 mM CaCl_2 (lanes 9-14).

CHAPTER 2

Association of Triclosan to Casein Proteins Through Solvent-Mediated High-Pressure Homogenization

*Adrienne Roach, John Dunlap, and Federico Harte. 2009. Journal of Food Science, 74(2):N23-29.**

ABSTRACT

The association of triclosan (TCS), a widely used hydrophobic compound, to the bovine casein micelle is investigated in this study. The use of high-pressure homogenization (HPH) at 0, 100, 200, and 300 MPa was introduced as a method for the dissociation of casein micelles in a skim milk/ethanol solution (1: 1, v/v) in the presence of TCS at 20, 80, and 160 mg/L where ethanol evaporation served as the final step for TCS association to caseins. The majority of TCS (> 80%) was associated with the caseins regardless of initial TCS concentration or applied pressure. TCS association to caseins was enhanced by 30% with continued pressurization to 300 MPa. Micellar dissociation and reassociation was found to be an irreversible process as evidenced by microscopic images. Pressurization to 300 MPa resulted in the formation of an integrated protein network of casein proteins and non-covalently linked whey proteins where the solubility of TCS was enhanced up to 40 times its reported water solubility at the highest initial TCS level of 160 mg/L. Reformed micelles exhibited Newtonian flow behavior at all pressure levels. This study provides evidence for the solubility enhancing quality of TCS through the solvent-mediated pressure/shear-induced dissociation of casein proteins.

* Manuscript was modified from its original published version to accommodate dissertation formatting.

INTRODUCTION

The casein micelle is an association colloid formed by the self-assembly of the four amphiphilic casein proteins, κ -, α_{s1} -, α_{s2} -, and β -casein, along with calcium phosphate nanoclusters. It is a natural nanocarrier for the transport of proteins and minerals to feed mammalian young. Although the actual structure of the casein micelle is not resolved, its structure has been described as a block-copolymer where the self-assembly of the caseins to micelles occurs via bridging by calcium phosphate and intermolecular hydrophobic interactions (Horne 1998). Micellar growth is limited by κ -casein, the calcium insensitive surface protein, which serves to stabilize the micelle within the milk serum preventing aggregation with adjacent micelles.

Nanoparticles formed from amphiphilic copolymers, both natural and synthetic, have been extensively explored as carriers for hydrophobic compounds (Sun and others 2001b; Tang and Du 2008). Biopolymers can be an appealing alternative to synthetic polymers as a delivery system based on commercial availability, relatively low cost, and biodegradability. Commonly used natural polymers include gelatin, collagen, starch, dextran, and chitosan (Sun and others 2001a, 2001b). However, there is limited research regarding the use of the casein micelle for similar commercial applications. Semo and others (2007) successfully achieved encapsulation of vitamin D through the natural self-assembly of casein micelles from a sodium caseinate powder in the presence of vitamin D by simulating their formation in the Golgi system of the mammary gland (Knoop and others 1979). Pan and others (2007) reported on the formation of casein-grafted dextran

nanoparticles by Maillard type reactions containing encapsulated β -carotene. Both studies utilized the natural tendency of the caseins to self-associate.

The use of high-pressure homogenization (HPH) has been explored for both pressure induced disruption and reassociation of casein micelles from skim milk evident by a reduction in micelle diameter (Hayes and Kelly 2003; Sandra and Dalglish 2005; Roach and Harte 2008) followed by an increase in micelle diameter as pressure increases (Roach and Harte 2008). Fluids at the homogenizing valve are subjected to a combination of physical stimuli, such as, turbulence, cavitation, high pressure, shear stress, and elevated temperatures. The reduction of casein micelle diameter by HPH is understood to be due to the pressure-induced solubilization of micellar calcium phosphate and the subsequent disruption of hydrophobic and electrostatic interactions (Sandra and Dalglish 2005; Roach and Harte 2008). Exposure of the hydrophobic residues of the individual casein proteins during homogenization and micellar reassociation provides a potential method for the binding and subsequent encapsulation of hydrophobic compounds.

The effect of organic solvents on casein micelle dissociation is also an area of increasing interest. The dissociative effect of ethanol is attributed to the increased solubility of the caseins due to a reduction in the cross-linking of phosphoseryl residues and decreased protein hydrophobicity (Zadow 1993; Huppertz and others 2004). The solvent enhancing quality of ethanol on the casein micelle has been primarily explored in relation to elevated temperatures and acidification (O'Connell and others 2001a, 2001b; Huppertz and others 2004). Mixtures of milk and ethanol (1:1, v/v) become clear upon heating to 65°C, an indication of micellar dissociation (Zadow 1993; O'Connell and

others 2001a, 2001b). The combination of forces at the homogenizing valve in the presence of an organic solvent, such as ethanol, has the potential to enhance micellar dissociation as temperature will increase during the homogenization process. However, to our knowledge, the combination of micelle disruption during HPH in the presence of ethanol has yet to be explored. The objective of this research was to determine the influence of a 2-step process on the incorporation of a hydrophobic compound, triclosan (TCS), into casein proteins through (1) HPH in the presence of an organic solvent, ethanol, to dissociate casein micelles followed by (2) ethanol evaporation to initiate micellar reformation and subsequent association of TCS to caseins.

MATERIALS AND METHODS

Milk source and hydrophobic probe

Pasteurized skim milk was purchased from a local grocery store and used as received. Triclosan (97%, TCS) was obtained from Sigma-Aldrich (St. Louis, Mo., U.S.A.) and used as the hydrophobic probe for encapsulation experiments. Individual TCS standards were prepared for each concentration in high-pressure liquid chromatography (HPLC) grade acetonitrile (Fisher Scientific, Atlanta, Ga., U.S.A.) and added to skim milk/ethanol mixtures. Less than 0.1% (v/v) of the co-solvent, acetonitrile, was added to milk samples. All other reagents were of HPLC or analytical grade.

HPH milk/ethanol mixtures

TCS standards were first dispersed in ethanol (95%) (Fisher Scientific) and skim milk was added at a 1:1 (v/v) ratio of ethanol:skim milk for a final TCS concentration of 20, 80, and 160 mg/L. After approximately 30 min of mixing, milk samples (25°C) were

treated by HPH at 0, 100, 200, and 300 MPa (Stansted Fluid Power, Ltd., Essex, U.K.). The instrumental setup consists of a bench top unit providing synchronized, 2-stage homogenization using 2 hydraulic intensifiers at a sample flow rate of approximately 180 mL/min during homogenization. To prevent adsorption of TCS to equipment surfaces, Teflon tubing was used and all machinery in contact with samples consisted of glass or stainless steel. To minimize the effects of shear-induced heating at the homogenizing valve as pressure increased, a water bath set at 2°C (Isotemp 3016D, Fisher Scientific, Pittsburgh, Pa., U.S.A.) was connected to the homogenizing block reducing temperature elevation by approximately 10 to 15°C per 100 MPa increase in pressure (determined by previous experiments not using the cooling unit). Additionally, a tube in tube heat exchanger connected to a water bath set at 2°C was placed immediately after the homogenizing block to remove residual shear-induced heat from the processed samples. Pressure treated samples were collected in glass vials to a volume of approximately 100 mL per pressure level which corresponds to a TCS mass of 2, 8, and 16 mg for each initial TCS concentration, respectively. Following pressurization, ethanol was removed from 100 mL samples by rotary evaporation (Büchi, Flawil, Switzerland) maintained at 60°C under constant vacuum. Evaporation was carried out for 15 ($\pm$ 1) min, until 50 mL of ethanol was recovered (50 mL was added to milk sample prior to processing).

Milk fraction separation

Casein micelles were separated from serum proteins after ethanol evaporation by ultracentrifugation ($200,000 \times g$; 30 min) using an Optima™ L-100K ultracentrifuge equipped with a fixed angle titanium rotor and polycarbonate tubes (Beckman Coulter,

Fullerton, Calif., U.S.A.). Supernatants were carefully poured from top of centrifuge tube and transferred to a vial for TCS extraction. The casein pellets were removed from the bottom of the centrifuge tube with a metal spatula and resuspended in a buffer solution (20 mM imidazole, 30 mM CaCl₂, pH 6.8) using a handheld rotary homogenizer unit (Polytron, Kinematica, Bohemia, N.Y., U.S.A.) at approximately 7000 RPM for 5 min prior to TCS extraction. Both supernatant and casein pellet were analyzed for TCS concentration.

TCS extraction and quantification

TCS extraction from milk fractions was carried out using a 2-step liquid–liquid extraction procedure. Initially, milk proteins were precipitated according to the method of Zhang and others (2006) for the determination of celecoxib, a nonsteroidal anti-inflammatory drug, in human milk. Protein precipitation was induced with a 2: 1 (v/v) ratio of acetonitrile to milk sample, followed by vortexing and centrifugation (10,000 × *g* for 15 min). To further reduce the amount of proteins loaded on the HPLC column, TCS extracted supernatants were frozen (−40°C) for a minimum of 6 h to separate the remaining water soluble proteins (frozen bottom layer) from the TCS/acetonitrile rich top layer that remained unfrozen. The freezing point profile of acetonitrile/water mixtures is presented in detail by Zarzycki and others (2006). This phenomenon provides a simple method for sample purification without complex extraction procedures or the risk of compromised analyte recoveries with filtration.

The TCS/acetonitrile layer was transferred to a HPLC vial as the bottom fraction remained frozen. Fifty microliters were injected on to the column for triclosan

identification and quantification by HPLC (Dionex, Salt Lake City, Utah, U.S.A.). The instrument consisted of a quaternary pump, solvent degasser, autosampler, and a diode array UV detector. Triclosan separation was achieved on a 150 × 4.6 mm C₁₈ column with a 5-μm particle size (Econosphere, Alltech Associates Inc., Deerfield, Ill., U.S.A.). HPLC conditions for separation were as follows: mobile phase of 70:30, acetonitrile: water, 1 mL/min flow rate, 50 μL injection volume, and UV adsorption at 220 nm for detection. A 5-point calibration curve was achieved using standards ranging from 0.1 to 200 mg/L. Calibration standards were analyzed at the beginning of each sample set and exhibited linearity (r^2 of > 0.99) throughout the course of the study. The efficiency of the method for the association of TCS to micelles and the percent of unassociated, or free TCS, was calculated as follows:

$$AE\% = (TCS_{\text{pellet}} / TCS_{\text{total}}) * 100$$

$$\% \text{ Unassociated TCS} = (TCS_{\text{serum}} / TCS_{\text{total}}) * 100$$

where AE is the association efficiency, TCS_{pellet} is the mass of TCS in the pellet, TCS_{total} is the total mass of TCS in both the serum and pellet, and TCS_{serum} is the mass of TCS in the serum phase.

Characterization of reformed micelles

Structural analysis by field-emission scanning electron microscopy (FESEM). The method of Dalgleish and others (2004) for structural analysis of the casein micelle by FESEM was used with modifications. A polished silicon wafer was immersed in milk samples and samples were allowed to affix to the wafer for approximately 5 min. Excess

sample was washed once using phosphate buffer (pH 7). The primary fixative, a 2% glutaraldehyde solution prepared in phosphate buffer, was added and allowed to react for an hour. The wafer was rinsed with phosphate buffer in preparation for the secondary fixative, 1% osmium tetroxide in phosphate buffer, which also was allowed to react for an hour. The wafer was rinsed 3 times with phosphate buffer and dehydrated by immersion in aqueous solutions of ethanol: 70%, 90%, 95% (repeated twice), and 100% (repeated 3 times) for approximately 5 min per concentration. Samples were critical point dried using carbon dioxide and stored in a desiccator at room temperature until sample imaging using a field-emission scanning electron microscope (LEO 1525, Zeiss, Germany) at an acceleration voltage of 5 kV. Samples consisted of milk solutions after pressurization to 0, 200, and 300 MPa before and after ethanol evaporation.

Particle-size analysis

Particle-size analysis was performed on pressure treated milk samples (0, 100, 200, and 300 MPa) before and after ethanol evaporation using photon correlation spectroscopy with a Delsa Nano™ C Particle-size analyzer (Beckman Coulter Inc.). Approximately 2.5 mL of an undiluted sample were used for analysis. Undiluted skim milk was used as a standard control. Three measurements were made for each sample and controls with 30 size accumulations for each measurement. Light scattering was monitored at 25°C.

Rheological properties

The flow properties (shear strain rate = 0 to 120 per sec) of samples after ethanol evaporation was determined without dilution using an AR-2000 rheometer (TA

Instruments, New Castle, Del., U.S.A.) equipped with a concentric cylinder system (rotor radius = 14 mm, rotor height = 42 mm, cup radius = 15 mm) and temperature set at 5°C. Skim milk subjected to the same heat treatment during evaporation (15 min at 60°C) was used as a control.

Whey and casein interactions

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Bio-Rad (Hercules, Calif., U.S.A.) mini-gel electrophoresis unit on pressure treated samples before and after ethanol evaporation. Samples were preheated (95°C for 5 min) in the presence (reducing) and absence (non-reducing) of 2-mercaptoethanol. Proteins were stained using Coomassie Brilliant Blue R-250. Skim milk was used as a protein standard.

Statistical analysis

Experiments were analyzed as a completely randomized design with 3 replications, where ANOVA yielded significant treatment effects and 90% confidence intervals for the mean were done using the Student's t-test. All data was analyzed using SAS software version 9.0 (SAS Inst., Cary, N.C., U.S.A.).

RESULTS AND DISCUSSION

TCS recovery from milk fractions

Calibration curves generated from milk and milk serum spiked with TCS exhibited good linearity ($r^2 = 0.99$) over a concentration range of 0.1 to 200 mg/L. TCS was extracted from both matrices according to the simple 2-step liquid–liquid extraction procedure previously presented. Protein peaks from remaining protein in sample after

extraction was well separated from TCS peak (Fig 9). Calculated TCS recovery from extraction ranged from 89% to 99% in total milk and from 85% to 103% in milk serum. Loss by other means, such as sorption to instrument parts and sample vials, was accounted for by collecting samples before and after each processing step and analyzing for total TCS concentration. There was no TCS loss during homogenization, marginal loss during the ethanol evaporation process (<0.1 to 0.3 mg/L), and no apparent sorption to polycarbonate ultracentrifugation tubes. TCS has a relatively high water vapor pressure of 5.2×10^{-6} mm Hg (Singer and others 2002), which explains the minimal loss during evaporation. Additional physical properties of TCS are listed in Table 1.

Association of TCS to caseins

HPH of skim milk in the presence of ethanol (1:1, v/v) followed by ethanol evaporation and protein separation, resulted in a decrease in the amount of TCS in the serum at each initial TCS level as pressure increased, except for the case of 20 mg/L at 200 MPa (Fig 10). On average, TCS serum levels decreased by 20% at 100 MPa, by 46% at 200 MPa, and by 52% at 300 MPa for 20, 80, and 120 mg/L TCS relative to the un-homogenized control. The reduction of TCS serum levels indicates the pressure-induced incorporation of TCS to the caseins separated from the serum by ultracentrifugation after ethanol evaporation. However, due to the hydrophobic nature of TCS, the majority of TCS present in the sample was associated with the casein pellet regardless of pressure and, therefore, treatment effects are not as apparent in the casein pellet in comparison to milk serum analysis. On average, there was 2.5 times more TCS associated with the serum at 0 MPa than at 300 MPa, but only 1.3 times more TCS associated with the pellet

at the same pressures. The increase in TCS pellet levels as pressure increases is presented as the association efficiency of TCS to the casein proteins in Fig 11. The association of TCS to the caseins is enhanced by 30% at pressures reaching 300 MPa. A previous study by our group concluded that micelles are disrupted at the homogenizing valve as pressure is increased to the 200 to 300 MPa range (Roach and Harte 2008). It is our hypothesis that similar dissociative effects are occurring in our samples containing ethanol during the homogenizing process followed by subsequent association of TCS to the caseins during ethanol evaporation.

The natural interaction of TCS with the caseins maybe favorable during pressure treatment as TCS readily associates with the casein micelle and this initial surface contact can promote the incorporation of TCS into the micelle as pressure increases at the homogenizing valve. Sample time at the homogenizing valve is in the order of milliseconds and the initial location of a desired encapsulant prior to homogenization could promote binding and play a significant role in the encapsulation process. Additionally, the concentration of TCS_{serum} was measured up to 3 times greater than its reported solubility of 10 mg/L at the higher initial TCS levels of 80 and 160 mg/L. It is likely that serum proteins and other serum components aided in enhancing the aqueous solubility of TCS. Whey protein α -lactalbumin and bovine serum albumin have been explored as functional nanoparticles for encapsulation purposes (Graveland-Bikker and de Kruif 2006; Rahimnejad and others 2006). Moreover, a sorption maxima was not observed as the initial concentration of TCS added to the system was increased, rather, there seems to be a fairly linear increase in both the amount of TCS_{serum} ($r^2 = 0.99$ to 1)

and TCS_{pellet} ($r^2 = 0.96$ to 0.99) at each pressure. This indicates that binding capacity of the micelles or the serum proteins was not reached.

Structure and particle-size analysis of pressure-treated ethanolic milk solutions

It is well known that ethanol has a destabilizing effect on casein micelles and can cause their immediate aggregation as seen in Fig 12A for the un-homogenized milk/ethanol samples. Increasing pressure to 200 and 300 MPa resulted in a significant reduction of this aggregated network (Fig 12B and 4C). Particle-size analysis (Fig 13) showed a reduction of micelle diameter from $15 (\pm 6.0) \mu\text{m}$ without pressure to $5.2 (\pm 1.0) \mu\text{m}$ at 100 and 200 MPa with a slight further reduction to $3.6 (\pm 1.2) \mu\text{m}$ at 300 MPa. The large standard deviation at 0 MPa is attributed to the instability of the skim milk ethanol mixture during measurements and size measurements in this range ($>7 \mu\text{m}$) exceed the upper detection limit of the instrument. After homogenization, particle size was reduced, on average, by 65%. The resulting dispersion was fairly stable evidenced by minimal phase separation upon standing at room temperature (<1.5 h). Also, the surface of the micelles homogenized at both 200 and 300 MPa appeared to be smooth (Fig 12) as opposed to samples without ethanol (Fig 14). This could possibly be due to the collapse of κ -casein in the presence of ethanol (de Kruif 1999).

Images obtained after ethanol evaporation revealed the opposite effect, particle size increased dramatically with increasing pressure (Fig 14A to 14C). Size analysis with photon correlation spectroscopy reflected this effect (Fig 15); however, the increase was not as apparent in comparison to microscopy images. Here, micelle diameter increased slightly by only 5%, from $1.9 (\pm 0.04) \mu\text{m}$, to $2.1 (\pm 0.08) \mu\text{m}$ at 100 MPa, by 23% at 200

MPa to $2.4 (\pm 0.05) \mu\text{m}$, and by 34% at 300 MPa to $2.6 (\pm 0.16) \mu\text{m}$. This inconsistency in size results between microscopy images and instrumental analysis is most likely due to the absence of spherical shaped micelles post homogenization and ethanol evaporation. In this case, microscopy images provide information as to the structural changes of the micelle imparted by dissociation and subsequent reformation that particle-size analysis alone would not detect.

Microscopy images (Fig 12 and 14) reveal that the reassociation of dissociated caseins is not a reversible process. This effect is most evident in the ethanol evaporated images of reformed micelles processed at 300 MPa where the presence of an integrated network, made up of most likely casein proteins, dominate the sample. O'Connell and others (2003) also report on the structural irreversibility of micelles reformed from heated mixtures of milk and ethanol which formed colloidal aggregates upon cooling. However, the micelle morphology in our microscopy images are markedly different from those of O'Connell and others (2003) where the effects of pressure are apparent in the formation of an integrated casein network in our samples as opposed to dispersed colloidal aggregates. Control milk, skim milk alone, was subjected to the same heating treatment for ethanol evaporation, 15 min at 60°C under constant vacuum and rotation, and was identical in diameter ($250 \pm 20 \text{ nm}$) to skim milk without treatment. This suggests that the increase in particle size was due to the extensive dissociation of the caseins during homogenization and subsequent rearrangement of the original micellar structure to a more open and integrated protein network as ethanol was evaporated from the sample.

In an effort to further characterize the network of reformed caseins, reducing and non-reducing SDS-PAGE of homogenized samples before and after ethanol evaporation was carried out to account for the covalent disulfide interaction of κ -casein with denatured whey protein. Protein analysis revealed the lack of covalent interactions between the caseins and whey proteins in all samples analyzed (Fig 16). Ethanol (>50%) (O'Connell and others 2003) and heat (>70°C) are well-known denaturants of β -lactoglobulin. The lack of covalent linkages between denatured β -lactoglobulin and κ -casein is likely the result of their hindered interaction due to the collapse of κ -casein on the surface of the micelle from the destabilizing effect of ethanol prior to homogenization. Alternatively, ethanol-induced changes in the structure of whey proteins can also interfere with the interaction of whey proteins with casein micelles (O'Connell and others 2003). There are, however, conflicting reports on the denaturation of β -lactoglobulin during high-pressure treatment and subsequent interactions with the casein micelle. Studies using the continuous process of high-pressure homogenization suggests that there is minimal association to casein proteins with this method (Hayes and others 2005; Sandra and Dalgleish 2005; Roach and Harte 2008) possibly due to the instantaneous heating at the homogenizing valve followed by immediate cooling reducing protein denaturation.

Rheology of reformed micelles

All samples displayed flow curves corresponding to Newtonian fluids when subjected to a shear strain rate from 0 to 120 per sec. Table 2 shows average viscosity, coefficient of determination for the Newtonian model, and coefficient of variation of the

observed viscosity values. A decrease in viscosity values was observed for samples subjected to increasing processing pressure in the 100 to 300 MPa range. Although the behavior of samples not subjected to HPH is not clear, increased pressure could be responsible for the weakening of protein–protein interactions from the rearrangement of micellar constituents during homogenization resulting in loosely associated proteins. Intermolecular interactions, such as weak or strong protein–protein interactions, and packing density are largely responsible for the rheological behavior of particle surfaces (Fox and McSweeney 2003).

CONCLUSIONS

We introduced the utilization of HPH as a method for the ethanol-mediated association of a widely used low molecular weight hydrophobic compound, TCS, to casein proteins in skim milk. Through natural binding, caseins were able to enhance the solubility of TCS through binding up to 80% without pressure and this association increased by 30% at pressures reaching 300 MPa. The dissociation of caseins in skim milk and ethanol (1:1) occurred during homogenization as a result of increased temperature at the homogenizing valve. The incorporation of TCS was initiated as ethanol was evaporated from the samples, both serving to concurrently reform the dissociated micelles and subsequently entrap TCS. Reformed micelles displayed a markedly different structure from the original micelles forming what appeared to be a pressure-induced integrated protein network consisting of a lattice structure of casein proteins and non-covalently linked whey proteins. Its formation was likely promoted by the alternating arrangement of the hydrophobic and hydrophilic blocks along the

individual casein proteins most evident at pressurization to 300 MPa. Reformed micelles also displayed an increase in viscosity in comparison to skim milk with the greatest increase at 100 MPa but decreased at increasing pressures most likely attributed to the change in particle morphology.

The enhanced solubility of TCS by the caseins through association is promising and provides a strong basis for the use of the casein micelle as a potential delivery system for TCS and possibly other low-molecular weight hydrophobic compounds using the method presented. This has applications in the food packaging industry for the use of TCS as an antibacterial agent in films (Sanches-Silva and others 2005), the pharmaceutical industry where the use of TCS is gaining interest for the treatment of certain cancers (Lu and Archer 2005) and malaria (Kapoor and others 2004), as well as the cosmetic industry where its inclusion in consumer products is most common. However, future studies are needed to characterize micellar reformation and fully elucidate the effects of treatments. The release of TCS by caseins will give an indication as to the nature of the interaction between the milk proteins and TCS as affected by pressure and micellar structure.

ACKNOWLEDGEMENTS

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REFERENCES

- Dalgleish DG, Spagnuolo PA, Goff HD. 2004. A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *Int Dairy J* 14(12):1025–31.
- De Kruif CG. 1999. Casein micelle interactions. *Int Dairy J* 9(3–6):183–8.
- Fox PF, McSweeney PLH. 2003. *Advanced dairy chemistry, Vol. 1 Proteins*. 3rd ed. New York : Part B. Kluwer Academic/Plenum Publishers.
- Graveland-Bikker JF, de Kruif CG. 2006. Unique milk protein based nanotubes: food and nanotechnology meet. *Trends Food Sci Technol* 17(5):196–203.
- Hayes MG, Kelly AL. 2003. High pressure homogenization of raw whole bovine milk (a) effects on fat globule size and other properties. *J Dairy Res* 70:297–305.
- Hayes MG, Patrick FF, Kelly AL. 2005. Potential applications of high-pressure homogenization in processing of liquid milk. *J Dairy Res* 75:25–33.
- Horne DS. 1998. Casein interactions: casting light on the black boxes, the structure in dairy products. *Int Dairy J* 8(3):171–7.
- Huppertz T, Grosman S, Fox PF, Kelly AL. 2004. Heat and ethanol stabilities of high-pressure-treated bovine milk. *Int Dairy J* 14(2):125–33.
- Kapoor MC, Reddy M, Krishnasasthy A, Surolia N, Surolia N. 2004. Slow-tight-binding inhibition of enoyl-acyl carrier protein reductase from *Plasmodium falciparum* by triclosan. *Biochem J* 381:719–25.
- Knoop AM, Knoop E, Wiechen A. 1979. Sub-structure of synthetic casein micelles. *J Dairy Res* 46(2):347–50.
- Lu S, Archer MC. 2005. Fatty acid synthase is a potential molecular target for the chemoprevention of breast cancer. *Carcinogenesis* 26(1):153–7.
- O'Connell JE, Kelly AL, Auty MAE, Fox PF, de Kruif KG. 2001a. Ethanol-dependent heat-induced dissociation of casein micelles. *J Agric Food Chem* 49(9):4420–3.
- O'Connell JE, Kelly AL, Fox PF, de Kruif KG. 2001b. Mechanism for the ethanol-dependent heat-induced dissociation of casein micelles. *J Agric Food Chem* 49(9):4424–8.
- O'Connell JE, Steinle S, Reiter F, Auty MAE, Kelly AL, Fox PF. 2003. Properties of casein micelles reformed from heated mixtures of milk and ethanol. *Colloids Surf A Physicochem Eng Asp* 213(2–3):265–73.

- Pan XY, Yao P, Jiang M. 2007. Simultaneous nanoparticle formation and encapsulation driven by hydrophobic interaction of casein-graft-dextran and beta-carotene. *J Colloid Interface Sci* 315(2):456–63.
- Rahimnejad M, Jahanshahi M, Najafpour GD. 2006. Production of biological nanoparticles from bovine serum albumin for drug delivery. *African J Biotechnol* 5(20):1918–23.
- Roach A, Harte, F. 2008. Disruption and sedimentation of casein micelles and casein micelle isolates under high-pressure homogenization. *Innov Food Sci Emerg Technol* 9(1):1–8.
- Sanches-Silva R, Sendón-García J, López-Hernández P, Paseiro-Losada . 2005. Determination of triclosan in food stuffs. *J Sep Sci* 28:65–72.
- Sandra S, Dalgleish DG. 2005. Effects of ultra-high-pressure homogenization and heating on structural properties of casein micelles in reconstituted skim milk powder. *Int Dairy J* 15(11):1095–104.
- Semo E, Kesselman E, Danino D, Livney YD. 2007. Casein micelle as a natural nano-capsular vehicle for nutraceuticals. *Food Hydrocolloids* 21(5–6):936–42.
- Singer H, Müller S, Tixier C, Pillonel L. 2002. Triclosan: occurrence and fate of a widely used biocide in the aquatic environment: field measurements in wastewater treatment plants, surface waters, and lake sediments. *Environ Sci Technol* 36(23):4998–5004.
- Sun Y, Watts DC, Johnson JR, Shukla AJ. 2001a. Biodegradable polymers and their degradation mechanisms. *Am Pharm Rev* 4(3):8–16.
- Sun Y, Watts DC, Johnson JR, Shukla AJ. 2001b. Biodegradable drug delivery systems. *Am Pharm Rev* 4(4):25–30.
- Tang YF, Du YM. 2008. Chitosan-based injectable thermosensitive hydrogel. *Prog Chem* 20(2):239–44.
- Zadow JG. 1993. Alcohol-mediated temperature-induced reversible dissociation of the casein micelle in milk. *Aust J Dairy Technol* 48(2):78–81.
- Zarzycki PK, Włodarczyk E, Lou DW, Jinno K. 2006. Evaluation of methanol-water and acetonitrile-water binary mixtures as eluents for temperature-dependent inclusion chromatography. *Anal Sci* 22(3):453–6.
- Zhang M, Moore GA, Gardiner SJ, Begg EJ. 2006. Determination of celecoxib in human plasma and breast milk by high-performance liquid chromatographic assay. *J Chromatogr B Analyt Technol Biomed Life Sci* 830(2):245–8.

APPENDIX II - FIGURES

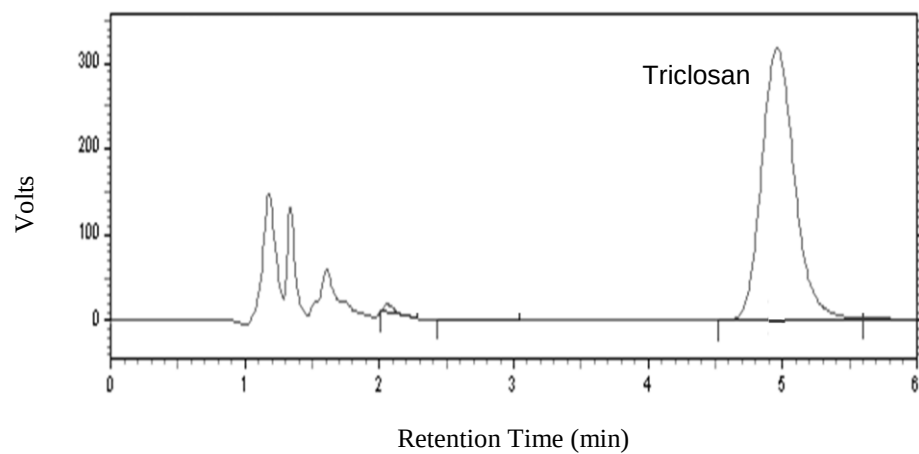


Fig 9. HPLC chromatogram of TCS extracted from skim milk. Peaks at 1-2.5 min are proteins that remained after the extraction process.

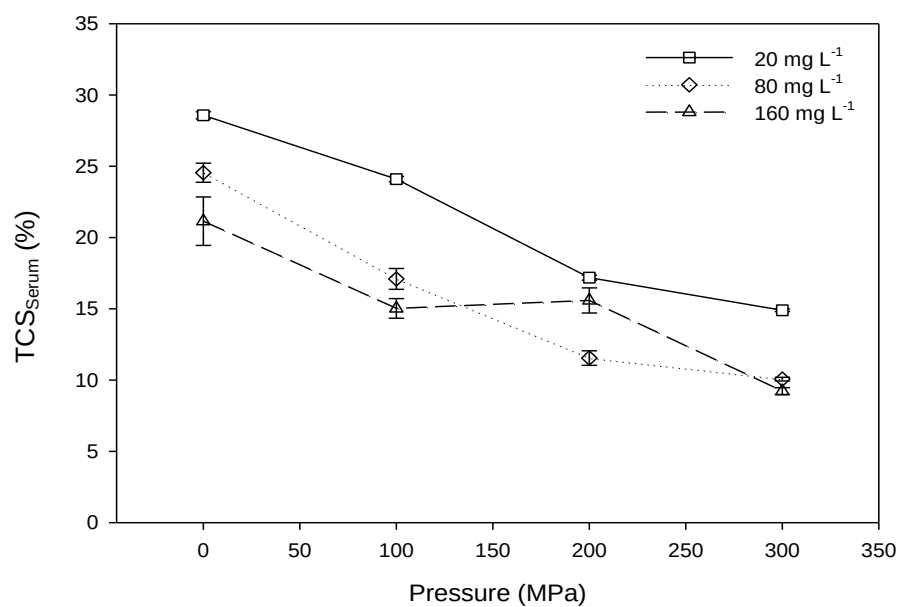


Fig 10. Percent of TCS present within the serum after pressure treatment at 0, 100, 200, and 300 MPa and ethanol evaporation. Data represent the amount of TCS not associated to the micelle/free TCS. Error bars represent the standard deviation of 3 measurements.

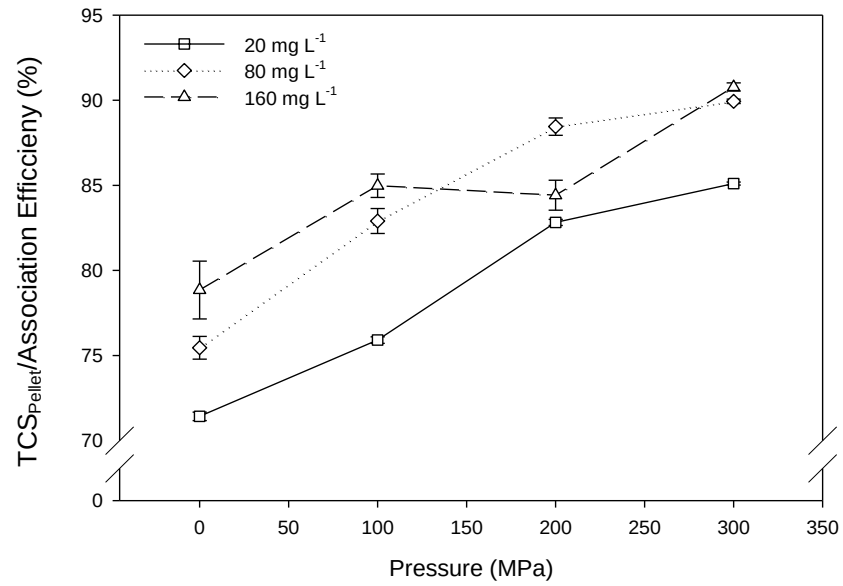


Fig 11. Association efficiency/or percent of TCS associated to the casein micelle after pressure treatment at 0, 100, 200, and 300 MPa and ethanol evaporation. Error bars represent the standard deviation of 3 measurements.

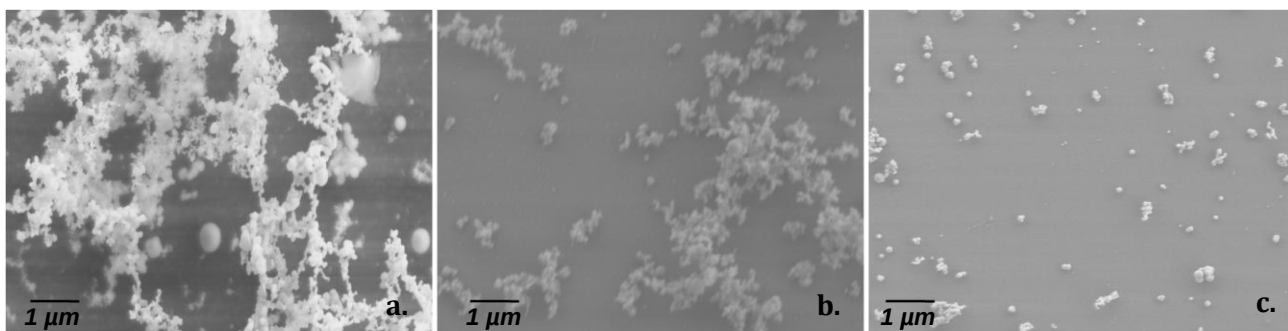


Fig 12. FESEM (Field Emission Scanning Electron Microscopy) micrographs of casein micelles in skim milk and ethanol (1:1, v/v) at a) 0 MPa, b) 200 MPa, and c) 300 MPa before ethanol evaporation. Bar is 1 μm .

Fig 12. FESEM (Field Emission Scanning Electron Microscopy) micrographs of casein micelles in skim milk and ethanol (1:1, v/v) at a) 0 MPa, b) 200 MPa, and c) 300 MPa before ethanol evaporation. Bar is 1 μm .

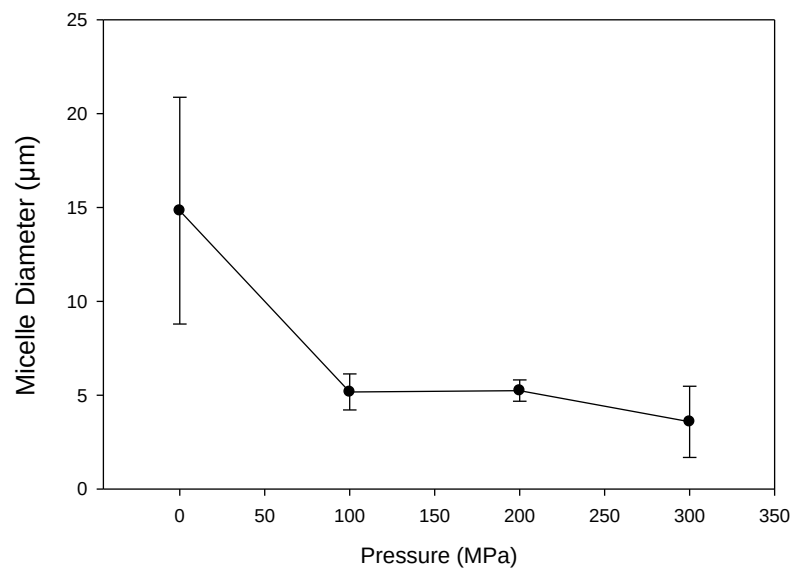


Fig 13. Particle size analysis representing the effect of pressure on the particle size reduction of casein micelles in skim milk and ethanol (1:1, v/v) after pressurization to 100, 200, and 300 MPa. Error bars represent the standard deviation of 3 measurements.

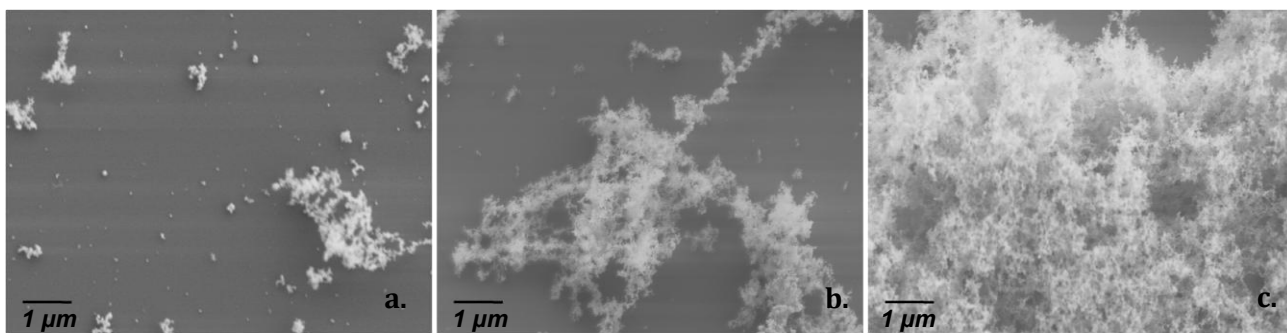


Fig 14. Casein micelles in skim milk and ethanol (1:1, v/v) at a) 0 MPa, b) 200 MPa, and c) 300 MPa after ethanol evaporation. Bar is 1 μm .

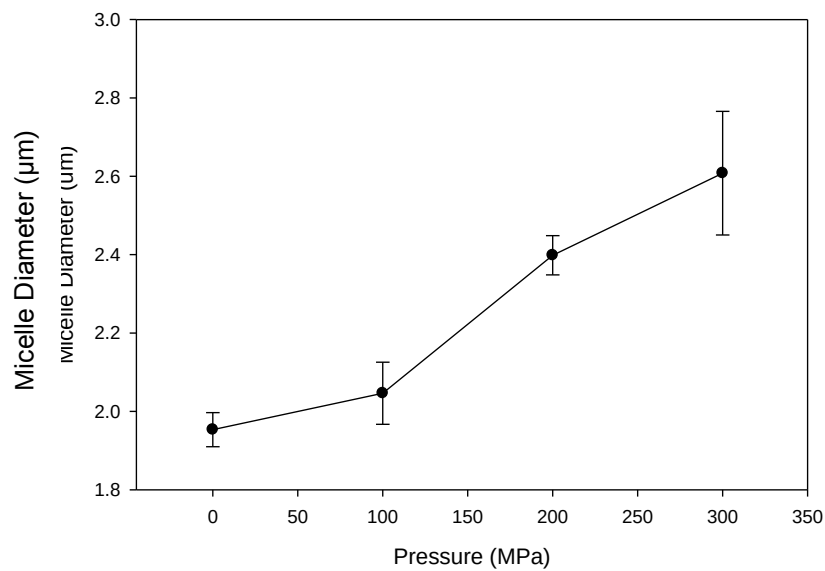


Fig 15. Particle size analysis demonstrating the increase in particle size of reformed casein micelles in skim milk after ethanol evaporation as affected by pressure at 0, 100, 200, and 300 MPa. Error bars represent the standard deviation of 3 measurements.

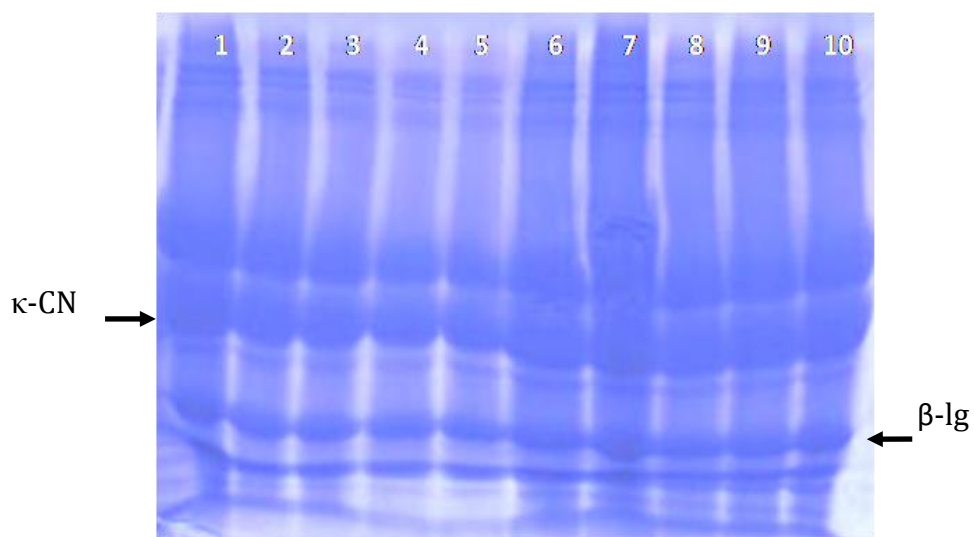


Fig 16. Non-reducing SDS PAGE of pressurized skim milk/ethanol solutions after ethanol evaporation. Lanes 1-5 are samples before ethanol evaporation: 1) skim milk (control), 2) 0, 3) 100, 4) 200, and 5) 300 MPa. Lanes 6-10 are samples after ethanol evaporation: 6) 0, 7) 100, 8) 200, and 9) 300 MPa, and 10) skim milk (control),

TABLES

Table 1. Structure and physicochemical properties of triclosan. (S_w is solubility in water; $\text{Log } K_{ow}$ is the coefficient of partition in octanol/water; Singer and others 2002).

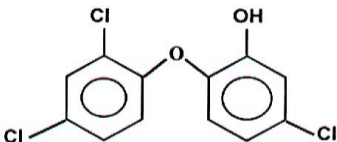
Chemical	Trade Names	Molecular Weight	S_w	$\text{Log } K_{ow}$
2,4,4'-trichloro-2-hydroxydiphenyl ether	Triclosan	289.5 g mol ⁻¹	10 mg L ⁻¹	5.4
	Irgasan			
	Microban			
	Lexol 300			
	Ster-Zac			

Table 2. Flow behavior index (n) of reformed casein proteins as determined by the power law relationship. Samples were subjected to HPH at 0, 100, 200, and 300 MPa and subsequent ethanol evaporation.

HPH Treatment (MPa)	k	n	r^2
0	0.011	0.92 ±0.02	0.999
100	0.011	0.95 ±0.01	0.999
200	0.008	0.97 ±0.02	0.998
300	0.011	0.89 ±0.03	0.999
Skim milk (ctl)	0.008	0.86 ±0.05	0.997

Skim milk (ctl) – Skim milk heated to equivalent time and temperature as ethanol evaporated samples (60°/15min)

k – Consistency coefficient (Pa sⁿ)

n – Flow behavior index

CHAPTER 3

Field emission scanning electron microscopy analysis of casein micelles subjected to pressure and ethanol treatment

ABSTRACT

Casein micelle imaging by field emission scanning electron microscopy (FESEM) provided a range of detailed information about the structural arrangement of the casein micelle as affected by two solvent-induced dissociative methods: 1) continuous high shear processing (CHSP) in the presence of ethanol (1:1) and 2) ethanol-mediated acid-induced micellar dissociation. Images from both methods reveal similar trends. Casein micelles in the presence of ethanol form chain-like aggregates that, in some cases, form an extensive gel network or interact via thin fragments with discrete micelles. The removal of ethanol after micellar disruption in both methods enhances the formation of this aggregated network, most likely made up of serum (whey) proteins and dissociated casein proteins that interact to form a protein matrix. The increased amount of this matrix at 300 MPa in comparison to 0MPa and 200 MPa in the samples processed by CHSP provides evidence for this theory. Surface topography of the micelles, in some of the images, reveal a textured appearance indicative of a complex outer layer. Additionally, images of skim milk alone provide support for the submicellar theory of casein micelle structure. These findings warrant further research but also provide a new look at the casein micelle and offer explanations as to the reactivity and the behavior of the micelle in different environments.

INTRODUCTION

Casein proteins are the major protein in milk representing 80% of the total protein fraction while whey proteins represent the remaining 20%. Caseins are responsible for the transport of vital minerals and protein to the feeding mammalian young. Its biological efficiency is attributed to the stability of its natural structural arrangement as well as its ability to be easily digested. The four individual casein proteins, α_{s1} -, α_{s2} -, β -, and κ -casein, along with calcium phosphate nanoclusters, are arranged in an association colloid known as the casein micelle. This quaternary protein structure has a spherical shape with a mean diameter of 200 nm (range of 50-500 nm) and a molecular weight of 1.3×10^9 Da (Fox and McSweeney 1998).

However, the actual structure of the casein micelle is under debate due to the inability to crystallize the individual proteins and decipher artifact from actual structure in microscopy images. One model suggests that the micelle is formed by spherical submicelles linked together by colloidal calcium phosphate where the inner submicelles are formed by hydrophobic proteins (caseins α_{s1} , α_{s2} , β) and κ -casein, the amphiphilic protein, predominates the outer submicelles and is responsible for the suspension of the micelle within the aqueous phase (Walstra 1990). A model suggested by Horne (1998), presents the micelle as a block copolymer where the amphiphilic nature of all the casein proteins are considered. Micellar assembly and growth occurs through a polymerization process of Ca^{2+} bridging on phosphorylated serine residues and hydrophobic bonding among hydrophobic amino acid residues. One of the most recent models presented by Dalgleish and others (2004) based upon scanning electron microscopy images suggests a

tubular arrangement of the caseins. Examining the surface details of the micelle revealed a complex structure of tubular structures as opposed to spherical subunits that created a rough surface and gaps between the substructures. Here they suggest that these gaps are actually pores theorizing that the interior of the micelle is readily accessible from its exterior, providing evidence of a bicontinuous water/protein system.

Elucidating the actual structure of the casein micelle is important in explaining its reactivity and behavior. Of particular interest is the effect of various treatments on the modification of micelle structure and post treatment stability. Functional properties such as aqueous stability, heat stability, coagulation, gelation, emulsification, film formation, foaming, and whipping properties are greatly affected by the casein structure (Munro 2004). The continued clarification of the structural properties of the casein micelle will allow a better understanding of its basic structure and to extend its functional properties to non-food applications such as drug delivery systems and controlled release polymers.

The objective of this study was to determine the surface characteristics of bovine casein micelles before and after subjection to two treatments: 1) continuous high shear processing (CHSP) in the presence of ethanol (1:1, v/v), and 2) ethanol mediated dissociation through acidification. It is hypothesized that understanding micellar disruption and subsequent reformation will lead to development of a natural system for use as a potential nano-carrier of low molecular weight hydrophobic compounds. Additionally, information concerning the structural characteristics of the micelle during each phase of dissociation and reassociation would provide insight as to how the casein micelle interacts with its surrounding environment.

MATERIALS AND METHODS

Sample Preparation

Micellar Dissociation Method I – CHSP

A solution of skim milk and ethanol at a 1:1 (v/v) ratio was pressure treated by CHSP at 0, 200 and 300 MPa (Stansted Fluid Power, Ltd., Essex, U.K.). The operating structure consists of a bench top unit providing synchronized homogenization using two hydraulic intensifiers. Basic experimental schematic and possible encapsulation mechanism of a hydrophobic probe is illustrated in Fig 17. To minimize the effects of heating and prevent temperature elevation to temperatures exceeding 95°C, temperature at the homogenizing valve was regulated by water bath (Isotemp 3016D, Fisher Scientific, Pittsburgh, PA) at a constant temperature of 4°C. In this case, the cooled water circulates around the homogenizing block and reduces temperature increase by approximately 10-15°C per pressure level with more heat removal at the higher pressure levels (>200 MPa). Processed samples were collected in glass vials to a volume of 100 mL per pressure level. Following processing, ethanol was removed from samples by a rotary evaporator maintained at 60°C under constant vacuum. Skim milk not containing ethanol was also processed by CHSP at 0, 200, and 300 MPa for comparison purposes.

Micellar Dissociation Method II – Ethanol-mediated Acid-induced Dissociation

Skim milk diluted in a buffer solution (20 mM imidazole and 30 mM CaCl₂) to 0.5% (v/v) was added to ethanol at a 1:1 (v/v) ratio. After sufficient mixing, approximately 15 minutes, milk samples were acidified to pH 3.0 to achieve micelle dissociation as indicated by a clear solution. (Previous particle size experiments indicate

that micelles are dissociated (<40 nm) through these means.) Micellar re-association was achieved through alkaline treatment with 1N KOH to increase the pH to 6.8, the average pH of skim milk. Fig 18 illustrates the experimental schematic and proposed mechanism for the encapsulation of a hydrophobic probe.

FESEM Imaging

The methods of Dalglish and others (2004) for structural analysis of the casein micelle by FESEM was followed with modifications. Polished silicon wafer was immersed in sample and allowed to adhere to the wafer for 5 minutes. Excess was removed with a Pasteur pipette and the wafer washed once with a phosphate buffer (pH 7). The primary fixative, a 2% glutaraldehyde solution prepared in Sorensen's phosphate buffer, was added and allowed to fix for an hour. Wafer was rinsed with phosphate buffer in preparation for the secondary fixative, 1% osmium tetroxide in phosphate buffer, which also reacted for an hour. Sample was rinsed 3 times with phosphate buffer and dehydrated in increasing concentrations of ethanol: 70%, 90%, 95% (repeated twice), and 100% (repeated 3 times). Contact time for each solution was approximately 5 min. As a final step samples were critical point dried using carbon dioxide and stored in a desiccator at room temperature until sample imaging.

RESULTS AND DISCUSSION

CHSP (Continuous High Shear Processing)

Without ethanol assisted dissociation

In order to fully elucidate the effects of ethanol mediated dissociation with CHSP, pressurized skim milk samples in the absence of ethanol were imaged for comparison

purposes (Fig 19a-c). Skim milk samples without pressure (Fig 19a) reflect two different casein micelle populations. Micelles associate in clusters as well as individual structures. The surface topology of the individual micelles are similar to that of Dalgleish and others (2004). In this case, micelles appear to have protruding structures that give the surface of the micelle a lumpy appearance. The authors attribute this effect as well as the presence of pores to the possible tubular nature of the casein micelle structure. They question whether, using a tubular model, the casein micelle can be considered a bicontinuous water/protein system. However, our images do not give the appearance or suggest the presence of pores. This is most likely due to the differences in resolution between our samples and those of Dalgleish and others (2004). The other population of micelles in this sample are associated in clusters and appear to be linked through thin strands. Dalgleish and others (2004) also noted the presence of these filaments in their samples and attribute this effect to the spreading of the sample on the hydrophobic carbon substrate resulting in the disassociation of the micelles. This is very likely in our case as well, given the hydrophobic nature of our silicon wafer used for mounting our substrate. Additionally, the spreading or dissociation of the micelle could possibly be the effects of dilution. Skim milk was diluted to 11% in a buffer solution. Although the buffer solution contained proportional amounts of calcium found in bovine milk (30 mM), these values are only an estimate and provide one value based on a range of concentrations. If the concentration of calcium in the buffer solution is less than the concentration of that in the micelle, then micelle dissociation can be encouraged by the migration of micellar calcium

to the aqueous solution. Micellar calcium phosphate serves to adhere the individual casein proteins and contributes to the spherical structure of the casein micelle.

Skim milk subjected to pressures of 200 MPa reveals dissociation of the original micellar structure (Fig 19b). We believe that the combination of forces at the homogenizing valve encourage the dissociation of the micelle into its submicellar constituents. Surface topology of the structures are not clear and therefore it is not possible to see if these micelles or submicelles have a similar “lumpy” appearance. Elevated pressures increase the solubility of micellar calcium phosphate and the intensity of the shearing force has the ability to disrupt hydrophobic bonds. Both occurrences have the ability to disrupt the casein micelle. Continuing sample processing to 300 MPa increases the effects of pressure induced dissociation but is also accompanied with an increase in temperature at the homogenizing valve. Temperature will increase by 20°C per 100 MPa increase at the homogenizing valve (Fig 20) (Roach and Harte (2008); Sandra and Dalgleish (2005). Elevated temperatures have the ability to reverse the pressure-induced dissociation of the casein micelle. Increased temperature (>60°C) reduces the solubility of calcium phosphate, encouraging migration into the micelle, and promotes hydrophobic bonding – both of which have the ability to reform the micelle. Partial reformation of the micellar structure can be seen in Fig 3c. Additionally, the surface topology of the “reformed” micelles are similar to the “lumpy” appearance of the control samples without pressure (Fig 19a).

With ethanol assisted dissociation.

Ethanol is known to cause the collapse of the outer κ -casein protein layer of the micelle and initiate micellar aggregation. This effect is similar to acid induced gelation of milk for the production of yogurt. Large ($>5\ \mu\text{m}$) chain-like aggregates of skim milk in ethanol (1:1, v/v) at 0 MPa reflect this effect (Fig 21a). Also present in the same sample are discrete aggregates of a shorter chain lengths consisting of spherical structures, 100-300 nm in diameter (Fig 21b). A textured surface topography is not apparent and micelles appear as smooth spherical surfaces. Comparable images were not found in the literature which leads us to question whether these structures are actually micelles or other components within the milk. It is also likely, however, that the collapse of κ -casein in the presence of ethanol creates the appearance of a smooth micellar surface. The removal of ethanol through rotary evaporation appears to shorten the chain-like aggregation of the micelles (Fig 21c). The surface topology of discrete micelles also seems to be affected by the evaporation of ethanol. The micellar surface appears to be less smooth but still consists of smaller spherical micelles (Fig 21d).

Prior to the removal of ethanol, pressure treated samples follow a predictable trend in comparison to the no pressure control. The large chain-like aggregates are not as prominent at 200 MPa (Fig 22a) and almost completely dispersed at 300 MPa (Fig 23a). It is most likely that the combination of shearing forces at the homogenizing valve and the effects of elevated temperature caused the ethanol-induced disruption of the casein micelle aggregates. However, the surface structure and micellar arrangement of micelles not associated with the larger chains are similar in both pressure treated samples as well

as the control (Figs 22b and 23b) . After the removal of ethanol through evaporation, we see the same but opposite trend of the pressure treated samples in comparison to the control. The presence of chain-like micellar aggregation increases as pressure increases (Fig 22c). At 300 MPa there is a large mass of this matrix (Fig 23c). Additionally, the discrete micellar structures, as seen in the 0 MPa sample, appear to interact with the chain like structures through connection by thin thread-like aggregates (Fig 22d) or entrapment (Fig 23d). The precise constituents if the matrix is not known but most likely made up of whey proteins found in milk serum, α -lactalbumin and β -lactoglobulin, and casein proteins not associated with the micelle. It is known that β -lactoglobulin will denature at high temperatures (>70 - 75°C) and interact with the surface of the casein micelle and form disulfide linkages with κ -casein, the outer micellar protein (Huppertz and others 2004). It is probable that the increased temperature at the homogenizing valve associated with increasing pressures (Fig 20) served to not only dissociate the casein micelle in the presence of ethanol but also promote the denaturation of serum proteins. Removal of ethanol encouraged reassociation of the micelles that were already coupled with denatured serum proteins creating a casein-whey matrix. This theory can be assessed by repeating the same experiment in the absence or minimal presence of whey proteins.

Ethanol-mediated Acid-induced Dissociation

Casein images (skim milk (0.5%) suspended in buffer in the presence of 50% ethanol) prior to acidification reflect micellar aggregation due to the presence of ethanol (Fig 24a). However, different from the smooth surface casein images prior to pressurization in 50% ethanol and 50% skim milk, micelles not associated with the chain-like aggregates have a

textured topography (Fig 24b) similar to that of skim milk (Fig 19). This could possibly be due to the stabilizing effect of the suspension buffer on the casein micelles even in the presence of such high concentrations of ethanol. Calcium chloride was used at a concentration of 30 mM in the buffer solution and could serve to prevent the collapse of κ -casein on the surface of the micelle. Then again, these “textured” micelles are not completely isolated from the chain-like aggregates that appear to be smooth on the surface. Why some micelles would have a textured topography and others a smooth surface deserves further investigation.

Acidifying the sample to a pH of less than 4, results in the formation of a clear solution during sample preparation which is indicative of micellar dissociation. Buffer and fixing solutions for sample imaging can alter dissociative effects of the acidified ethanol/buffer solution and hinder sample imaging at this stage. This factor was taken into account while imaging. Ideally, imaged micelles should not exceed 100 nm in diameter. However, micelle images obtained ranged from approximately 50-250 nm for discrete micelles (Fig 25a), not taking into account random aggregation of the discrete structures. This is possibly due to the reformation of dissociated micelles or that all initial micelles were not completely dissociated. Again, we see two different micelle morphologies, one of a textured or “lumpy” appearance (Fig 25b) and the other of smooth spheres that associate one with another (Fig 25c) forming a distorted open sphere. [In an effort to account for the possible adverse effects associated with differences within the sample and buffer and fixing solutions used, the pH of the first phosphate wash and primary fixative, glutaraldehyde, was adjusted to match that of the sample. All other procedures following

these initial steps were not adjusted. Unfortunately, these images (not shown) did not provide any additional information or an alternative method for fixing samples.] The “reformation” of the dissociated micelles is visibly seen (cloudy appearance) by increasing the pH of the system back to its original pH of approximately 6.8. Microscopy images of the reformed micelles reveal an interesting structure and a new representation of possible casein/whey protein interactions (Fig 26a-c). Most micelles appear as small individual spheres (Fig 26c) or clusters (Fig 26b) with no apparent surface morphology. They also coat the protein matrix, which is extremely large ($>30\text{ }\mu\text{m}$) in this case giving it a speckled appearance (Fig 26a). There are, however, two micelles in fig 26b and 26c that show a textured surface morphology and abandon the smooth surfaces seen in the other micelles. It is not clear why these textured micelles are a random occurrence in this sample and leads us to question whether the smooth structures are casein micelles.

Artifact Resolution

It was thought that alternative preparation steps may be necessary to resolve artifacts associated with the fixing and staining process. The main concern is the use of the primary fixative, glutaraldehyde, which is known to create a gel network within the casein protein matrix. The use of ethanol as a dissociative agent in our samples also causes casein aggregation and the appearance of a gel network as seen in the previous skim milk images prior to ethanol evaporation. To determine whether the network was due to the effects of ethanol or glutaraldehyde, skim milk (100%) and skim milk (0.5%) suspended in a buffer solution identical to that used in the acid-induced dissociation samples were also imaged. The presence of chain-like casein aggregates are absent in

both samples (Fig 27a-b) indicating no adverse effects of the glutaraldehyde fixative. The presence of so few micelles in the skim milk sample leads us to believe there is a problem with the initial adherence of the milk to the silicon wafer, longer contact time is possibly needed. Nonetheless, we see an interesting effect that possibly adds to the submicellar theory. The micelle strand about 250 nm in length appears to be made up of small spherical structures, less than 50 nm in diameter (Fig 27a). It is likely that the effects of the wafer or lack of sufficient adherence caused this extended structure. Condensation of this strand into a sphere would indicate the presence of submicelles within the casein micelle. Micrograph of the 0.5% skim milk solution reveals a good distribution individual casein micelles (Fig 26b).

CONCLUSION

The images of the casein micelles obtained reflect the diverse nature of these natural structures. Although many questions arose through this study and assorted results, it can be generally stated that micellar dissociation and reformation is not a completely reversible process. Micelles in the dissociated state will interact with serum proteins and form an intricate protein matrix. The analysis of this matrix and possible prevention of its formation, by the minimal presence of whey proteins, is needed for the continued exploration of using the casein micelle as an encapsulation system for low molecular weight hydrophobic compounds. However, this occurrence could find use within the food industry for dairy product enhancement and new textural applications.

REFERENCES

- Dalgleish, D. G., P. A. Spagnuolo, and H. D. Goff. 2004. A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *International Dairy Journal*. 14, 1015-1031.
- Fox, P. F. and P. L. H. McSweeney. 1998. Dairy chemistry and biochemistry. Chapman & Hall, NY.
- Horne, D.S. 1998. Casein interactions: Casting light on the black boxes, the structure in dairy products. *International Dairy Journal*. 8, 171-177.
- Huppertz, T., Fox, P. F., & Kelly, A. L. (2004). High pressure treatment of bovine milk: effects on casein micelles and whey proteins. *Journal of Dairy Research*, 71(1), 97-106.
- Munro, P. A. 2004. Caseins, functional properties and food uses. In Encyclopedia of dairy science. H. Roginsky, J. W. Fuquay, and P. F. Fox (Ed.). Academic Press, NY 1909-1915.
- Roach, A.; Harte, F. 2007. Disruption and sedimentation of casein micelles and casein micelle isolates under high-pressure homogenization. *Innovative Food Science and Emerging Technologies*, 9(1): 1-8.
- Sandra, S., & Dalgleish, D. G. (2005). Effects of ultra-high-pressure homogenization and heating on structural properties of casein micelles in reconstituted skim milk powder. *International Dairy Journal*, 15(11), 1095-1104.
- Walstra, P. (1990). On the stability of casein micelles. *Journal of Dairy Science*, 73(8) 1965-1979.

APPENDIX III - FIGURES

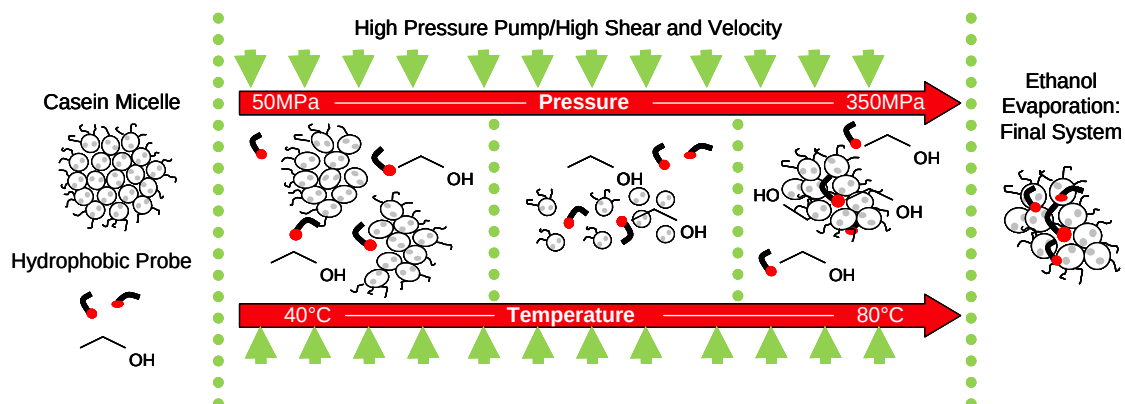


Fig 17. Experimental schematic of pressure-induced ethanol-assisted dissociation of the casein micelle through CHSP (continuous high shear processing). Ethanol and skim milk (1:1, v/v) is subjected to a combination of frictional forces at the homogenizing valve in the presence of a hydrophobic probe. Once exposed to the combined forces at the homogenizing valve, micelles dissociate due to the effects of pressure and friction. As pressurization is continued, the increase in temperature reverses the effects of pressure induced dissociation and results in partial micellar reformation. The removal of ethanol through evaporation encourages the association of the hydrophobic probe to the hydrophobic protein constituents of the micelle.

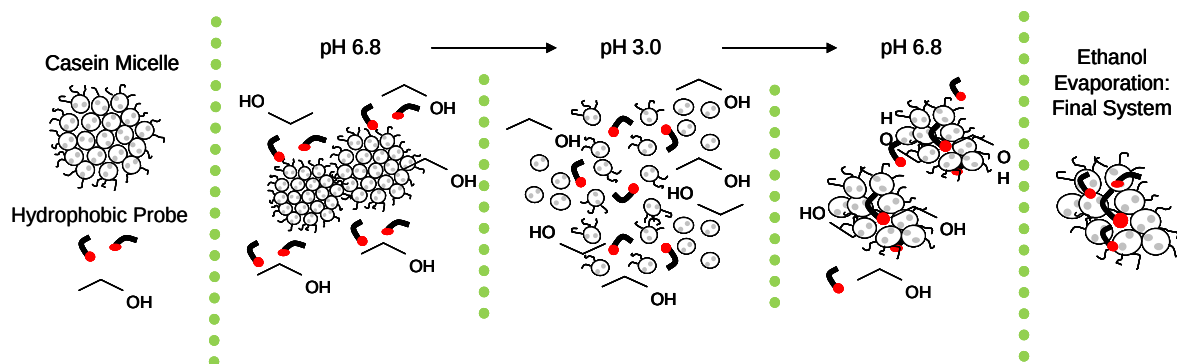


Fig 18. Ethanol-mediated acid-induced experimental schematic for micellar dissociation.

Skim milk diluted in a buffer solution (0.5%, v/v) is added to ethanol containing the hydrophobic probe at a 1:1 (v/v) ratio. Milk solution is acidified to pH 3.0 resulting in a clear solution indicative of micelle dissociation. Solution is treated with base to a pH of 6.8 and solution turbidity is regained, i.e., micellar re-formation. The removal of ethanol through evaporation encourages the association of the hydrophobic probe to the hydrophobic protein constituents of the micelle.

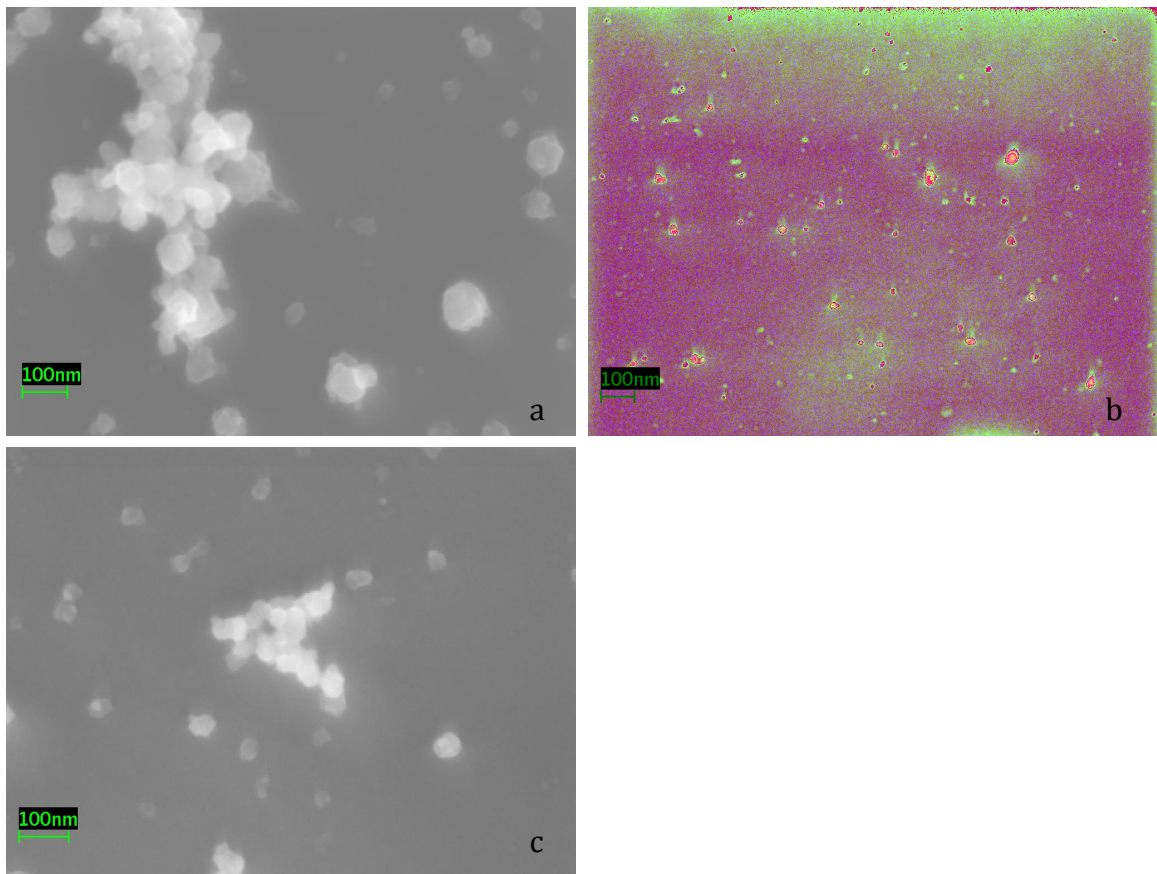


Fig 19. Casein micelles in skim milk subjected to pressure at a) 0 MPa, b) 200 MPa, and c) 300 MPa. Bar is 100 nm.

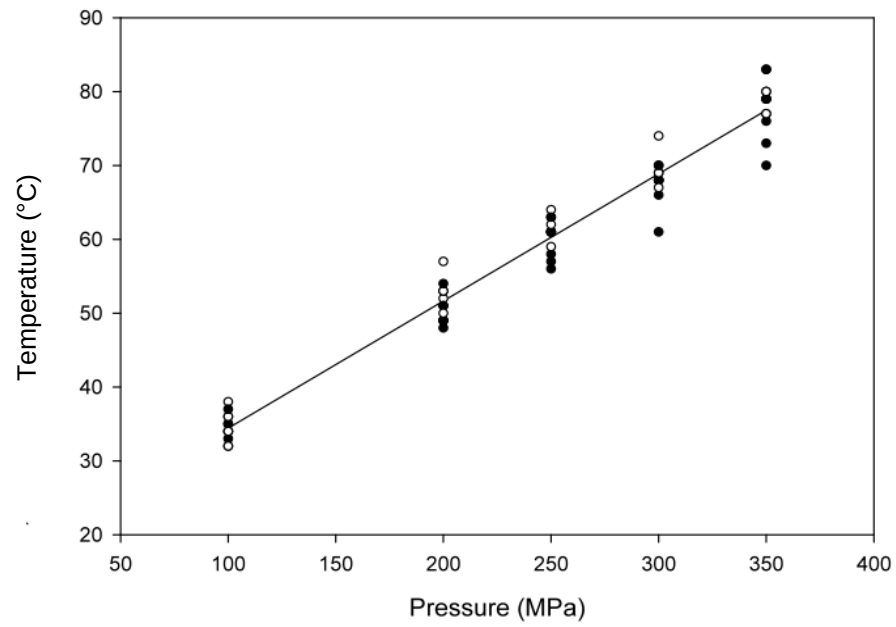


Fig 20. Temperature increases during continuous high shear processing. Data points at each pressure level represent individual experiments. Linear regression for all points in plot ($r^2 = 0.97$).

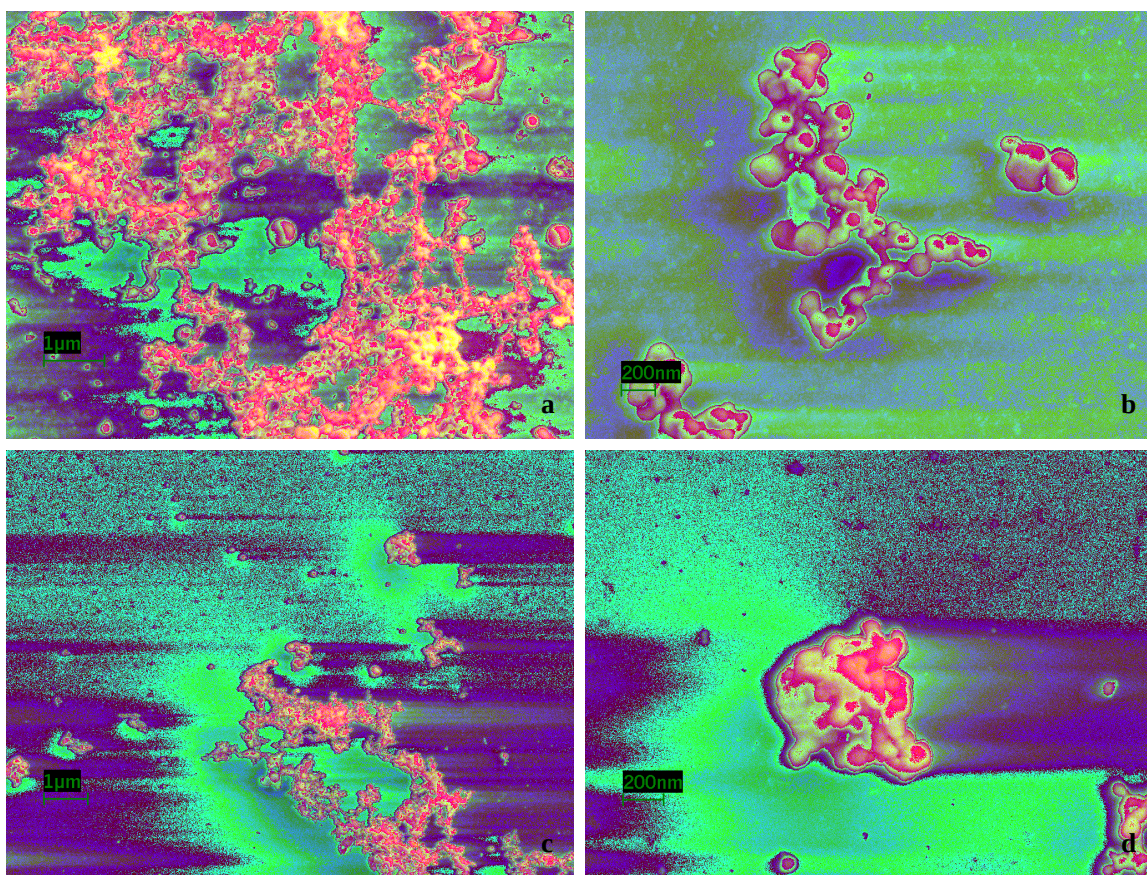


Fig 21. Casein micelles in skim milk and ethanol (1:1, v/v) at 0 MPa before ethanol evaporation at a) low and b) high magnification and after ethanol evaporation at c) low and d) high magnification. Bar is 1 μm and 200 nm at low and high magnification, respectively.

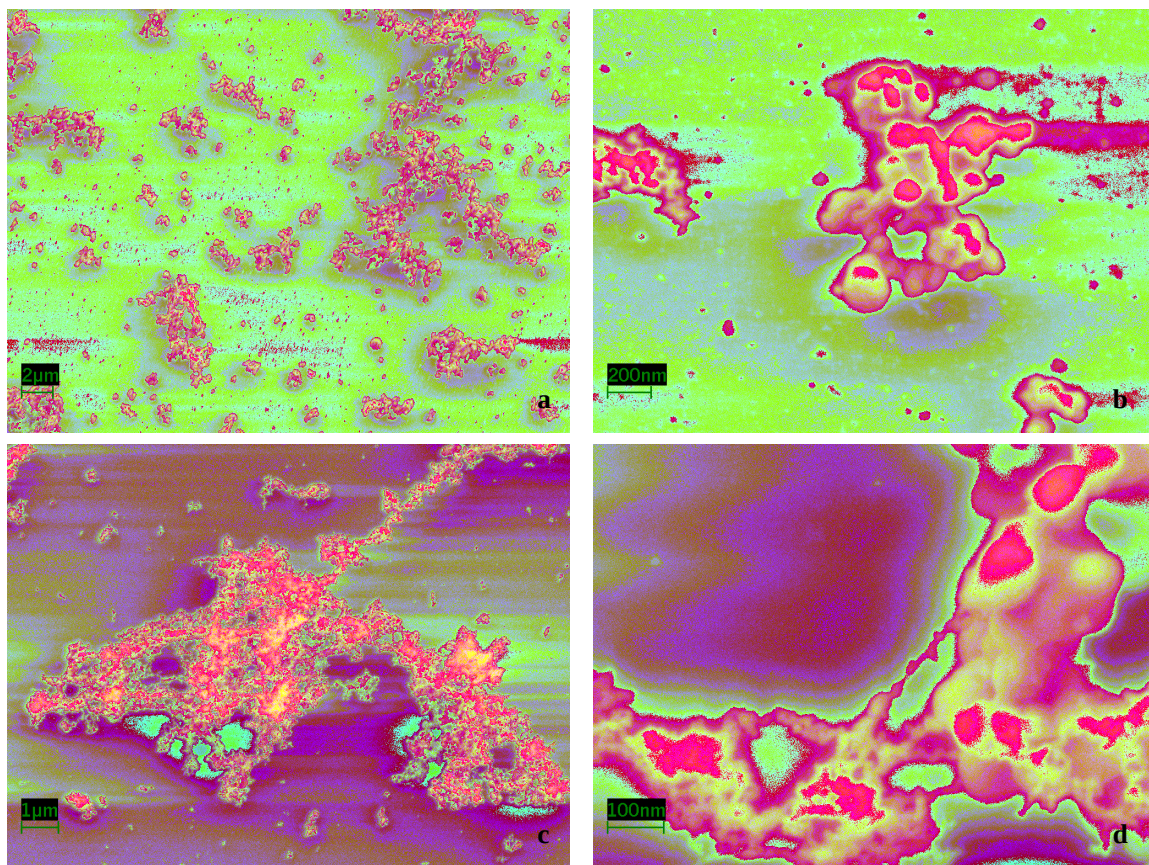


Fig 22. Casein micelles in skim milk and ethanol (1:1, v/v) at 200 MPa before ethanol evaporation at a) low (bar is 2 μm) and b) high magnification (bar is 200 nm) and after ethanol evaporation at c) low (bar is 1 μm) and d) high magnification (bar is 100nm).

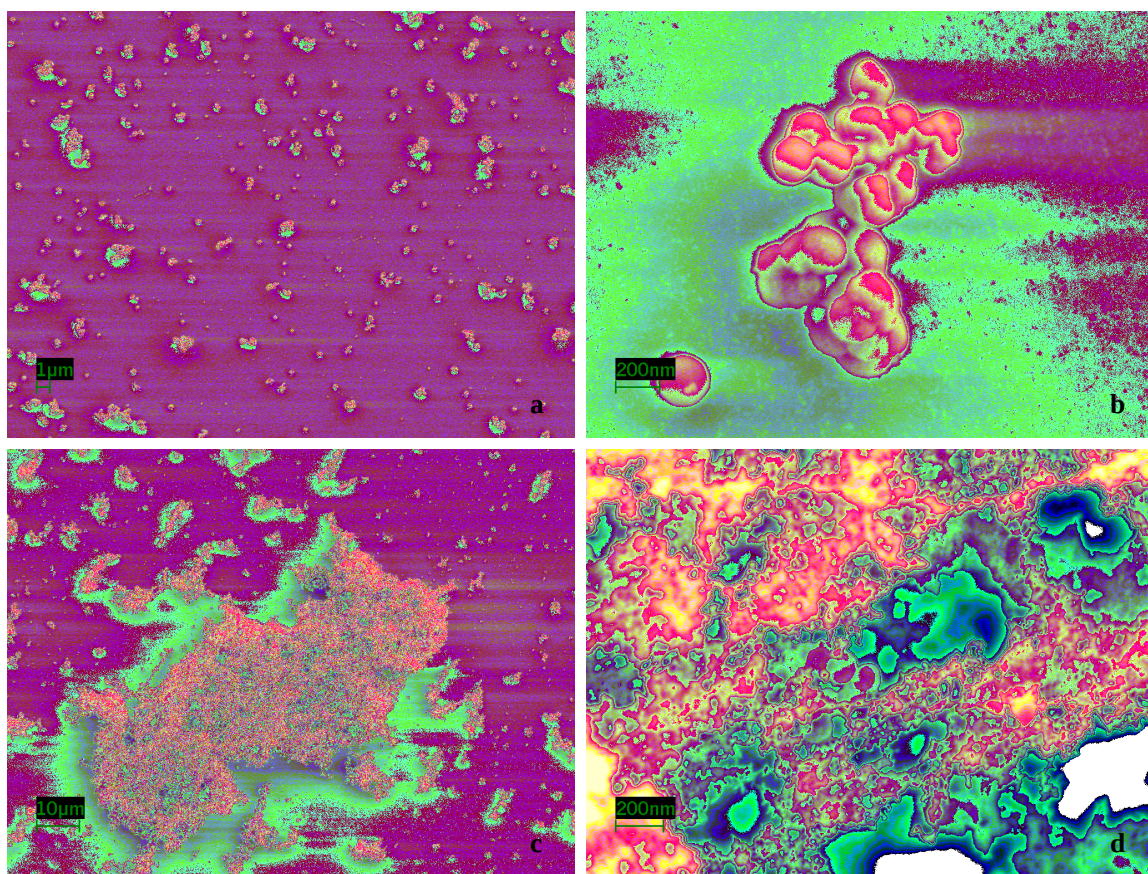


Fig 23. Casein micelles in skim milk and ethanol (1:1, v/v) at 300 MPa before ethanol evaporation at a) low (bar is 1 μm) and b) high magnification (bar is 200 nm) and after ethanol evaporation at c) low (bar is 10 μm) and d) high magnification (bar is 200 nm).

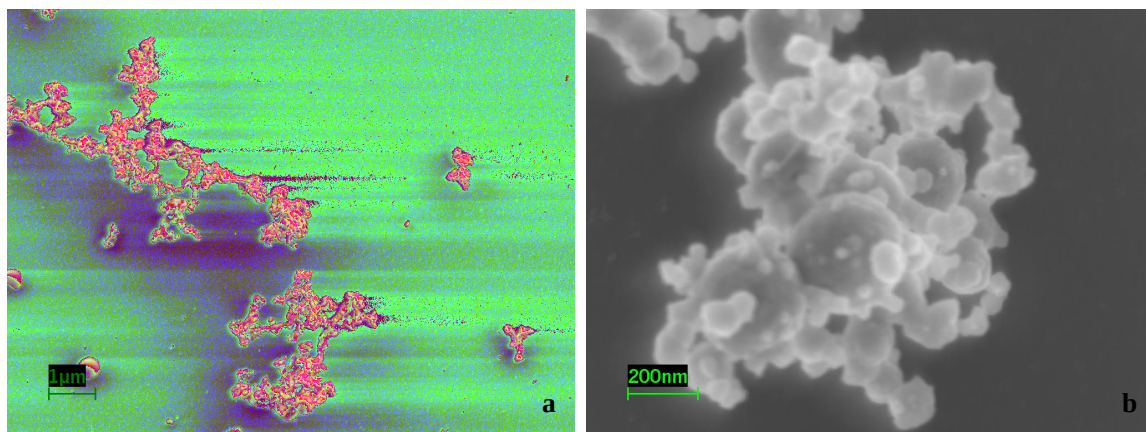


Fig 24. Casein images (skim milk (0.5%) suspended in buffer in the presence of 50% ethanol) prior to acidification at a) low (bar is 1 μm) and b) high magnification (bar is 200 nm). High magnification images reveal a textured surface topography of the casein micelle.

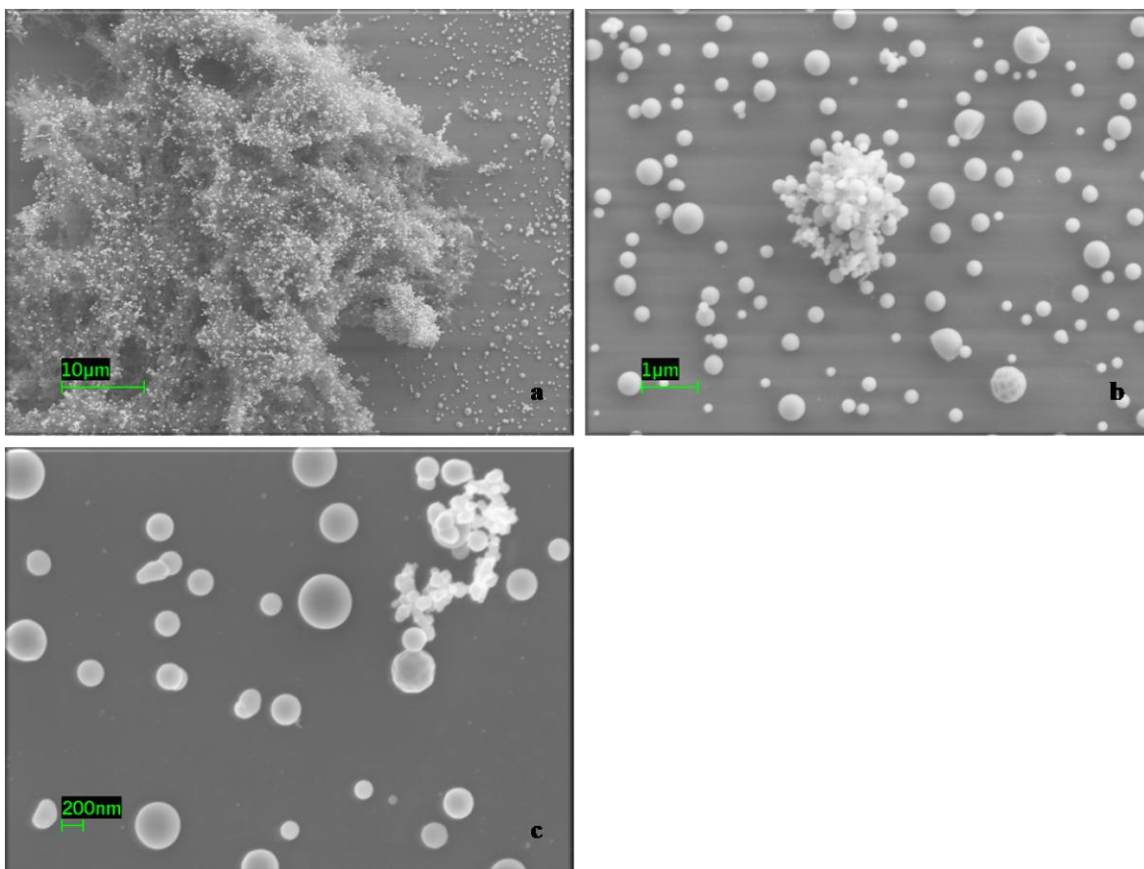


Fig 25. Images of the reformed micelles by alkaline treatment of the acid-induced dissociated casein suspension: a) large protein matrix speckled with casein micelles (bar is 10 μm), b) micellar clusters (bar is 1 μm) and c) small individual micellar spheres (bar is 200 nm).

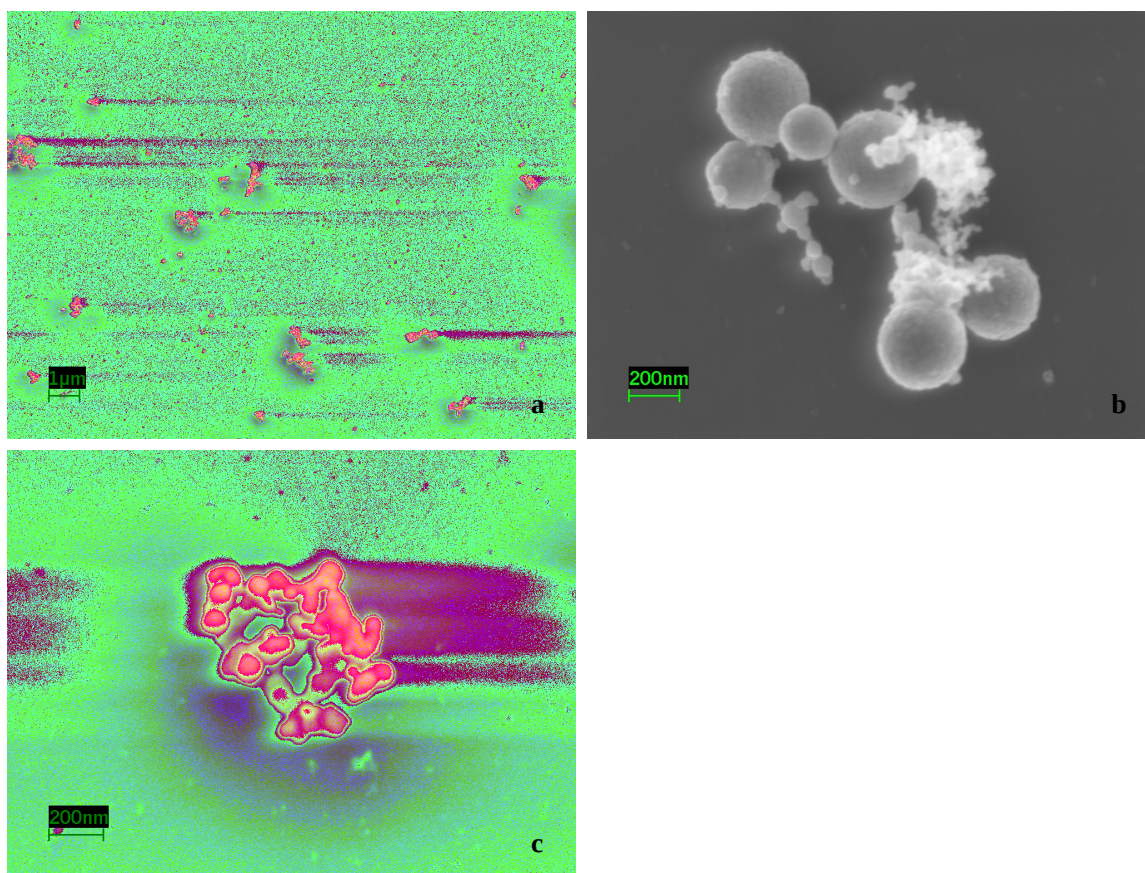


Fig 26. Casein suspension (skim milk (0.5%) suspended in buffer in the presence of 50% ethanol) subjected to acidification to initiate micellar dissociation: a) Low magnification image reflecting the overall micellar structure (bar is 10 μm), b) textured micelles (bar is 1 μm) and c) smooth micelles (bar is 200 nm).

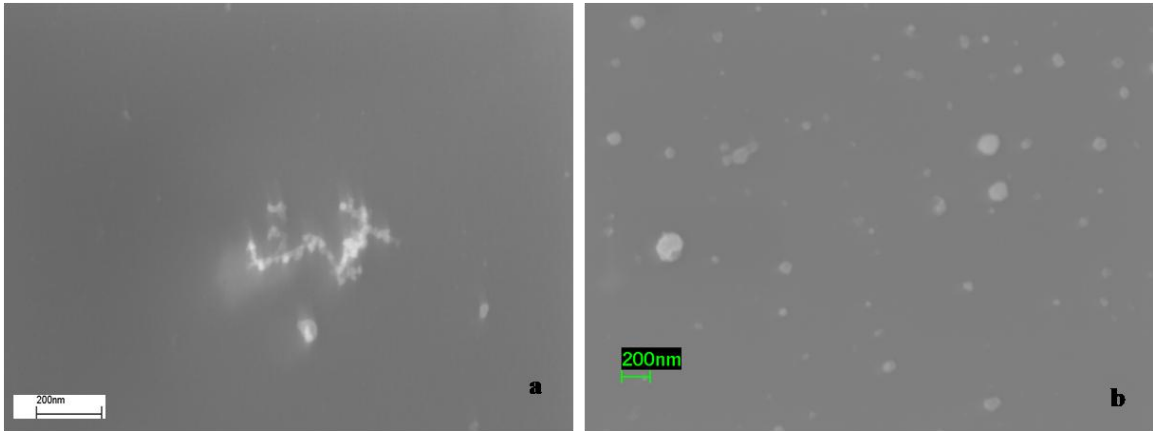


Fig 27. a) 100% skim milk and b) 0.5% skim milk (suspended in a buffer solution identical to that used in the acid-induced dissociation samples). The presence of chain-like casein aggregates are absent in both samples indicating no adverse effects of the glutaraldehyde fixative. Bar is 200 nm.

CHAPTER 4

**Release of triclosan from the casein micelle as affected by diffusion,
pH, and *in vitro* digestion**

ABSTRACT

The bovine casein micelle has been recently explored as a biopolymer for encapsulating hydrophobic compounds. The objective of this study was to investigate the release of triclosan, a hydrophobic antimicrobial, from the casein micelle under three conditions: 1) membrane dialysis from intact casein micelles in Franz diffusion cells, 2) under a pH range from pH 2 to 12, and 3) during *in vitro* digestion using simulated gastric and intestinal fluids. TCS (14 mg L^{-1}) was added to skim milk and equilibrated to milk constituents. Milk-solution was spray dried and later re-suspended in deionized water (1%, w/v) and subjected to the release methods previously described. The final concentration of TCS in the re-suspended milk-solution (pH7) was 8.6 mg L^{-1} , of which, 64% was encapsulated by casein micelles and 36% associated with the serum. The dialysis of triclosan across the membrane was delayed in the presence of milk, only 20% of the total amount of triclosan in the sample chamber was released into the receiving chamber as opposed to 80% in the control sample absent of milk protein. Changes in solution pH had a profound effect on the release of triclosan. At extreme pH values (pH<3 and pH>10) micelles dissociate, subsequently releasing triclosan into solution. The release of TCS from the caseins into the solution was increased up to 97% at pH 11 to 46% at pH 2. Maximal TCS loading onto micelles occurs at pH 4.6, the isoelectric point of casein proteins. During *in vitro* digestion, the release of triclosan is hindered in the gastric phase and released in the intestinal phase, an indication of bioaccessibility. These experiments demonstrate that the disruption of micelle integrity is required for the subsequent release of triclosan.

INTRODUCTION

The casein micelle has been described as a block copolymer micelle formed by self-assemblage of the 4 amphiphilic casein proteins, κ -, α_{s1} -, α_{s2} , and β -casein via bridging by calcium phosphate and intermolecular hydrophobic interactions (Horne 1998). Amphiphilic copolymers, both natural and synthetic, have been extensively explored as carriers for hydrophobic compounds (Sun and others 2001b; Tang and Du 2008) and are emerging as one of the most promising families of materials for use in micellar drug formulation and delivery (Liu and others 2006). Biopolymers can be an appealing alternative to synthetic polymers as a delivery system based on commercial availability, relatively low cost, and biodegradability. Although research is limited on the use of the casein micelle for carrier based applications, recent studies have highlighted its ability to encapsulate a range of poorly soluble compounds such as the encapsulation of vitamin D through the natural self-assembly of casein micelles (Semo and others 2007), the encapsulation of β -carotene in casein-grafted dextran nanoparticles (Pan and others, 2007), and the natural association of triclosan to the casein micelle enhancing the solubility of triclosan by 40 times (Roach and others 2009). The solubility enhancement of pharmaceuticals by milk has also been explored in earlier studies as a potential method to administer poorly soluble compounds (Macheras and others 1986; Macheras and others 1990) and more recently as an effective delivery vehicle for fat-soluble vitamins (Herrero-Barbudo and others 2009).

Over 40% of new drug candidates per year do not pass the developmental period because they have poor aqueous solubility (Liu and others 2006) and this typically

translates into low bioavailability. The proposed solution is the design of delivery systems capable of enhancing the bioavailability of these hydrophobic pharmaceuticals (Maestrelli and others 2006). Two major factors governing the success of a carrier to enhance bioavailability is the drug loading capacity and release behavior. Oral drug administration is the most convenient and desired method of drug delivery for routine administration (Quan and others 2008). Here release is regulated by the pH changes of the gastrointestinal tract, sink conditions which have the most profound effect on the carrier, and the presence of digestive enzymes. In vitro digestion methods can be an economical and efficient method for screening the relative bioaccessibility of compounds in various matrices and is usually a necessary prerequisite for focused animal and human studies (Failla and others 2008). Bioaccessibility is the first step towards assessing bioavailability, the amount of drug soluble in the intestinal tract and available for absorption, whereas bioavailability is the amount of drug that is actually absorbed, transported across the intestinal epithelia and present within the circulation system for cellular utilization (Perales and others 2007).

Triclosan (TCS) is a broad spectrum antimicrobial found in numerous consumer products for its antimicrobial properties. The Biopharmaceutics Classification System classifies TCS as a class IV drug, defined as having low solubility and low permeability. It has recently been investigated for its ability to prevent breast cancer (Lu and Archer) and its effectiveness in treating liver-stage malaria (Yu and others 2008). However, its full realization as a pharmaceutical is limited by its low oral bioavailability, a problem often encountered in Class IV drugs. Numerous studies have explored the use of carriers

and encapsulants to enhance the solubility of triclosan mainly for cosmetic applications but few have investigated the use of encapsulants to increase its oral bioavailability for pharmaceutical applications.

Our previous work has demonstrated the capacity of the casein micelle in binding triclosan using various methods (Roach and others 2009). The objective of the present study is to understand the release behavior of triclosan from the casein micelle under three conditions to evaluate its bioaccessibility: 1) membrane dialysis in Franz diffusion cells under sink conditions from intact casein micelles, 2) under a wide pH range (pH 2-12) that effects the solubility of both triclosan and properties of the casein carrier, and 3) during in vitro digestion using simulated gastric and intestinal fluids. Franz diffusion cells have been the standard system to study the release of semisolid drug formulations (Liebenberg and others 2004) and pH-responsive drug delivery to the intestine is well researched utilizing pH changes along the gastrointestinal tract. In most cases, coatings are used to prevent the release of drugs in the stomach and then allow drug release as the pH increases in the small intestine (McConnell and others 2008). Our aim is to assess the diffusibility of triclosan from milk components and provide some insight into the bioaccessibility of triclosan when ingested orally in the presence of milk and evaluate the capacity of the casein micelle to serve as a potential oral delivery system.

MATERIALS AND METHODS

Milk Source and Hydrophobic Probe

Pasteurized skim milk was purchased from a local grocery store and used as received. Triclosan (97%, TCS) was obtained from Sigma-Aldrich (St. Louis, MO) and used as the hydrophobic probe for encapsulation and release experiments. Individual TCS standards were prepared for each concentration in high pressure liquid chromatography (HPLC) grade acetonitrile (Fisher Scientific, Atlanta, GA) and added to skim milk. Less than 0.1% (v/v) of the co-solvent, acetonitrile, was added to samples. All other reagents were of HPLC or analytical grade.

Sample Preparation

The natural tendency of triclosan to associate with skim milk components, as described by Roach and others (2009), was used as the means of triclosan encapsulation. In this method, skim milk is equilibrated with triclosan dissolved in acetonitrile. Triclosan was dissolved in the acetonitrile at a concentration as to not add more than 0.1% (v/v) of the co-solvent, acetonitrile, to any given sample. Triclosan association to milk components was encouraged by adding triclosan (80 mg L^{-1}) slowly to skim milk with a syringe under continuous agitation with a magnetic stir bar at room temperature and allowed to mix for 1 hour. The triclosan-skim milk solution was spray dried (Büchi Cooperation, New Castel, Delaware; Mini Spray Dryer B-290) and stored in a vacuum desiccator until use. Spray drying parameters are as follows: 180°C inlet temperature and 100% aspiration at 10 mL per minute. The spray dried milk powder was dispersed/re-

suspended in their respective dissolution medium for experiments. The spray dry step was included to extend the commercial applicability of by having a shelf-stable product.

Particle Size Analysis

Particle size analysis was performed on the re-suspended spray dried sample using photon correlation spectroscopy with a Delsa Nano C Particle-size analyzer (Beckman Coulter, Inc., California). Approximately 2.5 mL of an undiluted sample were used for analysis. Undiluted skim milk was used as a standard control. Fifty size accumulations were collected for each sample (25°C) and done in triplicate.

Release Methods

Membrane Diffusion

The diffusion of triclosan across a dialysis disc membrane made of regenerated cellulose (12-14 kDa MWCO, Spectra/Por[®]) was carried out in Franz diffusion cells (PermeGear, Inc., Hellertown, PA). A schematic of the release apparatus is illustrated in Fig 28. The apparatus consists of: a 3 mL sample chamber, 33 mm dialysis membrane, 12 mL release chamber, magnetic stir bar, flange joint sample arm, and a metal clamp securing the sample chamber to the release chamber. Five hundred milligrams of the spray dried milk-solution was dispersed in 500 mL HPLC water with a handheld homogenizer (1%, w/v) and 3 ml of this solution was placed in the sample compartment. The release medium consisted of an aqueous solution of acetonitrile (30% and 40%, v/v) at a 12 ml volume. The acetonitrile/water mixture was chosen as a release medium because the release of the lipophilic TCS is reportedly faster in media containing a co-solvent, such as ethanol (Liebenberg and others 2004). The dialysis membrane is

compatible with a range of organic solvents as listed by the manufacturer. Care was taken to prevent the formation of air bubbles at the membrane-liquid interface between both the release chamber and sample chamber as this would impede diffusion. At each time point, 100 μ L was withdrawn from the sample arm of the apparatus. An equal volume of fresh release medium was replaced carefully by syringe immediately after removal. Triclosan concentration was measured by HPLC without any pre-treatment step. Control sample consisted of free triclosan added to the sample compartment and release medium was the same as in the treated sample. In this case, 8 mg L⁻¹ of TCS was dissolved in HPLC water and added to the diffusion apparatus and carried out concurrently with the treated samples. Experiments were conducted at room temperature (21°C).

pH Triggered Release

Spray dried milk-solution was dispersed in HPLC water (1%, w/v) as previously described. In two separate experiments, milk-dispersions (initial pH of 6.8) were adjusted to pH values of 6, 5, 4.6, 4, 3, and 2 with 1M HCl and to pH values of 7, 8, 9, 10, 11, and 12 with 1M KOH. Samples were magnetically stirred throughout the duration of the pH adjustment. At each pH value 10 mL aliquots were removed after pH stabilized (approximately 5 min) to measure total TCS concentration and TCS serum concentration. The amount of TCS in the serum was determined by precipitating the casein micelles through ultracentrifugation (Beckman Coulter, Inc., California) at 200,000 x *g* for 30 min and analyzing the supernatant for TCS concentration by HPLC. Prior to HPLC analysis in both the total sample and supernatant, TCS was extracted from the sample matrices for

purification purposes (explained in detail in a later section). Experiments were done in triplicate.

In vitro digestion

The *in vitro* digestion procedure of Perales and others (2007) was followed with moderate modifications. *In vitro* digestion was carried out in 2 phases: gastric and intestinal. Spray dried milk-solution was re-suspended in freshly prepared pepsin (porcine: cat no. P-7000, Sigma Aldrich, St. Louis MO) solution yielding 0.05 g pepsin per sample. The solution was adjusted to a pH of 2.0 prior to adding milk powder. Samples were incubated in a shaking water bath at 37°C for 1 hour at 120 rpm. Samples were removed from water bath and immediately placed on ice for 5 minutes and raised to a pH of 5.0 with 1M KOH to stop gastric digestion. Prior to pH adjustment, samples allocated for gastric digestion were removed, centrifuged (10,000 rpm, 15 min) and supernatant was later analyzed by HPLC to determine the amount of TCS released. Freshly prepared intestinal solution yielding 0.008 g pancreatine (porcine: cat no. P-1750, Sigma Aldrich, St. Louis MO) and 0.05 g bile (porcine: cat no. B-8631, Sigma Aldrich, St. Louis MO) per sample was added to the gastric digests allocated for both the gastric and intestinal phases. The pH was raised to pH 7.4 with 1M KOH and incubation continued for an additional 2 hr. To stop intestinal digestion, samples were placed on ice for 10 min. Intestinal digestates were centrifuged (8,000 rpm, 1 hr) and supernatant was analyzed by HPLC to determine the amount of TCS released. Low speed centrifugation for an extended period of time was chosen over short time high speed centrifugation as it provides a more accurate representation of *in vivo* conditions (Perales and others 2007).

TCS Extraction and quantification

TCS extraction from milk solutions and digestates was carried out using a two step liquid-liquid extraction procedure. Initially, milk proteins were precipitated according to the method of Zhang and others (2006) for the determination of celecoxib, a non-steroidal anti-inflammatory drug, in human milk. Protein precipitation was induced with a 2:1 (v/v) ratio of acetonitrile to milk sample, followed by vortexing and centrifugation ($10,000 \times g$ for 15 min). To further reduce the amount of proteins loaded on the HPLC column, TCS extracted supernatants were frozen (-40°C) for a minimum of 6 hr to separate the remaining water soluble proteins (frozen bottom layer) from the TCS/acetonitrile rich top layer that remained unfrozen. The freezing point profile of acetonitrile/water mixtures is presented in detail by Zarzycki and others (2006). This phenomenon provides a simple method for sample purification without complex extraction procedures or the risk of compromised analyte recoveries with filtration.

The TCS/acetonitrile layer was transferred to a HPLC vial as the bottom fraction remained frozen. Twenty-five μL was injected on to the column for triclosan identification and quantification by HPLC (Waters Corp, Millford MA). The instrument consisted of a quaternary pump, solvent degasser, autosampler, and a diode array UV detector. Triclosan separation was achieved on a 150×4.6 mm C18 column with a $5 \mu\text{m}$ particle size (Econosphere, Alltech Associates, Inc., Deerfield, IL). HPLC conditions for separation were as follows: mobile phase of 70:30 acetonitrile:water, 1 mL min^{-1} flow rate, $25 \mu\text{L}$ injection volume, and UV adsorption at 220 nm for detection. A five point calibration curve was achieved using standards ranging from 0.1 to 200 mg L^{-1} .

Calibration standards were analyzed at the beginning of each sample set and exhibited linearity (r^2 of >0.99) throughout the course of the study

Protein identification by gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide) was carried out using a Bio-Rad (Hercules, CA) mini-gel electrophoresis unit on the supernatants of samples subjected to pH triggered release and in vitro digestion. Samples were pre-heated (95°C for 4 min) in the presence of 2-mercaptoethanol. Proteins were stained using either Coomassie Brilliant Blue R-250 or Silver Stain. Coomassie Blue was used for samples subjected to pH triggered release and due to the need for a more sensitive technique or for the intestinal digestates from dilution effects, silver staining was used.

RESULTS AND DISCUSSION

TCS Dissolution

The dissolution profile of TCS from intact casein micelles and other milk constituents into the receiving chamber of Franz diffusion cells is presented in Fig 29. Data is represented as the percent of the total concentration of TCS released into the receiving medium, % release. Initially, the release of both un-bound TCS in the control sample (TCS_{Free}) and TCS associated to milk proteins ($TCS_{protein}$) behaves similarly. Within 20 hr, however, both samples begin to show marked differences in the release behavior of TCS into the receiving medium. The diffusion of TCS_{Free} across the dialysis membrane is relatively constant throughout the duration of the experiment and over 80% of the initial TCS amount in the sample chamber was recovered in the release chamber at

the final time point of 6 days. The remaining unrecovered TCS is likely due to the binding of TCS to the dialysis membrane and equilibrium effects at the later time points. The prolonged release of triclosan has also been encountered by Martinez-Palau and others (2008) in the release of triclosan from polymer matrices. Here, a small burst effect was observed at the initial stage of release from drugs believed to be located at the surface of the particles, followed by a sustained release of triclosan, reaching a plateau at 4-6 days. In our case, membrane material and the hydrophobicity of triclosan is the likely reason for such an extended release period. Given the duration of the study, TCS concentration was measured in the sample compartment of the milk and control sample at the final time point to account for TCS loss to other means and was found to be negligible. The particle size of casein micelles in the re-suspended milk solutions was 250 nm (± 100 nm) very similar to original size of casein micelles prior to spray drying (210 nm ± 75 nm).

The release of TCS from intact casein micelles – $\text{TCS}_{\text{protein}}$ – into the release chamber plateaus within the first 24 hr of the experiment where only 20% of the total amount of TCS is recovered in the release medium throughout the duration of the experiment. The initial release of triclosan in the treated sample is likely due to the presence of un-bound or free TCS not associated with milk constituents in the sample chamber. It is apparent that the presence of milk in the sample chamber has a profound effect on the release of triclosan into the receiving medium. Although the nature of the interaction is not clear and beyond the scope of this paper, TCS has a considerable attraction to milk components most likely casein proteins. Albeit minimal, approximately

5%, increasing the acetonitrile from 30% to 40% lessens this protein-drug interaction and encourages drug-solvent interactions. It is important to note that the increase in the amount of acetonitrile, did not affect the rate of TCS release in the TCS_{Free} sample. The slight enhancement of additional acetonitrile on the release of triclosan from the sample containing milk also gives an indication on the strength of the TCS-protein interaction as the release of TCS from the control sample is independent of acetonitrile concentration at the levels tested. This suggests that triclosan can be “pulled” from the milk solution by increasing the solvent quality of the release medium in relation to triclosan. Likewise, this is the method of triclosan extraction from milk for HPLC analysis only 75% acetonitrile is used in this case and able to “pull” or separate the majority of TCS from milk components.

Dissolution is a dynamic process dependent on the composition of the dissolution medium, the hydrodynamics of the experimental set-up (Nicolaidis and others 1999) and the physico-chemical properties of the drug carrier and the drug itself (Liu and Allen 2006). The composition of the release medium will greatly affect the dialysability of a compound and in the case of triclosan, a poorly soluble compound, the release medium should favor the full solubilization of triclosan. Liebenberg and others (2004) compared the release of salicylic acid and triclosan in an aqueous buffer (pH 5.8) in Franz diffusion cells and found the release of triclosan to be more rapid in the presence of 40-50% ethanol as opposed to release medium not containing a co-solvent. Swart and others (2005) used an aqueous solution of 80% ethanol as the dissolution medium for the release of triclosan from creams and gels in Enhancer® cells. Additionally, the behavior of the milk

components in preventing the diffusion of triclosan across the membrane has been reported in numerous studies in relation to milk and the release of other compounds. In dialysis assays to test the bioavailability of iron, caseins have shown to exert an inhibitory effect when compared with proteins from other food sources. Macheras and others (1986) studied the dissolution rate of various drugs ranging in solubility in low fat milk and found that milk retarded the release of all compounds tested. However, a binding/release mechanism was not proposed. The solubilization of lipophilic drugs to milk is likely due to non-specific binding to casein micelles and partitioning to lipid components if present (Nicolaidis and others 1999). In the iron bioavailability studies, the dialysis percentage of iron increases as casein is hydrolyzed (Perales and others 2007) suggesting that micellar degradation is needed to “release” bound compounds. The results from the release of triclosan in conditions favoring both dissociation and denaturation are presented in the following sections.

pH triggered release

At the natural pH of the re-suspended milk solution, ~pH 6.9, 64% of the total amount of TCS was associated to casein micelles and 36% associated with the serum. The amount of TCS associated to casein micelles was determined indirectly, calculated as the difference between total TCS and TCS in the supernatant post ultracentrifugation, i.e., TCS_{Serum}. The release of TCS from the caseins to the milk serum (Fig 30) was increased by 42%, 59%, 81%, and 97% as the pH was increased to 8, 9, 10, and 11, respectively, with no further increase at pH 12. As the pH was reduced to 4 from 6.9, in a separate sample, serum TCS levels were reduced significantly from 34% at a pH of 6 to 16%,

10%, and 13% at a pH of 5, 4.6, and 4, in that order. Interestingly, maximal TCS loading onto micelles occurs at pH 4.6, the isoelectric point of casein proteins. As pH was further reduced to 3 and 2, TCS was subsequently released from the caseins increasing TCS serum levels to 34% and 46%, respectively. Total TCS concentration (amount of TCS in the entire sample prior to ultracentrifugation) remained constant, indicating that the extreme changes in pH did not affect the recovery of TCS from the sample.

The release of triclosan from the micelle during pH adjustment is likely not due to the actual presence of free triclosan in solution, but rather the presence of TCS-casein conjugates from the liberation of the casein protein from the micelle under various pH's. Gel electrophoresis of the supernatants post ultracentrifugation at each pH supports this theory. Fig 31 illustrates the behavior of the casein proteins during changes in pH. The presence of whey proteins remains relatively the same throughout the entire pH range. The presence of TCS in the supernatant mimics the behavior of the casein proteins: at pH 4.6, the casein micelle precipitates out of solution indicated by the absence of casein protein bands, this is also the pH with the maximum amount of TCS loaded on to the micelle. Factors favoring the dissociation of the casein micelle at extreme pH values result in the breakdown of micellar integrity leading to its dissociation to submicellar components. These submicellar components bound to TCS form what we have termed the TCS-protein conjugate.

Our findings conflict a study by Liu and Guo (2008) where they observed self assemblage of casein molecules to form casein micelles in the pH range of 2.0 to 3.0 and 5.5 to 12.0. Our results do however, support the findings of Huppertz and others (2008)

in the alkaline disruption of the casein micelle at pH >10.0. Casein micelle disintegration is initiated when the solvent quality of the milk serum increases. Hydrophobic interactions and phosphoserine crosslinking is responsible for maintaining micellar integrity. Once the hydrophobic domains become thermodynamically favorable, there is little to hold the micelle together and it dissociates into submicellar components, described by Huppertz and others (2008) as individual nanoclusters or casein molecules. Ionic disruption occurs when the crosslinking of casein proteins along the phosphoserine residues is reduced and hydrophobic disruption occurs when protein-solvent interactions are more favorable than the cohesive protein-protein interactions among the hydrophobic protein regions. During alkaline treatment, the solubility of the calcium phosphate is reduced and ionic calcium and phosphate in the serum migrates into the micelle. So the disruption of the micelle at alkaline pH is unlikely due to the reduction in crosslinking, on the contrary, crosslinking is actually enhanced (Vaia and others 2006). However, as the pH is raised above the pI, the ionization of acidic amino acids increases and reduced on the basic amino acids. This increases the net-negative charge on the micelle encouraging intermolecular repulsion promoting micellar dissociation. As the pH increases, molecular repulsion also increases and protein-solvent interactions are more favorable than ionic interactions and protein-protein interactions. The migration of ionic calcium and ionic phosphate from the serum to the micelle during alkaline treatment increases the solvent quality of the milk by increasing the volume of water available to hydrate the exposed protein surfaces (Vaia and others 2006) resulting in micellar dissociation.

The acidic dissociation of the casein micelle at pH values below 4.0 has not been previously reported to the authors' knowledge. However, it is well known that during acidification calcium migrates from the micelle to the serum reducing the crosslinking along the serine residues. As the pH continues to drop below the pI, the net negative charge decreases and intermolecular repulsion is increased encouraging micelle dissociation. It appears from Fig 30 that the release of triclosan is more extensive or complete at extreme alkaline pH rather than extreme acidic pH. Likewise, gel electrophoresis band of the sample supernatants indicate a greater presence of casein proteins at alkaline pH rather than acidic pH (Fig 31).

The release of triclosan could either be due to this effect of pH on casein solubility, the effect of pH on the solubility of triclosan, or a combination of both. Triclosan is classified as a weak acid with a pKa of 8.1 (Tixier and others 2002) and its solubility is dependent on the pH of the dissolution medium (Loftsson and others 2005; Grove and others 2003). At alkaline pH (>8) the hydroxyl functional group is deprotonated and increases its aqueous solubility (Loftsson and others 2005). As the pH increases the solubility of the acid increases displaying a linear relationship until the limiting solubility of the ionized form is reached. The solubility of triclosan is increased dramatically when the compound is fully ionized in dissolution medium at a pH>10 (Gardiner and others 2008; Loftsson and others 2005) and solubility is decreased significantly at pH values below its pKa (pH 5<8) (Gardiner and others 2008). The dissolution behavior of free triclosan under acidic conditions (<5) is not clear but its activity as an antimicrobial sharply decreases at lower pH values. Here, triclosan was

used at a concentration of 8 mg L^{-1} , just below its reported aqueous solubility of 10 mg L^{-1} . Although it is likely that the increase of triclosan in the sample supernatant is due its subsequent release during micellar dissociation is it also probable that its increased solubility under alkaline conditions is pH induced.

In vitro bioaccessibility

During the gastric phase of digestion the release of TCS in samples containing milk was prevented (Fig 32), only 8% of the total amount of TCS was present in the supernatant. Interestingly, approximately 80% of the total amount of TCS was measured in the control sample absent of milk. It is likely that casein proteins precipitated under the acidic conditions of the gastric solution and prevented the release of TCS. This indicates that casein protein is a potential candidate to “protect” associated compounds from degradation in the acidic environment of the stomach. Casein is often described as a slow digesting protein as opposed to whey proteins, where the caseins precipitate under the acidic environment of the stomach. In this case, it is likely that the pepsin present in the gastric solution was not able to degrade the casein proteins during the gastric phase of digestion. Additionally, the solubility of triclosan under acidic conditions is limited because it is a weak acid, as mentioned in the previous section.

Conversely, over 85% of the total amount of TCS in the samples containing milk was released during intestinal digestion. Based on the results from the pH triggered release, only 60% of triclosan is released at the equivalent pH of 7 or 8. The solubility of a compound in water is seldom reflective of its solubility in the gastrointestinal tract. It is likely that the presence of digestive enzymes aided in degrading the micelle into

submicellar components. Gel electrophoresis of the supernatants of the intestinal digestate did not reveal any casein or whey protein bands (not pictured). This indicates that proteins were degraded to small peptide units. Ferranti and others (2004) describes casein proteolysis during in vitro digestion and characterized the sequence of casein degradation from intermediate oligopeptides to the formation of small peptides. Perales and others (2007) compared the dialysis percent of iron from protein-hydrolyzed formulas to non-hydrolyzed protein formulas and determined that a reduction in protein molecular weight reduces the number of ligands formed with iron and subsequently favors an increase in the release of iron into solution. Our results support these findings, casein dissociation and/or denaturation is necessary for the release of bound compounds. The release of TCS in the control sample remained constant in both the gastric and intestinal phases strengthening the theory that the casein micelle has the ability to bind TCS. Release behavior of TCS mimics the dissociation behavior of the casein micelle under conditions favoring micelle disruption.

Percentages of solubility can provide helpful information on the amount of a substance present in the intestinal lumen and potentially available for absorption, i.e, bioaccessibility (Perales and others 2007). The stomach is the first site where drugs administered orally can release. Compounds highly soluble at gastric pH can completely empty and limit the amount of compound available for intestinal absorption. Enteric coatings are used to delay the release of a drug in the stomach and encourage release as the complex enters the alkaline environment of the small intestine (McConnell and others 2008).

CONCLUSION

The release behavior of triclosan from the casein micelle under 3 different conditions is demonstrated in this study. The full diffusion of triclosan in Franz diffusion cells is prevented in the presence of milk indicating the role of intact casein micelles in binding triclosan. Triclosan that was measured in the release medium is believed to be the un-bound/freely diffusible fraction not associated with any protein or solid component of the milk. It is apparent that casein dissociation and degradation is needed for the subsequent release of triclosan. This was achieved through a pH triggered release and release during simulated gastric and intestinal digestion. At alkaline pH, not only is the solvency of the casein micelle enhanced favoring micelle dissociation but the aqueous solubility of triclosan is also enhanced. Protein-solvent and drug-solvent interactions are most favorable at $\text{pH} > 10$.

These studies demonstrate that the release of triclosan from the casein micelle is governed by two major mechanisms: casein micelle dissociation and casein degradation. Utilizing the pH changes along the gastrointestinal tract through in vitro digestion reveals the ability of the casein micelle in preventing gastric emptying of triclosan. Upon micellar denaturation through both enzymatic and pH effects, triclosan is released into intestinal digestate solution. This gives an indication of the relative bioaccessibility of triclosan. Here triclosan is present in the sample supernatant and available for uptake by the intestinal epithelia. At this stage of digestion, the transport of triclosan across a cellular membrane, such as Caco2 cell monolayers, is needed to fully access the extent of the oral bioavailability of the triclosan-protein conjugate.

REFERENCES

- Failla M, T Huo, and S Thakkar. 2008. In vitro screening of relative bioaccessibility of carotenoids from foods. *Asia Pacific Journal of Clinical Nutrition*, 17(S1): 200-203.
- Ferranti P, M Traisci, G Picariello, A Nasi, V Boschi, M Siervo, C Falconi, L Chianese, F Addeo. 2004. Casein proteolysis in human milk: tracing the pattern of casein breakdown and the formation of potential bioactive peptides. *Journal of Dairy Research*, 71(1): 74-87.
- Gardiner J, S Freeman, M Leach, A Green, J Alcock, and A D'Emanuele. 2008. PAMAM dendrimers for the delivery of the anti bacterial triclosan. *Journal of Enzyme Inhibition and Medicinal Chemistry*, 23(5): 623-628.
- Grove C, W Liebenberg, J Preez, W Yang, M Villiers. 2003. Improving the aqueous solubility of triclosan by solubilization, complexation, and in situ salt formation. *Journal of Cosmetic Science*, 54: 537-550.
- Herrero-Barbudo MC, F Granado-Lorencio, I Blanco-Navarro, B Perez-Sacristan, B Olmedilla-Alonso. 2009. Applicability of an in vitro model to assess the bioaccessibility of vitamins A and E from fortified commercial milk. *International Dairy Journal*, 19 (1): 64-67.
- Horne DS. 1998. Casein interactions: Casting light on the black boxes, the structure in dairy products. *International Dairy Journal* 8(3):171-177.
- Huppertz T, Vaia B, Smiddy MA. 2008. Reformation of casein particles from alkaline-disrupted casein micelles. *Journal of Dairy Research*, 75(1): 44-47.
- Liebenberg W, E Engelbrecht, A Wessels, B Devarakonda, W Yang, and M Villiers. 2004. A comparative study on the release of active ingredients from semisolid cosmeceuticals measured with Franz, Enhancer, or Flow-through cell diffusion apparatus. *Journal of Food and Drug Analysis*, 12 (1): 19-28.
- Liu J, H Lee, and C Allen. 2006 Formulation of drugs in block copolymer micelles: drug loading and release. *Current Pharmaceutical Design*, 12: 4685-4701.
- Liu Y, Guo R. 2008. pH-dependent structures and properties of casein micelles. *Biophysical Chemistry*, 136 (2-3): 67-73.
- Loftsson T, I Ossurardottir, T Thorsteinsson, M Duan, and M Masson. 2005. Cyclodextrin solubilization of the antibacterial agents triclosan and triclocarban: Effect of ionization and polymers. *Journal of Inclusion Phenomena and Macrocyclic Chemistry*, 52: 109-117.
- Lu S, Archer MC. 2005. Fatty acid synthase is a potential molecular target for the chemoprevention of breast cancer. *Carcinogenesis* 26(1):153-7.

- Macheras P, M Koupparis, C Tsaprounis. 1986. Drug dissolution in milk using the automated flow injection serial dynamic dialysis technique. *International Journal of Pharmaceutics*. 33: 125-136.
- Macheras P, M Koupparis, S Antimisiaris. 1990. Drug binding and solubility of milk. *Pharmaceutical Research*. 7(5): 537-541.
- Maestrelli F, Garcia-Fuentes M, Mura P, Alonso MJ. 2006. A new drug nanocarrier consisting of chitosan and hydroxypropylcyclodextrin. *European Journal of Pharmaceutics and Biopharmaceutics*, 63(2): 79-86.
- Martinez-Palau M, Franco L, Puiggali J. 2008. Microspheres of new alternating copolyesters derived from glycolic acid units for controlled drug release. *Journal of Applied Polymer Science*, 110(4): 2127-2138.
- McConnell EL, Short MD, Basit AW. 2008. An in vivo comparison of intestinal pH and bacteria as physiological trigger mechanisms for colonic targeting in man. *Journal of Controlled Release*, 130 (2): 154-160.
- Nicolaides E, E Galia, C Efthymiopoulos, J Dressman, and C Reppas. 1999. Forecasting the in vivo performance of four low solubility drugs from their in vitro dissolution data. *Pharmaceutical Research*, 16 (12): 1876-1882.
- Pan XY, Yao P, Jiang M. 2007. Simultaneous nanoparticle formation and encapsulation driven by hydrophobic interaction of casein-graft-dextran and beta-carotene. *Journal of Colloid and Interface Science* 315(2):456-463.
- Perales S, Barbera R, Lagarda MJ, R Farre. 2007. Availability of iron from milk-based formulas and fruit juices containing milk and cereals estimated by in vitro methods (solubility, dialysability) and uptake and transport by Caco-2 cells. *Food Chemistry*, 102(4): 1296-1303.
- Roach A, J Dunlap, and F Harte. 2009. Association of Triclosan to Casein Proteins Through Solvent-Mediated High-Pressure Homogenization. *Journal of Food Science*, 74(2):N23-29.
- Semo E, Kesselman E, Danino D, Livney YD. 2007. Casein micelle as a natural nanocapsular vehicle for nutraceuticals. *Food Hydrocolloids* 21(5-6):936-942.
- Sun Y, Watts DC, Johnson JR, Shukla AJ. 2001. Biodegradable drug delivery systems. *American Pharmaceutical Review* 4(4):25-30.
- Swart H, J Preez, M Villiers, A Lotter, and W Liebenberg. 2006. Correlation between the in vitro release from topical delivery vehicles and microbicidal activity of triclosan. *Pharmazie*, 61: 35-40.

Tang YF, Du YM. 2008. Chitosan-based injectable thermosensitive hydrogel. *Progress in Chemistry* 20(2):239-244.

Tixier C, H Singe, S Canonica, S Muller. 2002. Phototransformation of triclosan in surface waters: A relevant elimination process for this widely used biocide - Laboratory studies, field measurements, and modeling. *Environmental Science and Technology*, 36(16): 3482-3489.

Vaia, B, M Smiddy, A Kelly, T Huppertz. 2006. Solvent-mediated disruption of bovine casein micelles at alkaline pH. *Journal of Agricultural and Food Chemistry*, 54(21): 8288-8293.

Yu M, Kumar TR, Nkrumah LJ, Coppi A, Retzlaff S, Li CD, Kelly BJ, Moura PA, Lakshmanan V, Freundlich JS, Valderramos JC, Vilcheze C, Siedner M, Tsai JH, Falkard B, Sidhu AB, Purcell LA, Gratraud P, Kremer L, Waters AP, Schiehser G, Jacobus DP, Janse CJ, Ager A, Jacobs WR Jr, Sacchettini JC, Heussler V, Sinnis P, and Fidock DA. 2008. The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites. *Cell Host Microbe*, 4(6): 509-11.

Zarzycki PK, Wlodarczyk E, Lou DW, Jinno K. 2006. Evaluation of methanol-water and acetonitrile-water binary mixtures as eluents for temperature-dependent inclusion chromatography. *Analytical Sciences* 22(3):453-6.

APPENDIX IV - FIGURES

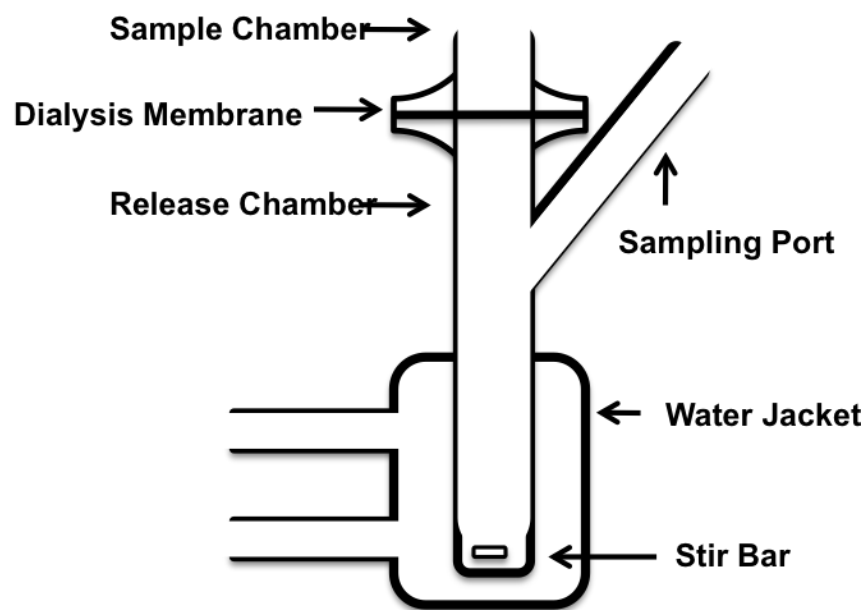


Fig 28. Franz diffusion cell schematic: sample chamber, dialysis membrane (12-14 kDa), release chamber, sample arm, water jacket (not utilized in experiment), and magnetic stir bar.

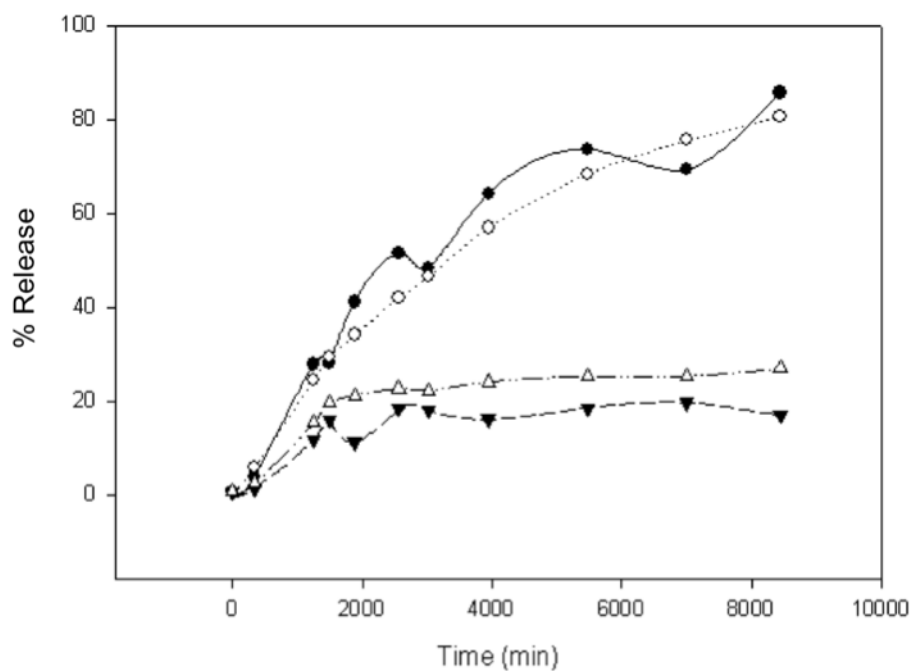


Fig 29. Dissolution profile of triclosan represented as percent of total triclosan released into release chamber. Control (circles): Percent triclosan released into release chamber from sample chamber containing triclosan absent of milk (i.e., free triclosan). Sample treatment (triangles): Percent triclosan released into release chamber from sample chamber containing triclosan in the presence of milk (i.e., triclosan “bound” initially). Closed data points are the release in 30% acetonitrile and open data points in 40%. Final time point is 6 days from initial time point.

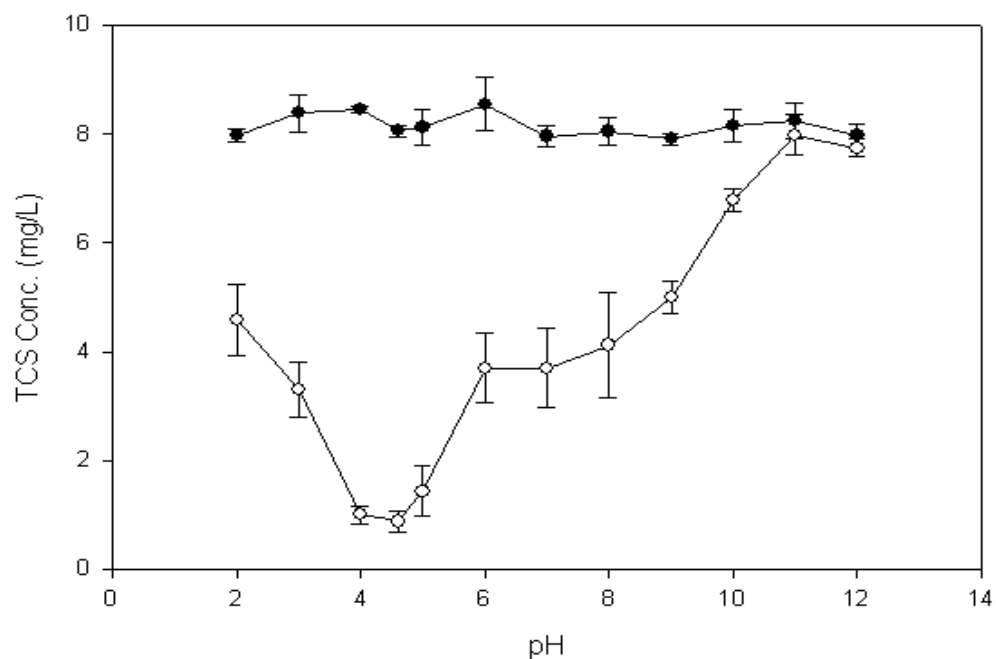


Fig 30. Triclosan concentration in sample supernatants (100,000 x *g*, 30 min) illustrating the release of triclosan into milk serum as triggered by changes in solution pH; total amount of TCS at each pH (closed circles), amount of triclosan released into serum at each pH (open circles).

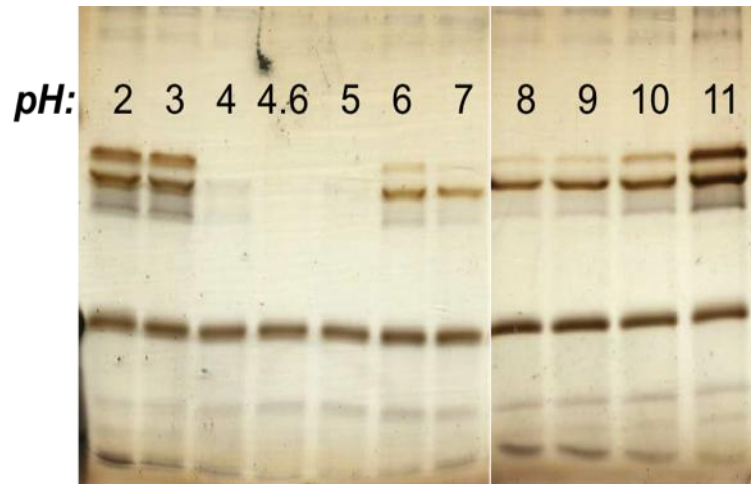


Fig 31. SDS PAGE of sample supernatants (100,000 x *g*, 30 min) from pH adjusted milk solutions containing triclosan. Note the lack of casein proteins in supernatant at pH 4, 4.6 (casein isoelectric point), and 5.

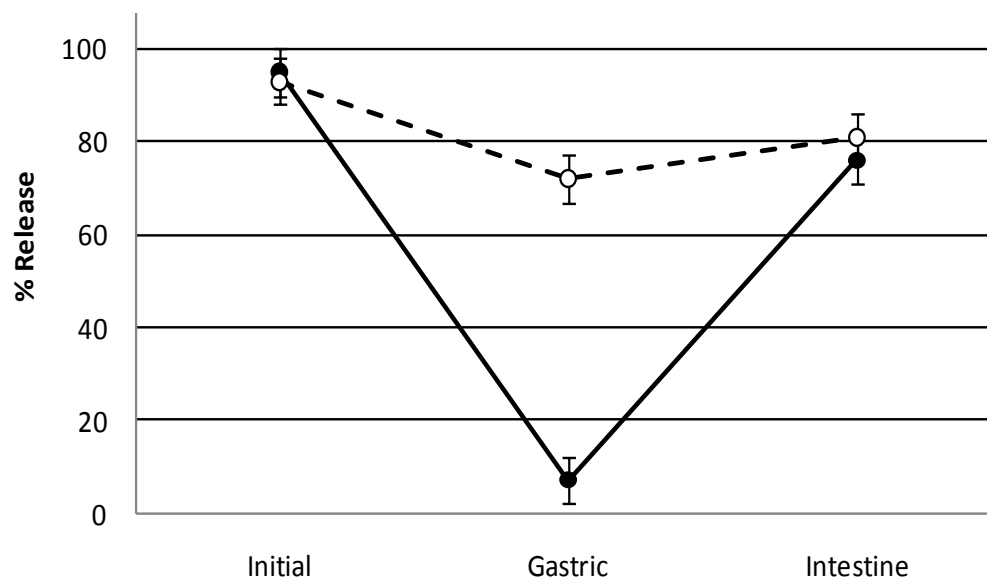


Fig 32. Release of triclosan in simulated gastric and intestinal digestive solution supernatants (8,000 x *g*, 15 min). Open circles - control sample not containing milk; closed circles - samples containing milk.

Chapter 5

Delivery of triclosan to *Plasmodium* infected hepatocytes via casein proteins/peptides as analyzed by *in vitro* imaging: A qualitative exploration

ABSTRACT

The use of casein proteins as an intercellular delivery molecule for triclosan is investigated in this study. The growth and development of *Plasmodium berghei* parasites as affected by a triclosan-protein drug complex within human liver cells is determined using *in vitro* imaging techniques. Samples consisted of milk, serum proteins isolated from milk, and β -casein micelles subjected simulated *in vitro* digestion (with the exception of β -casein micelles) in the presence of triclosan. Triclosan was added at three concentrations: 0.2, 2.0, and 20 mg L⁻¹ for each treatment regimen. Drug treatments (triclosan-protein) were added to infected cells and analyzed for parasite maturation at 48 and 72 hours post infection during the shizont stage of development and cellular detachment, respectively. The toxicity of treatments on host cells was analyzed at 48 hours post infection by thymidine incorporation. High triclosan concentrations were most effective in both standard controls and treatment samples in preventing parasite maturation. However, triclosan in free form was acutely toxic to host cells, whereas triclosan in the presence of milk proteins offered a protective effect preventing cellular death. The harmful effects of free triclosan on host cells can be avoided or reduced if applied to cells in the presence of a carrier molecule, likely due to the controlled release of triclosan. This study presents a potential delivery mechanism for triclosan and offers new information on the *in vitro* behavior of casein proteins.

INTRODUCTION

Malaria affects 300-500 million people worldwide resulting in over one million deaths per year (Owotade and Greenspan 2008). This equates to approximately one death every 30 seconds. Africa bears the majority of the disease burden due to the predominance of *Plasmodium falciparum*, the mosquito responsible for transmitting the most severe form of malaria (Owotade and Greenspan 2008). Malaria has been termed a neglected disease because there is a severe lack of attention and resources in poor countries to facilitate its eradication. There is an immediate need for new drugs against increasingly resistant strains and preventative mechanisms such as bed nets and insect control to facilitate its eradication.

Triclosan is commonly used in consumer products such as hand soap and toothpaste as an antimicrobial but has recently been investigated for its activity against malaria (Surolia and Surolia 2001; Yu and others 2008). However, the major limitation associated with triclosan is its hydrophobicity and low oral bioavailability. Many pharmaceuticals have this limiting issue, which prevents their utilization (Liu and others 2006). Oral drug administration is often favored over other routes for a number of reasons and in the case of malaria, drug administration by mouth is more conducive to the rural areas in which malaria is most heavily present.

Synthetic polymers, block copolymer micelles in particular, are becoming popular in encapsulating poorly soluble drugs and facilitating oral drug delivery (Liu and others 2006). Interestingly, the casein micelle - the major protein in milk responsible for transporting calcium and other essential nutrients to the feeding mammalian young - has

been described structurally as a block copolymer micelle (Horne 2002). Studies in our laboratory have shown that the casein micelle has a natural capacity to bind triclosan (Roach and others 2009) and could potentially serve as an oral delivery system as a means to enhance its bioavailability.

Triclosan is most effective against liver-stage *Plasmodium* parasites as FabI plays a vital role in the parasite development and maturation at this stage (Yu and others 2008). When FabI is inactivated in liver-stage parasites they are unsuccessful in developing to the cytomere stage, the parasitophorous vacuole membrane (PVM) is not degraded normally, merozoite development is abated, hepatocytes do not detach from neighboring cells, and merozoite formation is delayed or prevented (Strum and Heussler 2007). It has been well proven that blood-stage parasites do not rely on FAS-II activity in the apicoplast rendering triclosan ineffective at this stage of the disease where the clinical manifestations of the disease are present (Yu and others 2008). Therefore, the anti-malarial efficacy of triclosan is strictly restricted to preventing liver-stage parasite development. Note: Information and schematic of the *Plasmodium* parasite life cycle is detailed in Appendix VI as excerpts from the following journal articles: “*Live and let die: manipulation of host hepatocytes by exoerythrocytic Plasmodium parasites*” by A. Strum V. Heussler, Medical Microbiology and Immunology, 2007, 196: 127-133 and “*Alteration of the Parasite Plasma Membrane and the Parasitophorous Vacuole Membrane during Exo-Erythrocytic Development of Malaria Parasites*” by A. Sturm, S. Graewe, and B. Franke-Fayard, S. Retzlaff, S. Bolte, B. Roppenser, M. Aepfelbacher, C. Janse, and V. Heussler, Protist, 2009, 160(1): 51-63.

The objective of this study was to evaluate the capability of casein as a suitable carrier for triclosan and the potential use of this triclosan-protein complex as an antimalarial agent. It was our desire to determine if the triclosan-protein complex will be as effective against liver-stage parasites as free triclosan as measured by the prevention of parasite development through *in vitro* live imaging during malaria liver-stages: shizont phase, presence of detached host cells and merozoite formation. The effect of treatments on host cell toxicity was also determined.

MATERIALS AND METHODS

Drug treatments

Shelf-stable (milk pasteurized by ultra high temperature) skim milk (0.1% fat) was used as received for all experiments using milk. Milk serum was prepared by acid precipitation of the casein micelle fraction (pH 4.6) and the solution was centrifuged to separate micelle fraction from the serum proteins. Supernatant was carefully removed and pH was return to the initial milk pH of 6.7. The association of TCS (Sigma Aldrich, Frankfurt, Germany) to milk and serum components are explained by Roach and others (2009). Here, adherence of TCS to milk components is achieved simply by equilibrating TCS with the desired milk solution for approximately 1 hr.

β -CN (casein) micelles were prepared from a gel electrophoresis standard of lyophilized β -CN (Sigma Aldrich, Frankfurt, Germany). A saturated stock concentration of β -CN powder was dissolved in deionized water at 1200 mg L⁻¹. Solution was centrifuged to remove large insoluble particulates and supernatant was filtered (0.2 μ m) to remove large aggregates. TCS incorporation was achieved by dissolving TCS in

deionized water and adding the β -CN stock solution to reach a final concentration of 40 mg L⁻¹. β -CN was added slowly with a syringe to the aqueous TCS mixture under continuous magnetic stirring and allowed to equilibrate for 15 min after the β -CN solution was added.

Three different concentrations of TCS were used for each carrier matrix: 0.2 mg L⁻¹ (low), 2.0 mg L⁻¹ (medium), and 20 mg L⁻¹ (high) which are equivalent to 1 $\times$, 10 $\times$, and 100 $\times$ the IC₅₀ of triclosan measured in a previous experiment (680 nm). Where the IC₅₀ is the amount of drug needed to kill 50% of a population. In each case, drug treatment was added to infected hepatocytes grown in a 24 well plate (detailed in next section) at a 1:10 (v/v) dilution of drug treatment to media in 1 mL sample wells. (Note: β -CN samples were not subjected to *in vitro* digestion.)

In vitro Digestion: Milk and Milk serum

In vitro digestion was carried out on milk and milk serum samples containing TCS to simulate oral ingestion. The methods of Perales and others (2007) as revised by Roach and others (2009), were followed. Here, *in vitro* digestion was carried out in 2 phases: gastric and intestinal. Milk solutions were added to freshly prepared pepsin solution yielding 0.08 g pepsin (porcine: cat no. P-7000, Sigma Aldrich, Frankfurt, Germany) per sample. The solution was adjusted to a pH of 2.0 prior to adding samples. Samples were incubated in a shaking water bath at 37°C for 1 hr at 120 rpm. Samples were removed from water bath and immediately placed on ice for 5 min and raised to a pH of 5.0 with 1M KOH to stop gastric digestion. Freshly prepared intestinal solution yielding 0.008 g pancreatine (porcine: cat no. P-1750, Sigma Aldrich, Frankfurt,

Germany) was added to the gastric digests. *Previous experiments with bile were toxic to host cells and thus removed in the intestinal phase.* The pH was raised to pH 7.4 with 1M KOH and incubation continued for an additional 2 hr. To stop intestinal digestion, samples were placed on ice for 10 min. Intestinal digestates were centrifuged (10,000 rpm, 15 min) and supernatant was removed and stored at -20°C until use in cell culture assays. Prior to adding drug treatments to HepG2 cells, the solution was heated to 99°C for 10 min to deactivate enzymes.

HepG2 Cell culture

Human liver carcinoma cells, HepG2 (ATCC HB-8065 Hep G2), were grown in horizontal culture flasks in MEM buffer. Cells were removed from culture flask with accutase and seeded in 24 well plates to yield 10^5 cells per mL. Wells were refreshed twice a day with buffer and stored at 37°C. After 24 hr of growth, wells were checked for attachment to the bottom of the well and a uniform cell layer.

Parasite infection

The salivary glands of infected mosquitoes expressing green fluorescent protein, (GFP) or red fluorescent protein (mCherry) were extracted and stored in cold PBS (peptone buffer solution, pH 7.1) and disrupted with a handheld homogenizing unit once all salivary glands were collected for a given experiment, approximately 1 mosquito per well. Sporozoites were counted, typically yielding 10^4 sporozoites per mL, and dispersed evenly in the desired well plates containing HepG2 cells. To ensure a good infection rate, well buffer was not replaced until 6-15 hr post infection. Twenty-four hours post infection, drug treatments were added to wells and replenished twice a day. Drug

treatments consisted of: Milk digestates, serum digestates, β -CN, and Free TCS each at 3 different TCS concentrations (0.2, 2.0, and 20 mg L⁻¹) for a total of 12 treatments.

Parasite development

Shizont stage

Density slicing was carried out at 48 hpi (hours post infection). Parasites in the shizont stage are viewed under fluorescent microscopy and a representative number of images are taken and overlaid image is created. Computer software takes a binary image and the area of the parasites are calculated and averaged. The smaller the area of the shizont, the less developed the parasites are and is an initial indication on the effectiveness of drug treatments.

Hepatocyte detachment and merosome formation

At 72 hpi, detached liver cells and merosomes are counted with fluorescent imaging. Matured parasites cause the detachment of adjacent liver cells or exist as independent sacs filled with merozoites that are exuded from the cell (merosomes). Detached cells and merosomes are collected by gently agitating attached cells (pipetting the media at 4 corners within the well) and transferring the supernatant to a new 24 well plate. If desired, a nuclear stain is added prior to removing the supernatant in order to differentiate between detached liver cells and merosomes. Detached cells will contain a nucleus and merosomes will not, only the nuclei of merozoites are visible. The fewer the cell count the more effective the drug treatment.

Host cell proliferation

The viability of the host liver cells is analyzed with a thymidine incorporation assay. Here, hepatocytes are grown in 96 well plates and drug treatments are added as in infected cells but cells are not infected with parasites. At 48 hr of drug treatment (drugs and media added twice a day), a radioactive solution of thymidine is added to cells and allowed to incubate for 12 hr. Cells are lysed by freezing-thawing the 96 well plate. Solutions are filtered and the radioactivity of the supernatant is analyzed by a scintillation counter. The higher the scintillation count, the more viable the cells, i.e., the more thymidine incorporated into the cell.

Immunofluorescence

Hepatocytes were grown in cover slips in a separate 24 well plate and infected with *wild type* parasites (absent of fluorescent proteins). Immunofluorescent staining was carried out at 48 hr. Slides of stained cells were prepared and analyzed under fluorescent microscopy to view and compare the size of the PVM (parasite vacuole membrane) and the size of the host cell nucleus as effected by treatments.

RESULTS AND DISCUSSION

The antiparasitic activity of triclosan standards is only apparent at the highest triclosan concentration (TCS_{High}) of 20 mg L^{-1} (Fig 33 and 34). Parasite development at 48 hr is severely delayed as evident by the reduced area measured by density slicing at the shizont stage in comparison to the other TCS standards at lower concentrations and controls not containing triclosan (Fig 33). Immunofluorescent imaging (Fig 35) shows a significantly smaller PVM (parasite vacuole membrane) at the highest TCS level, also

indicative of hindered growth. At 72 hr, the presence of detached liver cells and merozoites is reduced in both the TCS_{High} and TCS_{Med} with the greatest effect in the TCS_{High} sample (Fig 34). The presence of detached cells and merozoites is one of the final stages of liver-stage development vital to the life cycle of the parasite. The retardation of parasite maturation is directly dependent on triclosan concentration. There is no effect at the lowest concentration (TCS_{Low}) when compared to the media only control.

There is no enhanced efficacy of triclosan in the presence of the milk proteins. Samples containing the lowest level of triclosan (Milk_{Low}, Serum_{Low}, and β -CN_{Low}) did not have any increased ability in delaying sporozoite development (Fig 36-41); these samples behaved similarly to the media control. However, the antiparasitic activity of triclosan at the high treatment level is dependent on the original sample matrix (milk, milk serum, or β -CN) as triclosan is most effective at this concentration. Sporozoites fully matured to the 48 hr shizont stage measured by *in vitro* parasite imaging at all triclosan concentrations in milk samples (Fig 36). At the high triclosan level (Milk_{High}), parasite development was delayed at 72 hr, noted by the reduced presence of detached liver cells and merozoites (Fig 37). Growth was hindered after 48 hr as matured shizonts did not develop to merozoites, which initiate the detachment of adjacent liver cells or extrude themselves from liver cells. This delay in development between the 48 and 72 hr stage is also seen in the β -CN_{High} sample (Fig 40 and 41) but not the Serum_{High} sample (Fig 38 and 39). Shizont development is also slightly hindered in the β -CN_{High} sample, but to a lesser degree of significance in comparison to TCS_{High} (Fig 40).

The most apparent difference in the treated samples is the behavior of triclosan in the presence of serum proteins. Here there is no clear effect of triclosan at any concentration (Fig 38 and 39). The reason for this effect is not evident but appears that the presence of whey protein inhibits triclosan activity. Triclosan and whey proteins may form a complex that either prevents it from entering into the cell or once entered, the compound does not dissociate and triclosan is not available for utilization. This argument is strengthened by order of triclosan efficacy at the high concentration: Free TCS> β -CN>Milk>Serum, where the treated sample containing the least amount of whey proteins is the most effective, β -CN.

Host cell toxicity

While free triclosan is fully capable of preventing parasite development at 20 mg L⁻¹ it is also acutely toxic to host cells (Fig 42a). The cellular proliferation assay shows no effect of cell growth at the lowest TCS concentration but a sharp reduction in cell number at the medium concentration and practically no cell growth of host cells at the highest TCS concentration. Death of liver cells *in vitro* at this capacity *in vivo*, would most likely result in liver failure and be fatal to the animal host. However, *in vivo* studies have found TCS to be non-toxic after long-term (3 mo) oral administration at 1000 mg L⁻¹ in Sprague-Dawley rats, leading to blood levels ranging from 26 to 87 mg L⁻¹ (90 – 300 μ M) (Lu and Archer, 2005). Here toxicity issues could be due to the nature of *in vitro* studies where drug treatment is does not correlate with *in vivo* behavior.

Interestingly, TCS is less toxic to host cells in the presence of milk protein (Fig 42 b-d). The level of toxicity is difficult to determine due to variability between replications,

but it is apparent that cellular proliferation is not hindered as much in samples containing milk, milk serum, and β -CN when compared to the same amount of TCS without protein. The protective effect could be from the slow dissociation of TCS from the TCS-protein complex. As mentioned, in the case of the serum samples, it is not clear if triclosan even enters the cell as parasite development progresses similar to controls at both 48 and 72 hr. The protective effect here could be due to the lack of triclosan present within the cell or its unavailability within the cell. However, in the milk and β -CN samples, the effect of triclosan is seen at either 72 hr or at both 48 and 72 hr of parasitic development, respectively. This indicates that triclosan does enter the cell and because it behaves differently from the free triclosan control, most likely exists as a TCS-protein complex. The rate of diffusion and the subsequent effect on parasites and host cells could be determined by extending the read-out time points for development and cell proliferation past 72 hr but was not explored in this study.

CONCLUSION

An effective drug to treat malaria should pass a host of qualifications, these include the ability to arrest parasite development, prevent liver-stage disease progression, and not be toxic to host cells. The closest treatment regimen to meeting these qualifications in the current study was the use of β -CN as a carrier for triclosan at the high concentration as this drug level is only effective against parasite development. β -CN_{High} prevented the full maturation of sporozoites to shizonts at 48 hr and retarded the detachment of liver cells and reduced the presence of merozoites at 72 hr. It also appeared to be less toxic to host cells in comparison to those exposed to free triclosan at

the same concentration. It is therefore possible that the damaging effects of free triclosan on host cells can be avoided or reduced if applied to cells in the presence of a carrier molecule where the controlled release of triclosan can be effective against parasites at various stages of development. Whether the casein micelle can serve in this capacity remains to be seen but this study offers a promising alternative and potential delivery mechanism for triclosan and sheds new light on the *in vitro* behavior of casein proteins.

REFERENCES

- Horne DS. 2002. Casein structure, self-assembly and gelation. *Current Opinion in Colloid and Interface Science*, 7(5-6): 456-461.
- Liu J, H Lee, and C Allen. 2006. Formulation of drugs in block copolymer micelles: drug loading and release. *Current Pharmaceutical Design*, 12: 4685-4701.
- Lu S, Archer MC. 2005. Fatty acid synthase is a potential molecular target for the chemoprevention of breast cancer. *Carcinogenesis* 26(1):153-7.
- Owotade FJ and JS Greenspan. 2008. Malaria and oral health. *Oral diseases*, 14: 302-307.
- Roach A, J Dunlap, and F Harte. 2009. Association of Triclosan to Casein Proteins Through Solvent-Mediated High-Pressure Homogenization. *Journal of Food Science, In Press*, available online January 27, 2009.
- Strum A and V Heussler. 2007. Live and let die: manipulation of host hepatocytes by exoerythrocytic *Plasmodium* parasites. *Medical Microbiology and Immunology*, 196: 127-133.
- Surolia N and A Surolia. 2001. Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of *Plasmodium falciparum*. *Nature Medicine*, 7(2): 167-173.
- Yu M, Kumar TR, Nkrumah LJ, Coppi A, Retzlaff S, Li CD, Kelly BJ, Moura PA, Lakshmanan V, Freundlich JS, Valderramos JC, Vilcheze C, Siedner M, Tsai JH, Falkard B, Sidhu AB, Purcell LA, Gratraud P, Kremer L, Waters AP, Schiehser G, Jacobus DP, Janse CJ, Ager A, Jacobs WR Jr, Sacchettini JC, Heussler V, Sinnis P, and Fidock DA. 2008. The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites. *Cell Host Microbe*, 4(6): 509-11.

APPENDIX V - FIGURES

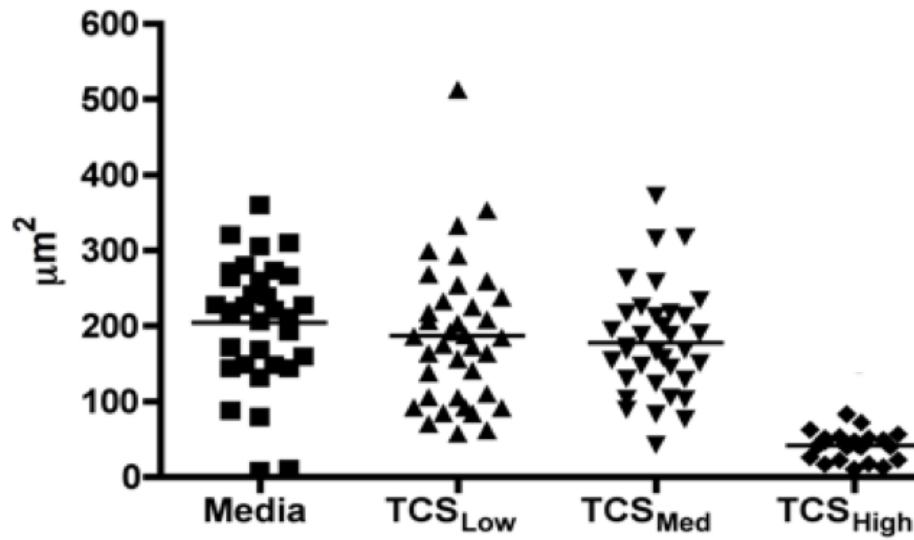


Fig 33. Density slice of parasites in the schizont stage of development at 48 hr post infection. Data represents parasite growth in the media control and as effected by triclosan standards at low, medium, and high concentrations; 0.2, 2.0, and 20 mg L⁻¹, respectively. Data points represent the average (experiment repeated 3 times) area of individual shizonts and horizontal bar is the mean area of all data points.

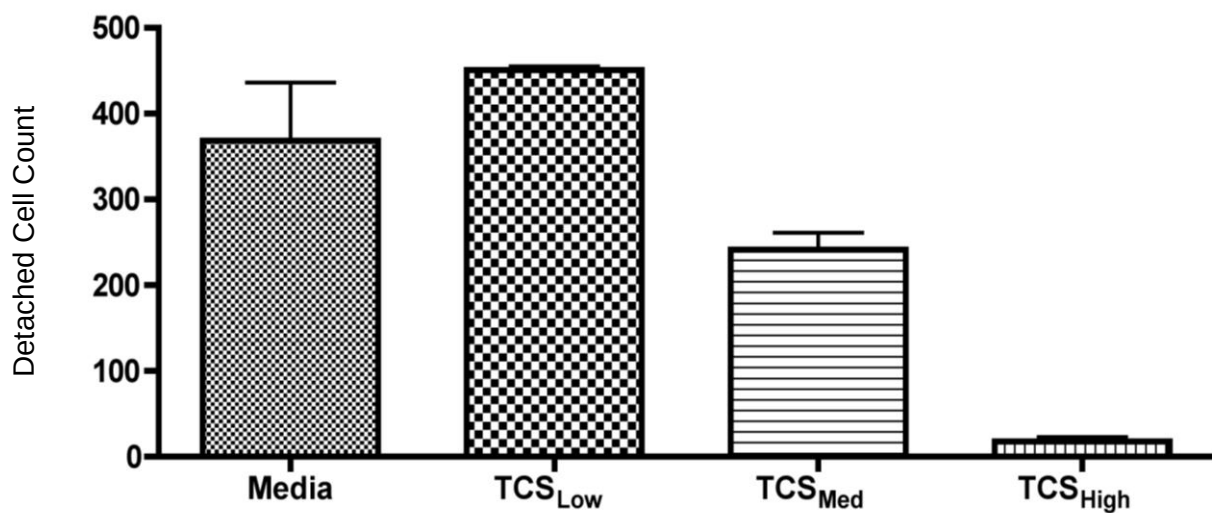


Fig 34. Presence of detached cells and merosomes in media control and triclosan standards (low, med, and high) 72 hr post infection. Bars represent the average count of 3 independent experiments and error bars are the standard deviation between experiments.

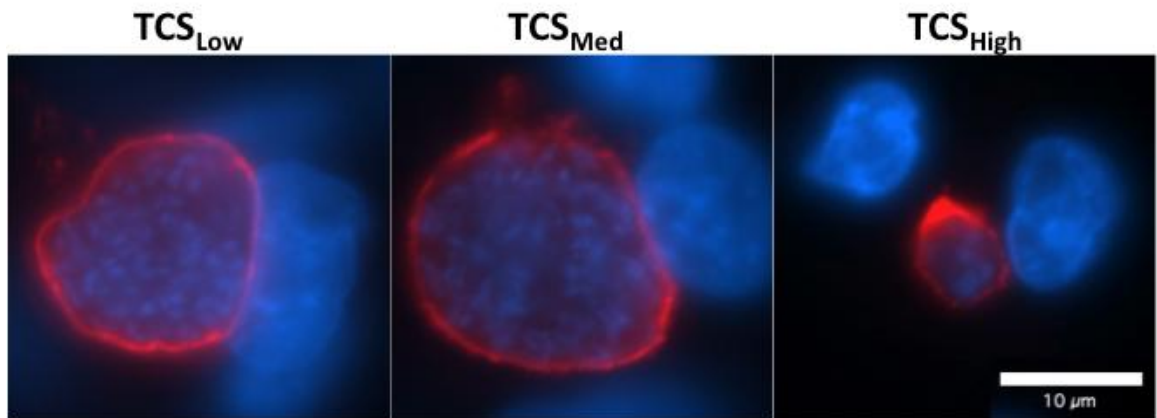


Fig 35. Immunofluorescent staining at 48 hr of wild type *P. berghei* infected hepatocytes treated with triclosan standards. PVM (parasite vacuole membrane) stained in red with chicken antibody and nuclei of host cell and parasites stained in blue with hoescht DNA stain. Bar is 10 μm .

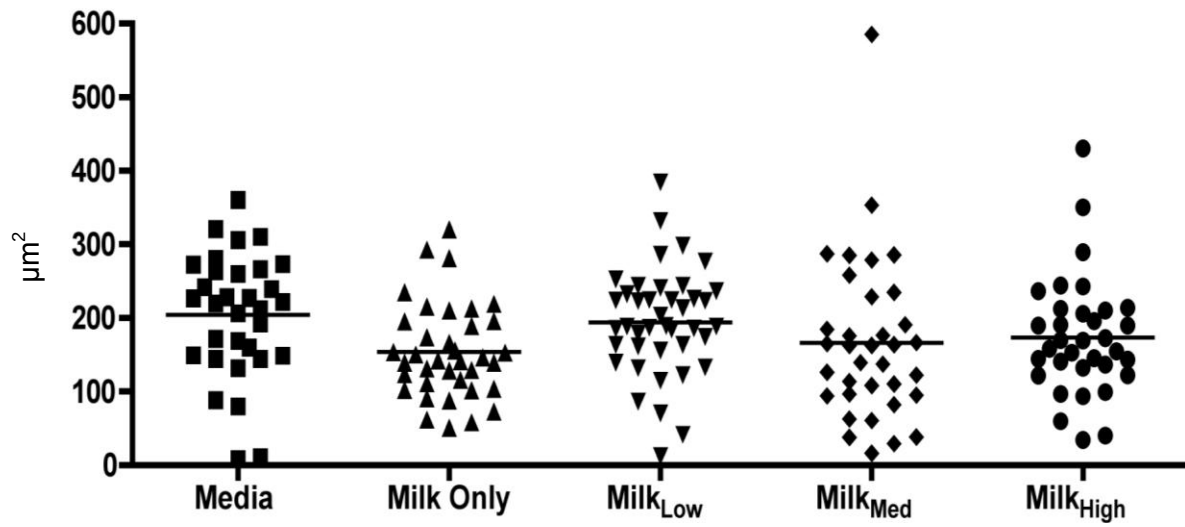


Fig 36. Density slice of parasites in the schizont stage of development at 48 hr post infection. Data represents parasite growth in the media control, milk only control and as effected by triclsoan associated to milk digestates. Data points represent the average (experiment repeated 3 times) area of individual shizonts and horizontal bar is the mean area of all data points.

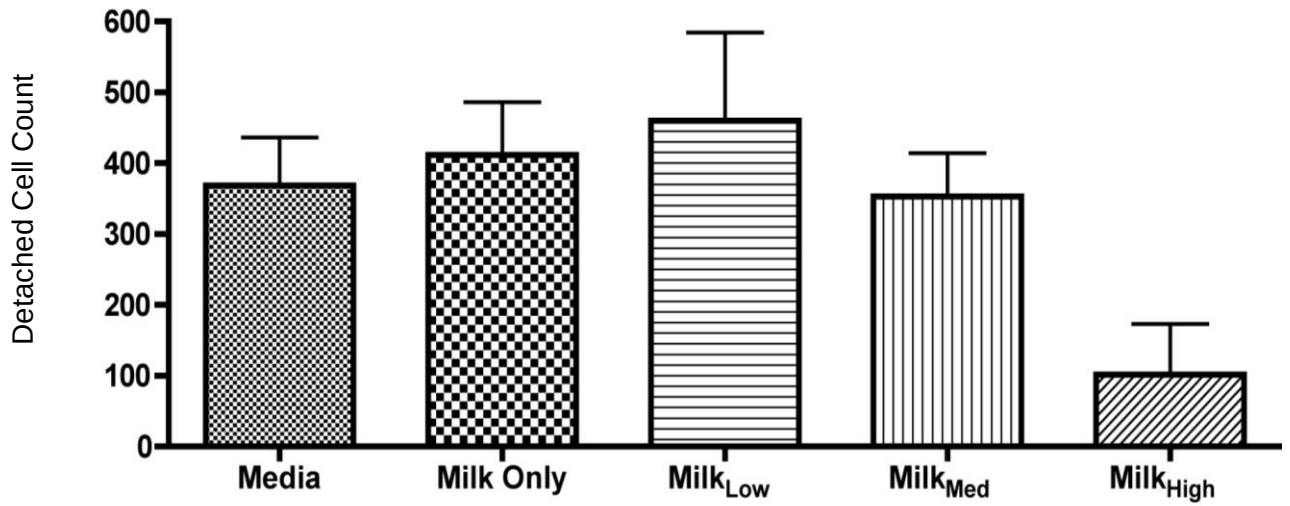


Fig 37. Number of detached cells and merosomes in media control, milk only control and triclosan associated to milk digestates at 72 hr post infection. Bars represent the average count of 3 independent experiments and error bars are the standard deviation between experiments.

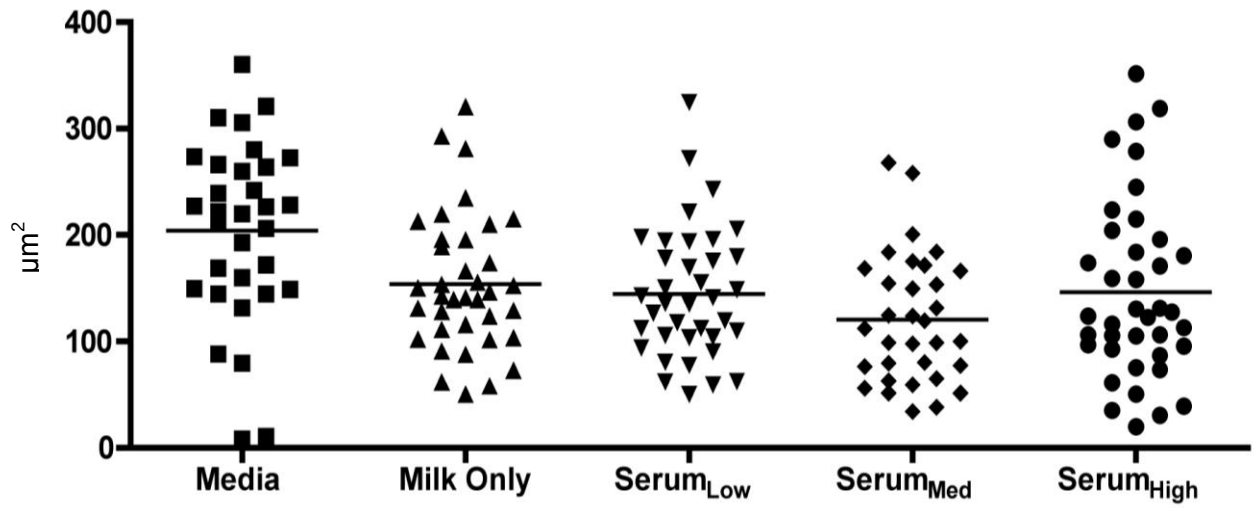


Fig 38. Density slice of parasites in the schizont stage of development at 48 hr post infection. Data represents parasite growth in the media control, milk only control and as effected by triclsoan associated to milk serum digestates. Data points represent the average (experiment repeated 3 times) area of individual shizonts and horizontal bar is the mean area of all data points.



Fig 39. Number of detached cells and merosomes in media control, milk only control and triclosan associated to milk serum digestates at 72 hr post infection. Bars represent the average count of 3 independent experiments and error bars are the standard deviation between experiments.

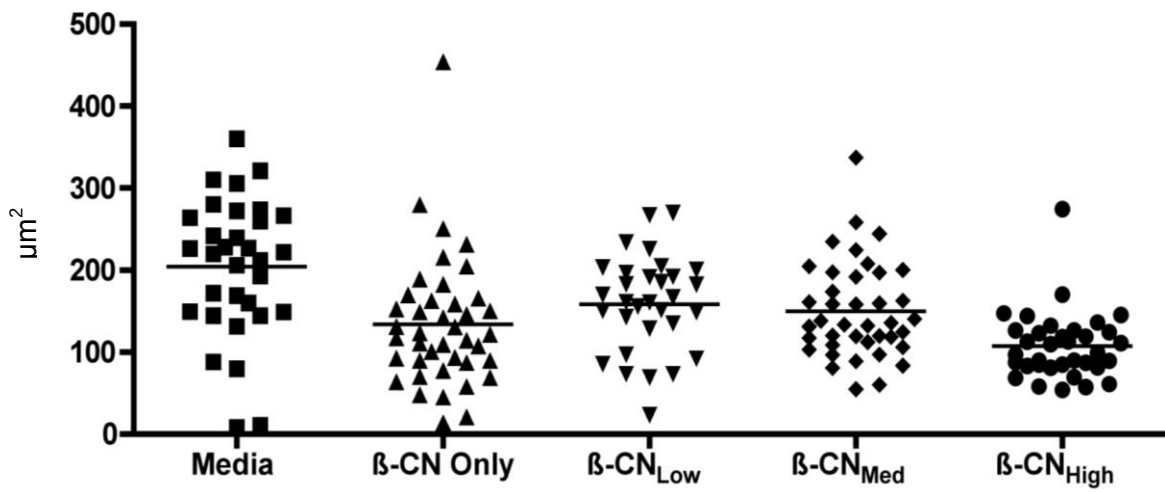


Fig 40. Density slice of parasites in the schizont stage of development at 48 hr post infection. Data represents parasite growth in the media control, milk only control and as effected by triclsoan associated to β -CN micelles. Data points represent the average (experiment repeated 3 times) area of individual shizonts and horizontal bar is the mean area of all data points.

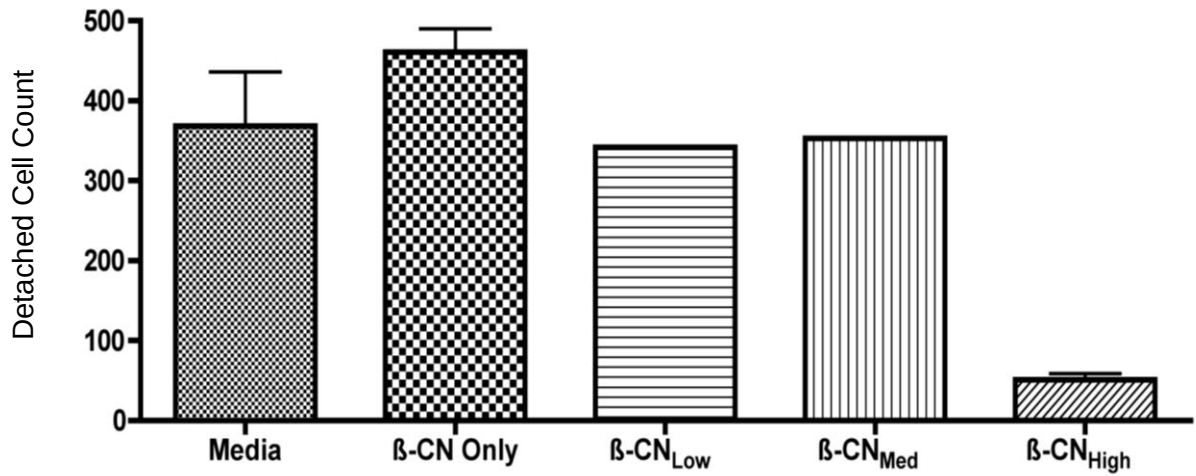
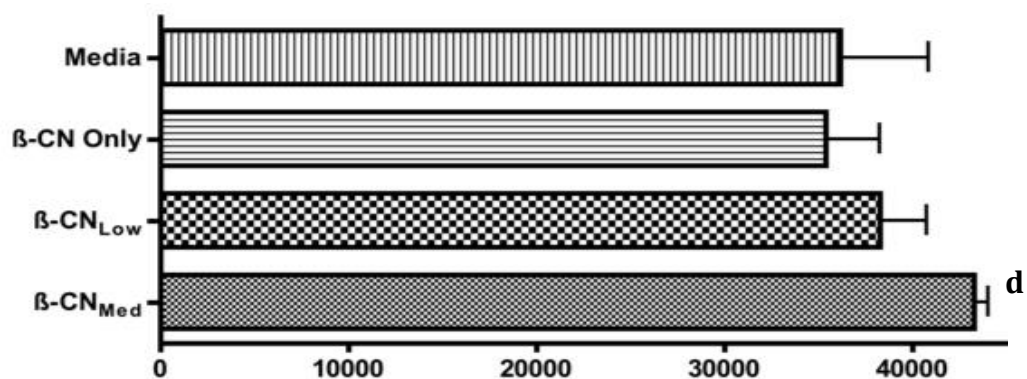
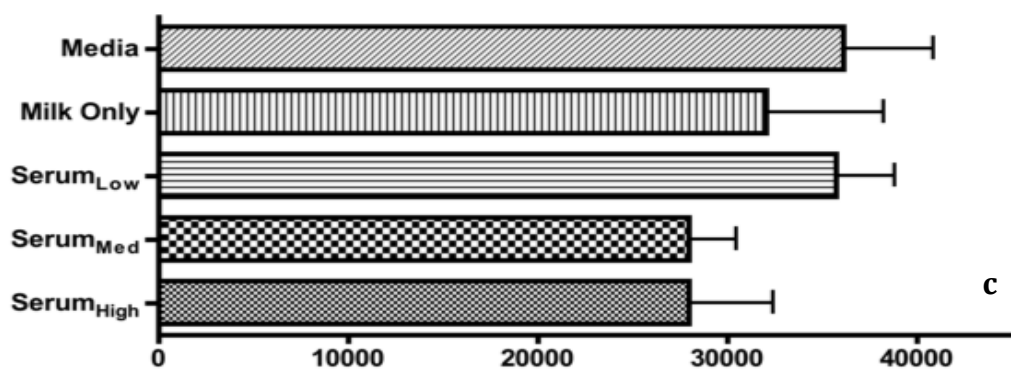
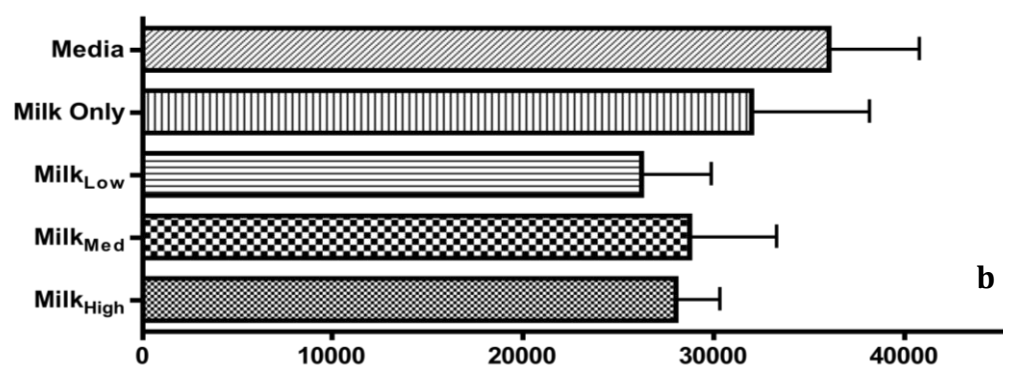
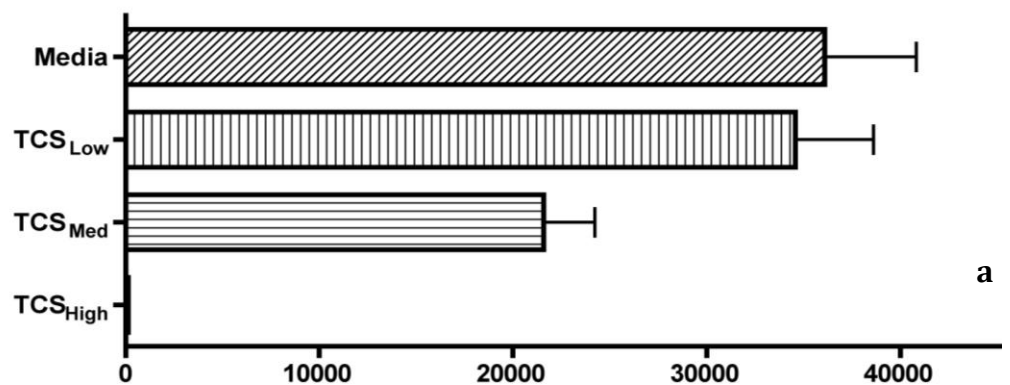


Fig 41. Number of detached cells and merosomes in media control, milk only control and triclosan associated to β -CN micelles at 72 hr post infection. Bars represent the average count of 3 independent experiments and error bars are the standard deviation between experiments.



Becquerel

Fig 42. Thymidine incorporation assay measuring the effect of treatments on host cell viability. Thymidine was applied to host cells 48 hrs after initial drug treatment. The presence of thymidine within the cell was measured by lysing cells after 12 hours of incubation. Amount of thymidine present within in cells was measured by a scintillation counter in Becquerel units measuring the toxic effects of treatments on host cells in the presence of a) triclosan standards (free triclosan), b) milk digestates, c) serum digestates, and d) β -CN micelles at low, medium, and high triclosan concentrations.

APPENDIX VI – *PLASMODIUM* LIFE CYCLE

Excerpts from journal articles outlining the life cycle of the *Plasmodium* parasite to provide background information for Chapter 5.

A. Strum and V. Heussler. 2007. Live and let die: manipulation of host hepatocytes by exoerythrocytic *Plasmodium* parasites. *Medical Microbiology and Immunology*, 196: 127-133.

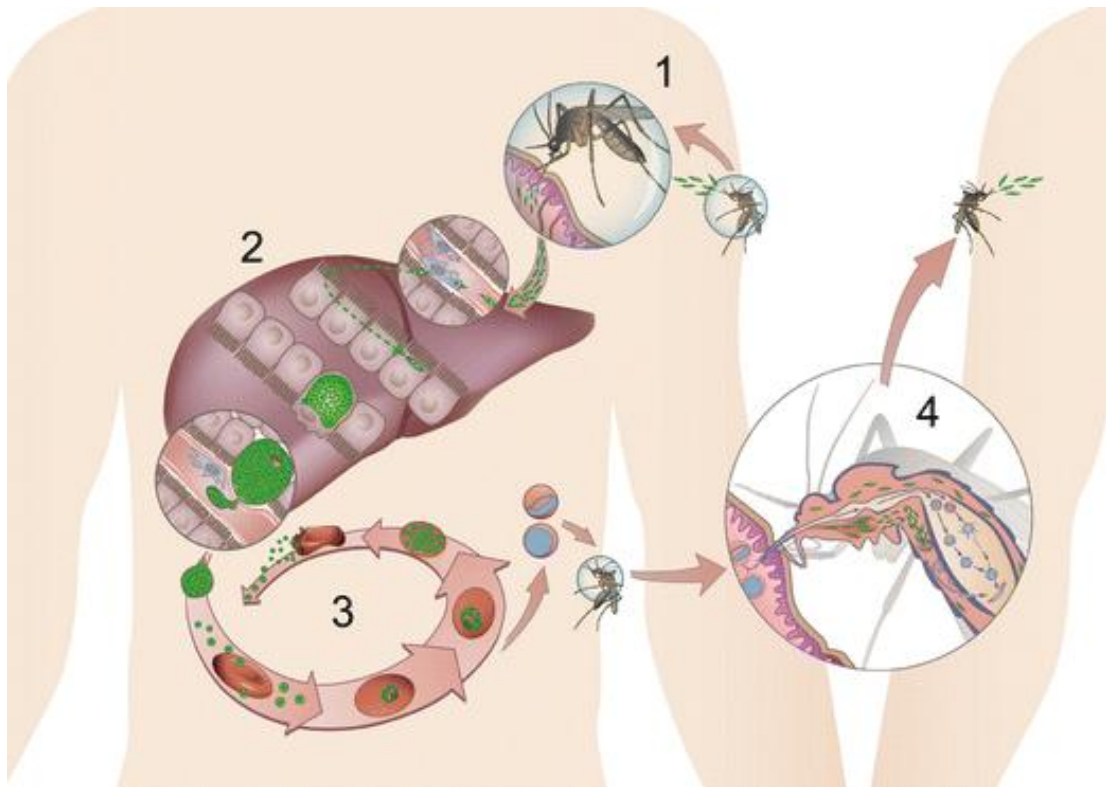


Fig. 4 Revised life cycle of *Plasmodium* parasites in mammals. 1 Inoculation of *Plasmodium* sporozoites during blood meal of an infected *Anopheles* mosquito. 2 Sporozoites reach the liver with the blood stream and glide along sinusoidal endothelia before breaching through Kupffer cells and several hepatocytes before finally developing

into liver schizonts within an hepatocyte. Upon merozoite formation, merozoites are extruded into liver sinusoids. 3 Liver-derived merozoites are liberated and infect red blood cells. Repeated infection cycles occur with some parasites developing into gametocytes. 4 Fertilisation in the mosquito gut and development to infective sporozoites in the salivary glands. Picture courtesy of Maria Mota

The Plasmodium liver stage

Plasmodium-infected hepatocytes are ideal targets for the development of anti-malarial measures, as liver infection is still clinically silent. In addition, Plasmodium parasites remain for several days in hepatocytes that should allow enough time for the immune system of a vaccinated individual to eliminate the infection at this early stage. Research performed in humans and mice has shown that irradiated or genetically attenuated sporozoites are able to confer protection against challenge with wild type parasites [5–8]. In spite of this knowledge and the recent effort to mass-produce irradiated sporozoites for vaccination trials [9, 10], a commercial vaccine against malaria is still not available. It is therefore of decisive importance to gain more detailed knowledge about the observed protective immune responses and the parasite survival strategies within hepatocytes.

The *P. falciparum* liver stage still remains a black box [11], because in vitro infection of human hepatocytes [12, 13] and in vivo experiments using primates or humanized mice [14] rely on *P. falciparum*-infected *Anopheles* mosquitoes and all aspects are difficult to perform, since they need to be carried out under high (S3) safety

laboratory conditions. Therefore, mouse models using *P. berghei* and *P. yoelii* infections have successfully been introduced and the generation of fluorescent rodent parasite strains has opened new avenues to investigate migration and development in the mammalian host [15, 16]. In vitro live imaging of infected cell cultures (Fig. 1) and intravital imaging of surgically exposed livers of infected mice (Fig. 2) allowed us to investigate intracellular development of the parasite and to follow its journey from the skin into the liver and back to the blood stream [3, 17–19].

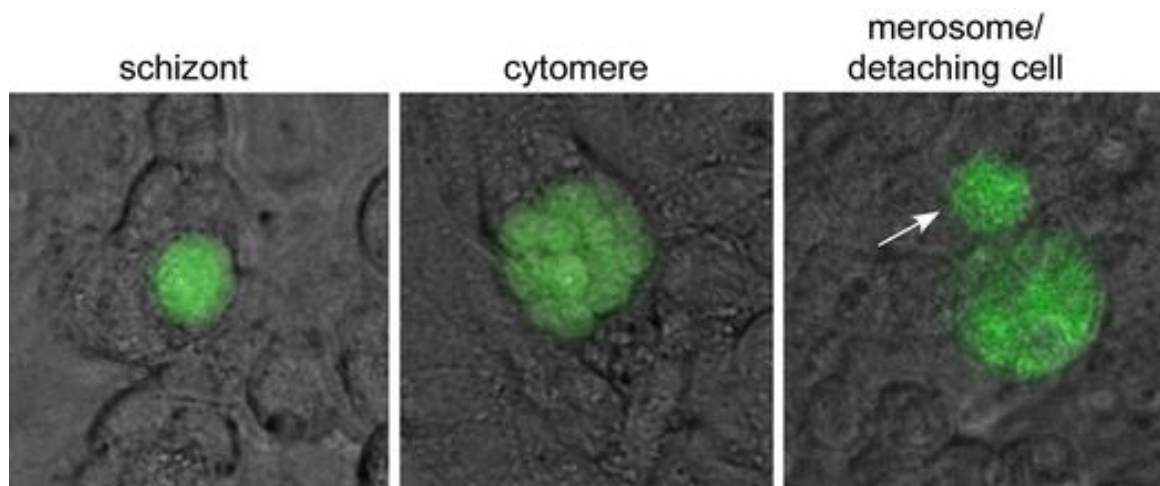


Fig. 1 In vitro live imaging of GFP-expressing *P. berghei*-infected HepG2 cells (merger of IFA and phase contrast image). Different developmental stages of liver stage parasites: schizont stage, cytomere stage and detached infected cell with a budding merosome (arrow)

A. Sturm, S. Graewe, and B. Franke-Fayard, S. Retzlaff, S. Bolte, B. Roppenser, M. Aepfelbacher, C. Janse, and V. Heussler. 2009. Alteration of the Parasite Plasma Membrane and the Parasitophorous Vacuole Membrane during Exo-Erythrocytic Development of Malaria Parasites. *Protist*, 160(1): 51-63.

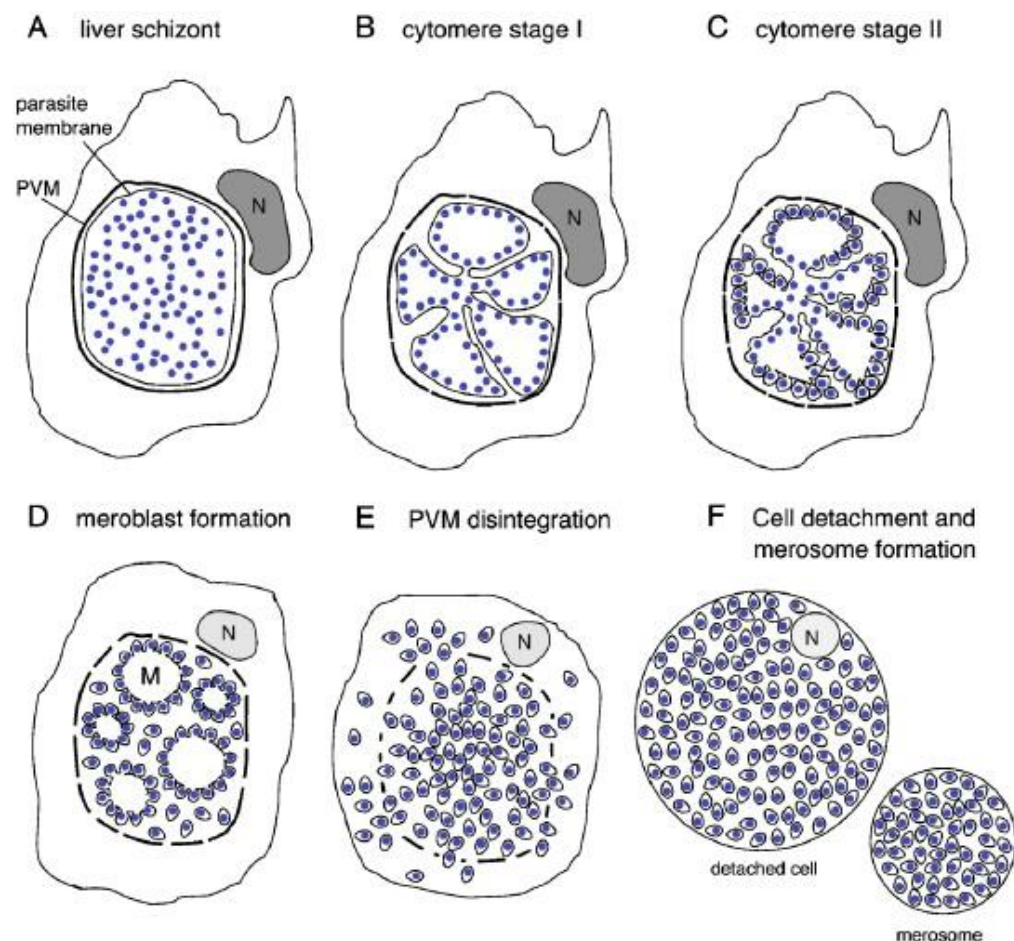
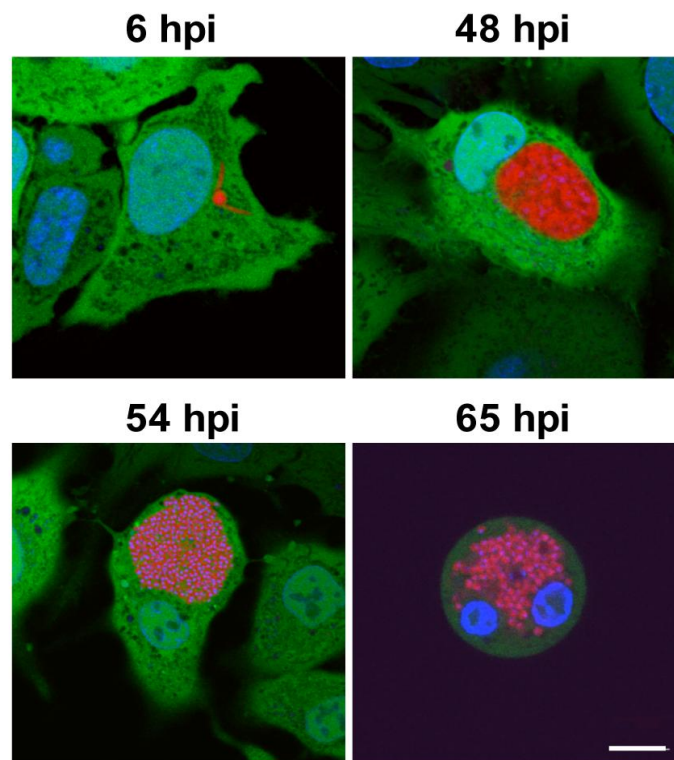


Figure 8. Schematic representation of late liver stage development: (A) late liver schizont residing in a PV next to the host cell nucleus (N); (B) during the cytomere stage, the

parasite membrane invaginates, surrounding large groups of parasite nuclei. At this stage the PVM has already changed its permeability (cytomere stage I); (C) invagination of the parasite membrane continues, now surrounding single parasite nuclei (cytomere stage II); (D) repeated membrane invaginations result in development of merozoite-producing meroblast (M) (meroblast formation); (E) successive disruption of the PVM results in merozoite release into the host cell cytoplasm (PVM disintegration); (F) during this process the infected cell detaches and from here merozoite-filled vesicles (merosomes) start to bud off (cell detachment and merosome formation). Note the difference between detached cell and merosome: detached cell still contains host cell nucleus whereas merosome is a host cell membrane-derived vesicle, containing merozoites but no host cell nucleus.

Graewe S, S. Retzlaff, N. Struck, C. Janse, V. Heussler. 2009. Going live: a comparative analysis of the suitability of the RFP derivatives RedStar, mCherry and tdTomato for intravital and in vitro live imaging of Plasmodium parasites. Biotechnology Journal, 4(6): 895-902.



6 hpi: Parasite in sporozoite stage

48 hpi: Parasite in shizont stage

54 hpi: Merozoites within host cell at beginning stages of detachment

65 hpi: Detached host cell containing merozoites

CONCLUSION

The unique structure and remarkable functionalities of the major protein in milk, the casein micelle, will be indispensable in the quest for sustainable and biocompatible materials for our everyday use and select novel applications. Exploring methods for micelle dissociation and properties of the reformed micelles will prove to be an essential element in the complete realization of the capabilities of the casein micelle. Although its quaternary structure remains somewhat of a mystery, the individual casein proteins (κ -, β -, α_{s1} -, and α_{s2} -casein), and their interactions with each other, colloidal calcium phosphate, and its surrounding environment will prove to offer a number of new possibilities extending beyond the food industry. This dissertation outlines

VITA

Adrienne Lysandra Roach was born on August 25, 1979, in Rochester, New York. In 1985 she, along with her family, moved to Huntsville, AL. She attended Chapman Elementary, Liberty Middle and graduated from Sparkman High School 1997. She entered Alabama A&M University in August of 1997 and graduated with a Bachelor's of Science in Biology with a minor in Chemistry in 2001. Upon graduation she relocated to Knoxville, TN where she worked as an intern and later employee of Shaw Environmental and Infrastructure. In August of 2003, she entered graduate school at the University of Tennessee in Knoxville as a Master's student in Biosystems Engineering Technology. Her main area of focus was carrier mediated chemical transport in porous media and onsite wastewater technology. Adrienne received her Masters degree in December, 2005. In August of 2006 she became a doctoral student in Food Science and Technology in the Food Biopolymers Protein Group under the direction of Federico Harte. Her focus was on functional properties of the casein micelle and its use as a drug delivery system. She plans to continue researching various aspects of protein polymers as a postdoctoral fellow at Oak Ridge National Lab.