Self-assembled Casein Nanostructures to Deliver Novel Functionalities

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I am submitting herewith a dissertation written by Kang Pan entitled "Self-assembled Casein Nanostructures to Deliver Novel Functionalities." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

Qixin Zhong, Major Professor

We have read this dissertation and recommend its acceptance:

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Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
Self-assembled Casein Nanostructures to Deliver Novel Functionalities

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

Kang Pan
August 2015
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ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my major advisor Dr. Qixin Zhong. Firstly, he was very “brave” to accept me as a Bachelors’ student to the PhD program in Department of Food Science and Technology at University of Tennessee. He was even “braver” to allow me to finish in 4 years with a concurrent Master of Science degree in Statistics. I am really convinced by his confidence, and I am not sure whether I would do this if I were a professor. Secondly, Dr. Zhong has been extremely patient with my progress. I cannot forget that my first manuscript was revised extensively by him for more than 20 times and it took a year from the first draft to the submission. However, he never criticized me for my writing, but only encouraged me instead. Thirdly, I am impressed by his attitude towards research and his enthusiasm for work. He set an excellent example for me and I could not find a reason to not work hard when I know how hard-working he is.

I am very grateful to my committee members, Dr. Svetlana Zivanovic, Dr. Seung Joon Baek, and Dr. Hamparsum Bozdogan for their time and efforts in helping me throughout my research and my study. Dr. Zivanovic is a very kind and elegant professor. She always tried to guide me step by step if I don’t have the right answer for her questions. As a graduate coordinator, she really cares about our students and we can feel that she is on the same side with us. I want to thank Dr. Baek for allowing me to use his lab for research. He has been very active and helpful when I was asking for help, he will put his work aside when I approached him and took me to the right place finding the right person, so I can get what I need almost instantaneously. I also want to express me special thanks to Dr. Bozdogan, who is also the advisor for my Statistics program. I understand how hard it would be for him to be on my committee since there is a huge difference
in our background. However, he has been very supportive and willing to take the challenge with me.

I’d like to thank all my labmates, either still in the lab or having graduated: Minfeng Jin, Bhavini Shah, Dan Xiao, Wan Wang, Yue Zhang, Qiumin Ma, Jia Xue, Linhan Zhang, Xueqian Shi, Yun Zhang, Bai Qu, Kangkang Li, Dan Su, Corina Rei, Xin Miao, Dr. Yangchao Luo, Dr. Yongguang Guan, Dr. Gang Liu, Dr. Jiwang Chen, Dr. Jin’e Wu, Dr. Jianhui Xiao. I am very lucky to meet you all here at UTK, and I am sure we will come across with each other again in our future.

I also want to thank all the faculty, staff and graduate students in our department, who made my life easier and memorable. I will also give my thanks for the professors in Chemistry, Material Science & Engineering, Physics, and Biochemistry & Cellular & Molecular Biology Departments for allowing me to use the instrument and provided me with their insightful discussions.

Last but not least, I reserve my deepest gratitude to my family members. They have always being a very important reason for me to keep working and learning. I would not be here without their love for me and my love for them.
ABSTRACT

The nanoscale micellar structure of caseins can be manipulated by mechanical forces, solvent quality, and pH to reduce turbidity, encapsulate hydrophobic compounds, and improve dispersion stability and rheological properties. The goal of this dissertation was to control the nanostructure of self-assembled caseins to deliver functionalities. The combination of treatments at pH 11.0 and acidification with citric acid was first studied to produce translucent skim milk dispersions at pH 5.5-7.0, resulting from the significantly reduced dimensions of reassembled casein nanoparticles. Using sodium caseinate (NaCas), soluble soybean polysaccharide (SSPS), and high-speed homogenization, a food grade delivery system was studied to encapsulate thymol as transparent nanodispersions. The dispersions had the improved anti-listerial activity in milk than free thymol and SSPS enabled stability at an acidity around the isoelectric point of NaCas. To improve the encapsulation efficacy, a novel method was developed to encapsulate curcumin with improved dispersibility and bioactivity by spray-drying warm aqueous ethanol solution with co-dissolved NaCas and curcumin. Utilizing pH-dependent solubility properties of curcumin and self-assembly properties of NaCas, another scalable low-energy and organic solvent free encapsulation technology was studied. Curcumin was successfully encapsulated in self-assembled casein nanoparticles by pH-cycle treatment under only mild stirring, and showed significantly improved anti-proliferation activity against human colorectal and pancreatic cancer cells. The principle was then applied to stabilize zein by mixing with NaCas at pH 11.5 and the subsequent neutralization enabled the co-assembly of zein and NaCas, showing the promise of this novel method to prepare water dispersible zein nanoparticles for applications such as delivery systems and edible packaging films. Lastly, intrinsically disordered β [beta]- and κ [kappa]-caseins were found to form amyloid-like fibrils at pH 2.0 and 90 °C, which had a Young’s modulus of $2.35 \pm 0.29$ GPa and $4.14 \pm 0.66$
GPa, respectively, as measured using quantitative nanomechanical atomic force microscopy. The
dispersion with β-casein fibrils had a viscosity more than 10 and 5 times higher than those of κ-
casein and β-lactoglobulin, respectively, at 0.1 s\(^{-1}\) [1/second] and comparable concentrations. Self-
assembled casein nanostructures in the present dissertation may found novel applications as
functional materials.

**Keywords:** casein nanoparticle; self-assembly; delivery system; zein dispersibility; nanofibril
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Chapter 1. Introduction and literature review
1.1 Abstract

The development of nanotechnology has been increasing rapidly. The improved physical, chemical, and biological functionalities also boosted the research and development of nanomaterials in consumer goods. Organic engineered nanoparticles (ENPs) processed from the bulk food material have been one of the hot zones attracting attentions to produce functional materials for applications in food matrices due to their easy incorporation into the current food systems as well as their potentially low toxicity compared with inorganic nanoparticles. In this review, various technologies available for processing of organic ENPs will be reviewed. The identification and characterization techniques will be discussed for their suitability for ENPs with different properties and to fulfill characterization purposes. The utilizations of ENPs in the current and the future complex matrices with designed physicochemical and biological functionalities will then be discussed. A systematic knowledge of the whole scheme of ENPs (fabrication, characterization, and utilization) will be beneficial to the better understanding of ENPs in different systems as well as fostering the development of more innovative and safe functional materials.

1.2 Introduction

Nanotechnology has been used increasingly to improve the functionalities of a wide range of consumer products. Materials being engineered into nanoscale possess unique physicochemical and biological properties that might be related to the significantly increased surface area and thus surface activity. Engineered nanomaterials (ENMs) manipulated into forms like nanoparticles, nanofibrils, nanotubes, and nanoemulsions have been developed for applications in food systems as packaging materials, functional ingredients, etc. However, there has no consensus about the definition of nanomaterial presently, and different particle systems with sizes lower than 100 nm and up to several hundred nanometers have been reported to be in nano-scale (Abd El-Salam &
El-Shibiny, 2012; Q. R. Huang, Yu, & Ru, 2010; D. J. McClements & Rao, 2011). Although there are some government service departments such as National Nanotechnology Initiative of the United States (www.nano.gov) and European Commission (Bleeker, et al., 2013; Kreyling, Semmler-Behnke, & Chaudhry, 2010) among others recommended using “nano” to refer to the structures such as spheres, rods, and fibers with at least one dimension less than 100 nm, there are also some professional societies give rather imprecise definitions (Joachim, 2005).

Many technologies have been developed to produce ENMs for uses in food matrices. The development of food nanotechnology may bring benefits such as more nutritiously balanced and safer foods with better sensory properties and lower prices (Szakal, Tsytsikova, Carlander, & Duncan, 2014). Various technologies based on different mechanisms can be used to produce nanoparticles and there is no one that can be considered better than the others, and the best fit method needs to be chosen according to specific system and environmental requirements. By paring with suitable technologies, organic materials containing organic carbons (such as proteins, polysaccharides, carbon-based polymers, and lipids), different from inorganic materials (such as silica and metallic elements like silver or copper), may be processed into food grade ENPs that can be used in food or nutraceutical matrices. However, beside all the benign aspects that nanotechnology may bring to us, there is also an increasing concern about the potential toxicity of ENMs to human health or environment (Szakal, et al., 2014). Therefore, ENMs identification and characterization become crucial in terms of regulations on safety of food systems. Because of their nanometer dimensions, direct observation is not possible and also makes most conventional instruments like optical microscopy less useful. Techniques with much higher resolution and sensitivity are therefore in need to identify, isolate, and characterize ENMs/ENPs. Although many technologies can be utilized to study inorganic ENPs, few systematic methods can be directly
applied to study organic ENPs due to their similar physicochemical properties with the bulk food systems containing them, which are also rich in organic materials.

In this review, available methods that are suitable for organic ENPs preparation will be introduced and discussed. Characterization is a very important aspect in nanostructure innovations that connects particle processing with their functionalities or utilizations, therefore the following section will discuss the available techniques to characterize the physical, chemical, and biological properties of organic nanoparticles in complex matrices. The third section will discuss the utilizations of organic ENPs to deliver stability, sensory, bioactivity, and other functional properties in food and nutraceutical sectors. The last sections will cover the background information specific to the materials being studied for this thesis, focusing on casein nanostructures and model bioactive compounds.

1.3 Methods of fabricating organic nanoparticles

Reduction of dimension is one of the main goals to achieve when designing a functional ENPs system. For food applications, nanoparticles can be engineered through both top-down and bottom-up routes. Top-down methods are achieved by applying mechanical forces to “break-down” the macrostructures, reducing the dimension of bulk materials using operation units such as high pressure/speed homogenization. Bottom-up methods refer to nanostructure formation from smaller particles or molecules through self-assembly or particle attractions/aggregations. Based on the consumption of energy for the particle formation, top-down and bottom-up approaches are called high energy and low energy methods respectively.
1.3.1 Top-down methods

For particles that are dispersible in liquid and can be disrupted by mechanical forces, such as coarse emulsions with large droplet sizes and was formed by blending two immiscible phases stabilized by surfactants, the reduction of size is commonly achieved by applying shear or normal forces to overcome the Laplace pressure, which increases as the size decreases and therefore defines the limit of size reduction by mechanical forces (D. J. McClements, 2011).

1.3.3.1 High-pressure homogenization

High-pressure homogenization (HPH) systems can be classified into three categories and are one of the most popular methods to create fine dispersions in food industry (Schultz, Wagner, Urban, & Ulrich, 2004). Radial diffuser HPH is the most commonly used set-up (Figure 1-1a), which diffuses and deflects the radially incoming fluid for $90^\circ$ by the valve face, and a mobile valve seat which changes slit width, creating a variation of homogenization pressure at the constant flow rate. Different valve types can be adopted such as flat-valve, tooth-valve, and cutting-edge-valve (Schultz, et al., 2004). For counterjet dispergators configuration (Figure 1-1b, the incoming fluid is divided into two streams towards narrowed compartment to achieve an increased flow velocity before they are brought together to disrupt and reduce the particle dimension by the turbulent flow and cavitation. For axial flow nozzle systems (Figure 1-1c), the fluid is forced through a configurable orifice in a valve to break the bigger particles. Because of the absence of no movable parts like the mobile valve seat in radial diffuser type homogenizer (Figure 1-1a), axial flow nozzle system can be configured to higher pressures and multiple passes to further reduce the dimension of particles (Schultz, et al., 2004).
1.3.1.2 High-speed homogenization

High-speed or high-shear homogenization processes dispersions through direct application of shear forces. Both rotor-stator and disc set-up can be applied in a typical high-speed homogenization set-up (Urban, Wagner, Schaffner, Roglin, & Ulrich, 2006). A rotor-stator configuration shears the dispersion phase in the space between the rotor and the stator, generating laminar or turbulent flows for rotor and stator surfaces that are smooth or toothed respectively. In a disc configuration, the coarse dispersion is directly blended into continuous phase by a high speed rotating disc head, generating a low to medium pressure turbulent flow, which is better for solid particles, emulsions with high viscosity, or ingredients that are stress sensitive (El Kinawy, Petersen, Helmdach, & Ulrich, 2012).

1.3.1.3 Membrane/microchannel emulsification

Membrane/microchannel emulsification technique works by forcing coarse dispersions through pores/microchannels in a membrane into a flowing continuous phase premixed with surfactant (David Julian McClements, 2004). Because of its inherent advantage of lower energy input and the simplicity, membrane/microchannel emulsification received increasing attention to produce possibly mono-distributed dispersions (Chuah, Kuroiwa, Kobayashi, & Nakajima, 2014; El-Abbassi, Neves, Kobayashi, Hafidi, & Nakajima, 2013). The dimension of dispersion can be controlled by parameters such as pore size, interfacial tension, types of surfactant, trans-membrane pressure, as well as the flow rate of the continuous phase.
1.3.1.4 Ultrasonication

Ultrasonication produces nanoparticles by applying high intensity ultrasonic waves with a frequency higher than 20 kHz, and has been used by the food industry to reduce the droplet/particle size and to homogenize dispersed phase into a continuous phase directly (D. J. McClements, 2011). However, the relatively small working zone limits the application of this technique for large volume samples.

1.3.2 Bottom-up methods

In bottom-up methods, physical chemistry is used to bring dissolved molecules or dispersed smaller particles together to form larger structures through predominantly physical forces instead of chemical synthesis methods to meet the regulations imposed on materials. Possible biological methods can also be used such as enzymatic reactions. Many methods have been studied with the most common ones discussed in this section.

1.3.2.1 Precipitation

For particles formed through precipitation methods, the structure forming molecules are dissolved in a solvent first before they are dispersed into another miscible solvent, which will bring down the quality of the final solvent and lead to the salting out and precipitation of the molecules. A widely studied food biopolymer example is prolamine zein proteins, which dissolve in aqueous ethanol solutions with appropriate portions of alcohol and precipitate out when the ethanol concentration is reduced by mixing with deionized water (Figure 1-2). The resultant particle dimension ranges from <100 nm to several hundred nanometers, and can be controlled by varying concentrations of zein and ethanol (Zhong & Jin, 2009). Several lipophilic compounds such as essential oils, fatty acids, and curcumin have been co-dissolved in aqueous ethanol solution with
prolamins and encapsulation can be simultaneously achieved through this anti-solvent precipitation method (Parris, Cooke, & Hicks, 2005; Patel, Hu, Tiwari, & Velikov, 2010; Zhong, Tian, & Zivanovic, 2009).

1.3.2.2 Self-assembling and coacervation

The dissociation and assembling behavior can be utilized to form nanoparticles in a controlled manner by changing parameters such as solution acidity and temperature. For example, when dissolved in warm aqueous ethanol solution, the micellar structure of caseins can be disrupted while re-association can occur again upon cooling (O'Connell, Kelly, Auty, Fox, & de Kruif, 2001). The subsequent removal of organic solvent through spray drying can be used to encapsulate lipophilic compounds with nano-scale dimensions (Pan, Zhong, & Baek, 2013). The acidity or pH of the solvent affects the protonation/deprotonation of hydrogen atoms, thus affecting the water solubility of small and macro-molecules. Another example of casein showed that caseins dissociated into smaller aggregates with extended chains at alkaline conditions, and the dissolution of hydrophobic molecules such as curcumin at high pH conditions improved their interactions with caseins, and the subsequent neutralization enabled the encapsulation of curcumin in the self-assembled nanoparticles (Pan, Luo, Gan, Baek, & Zhong, 2014). The mechanism can thus be used to encapsulate bioactive compounds that are sensitive to heat without uses of organic solvent.

Coacervation or co-assembling of different molecules by physical forces is another way to produce nanoparticles, predominantly through electrostatic attractions between two oppositely charge molecules or particles. Proteins with different isoelectric point or proteins/polysaccharides complexes are commonly used based on this mechanism. For example, proteins will become net positively charged below their isoelectric points while proteins with a much lower isoelectric point or polysaccharides lack amide groups still remain as net negatively charged during the acidification.
The predissolved and homogeneous mixture can thus form complexes or coacervates with nanoscale dimensions, and different biopolymers can be used as additional coatings to form multilayered delivery systems (Y. Luo, Pan, & Zhong, 2015; Yeo, Bellas, Firestone, Langer, & Kohane, 2005).

1.3.2.3 Emulsions and nanoemulsions

Most emulsions have composites with significantly different polarity between dispersed and continuous phases, and the systems are stabilized with surfactants by reducing the surface tension. Emulsions or Nanoemulsions not only can be prepared through high-energy methods mentioned above, they can also be prepared using low-energy methods by controlling interfacial properties (D. J. McClements, et al., 2011). Self-emulsifying nanoemulsions can be prepared by dispersing oil/water phase into continuous water/oil phase with enough water/oil soluble surfactants, and the process needs only gentle shaking or stirring to form a thermodynamically stable system. Phase inversion method is another technique can be used to prepare nanoemulsion systems. During the processing, an initial O/W or W/O emulsion is transformed to a W/O or O/W emulsion respectively upon heating above the critical temperature, and can be transformed back to O/W or W/O emulsion upon cooling. During the thermal processing, surfactants and oil phase are redistributed and O/W emulsion can be inverted into a W/O emulsion, or vice versa, by the increased volume fraction of the continuous phase, and the dimension of droplet size can thus be reduced significantly (D. J. McClements, et al., 2011).

1.3.2.4 Template methods

Template based nanoparticle formation represents a group of methods to produce nanoparticles using templates such as porous membrane or emulsion systems and turn the solutions dissolved with target materials into spatially defined particles (Hulteen & Martin, 1997). Different
nano-architectures can be achieved by using different template systems. For example, bulk materials can be filled into porous membranes pretreated to have desired mono-distributed pore sizes, and the subsequent polymerization or solidification ensures the formation of nanowire or nanotube structures with defined dimensions (Hulteen, et al., 1997). Using nano-emulsion based template, the dispersion can be emulsified into nano-sized droplet, and the curing processing delivers nanosphere or nanocapsule particles after environmental changes such as pH, temperature, or enzymatic activity, or by solvent evaporation (Anton, Benoit, & Saulnier, 2008). One common example is the preparation of solid lipid nanoparticle, which was initially formed as O/W emulsion at elevated temperature and the lipid nanoparticle can be solidified after the system is fast cooled down below the melting temperature of internal core material (Muller, Mader, & Gohla, 2000).

1.4 Characterization of organic nanoparticles

The identification, isolation, and characterization of ENPs in foods pose significantly challenges because of the low usage level. It is even more difficult for organic ENPs compared with inorganics ones due to their similarity to the bulk food matrices which are composed of mainly organic matters as well. This section reviews the available techniques that are suitable for characterizations of organic ENPs for their physicochemical, and biological properties.

1.4.1 Dimension and morphology

Dimension and morphology of ENP affect the colloidal stability and visual appearance of ENPs. A smaller particle size usually corresponds to a reduced turbidity and the stronger Brownian movement, leading to a clearer appearance and better colloidal stability, which is a very important parameter to consider when applying ingredients for uses in clear aqueous systems like sports beverages. Therefore, size distribution and morphology of ENPs need to be characterized to
understand their structures and to predict the physicochemical stabilities of the systems. Many technologies have been developed for size and morphology characterizations, and there is no single universally applicable technique that is able to fully characterize the dimension and morphology of ENPs, and a combination of different techniques is usually needed for more precise characterizations, especially for food systems with complex natures.

1.4.1.1 Electron microscopy

Microscopy based methods visualize objectives through the reflection or absorption of light or electrons by the surface of objects, working as a convenient way for direct observation of particle dimensions and tracking of particle movement equipped with high sensitive cameras (Crocker & Grier, 1996; Jaqaman, et al., 2008). However, conventional optical microscopes are not suitable for ENPs observations because of the weak reflection of visible light from particles with nanometer scale dimensions. Electron microscopes use electrons to visualize the structures of nanostructures. Because of the much shorter wavelength of the electron beam compared with visible light, a higher resolution image could be obtained compared with the conventional light microscope. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are two most commonly used electron microscopes. SEM images nanostructures by detecting the backscattered or secondary electrons when the surface of the specimen is being shot by the electron beam; while in TEM, electrons focused on the specimen are being absorbed or reflected by the specimen differently depending on the electron density of different atoms, and the electrons passed through are collected, magnified, and visualized (David B. Williams, 1996; Joy, 1997). Because of the low conductivity and the low contrast between the samples and the background, coating and staining are usually applied before organic samples are being imaged by SEM and TEM respectively, which in turn may alter the native structures of ENPs. Since SEM and TEM are
operated under high vacuum environment, samples are also needed to be dried before imaging, therefore some biological samples that are easy to collapse may not be suitable for SEM and TEM imaging as well. To overcome the difficulty of sample preparation for biological materials, some other techniques are developed to obtain the structural information of ENPs in their original state. Environmental scanning electron microscopy (ESEM) uses detectors that can operate in higher pressure and the gas in the chamber can be ionized, therefore humid samples can be imaged directly without needs of intense drying and coating (Danilatos, 1997). However, the contrast is relatively low when the humidity increases, and there is also a possibility to have more artifacts like drifting. Another alternative is to use cryo-TEM, which images samples in their frozen state on a cold stage and environment. This is particularly useful to observe the structures of soft particles that may collapse if dried slowly in atmosphere environment (Marchin, Putaux, Pignon, & Leonil, 2007; Trejo, Dokland, Jurat-Fuentes, & Harte, 2011).

1.4.1.2 Atomic force microscopy

Atomic force microscopy (AFM) is an emerging technology developed after the invention of scanning tunnel microscopy. Contacting or non-contacting (or tapping) modes can be operated to map the surface structure along with the height information by measuring the force between specimen surface and the oscillating cantilever, which could then be mapped into 3-D structures of samples being imaged. The ease of sample preparation and the ability to be operated in ambient or liquid condition make AFM an irreplaceable experimental technique to study biomacromolecules or even living cells in situ (Gad, Itoh, & Ikai, 1997; Karrasch, Hegerl, Hoh, Baumeister, & Engel, 1994; Morris, et al., 2001; Yang, et al., 2007). Using the newly developed Peak-Force Quantitative Nanomechanics (PF-QNM) technology, simultaneous nanomechanics analysis can be done with the dimension or morphology characterizations, making it a potentially
powerful tool to distinguish different materials in composite ENPs (Adamcik, et al., 2012). However, the low scanning speed and significant smaller scanning area compared with EM based techniques make the operating of AFM very tedious and time consuming.

Although high resolution structural information can be obtained using microscopy based methods, being only able to study a portion of the whole system may make the results not representative and provide a lower statistically power to find real differences between samples due to the limited number of particles can be measured. Another disadvantage of microscopy based techniques is that they are destructive methods, which means that the samples cannot be used for other analysis after being imaged. Therefore, some non-destructive spectroscopy and chromatography based techniques can be considered as alternatives to characterize the dimension and morphology of ENPs and to obtain more representative results by measuring samples in larger quantity or volume.

1.4.1.3 Light scattering

Indirect measurement of particle dimension and morphology could be done by using scattering based techniques, which usually generate results faster than the microscopy based techniques considering the time required for sample preparation (like drying) and equipment initialization (like chamber vacuuming). Light scattering techniques are important to gather the size and shape information about polymer solution systems. The relationship between the incident laser intensity ($I_0$) and the *excess* scattered laser intensity ($I_{ex}$) for “small” molecules ($qR_g \ll 1$) can be expressed by the following equations (Hiemenz & Lodge, 2007):

\[
I_{ex} = I_s^{solution} - I_s^{solvent} = I_0 \frac{4\pi^2 n_0^2}{r^2 \lambda^4 N_a v} \left( \frac{dn}{dc} \right)^2 \frac{c}{M} + 2Bc + \ldots
\]
where \( I_s \) is scattered intensity, \( n_0 \) is the refractive index of the solvent, \( \lambda \) is the incident laser wavelength, \( N_{av} \) is the Avogadro's number, \( c \) is the solution concentration, \( dn/dc \) is the change in refractive index of the solution with the change of concentration, \( r \) is the distance from the dipole, \( M \) is the molecular weight, and \( B \) is the second virial coefficient. Some items can be groups as:

\[
\frac{I_{ex} r^2}{I_0} = R_\theta
\]

and

\[
\frac{4\pi^2 n_0^2}{\lambda^4 N_{av}} \left(\frac{dn}{dc}\right)^2 = K
\]

where \( R_\theta \) is the so-called Rayleigh ratio, which depends only on the solution and \( \lambda \), but not the instrument used; and \( K \) represents a group of purely optical factors. Thus the equation can be re-wrote and expressed as:

\[
\frac{Kc}{R_\theta} = \frac{1}{M} + 2Bc + \cdots
\]

The equation can then be applied for polymer with a finite size when the form factor \( P(\theta) \) is away from 1, and Zimm equation can thus be obtained as:

\[
\frac{Kc}{R_\theta} = \frac{1}{M} \left(1 + \frac{q^2}{3} R_g^2 + \cdots\right) + 2Bc + \cdots
\]

where \( R_g \) is the radius of gyration of the polymer.

Through the construction of Zimm plot by varying laser angles and sample concentrations, two extrapolations to zero scattering angle (\( \theta=0^\circ \)) and to zero concentration (\( c=0 \)), the molecular weight \( M \) can be solved. The slopes of angle extrapolation and concentration extrapolation will provide values of \( R_g \) and \( B \) respectively (Hiemenz, et al., 2007). There theories and equations set
the basis for static light scattering (SLS), which is a very useful technique to determine the radius of gyration, molecular weight, and even information of molecular shapes information of polymers in solutions simultaneously.

Dynamic light scattering (DLS, or photon correlation spectroscopy) is one of the most extensively used light scattering techniques for dimensional characterizations of ENPs, which measures the hydrodynamic radius by collecting the fluctuation of laser light intensity over time caused by the Brownian motion of ENPs (Berne & Pecora, 2000). The second order autocorrelation of scattered light of particles at different time (t) could be generated by the following equation (Hiemenz, et al., 2007):

\[ g^2(q; \tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2} \]

where I is the detected intensity, \( \tau \) is the delay time, and q is the wave vector which could be obtained by the equation:

\[ q = \frac{4\pi n_0}{\lambda} \sin \left( \frac{\theta}{2} \right) \]

where \( \lambda \) is the incident laser wavelength, \( n_0 \) is the solvent refractive index, and \( \theta \) is the angle between the detector and the direction of the incident light.

DLS can be applied to measure sample size distributions that are either monodispersed or polydispersed. The mean hydrodynamic diameter can then be calculated based on Stokes-Einstein equation (Frisken, 2001). It should be noted that the samples need to be diluted sufficiently to prevent the interference on Brownian motion among particles or secondary scattering of light by multiple particles. However, the significant dilution of samples poses another issue worth of
attention, since some particles may have a concentration dependent structural properties, which may make the results not representative of their original state.

Small angle scattering (SAS) as an advanced scattering based technique is another very powerful tool to analyze the structures of macromolecules and biological samples such as proteins, polysaccharides, lipids. Small angle x-ray scattering (SAXS) and small angle neutron scattering (SANS) are two typical techniques in the category of SAS using x-ray and neutron beams as light sources. The scattered intensity being collected at small angles (from 0.1 to 10°) at various sample concentrations can be used to calculate the form factor and therefore the structural information could be obtained in addition to information about size (Hayter & Penfold, 1983; Kratky, 1963; Svergun & Koch, 2003).

1.4.1.4 Chromatography and ultracentrifugation

Size exclusion chromatography (SEC) is categorized as a gel filtration chromatography method, which has wide applications to characterize the size and the molecular weight distribution of synthetic and biological macromolecules since the native solvent of the macromolecules like water can be used directly as the mobile phase (Mori & Barth, 1999). The column is packed with porous beads as stationary phase and particles can be separated based on their shape and size when they are passing through pores in stationary beads with different retaining behaviors. Different detection modules such as light scattering, UV-Vis, refractive index detector, and inductively coupled plasma-mass spectrometry (ICP-MS) can be attached to SEC to collect real time data specific to each fraction of the whole particle system (Helfrich, Bruchert, & Bettmer, 2006; Wen, Arakawa, & Philo, 1996). However, the size range of particles can be analyzed by SEC is relatively small, therefore might be not suitable for analysis of systems with the presence of aggregates (J. Liu, Andya, & Shire, 2006). Analytical ultracentrifugation (AUC) as an alternative non-destructive
technique is capable to cover a much wider range of molecular weight (up to 10 GDa) without requirement for extensive dilutions, and the simultaneous conformational analysis can be done with the sedimentation velocity and equilibrium signals (Bootz, Vogel, Schubert, & Kreuter, 2004; J. Liu, et al., 2006). Another very promising separation and characterization technique is field flow fractionation (FFF), which fractionates samples by applying a field perpendicular to the direction of flow. Fields that developed includes semi-permeable membrane, centrifugal, thermal-gradient, electrical, and magnetic etc., and can separate samples based on size, density, chemical composition, surface electric, and magnetic properties (Giddings, 1993; J. Liu, et al., 2006).

1.4.2 Surface properties

Surface property is an important parameter to predict the colloidal stability and to design functionalities of ENPs in complex systems. Main forces affecting the stabilization of colloidal particles include electrostatic repulsions/attraction, steric repulsion, hydrophobic interaction and van de Waals force etc. (Ninham, 1999). Understanding the interplay of different forces is crucial to characterize and predict the colloidal stability of ENPs in solutions.

1.4.2.1 Surface charge

Zeta-potential is commonly used as one of the indicators for colloidal stability, which measures the mobility of charged particles with applied electrical potential (Hunter, 2013). High magnitude of zeta-potential stabilizes particles that are similarly charged, either negatively or positively depending on the surface chemical groups. Although a zeta-potential with a magnitude higher than 30 mV can be used as a rough cutoff for being stable (S. Y. Lin, Wu, & Chen, 2006), no single value can be used to determine the true stability of a colloidal system, and a small magnitude of zeta-potential does not always indicate a poor colloidal stability (Zimmermann & Muller, 2001), therefore other parameters should be taken into account such as size distribution.
and chemical properties. Zeta-potential can also be used to verify the successful deposition of additional layers for multi-layer ENPs by monitoring the alternative zeta-potential changes (Biggs, et al., 2007; Schneider & Decher, 2008; Sukhorukov, et al., 1998). Since zeta-potential can be affected significantly by salt concentration and pH (Gustafsson, Mikkola, Jokinen, & Rosenholm, 2000), attention should be paid to sample preparations to ensure the same solvent property is maintained to obtain representative results.

1.4.2.2 Surface hydrophobicity

Surface hydrophobicity influences intermolecular interactions between ENPs with other ENPs or between ENPs with ligands. Molecules with strong surface hydrophobicity causes flocculation if not well diluted or well separated by strong electrostatic or steric repulsions, therefore will affect the functionality and stability of ENPs in complex matrices, especially in highly concentrated systems. No consensus has been reached on the standard method to quantify hydrophobicity, and fluorescence probes based methods gained popularity because of their easy accessibility and simplicity (Haskard & Li-Chan, 1998). When binding with the hydrophobic groups of ENPs such as protein nanoparticles, the maximum emission wavelength and fluorescence intensity of hydrophobic probes will be changed because of the change of their environment (Hayakawa & Nakai, 1985; Kato & Nakai, 1980). Some hydrophobic probes can be used include anionic molecules such as 1-(anilino)-naphthalene-8-sulfonate (ANS-), 6-(p-toluidinyl)naphthalene-2-sulfonate (TNS-), cis-parinarate (CPA-), and also some neutral molecules like diphenylhexatriene (DPH), 6-propionyl-2-(N,N-dimethylamino) naphthalene (PRODAN) (Cardamone & Puri, 1992; Haskard, et al., 1998; Tsutsui, Lichan, & Nakai, 1986; Weber & Farris, 1979). Another method can be used to measure hydrophobicity of ENPs is hydrophobic chromatography, which not only measures the hydrophobicity, but also separates
particles based on their interactions with the gel matrix due to their differentiated surface hydrophobicity (Roettger & Ladisch, 1989).

1.4.2.3 Surface composition

The surface composition is important when a special function of ENPs is after, such as surface functionalized ENPs for uses in site specific delivery by attaching special ligand, antibody, and aptamers (Kocbek, Obermajer, Cegnar, Kos, & Kristl, 2007; Thorpe, 2004; Tong, Yala, Fan, & Cheng, 2010). Surface elemental analysis can be done by attaching other modules to EMs, such as energy dispersive X-ray spectroscopy (EDS) or X-ray photoelectron spectroscopy (XPS). The differences in electron scattering and absorption by different chemical elements or atoms can be collected to determine the surface composition. For ENPs designed with special molecular arrangement to achieve certain functions such as colloidal stability, the surface molecular composition can potentially be determined by selectively removing the surface molecules using enzymes, changing ionic strength of the solvent by adding salts, or by adjusting pH to weaken the electrostatic attractions between different components, and subsequent analysis of the collapsed/detached molecules (Chen & Zhong, 2014). A classic example is the surface molecular characterization of casein micelle. Although the exact model of casein micelle has not been determined, the surface of the micelle is agreed to be rich in κ-casein, which was confirmed by the fact that casein micelle will aggregated after using chymosin to selectively cut the macropeptide (residue 106-169), which can then be identified by analyzing peptide composition remained in the serum (Dalgleish & Corredig, 2012). For emulsion systems, surface macromolecules could be displaced from the droplet surface by small surfactant molecules such as Tween 20 through competitive absorption, and the detached molecule can be subsequently qualitatively and quantitatively determined (Dickinson & Tanai, 1992; Mackie, Gunning, Wilde, & Morris, 2000).
Although it is possible to indirectly access the surface molecular structure by the aforementioned methods, locating the exact position of molecules that are interested in is not easy and accurate. AFM with its advantage of sub-nanometer resolution can be modified to map the detailed molecular structure of ENPs. Functionalized AFM tips with special chemical groups or antibody attached have been developed for elemental analysis or probing specific proteins by monitoring the changes of force curve due to specific binding between specific receptor and ligands (Dufrene & Hinterdorfer, 2008; Dupres, Verbelen, & Dufrene, 2007; Hinterdorfer & Dufrene, 2006). Recently development of AFM coupled with infrared spectroscopy with molecular level resolution also makes AFM-IR a powerful tool for nanoscale compositional mapping (Dazzi, et al., 2012; Lo, Prater, Dazzi, Shetty, & Kjoller, 2012).

### 1.4.3 Internal structures

For ENPs developed by extensive internal structure modifications such as crosslinking, or nanocomplex systems with mixed particles and entangled chain conformations, or delivery systems for bioactive compounds, the internal structures of the ENPs are of interest to understand the mechanism of molecular assembling and to predict their functionalities *in vitro* and *in vivo*.

#### 1.4.3.1 Electron microscopy

TEM and cryo-TEM can be used to differentiate two components that have distinct ability to absorb or reflect electrons, and thus the internal structures can be visualized at high resolutions. For example, because of the distinct difference in electron density between Ag and chitosan, the position and morphology of Ag embedded inside chitosan nanoparticle can be clearly visualized as shown in Figure 1-3 (Sun & Li, 2004; Yue, et al., 2011). Confocal laser fluorescence microscopy scans fluorescence emission from particles that were treated with different fluorescence markers. The technique has the advantage to focus and scan the inner structure of ENPs by a point-to-point
mechanism and the three dimensional structure can be constructed (Tiede, et al., 2008). This technique is especially useful for study the uptake of ENPs by cells and the visualization of delivery system structures by premixing different fluorescence markers with each component before they are integrated into ENPs (Kim, Ohulchanskyy, Pudavar, Pandey, & Prasad, 2007; Lamprecht, Schafer, & Lehr, 2000; Y. C. Luo, Teng, Wang, & Wang, 2013; Yun Zhang, Lin, & Zhong, 2015).

1.4.3.2 Spectroscopy based methods

Besides direct observations of macro-structure through microscopes, the internal micro-structure and interactions between different molecules of ENPs can also be probed by spectroscopy based methods. During the formation of ENPs, it is important to understand the structural changes of both encapsulant and compounds being encapsulated. The changes might be related to secondary structural breakdown or formation for protein materials, or crystal structure modifications at a broader scope for macromolecules. Fourier transform infrared spectroscopy (FTIR) can be used to identify the chemical group modification and interactions within a molecule complex and to resolve the secondary structure of proteins or peptides after applying spectra processing and fitting algorithms (K. Fu, Griebenow, Hsieh, Klibanov, & Langer, 1999; van der Weert, et al., 2000). Circular dichroism (CD) can be used to both qualitatively and quantitatively determine the secondary structural changes of proteins and peptides during a process like heating, pH adjusting, as well as addition of small molecules or ionic salts (Johnson, 1988; Sreerama & Woody, 2000). The structural changes before and after a process can be easily identified by looking at the peak position and shape changes of the spectra, and the quantitative changes of secondary structures can be solved by applying different fitting algorithms (Sreerama, et al., 2000). X-ray crystallography (XRD) is commonly used as a way to confirm the successful encapsulation
of small crystal molecules like curcumin, β-carotene (Pan, Zhong, et al., 2013; L. H. Zhang, Hayes, Chen, & Zhong, 2013). The absence of sharp crystalline peaks corresponding to their original crystal structure indicates that target molecules are either being embedded inside the wall material or are interacting and binding with the wall materials through which the crystallization has been inhibited.

1.4.3.3 Differential scanning calorimetry (DSC)

DSC applies heat to access the molecular mobility of samples either in solid or in liquid form. The changes of side chain thermodynamics can be determined by looking at the changes in melting or glass transition behavior. For example, DSC can be used as a complementary technique to confirm the successful encapsulation and the disappearance of crystal structure of small molecules with the absence of melting or crystallization peaks in a thermo curve (Pan, Zhong, et al., 2013). DSC has also been widely used to measure the miscibility of two macromolecules by looking at the shifting of glass transition temperatures. A good miscibility is indicated by the absence of individual glass transition temperatures and the presence of a new transition curve with on-set and ending temperatures spreading wider than the individual ones, and a new transition temperature will appear in between of individual temperatures (Herrera, et al., 2005; Sakurai, Maegawa, & Takahashi, 2000). This technique has an advantage compared with other spectroscopy based methods since the lighting source, either as optic, x-ray, or neutron, may be interfered or blocked by the surface structure of the ENPs, leading to a limited accessibility of the inner core structure, especially for dry solid ENPs. However, the good penetration property of heat makes DSC a very useful technique to study interactions between different molecules.
1.4.3.4 Light scattering and electrophoresis

SLS, SANS and SAXS, which provide the dimensional information of ENPs with higher precisions, are power tools to study the structures or conformation of ENP molecules in addition to the size and aggregation behavior, even for polydispersed colloidal systems (Hayter, et al., 1983; Holt, de Kruif, Tuinier, & Timmins, 2003; Kotlarchyk & Chen, 1983). Gel electrophoresis is a widely used technique to separate biopolymers based on their electrophoretic mobility, especially proteins, peptides, and nucleic acids (Shapiro, Vinuela, & Maizel, 1967). Polyacrylamide gel electrophoresis (PAGE) can be run in both native and denatured mode with the higher order structures being preserved or disrupted respectively. PAGE without uses of denaturation agents are called Natave-PAGE, and the migration pattern depends on the charges as well as the hydrodynamic sizes of the ENPs. Urea and SDS are two most commonly used detergent to denature the higher dimensional structures for identification of the molecular weight and number of nucleic acids or proteins respectively, which are called Urea/SDS-PAGE. Reducing agents such as dithiothreitol (DTT) or 2-mercaptoethanol can be added to further break the disulfide bonds and are called reducing SDS-PAGE. Information about stabilizing forces such as hydrogen bonds and disulfide bonds can thus be identified by combined running of native PAGE, native SDS-PAGE, and reducing SDS-PAGE and comparing the band shifting after sequentially breaking of different interactions.

1.4.4 Biological properties

One of the benefits of ENPs is to deliver bioactive compounds while maintaining their original bioactivity. Therefore, the bioactivity of the ENPs as well as the encapsulated compounds need to be evaluated.
1.4.4.1 Antioxidant activity

Antioxidant activity is one of the most important bioactivities that nutritional foods are targeting for, and can be qualitatively and quantitatively measured by different methods based on two different mechanisms of reactions: Hydrogen atom transfer and electron transferring. Hydrogen atom transfer based methods measures the ability of antioxidant to quench free radicals by donating hydrogen atom, and includes methods like oxygen radical absorption capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), total oxyradical scavenging capacity assay (TOSCA), Crocin bleaching assay, inhibition of linoleic acid oxidation, inhibition of LDL oxidation assay. Another mechanism is based on electron transferring from antioxidant compound to reduce any compounds like metals, carbonyl groups, and radicals, and includes assays such as Trolox equivalence antioxidant capacity (TEAC) assay / 2,2’-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) assay, total phenolics assay by Folin-Ciocalteu reagent assay, copper(II) reduction capacity assay, FRAP (ferric reducing antioxidant potential) assay, total oxidant scavenging capacity (TOSC) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and N,N-dimethyl-p-phenylenediamine radical (DMPD+) scavenging assay (Gulcin, 2012; D. J. Huang, Ou, & Prior, 2005; Prior, et al., 2003; Re, et al., 1999; Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Byrne, 2006). Usually a combined use of assays from different categories is recommended to better understand the chemistry behind the reactions and to fully describe the antioxidant capacity of a bioactive compound.

1.4.4.2 Digestion, absorption, and toxicity

Digestion of organic ENPs is mainly due to enzymes in alimentary tract before their absorption by epithelia cells after being administrated orally. Because of the changed surface structure, the digestion and absorption behavior of ENPs may be altered, and the potential toxicity
of ENPs is a rising concern in applications of nanotechnology in food industry (Szakal, et al., 2014). *In vitro* digestibility test can be done by mixing ENPs in buffer solutions prepared according to recipes to simulate gastric and small intestinal (GI) fluids (T. T. Fu, Abbott, & Hatzos, 2002; Y. Zhang & Zhong, 2015). The resultant mixtures can be taken for compositional and physical chemical analysis using methods aforementioned such SDS-PAGE for protein degradation and EM observation for particle dimension and morphology etc.. Because of the possible interference on the analysis by enzymes introduced in the simulated GI fluids, ENPs separation using centrifugation or chromatography might be needed before the analysis are conducted. *In vitro* intestinal absorption of ENPs before and after digestion can be studied using epithelial cells such as Caco-2 monolayer cell model, and the transportation rate as well as the transport mechanism (active versus passive) can be determined by measuring the concentration of ENPs in both sides of the monolayer cells (Davda & Labhasetwar, 2002; Y. C. Luo, et al., 2013). *In vivo* digestion can be performed using animal models and the fluids in gastric, small intestinal, and colon can then be collected and analyzed to understand the fate of ENPs in each alimentary tract. *In vivo* absorption, and potential toxicity effects of intact ENPs (such as ENPs coated with polysaccharides that will not dissolve or digested by GI fluids) can be determined by direct organs analysis, blood collecting, and urinary excretion etc. (Alger, Momcilovic, Carlander, & Duncan, 2014). The amount of the ENPs being adsorbed and circulated can be monitored in a certain time of period to evaluated the retention and potential toxicity of ENPs.

### 1.5 Utilizations of organic nanoparticles

A wide range of ENPs have been developed for applications in complex food or nutraceutical matrices to improve the functionalities such as sensory properties including dispersion stability, gelation, viscosity, and turbidity. Protection of labile compounds is another
important application of using ENPs as delivery systems to prevent the compounds from degradation caused by environmental stresses, and a location and rate controlled release properties can be achieved. Organic ENPs used as edible packaging materials and used to stabilize Pickering emulsion and foams are emerging applications of nanotechnology in food systems, which are evidenced by their promising effect compared with conventional materials.

1.5.1 Dispersibility and Sensory properties

Sensory properties are a major concern directing product development of consumer packaging products, which may be influenced by physical chemical properties like dispersion stability, gelation, viscosity, turbidity, and color of food components. Since turbidity and dispersion stability can be improved by reducing the particle dimension, nanotechnology has been used as a powerful tool by making food material from bulk phase or microparticle to nano-sized particles. Turbidity is caused by the reflection of light from large particles, and therefore reducing particle dimension is directly related to the decreasing of turbidity. For example, casein micelles contribute to the opaque appearance of milk because of the relatively large particle size and their association with fat globules. By breaking the micelles into particles of average size smaller than 100 nm, a translucent appearance was achieved, which enabled application of milk powder in transparent or translucent beverages without affecting their visual appearance significantly (Pan & Zhong, 2013). Through the utilization of emulsion and encapsulation technologies, aqueous dispersibility/stability of lipophilic compounds has been improved dramatically after being engineered into nanoemulsions or nanoparticles, enabling numerous applications of innovative ingredients in food systems especially in beverages. Curcumin is one of the most extensively studied bioactive compounds which has negligible solubility in water with very strong antioxidant and potential anti-cancer activity (Soudamini & Kuttan, 1988). Biopolymer based curcumin
encapsulation systems showed significantly improved water dispersibility with clear appearance and also with potentially improved cell uptake properties (Pan, Luo, et al., 2014; Pan, Zhong, et al., 2013). Studies about essential oil encapsulation also showed promising effect for applications in beverages as natural and organic antimicrobial agents (Y. Luo, et al., 2014; Pan, Chen, Davidson, & Zhong, 2014; Xue, Davidson, & Zhong, 2013). Viscosity of a colloidal system is affected by the concentration, molecular size and chain configuration. Chain breakage is considered as an indicator of decrease in viscosity and causes the weakening of gel strength (Albersheim, Neukom, & Deuel, 1960; Toyohara, Sakata, Yamashita, Kinoshita, & Shimizu, 1990). However, when self-assembled into long nanofibrils, systems with the same concentration of material can have a dramatically increased viscosity and a lower gelling concentration (Krysmann, et al., 2008; G. Liu & Zhong, 2013). The functional nanofibril is promising to be used as thickening agents to replace starch by increasing the content of protein while decreasing the content of carbohydrates. Nanotechnoloy also enabled the applications of more stable natural colors and flavors, especially lipophilic compounds, to replace synthetic colors (Matalanis, Jones, & McClements, 2011; Szente, Mikuni, Hashimoto, & Szejtli, 1998; Y. Zhang & Zhong, 2013).

1.5.2 Protection of labile compounds from degradation by environmental stresses.

A lot of bioactive compounds are sensitive to environmental stresses such as heat, UV radiation, and extreme pH conditions. ENPs can be used to provide additional protection to labile compounds against degradation. Both hydrophilic and hydrophobic compounds have been shown to have significantly improved stability against processing after being encapsulated in biopolymers (Guan & Zhong, 2014; Wu, Guan, & Zhong, 2015). ENPs can also be designed with structures to circumvent the digestion/degradation of inner materials in gastric or intestinal environment by applying another layers of indigestible compounds such as polysaccharides. This mechanism is
especially useful for delivery of bioactive polyphenols, proteins or peptide, and RNA or DNA for therapeutic uses by preventing them against enzymatic attacks in GI tracks (Sinha & Kumria, 2001). The developed ENPs could have colon specific delivery property for treatment of colon related diseases such as bowl disease (Y. C. Luo & Wang, 2014).

1.5.3 Delivery and controlled release:

ENPs can be manipulated to have special release properties depending on intended applications. Based on the releasing behavior or speed, delivery systems can be classified as gradual release and triggered or burst release systems. Gradual release can be accomplished either through gradual dissolving and disintegration of particle structures (Figure 1-4a), or through migration of inner material based on the diffusion mechanism for insoluble ENPs with defined geometry (Figure 1-4b) (Siepmann & Siepmann, 2012; Uhrich, Cannizzaro, Langer, & Shakesheff, 1999). Triggered release can be initiated through the change of environmental conditions such as pH, temperature, enzymes, or light (Figure 1-4c) (Chung, et al., 1999; Fomina, McFearin, Sermsakdi, Edigin, & Almutairi, 2010; Murthy, et al., 2003; Veronese, et al., 2005). The biological or chemical reactions could be used as triggers to design ingredients with site specific burst release functions to improve bioactivity/bioavailability of the encapsulated compounds, and can also be utilized to develop novel indicators for process control of proper heating and storage of food products. Particles designed using layer-by-layer deposition is commonly used to deliver orally administrated drugs and nutraceuticals based on the acidity difference in stages of alimentary tracts. Surface coating using acid and enzyme stable polysaccharides suppressed the release in gastric condition and enhanced the retention and stability of bioactive compounds for release in small intestinal (Y. Zhang, et al., 2015). Tumor cells have lower pH of around 6.5 compared with physiological pH around 7.4, based on the difference in acidity, polymeric micellar systems and
multiplayer polyelectrolyte nanoparticles were demonstrated to have a burst release effect near tumor cells, thus increased the delivery efficiency and lowered the side effects on normal cells (Du, Sun, Song, Wu, & Wang, 2010; Poon, Chang, Zhao, & Hammond, 2011).

1.5.4 Edible packaging/coating

Edible packaging/coating have a lot of applications in foods, especially in fresh produce that have direct contact with open environment and easy to be contaminated. Packaging incorporating nanotechnology is easier to be accepted as a packaging component rather than direct addition into bulk food phase for consumption, and may have less safety concern if no migration of ENPs into food is expected, therefore might be more welcome from product development perspective (Duncan, 2011). Using ENPs as a component of film and packaging material has the advantage to produce clear or transparent coatings, which will not affect the perception of native food properties. Mechanical properties can also be improved using ENPs. For example, chitosan and chitosan/tripolypophosphate ENPs were found to improve the mechanical strength and barrier effect as well as thermal stability of edible cellulose-based films without affecting its homogeneity (de Moura, et al., 2009; de Moura, Lorevice, Mattoso, & Zucoletto, 2011).

1.5.5 Functional particles: Pickering emulsions and foams

Pickering emulsion is a type of emulsion system that is stabilized by solid particles or nanoparticles. The long term stability of Pickering emulsion is achieved by the irreversible adsorption of particles to the water-oil or water-air interface. Numerous food grade materials have been developed and utilized to prepare and stabilize Pickering emulsions, such as particles or nanoparticles of lactoferrin, pea proteins, corn proteins (zein), soy proteins, cellulose, starch, and lipids, which can be used for both O/W and W/O emulsions (de Folter, van Ruijven, & Velikov, 2012; Gupta & Rousseau, 2012; Kargar, Fayazmanesh, Alavi, Spyropoulos, & Norton, 2012; Lee,
Blaker, Murakami, Heng, & Bismarck, 2014; Liang & Tang, 2014; F. Liu & Tang, 2013; Shimoni, Levi, Tal, & Lesmes, 2013; Tan, et al., 2012). Incorporation and stabilization of bubbles and foams are gaining more attention for utilizations to provide special textual characteristics of foods such as ice cream (Goff & Vega, 2007). Due to the high free energy and the thermodynamic instability of air-liquid interface, bubbles coalescence and Ostwald ripening may occur to reduce the interfacial area, causing the instability of foams. Stabilization of aerated systems is typically more difficult to achieve than emulsions because of the low density of bubbles and the diffusive mass transport between different sized bubbles due to the local Laplace pressure gradients (Dickinson, 2010). Rigid and strong particles are needed to give structural stability to the air and water interface. This requires compounds with strong and compact structure in the lamella between each bubble and can stay stable within a long time. Numerous systems have been studies using inorganic nanoparticles such as silica to stability bubbles, which provided supreme stability for days or weeks (Binks & Horozov, 2005; Dickinson, Ettelaie, Kostakis, & Murray, 2004; Gonzenbach, Studart, Tervoort, & Gauckler, 2006). However, considering the normally large quantity consumption characteristics of foods such as ice cream, the applications or inorganic ENPs in food systems would be questionable. Some biopolymer originated materials such as hydrophobic cellulose which is commonly used as coating material in pharmaceutical products have been developed and showed excellent long term foam stability that is comparable to inorganic nanoparticles (Wege, Kim, Paunov, Zhong, & Velev, 2008). Protein based particles or nanoparticles are promising alternatives to inorganic and chemically modified biopolymer nanoparticles for applications in bulk food systems. Several protein nanoparticles such as ovalbumin, β-lactoglobulin processed by heat have shown significantly improved foam stabilization ability than the native proteins, which could be contributed from the increased surface
hydrophobicity after pre-heating and particle aggregations (Croguennec, Renault, Beaufils, Dubois, & Pezennec, 2007; Moro, Baez, Busti, Ballerini, & Delorenzi, 2011).

1.6 Nanostructure of caseins and applications as delivery systems

1.6.1 Structure of casein micelles

Bovine milk is an important part of our daily diet for its rich contents such as milk protein, Vitamins, minerals, and sugars. Caseins is the major milk protein components of milk, consisting of about 80% of the bovine milk proteins. Native caseins in bovine milk present in spherical micellar form, with diameters in the range of 50-250nm (Udabage, McKinnon, & Augustin, 2003). There are four different types of caseins, αs1-, αs2-, β-, and κ-caseins with an approximate ratio of 11:3:10:4 and differs in different genetic variants (Chianese, et al., 1996; Erhardt, 1996; Holt, 1992). Properties of different types of caseins vary depending on their amino acid sequences. αs1-casein is the highest negatively charged casein and has a higher content of phosphate than other types of caseins, while αs2-casein is the most Ca^{2+} sensitive among the four types; β-casein has the highest hydrophobicity because of the high portion of prolines, and κ-casein has a calcium insensitive segment called glycomacropeptide, which is hydrophilic and stabilizes the casein micelles by providing electrostatic and steric repulsions in their native state in milk (Walstra, Wouters, & Geurts, 2006).

Although it is agreed that the surface of casein micelles is rich in κ-casein, the internal structure of casein micelles is still under debate, and there are two major hypothetical models that gained popularity during the past decades (Farrell, Malin, Brown, & Qi, 2006; Home, 2006). The first model being proposed claims that intact casein micelles are composed of submicelles that are connected by calcium phosphate (Schmidt, 1982). The submicellar model was first being postulated from the observations that sodium caseinate (NaCas), which is produced by acid
precipitation and therefore is depleted of calcium phosphate, form smaller aggregates linked by noncovalent forces (Dalgleish, et al., 2012; Waugh, Creamer, Slattery, & Dresdner, 1970). The direct observation of submicelle was first being reported using electron microscopy, however was challenged recently due to the possible artifacts caused by staining and fixations (McMahon & Oommen, 2008; Schmidt, 1982). Another models proposed that each small cluster of calcium phosphate can be called nanoclusters with a radius of 2.3 nm, which are bounded by about 50 phosphopeptides (Holt, 1998). The micellar structure can then be formed with more highly phosphorylated αs1-casein and αs2-casien by crosslinking of the calcium phosphate nanoclusters (De Kruif & Holt, 2003).

The structure of casein micelle can be affected by several parameters during processing or storage. Dissociation of casein micelle was found to increase at a lower temperature, and β-casien accounted for most of the increase (~46%) (Downey & Murphy, 1970). Change of pH condition greatly affects the integrity of casein micelle, both slightly decreased pH and elevated pH to alkaline condition were reported to correlated with the dissociation of casein micelle, which lead to differences in solubility of calcium phosphate and the electrostatic interactions (Huppertz, Vaia, & Smiddy, 2008; Y. Liu & Guo, 2008; Zadow, 1993). Solvent quality also has a significant influence on the dissociation of casein micelles, and dissociation of casein micelle was observed by heating to above 60 °C in aqueous solution with ethanol concentration >35%, due to the enhanced solvent quality and shifted the pKa values of phosphosrine (John, Kelly, Fox, & de Kruif, 2001; Trejo & Harte, 2010). High pressure homogenization is a commonly used technology to process milk products, which can significantly reduce the size of casein micelles by disrupting the micellar structure with high energy input (Huppertz & de Kruif, 2006; Huppertz, Fox, & Kelly, 2004; Huppertz, Kelly, & de Kruif, 2006; Orlien, Knudsen, Colon, & Skibsted, 2006). Since
calcium and calcium phosphate are critical to the integrity of casein micelle, addition of chelating agents are also quite effective to dissociate the micelles and prevent their re-associations, such as sodium polyphosphate (Pitkowski, Nicolai, & Durand, 2008), EDTA or Citrate (S. H. Lin, Leong, Dewan, Bloomfield, & Morr, 1972; Udabage, McKinnon, & Augustin, 2000; Ward, Goddard, Augustin, & McKinnon, 1997), and urea (McGann & Fox, 1974).

1.6.2 Nanostructured casein as delivery systems

In addition to being an important source of calcium and essential amino acids, caseins also have very good emulsifying property that is related to its balanced content of hydrophobic and hydrophilic amino, which makes it a naturally-occurring amphiphilic block copolymer (Walstra & Jenness, 1984). Therefore casein nanostructures can be utilized for fabricating delivery systems of food and pharmaceutical compounds (Brugman, et al., 2004). By applying high pressure homogenization, reconstituted casein micelles were used to encapsulate vitamin D$_2$ in nanoparticles formed upon depressurization (Semo, Kesselman, Danino, & Livney, 2007), and the nanostructure was able to provide a partial protection against degradation induced by UV-light. Bovine β-casein was found to self-associate to form micelles in aqueous solution, and are capable of encapsulating lipophilic drugs in its hydrophobic core by forming nanoparticle with diameters below 100 nm (Shapira, Assaraf, Epstein, & Livney, 2010). Curcumin, a polyphenolic compound extracted from rhiizome of turmeric, was successfully encapsulated in both camel β-casein and bovine casein micelles through hydrophobic interactions, and water dispersibility of curcumin was increased more than 2500 folds with significantly improved antioxidant and cytotoxicity against HeLa and human leukemia cancer cell lines (Esmaili & Ghaffari, 2011; Sahu, Kasoju, & Bora, 2008).
1.7 Model bioactive compounds

There are numerous compounds, synthesized or naturally occurring, have been identified with various bioactivities. Plants are one of the major sources for natural bioactive molecules extractions, and the corresponding phytochemicals isolated have tremendous applications in food, nutraceutical, and pharmaceutical industries. Essential oils (EOs) are a group of bioactive compounds that contributes to the flavor and aroma of plants, and can be extracted from various plant parts (Burt, 2004). EOs were traditionally used as seasonings in foods and was recently exploited for their effective antimicrobial activities in commercial products, and some of them have been classed as GRAS food additives in US such as EOs extracted from Thyme, Rosemary, Sage, Cinnamon bark etc. (Burt, 2004). Affected by the extraction methods, the composition of EOs varies a lot and may contain hundreds of components. Thymol is the major component of thyme oil, which is extracted from herb thyme (Johny, Darre, Donoghue, Donoghue, & Venkitanarayanan, 2010). The strong antimicrobial activity of thymol was found to be related to the depolarization effects on the cytoplasmic membrane of bacterial (Xu, Zhou, Ji, Pei, & Xu, 2008). Another type of phytochemicals is polyphenols, which are the secondary metabolites of plants protecting plants from ultraviolet radiation and against pathogens (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004). Curcumin is one of the polyphenols that attracted tremendous attentions from researchers because of its significantly high activity to reduce cytotoxic and tumor, and its high antioxidant as well as anti-carcinogenesis against a wide range of cell lines (Babu, Shylesh, & Padikkala, 2002; Ruby, Kuttan, Dinesh Babu, Rajasekharan, & Kuttan, 1995). However, due to the low solubility of the phytochemicals, their applications in food or pharmaceutical industries are limited, especially in aqueous systems such as beverages. Therefore,
the utilization of nanoencapsulation technology will be studied in this thesis to improve their dispersibility and bioactivity, using thymol and curcumin as two model molecules.

1.7 Concluding remarks

ENPs produced from organic materials are promising to be used as edible functional materials compared with inorganic ENPs. Innovations in designing and manipulation of organic ENPs may bring more opportunities to formulate and develop functional foods with exceptional physicochemical, and biological properties. Current technologies commonly used to process ENPs were summarized as top-down and bottom-up methods. Characterizations are very important to understand the structures, interactions, and safety of ENPs, which are useful to predict and verify the efficacy of the designed systems. Wide ranges of utilisations of ENPs are evidenced by their applications to improve physical, chemical, and biological stabilities, and uses as delivery systems with controlled release properties, improving packaging mechanical and functional properties, as well as stabilization of Pickering emulsion and foam. By applying novel ideas for fabrication, characterization, and utilization of ENPs, more functional foods with superb physicochemical properties and balanced nutritional value may be developed in the future.

1.8 Hypothesis and overview of dissertation research

The overall hypothesis of this dissertation is that nanostructures can be controlled by programming the self-assembly properties of caseins after dissociation or hydrolysis. The nanostructures can be utilized to improve the clarity and stability of casein dispersions, to encapsulate and improve the dispersibility and bioactivity of lipophilic compounds, to stabilize macromolecules through co-assembly, and to form nanofibril materials with improved functionalities such as viscosity.
To test the hypothesis, Chapter 2 was focused on the structural changes of casein micelles in re-constituted skim milk dispersion. Supramolecular structures of casein micelles were dissociated by elevating pH to 11.0 and the subsequent acidification using citric acid was used to control the re-association of caseins. The following Chapter 3-6 were focused on improving the bioactivity and/or dispersibility of lipophilic bioactive compounds and macromolecules. Inspired by the excellent surface activity of caseins and their self-assembly properties, as well as the strong antimicrobial activity of essential oil components, Chapter 3 was focused on the encapsulation of thymol by NaCas applying high-speed homogenization. The interactions between caseins and thymol were studied and the stability of the delivery system from pH 3.0 to 7.0 was studied by addition of soluble soybean polysaccharide. The antilisterial activity of encapsulated thymol was compared with free thymol in modeled milk systems. A novel encapsulation method was then developed in Chapter 4, disrupting the nanostructure of NaCas in warm aqueous ethanol solution and the subsequent spray-drying were used to encapsulate the co-dissolved curcumin studied as an example of lipophilic compounds. The encapsulated curcumin was evaluated for water dispersibility and bioactivity. To potentially eliminate ethanol in encapsulation, research findings on structural changes of casein micelles in Chapter 2 were studied in Chapter 5 to develop a scalable low-cost, low-energy, and organic solvent-free encapsulation technology. This was done by dissolving NaCas and curcumin at pH 12.0 and subsequent acidifying to pH 7 to encapsulate precipitated curcumin in the self-assembled casein nanoparticles. The encapsulated curcumin was tested for anti-proliferation activity against human colorectal and pancreatic cancer cells in vitro. Similarly, Chapter 6 applied the pH-cycle method to stabilize zein, a group of very hydrophobic protein extracted from maize. The produced zein-NaCas nanoparticles were compared with those prepared with a conventional anti-solvent precipitation method involving ethanol. The last chapter
(Chapter 7) studied the ability of caseins to assemble into nanofibril structures after acid hydrolysis, and physicochemical properties of the nanofibrils were studied. The formed nanofibrils can potentially be used as functional materials such as thickeners to provide both nutrition and functional properties.
References


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Appendix

Figure 1-1. Three categories of high-pressure homogenizers: (a) a radial-diffuser in the example of a flat-valve, (b) a counter-jet-dispergator in the example of a microfluidizer, and (c) an axial flow nozzle system in the example of an orifice valve. (Adapted from Schultz et al., 2004 (Schultz, et al., 2004).)
Figure 1-2. Principle of the liquid–liquid dispersion process to produce zein nanoparticles (Adapted from Zhong et al. 2009 (Zhong & Jin, 2009).)
Figure 1-3. TEM images of Ag-Nanoparticles (a) and Ag-Chitosan-Nanoparticles (b). (Adapted from Yue et al. 2011 (Yue, et al., 2011).)
Figure 1-4. Schematic mechanisms of controlled release: (a) delayed dissolution, (b) diffusion controlled, and (c) triggered release.
Chapter 2. Improving clarity and stability of skim milk powder dispersions by dissociation of casein micelles at pH 11.0 and acidification with citric acid
A version of this chapter was originally published by Kang Pan and Qixin Zhong:

Pan, K., & Zhong, Q. (2013). Improving clarity and stability of skim milk powder dispersions by dissociation of casein micelles at pH 11.0 and acidification with citric acid. *Journal of Agricultural and Food Chemistry*, 61(38), 9260-9268. My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing.
2.1 Abstract

Casein micelles in milk cause turbidity and have poor stability at acidic conditions. In this study, skim milk powder dispersions were alkalized to pH 10.0 or 11.0, corresponding to reduced particle mass. In the following acidification with hydrochloric or citric acid, the reformation of casein particles was observed. The combination of treatment at pH 11.0 and acidification with citric acid resulted in dispersions with the lowest turbidity and smallest particles, which enabled translucent dispersions at pH 5.5-7.0, corresponding to discrete nanoparticles. The concentration of ionic calcium was lower when acidified with citric acid than hydrochloric acid, corresponding to smaller particles with less negative zeta potential. The pH 11.0 treatment followed by acidification with citric acid also resulted in smaller particles than the simple chelating effects (directly implementing sodium citrate). The produced casein nanoparticles with reduced dimensions can be used for beverage and other novel applications.

Keywords: skim milk powder, dispersibility, alkaline dissociation, casein micelles, citric acid, nanoscale structure
2.2 Introduction

Bovine milk is an important source of proteins, vitamins, minerals, and energy. Caseins consist of about 80% of the bovine milk proteins, with the rest being whey proteins (Fox, 2003). There are four types of caseins (\(\alpha_s1\), \(\alpha_s2\), \(\beta\), and \(\kappa\)) that exist in bovine milk as mostly spherical casein micelles with a diameter of 50-250 nm (Udabage, McKinnon, & Augustin, 2003). Although the internal structure of casein micelle is still being debated, it is generally agreed that hydrophobic interactions and bridging by calcium phosphate are important to the micelle structure (Fox & Brodkorb, 2008; Udabage, McKinnon, & Augustin, 2000). The \(\kappa\)-casein is located mostly on the outer surface of micelles, with the calcium-insensitive segment (glycomacropeptide) not being associated with other caseins (Waugh & von Hippel, 1956). The glycomacropeptide protrudes from the micelle surface by about 10 nm and acts as a “hairy layer” providing repulsive steric interactions that stabilize casein micelles against aggregation in bovine milk with acidity of about pH 6.8 (Dalgleish, 1998; Holt & Dalgleish, 1986; Tuinier & De Kruif, 2002).

Fabrication of casein structures is important for several applications. Casein micelles are sufficiently big to scatter visible light, and disruption of casein micelles is required to incorporate milk protein ingredients in beverages that are transparent or translucent. There also has been great interest in applying dairy proteins as encapsulants to deliver bioactive food components in food matrices (Gunasekaran, Ko, & Xiao, 2007; Haham, et al., 2012; Rosenberg & Sheu, 1996; Semo, Kesselman, Danino, & Livney, 2007). Particularly, because nanoscale delivery systems have unique properties such as dispersion stability and low turbidity, dissociation of casein micelles to smaller structures may be important for developing relevant applications.

There are several methods that have been studied to control casein structures. High pressure treatment is a well-studied physical method (Huppertz & de Kruif, 2006; Huppertz, Fox, & Kelly,
that changes the arrangement of water molecules around proteins (Hvidt, 1975) and thus hydrophobic interactions between caseins. However, the process is energy consuming because a pressure of 150 MPa is needed to trigger the dissociation of casein micelles (Orlien, et al., 2010). When heated to above 65 °C in aqueous ethanol with more than 35% ethanol, dissociation of casein micelles was observed because of the improved solvent quality and the shifting of pKa values of phosphoserine (John, Kelly, Fox, & de Kruif, 2001; J.E. O’Connell, Kelly, Auty, Fox, & de Kruif, 2001).

Dissociation of casein micelles at acidic or alkaline pH has also been studied at ambient conditions without applying high energy and ethanol. Dissociation of casein micelles was observed during acidification from neutral pH to about pH 5.4, followed by re-association upon further acidification (McMahon, Du, McManus, & Larsen, 2009). The dissociation during acidification is favored at a lower temperature (Dalgleish & Law, 1988; Singh, Roberts, Munro, & Teo, 1996; Vreeman, van Markwijk, & Both, 1989). The alkaline dissociation of casein micelles has been studied by Vaia et al. (Vaia, Smiddy, Kelly, & Huppertz, 2006), with a higher dissociation extent at a higher temperature and a faster dissociation rate at a higher pH. The authors also reported that the alkaline disintegration of casein micelles was largely reversible and the re-associated caseins could closely resemble the properties of native casein micelles if controlled properly. The reversibility of casein micelle structure after alkaline disruption is probably caused by the incomplete dissociation at the studied pH (up to pH 10.0) conditions and the increased solubility and activity of calcium during reformation.

Additionally, several calcium-chelating agents such as ethylenediaminetetraacetate (EDTA), phosphate, and citrate have been reported to be effective in dissociating casein micelles (Lin, Leong, Dewan, Bloomfield, & Morr, 1972; Odagiri & Nickerson, 1964; Udabage, et al.,
2000). These compounds are competing with calcium in casein micelles that is present as colloidal calcium phosphate (Fox, et al., 2008; Horne, 1998). Colloidal calcium phosphate plays an important role in the association of calcium-sensitive caseins and, together with hydrophobic interactions, is critical to the structure of casein micelles. The loss of calcium in micelles causes the dissociation of β- and κ-caseins from casein micelles (Lin, et al., 1972).

The existing literature suggests the possibility of dissociating casein micelles at alkaline conditions and controlling the reformation of casein micelles during the subsequent acidification process, which can be further controlled by chelating agents such as citrate. In this work, our overall objective was to study the structural reformation after acidification of alkaline-treated dispersions constituted from skim milk powder, with and without citrate. Skim milk powder was chosen because of the need to improve stability, especially at acid pH. Particularly, the controlled reformation of casein micelles to smaller dimensions is desired because nanostructures reduce turbidity and improve the stability against gravitational sedimentation due to the significance of Brownian motion. To understand structure reformation, dispersions were characterized for macroscopic turbidity, hydrodynamic diameter using dynamic light scattering (DLS), morphology using atomic force microscopy (AFM), and ionic calcium concentrations. The engineered nanostructures enable the expanded use of milk protein in the beverage, cosmetic, and pharmaceutical industries where transparent appearance is desired. The improved understanding of nanoscale structure formation during acidification may also be significant to develop relevant nanoscale delivery systems discussed previously.
2.3 Materials and methods

2.3.1 Materials

Skim milk powder (Carnation® nonfat dry milk) was a product from Nestlé Baking Inc. (Solon, OH). According to the label, the product had 34.8% protein, dry-basis, and was a pasteurized product. Other chemical grade reagents were purchased from Fisher Scientific (Pittsburgh, PA) or Bio-Rad Laboratories, Inc. (Hercules, CA).

2.3.2 Sample preparation

Sample preparations were conducted at room temperature (21 °C). Skim milk powder was hydrated at 9% w/w in deionized water for 6 h. The control samples were acidified directly using 4.0 M HCl. To dissociate casein micelles, samples were adjusted to pH 10.0 or 11.0 with 4.0 M NaOH and were incubated for 1 h (Vaia, et al., 2006). The reformation of casein micelles was studied by acidification to pH 7.0, 6.5, 6.0, and 5.5 with 2.0 M citric acid or 4.0 M HCl under vigorous agitation using a stirring plate. To simplify description, the pH cycle treatments in Table 2-1 are referred as different routes hereafter. Approximately 300 µL of 4.0 M NaOH was used to adjust 20 mL 9% w/w skim milk dispersion to pH 11.0 and 150 µL of 2.0 M citric acid was needed to subsequently acidify the dispersion to pH 7.0. Another set of samples were prepared by dissolving 0-60 mM dihydrate sodium citrate in the skim milk dispersion that was adjusted to pH 7.0 using 1.0 M NaOH or HCl after vigorous stirring for 2 h. To prevent microbial spoilage, 0.05% w/w sodium azide was added to each sample. Three independent replicates were prepared for each sample.
2.3.3 Turbidity measurement

To determine the normalized turbidity (Equation 1) using the literature method (Vaia, et al., 2006), a milk serum sample was prepared by centrifugal ultrafiltration. The 6 mL of the 9% skim milk dispersion at pH 7.0 was placed in a Vivaspin 6 centrifuge tube (Vivaproducts, Inc., Littleton, MA) with the bottom mounted with an ultrafiltration membrane with a molecular weight cut-off of 10,000 kDa, and the centrifugation was carried out at 10,000g for 20 min (model 4540, Eppendorff, Hamburg, Germany). The absorbance of milk serum \( \text{Abs}_{\text{serum}} \) and skim milk dispersions \( \text{Abs}_{\text{sample}} \) was measured at 600 nm using a UV-vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA).

\[
\text{Normalized turbidity} = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{serum}}}{\text{Abs}_{\text{untreated}} - \text{Abs}_{\text{serum}}} \quad (1)
\]

where \( \text{Abs}_{\text{untreated}} \) is the absorbance of skim milk dispersion without pH adjustment.

2.3.4 Particle size and zeta-potential

Zeta-potential and particle size distribution were measured for samples adjusted to pH 7.0 using a Delsa Nano analyzer (Beckman Coulter, Inc., Atlanta, GA). Particle size of samples directly adjusted to pH 9.0 – 11.0 and subsequently acidified from pH 11.0 to pH 7.0-10.0 using 4.0 M HCl or 2.0 M citric acid was also measured. Samples were diluted 100 times using deionized water adjusted to the corresponding pH using 0.05 M NaOH. Particle size distributions were used to calculate the volume-length mean particle diameter \( D_{4,3} \) using Equation 2.

\[
D_{4,3} = \frac{\sum n_i D_i^4}{\sum n_i D_i^3} \quad (2)
\]

where \( n_i \) is the number of particles corresponding to diameter \( D_i \).
2.3.5 Morphological studies

The skim milk dispersions at pH 5.5 and 7.0 were diluted to an overall solids content of 10 ppm in deionized water adjusted to the same pH using 0.05 or 1.0 M NaOH or HCl. Two μL of each diluted sample was spread evenly onto freshly cleaved mica sheets that were mounted on sample disks (Bruker Corp., Santa Barbara, CA) for AFM. A rectangular cantilever having an aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m (FESPA, Bruker Corp., Santa Barbara, CA) and a Multimode microscope (Bruker AXS, Billerica, MA) were used to scan the sample. Images were generated at the tapping mode with a preset scan area of 2.0×2.0 μm at a scanning speed of 1 Hz. The dimension of particles was analyzed using the particle analyzer function of the NanoScope Analysis software (Version 1.4, Bruker Corp., Santa Barbara, CA).

2.3.6 Determination of ionic calcium concentration

The concentration of ionic calcium in skim milk dispersions was determined with a calcium-selective electrode (Denver Instrument Company, Bohemia, NY). The standard curve was established using a series of solutions with 10-1000 ppm CaCl₂ and 0.08 M KCl in distilled water. Results were normalized by the concentration of ionic calcium in skim milk dispersion directly adjusted to pH 7.0 using 1.0 M NaOH.

2.3.7 Analytical ultracentrifugation (AUC)

A Beckman XL-I analytical ultracentrifuge (Beckman Coulter, Inc., Palo Alto, CA) was used in AUC experiments according to the procedures in our earlier work (Liu & Zhong, 2012). Briefly, samples were diluted to a protein concentration of 1.2 mg/mL in deionized water and readjusted to the target pH. The centrifugation was performed at 50000 rpm and 25 °C. The
SEDFIT software of the instrument was used to analyze data using the continuous $c(s)$ distribution model, with the anhydrous frictional ratio ($f/f_0$) set at 1.2. Because molecular weights ($M_w$) of individual caseins are higher than 19 kDa (Leonil, et al., 1995), signals corresponding to a $M_w$ higher than 19 kDa was compared and further used to calculate the number average $M_w$ of the mixture using the following equation.

$$M_w = \frac{\sum_{i=1}^{n} M_{wi} p_i}{\sum_{i=1}^{n} p_i}$$

(3)

where $M_{wi}$ and $p_i$ represent the $M_w$ and mass percentage of the $i^{th}$ fitted peak.

### 2.3.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed under the reducing conditions using a Mini Protein Tetra cell (Bio-Rad Laboratories, Inc., Hercules, CA) at a constant voltage of 200 V. The 15% Tris-HCl gel (Ready Gel Precast Gel) was from Bio-Rad Laboratories, Inc. (Hercules, CA). The gel was stained with Coomassie blue.

### 2.3.9 Occurrence of Maillard reaction

The possible Maillard reaction between dairy protein and lactose at the studied conditions can be qualitatively compared for absorbance at 420 nm ($Abs_{420}$) (Lerici, Barbanti, Manzano, & Cherubin, 1990). Dispersions were adjusted to pH 4.6 with 4.0 M HCl to precipitate caseins. After centrifugation at 10,000g for 20 min (model 4540, Eppendorf, Hamburg, Germany), the supernatant was transferred and was measured for $Abs_{420}$ using a UV-vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA).
2.3.10 Statistical analysis

Statistical analyses were performed using the JMP Statistical Software (SAS Institute, Cary, NC). One-way analysis of variance was carried out. Differences between pairs of means were compared using the Tukey test. The significance level P was set at 0.05.

2.4 Results and discussion

Although skim milk powder contains whey proteins, lactose and small quantities of lipids, caseins are mostly responsible for physicochemical changes observed in the present study. This can be ascribed to the fact that whey proteins do not usually precipitate at the pH range (5.5-11.0) presented below. The discussion hereafter is thus focused on casein only. Furthermore, it is expected that casein structures in skim milk powder dispersions are different from native casein micelles in bovine milk because of the processes used to prepare powder and reconstitution. We use “casein micelles (CM)” nevertheless hereafter for simplicity. Likewise, casein structures reformed from dissociated CM may not resemble CM because of the possible changes in composition (types of caseins and amounts of colloidal calcium phosphate), but they are called “reformed CM (rCM)” for simplicity and consistency with the literature (Huppertz, Vaia, & Smiddy, 2008; J.E. O’Connell, et al., 2003). The structures of CM and rCM are sensitive to the environment and could be changed when samples were diluted for characterization using a specific technique. Additionally, the skim milk powder used in this study is expected to be different from that directly spray-dried from skim milk, because surfactants such as lecithin can be used to improve product properties (Tran Le, et al., 2007). Heat treatment conditions used to produce skim milk powder can also significantly impact the properties of milk powder dispersions (Tran Le, et al., 2007), although it was shown that preheating at 90 °C for 10 min did not impact the alkaline
disruption of casein micelles (Vaia, et al., 2006). These variables were not investigated in the present study.

2.4.1 Structure characteristics at alkaline pH

The dispersions showed reduced turbidity when samples were gradually adjusted to pH 11.0 (Figure 2-1a), which agrees with Huppertz et al. (Huppertz, et al., 2008) who studied up to pH 10.0. The turbidity increased only slightly when acidified with HCl to pH 7.0 and was practically unchanged when acidified with citric acid (Figure 2-1a). This contrasts with the almost complete recovery of sample turbidity after the pH cycle (from 7.0 to 10.0 then to 7.0) found by Huppertz et al. (Huppertz, et al., 2008). When hydrodynamic diameters were measured, the reduction of $D_{4,3}$ was observed when pH was increased from 7.0 to 10.0, followed by an increase at pH 11.0 (Figure 2-1b). When samples were acidified from pH 11.0, $D_{4,3}$ gradually decreased for both acid treatments (Figure 2-1b). The data in Figure 2-1 showed alkaline treatments at pH 10.0, as in the work of Huppertz et al. (Huppertz, et al., 2008), and 11.0, in the present study, had different effects on casein structure evolution.

Because turbidity is both a function of particle dimension and density (Giroux, Houde, & Britten, 2010), AUC was used to study samples alkalized to pH 7.0-11.0 and acidified from pH 11.0 to 7.0 using HCl, with the structures bigger than 19 kDa summarized in Table 2-2. A monotonic decrease in particle mass was observed with an increase in pH during alkalization, which suggests a greater extent of CM dissociation at a higher pH. At pH 11.0, there was on average about 26 caseins per particle, based on an average MW of 25 kDa for caseins, that is about one-third of particle mass at pH 10.0. The bigger $D_{4,3}$ at pH 11.0 (Figure 2-1b) than that at pH 10.0 can then be caused by fewer loosely-associated caseins that take a larger volume. Physically, the dimension difference between pH 10.0 and pH 11.0 treatments can be contributed by basic amino
Acid residues such as lysine and tyrosine whose side chain pKa is 10.5 and 10.1, respectively (Walstra & Jenness, 1984). At pH 11.0, caseins are (negatively) ionized to a greater extent than at pH 10.0, which increases the hydrodynamic diameter of polyelectrolytes and the intra-particle repulsion, corresponding to the lowered turbidity (Figure 2-1a) and increased dimension (Figure 2-1b).

When samples were acidified from pH 11.0 to pH 7.0, the particle mass increased gradually (Table 2-2), which indicates the re-association of caseins, as reported by Huppertz et al. (Huppertz, et al., 2008). The reduced dimension during acidification (Figure 2-1b) can be interpreted by the increased particle density as the weakened electrostatic repulsion between basic amino acid residues decreases the excluded volume of associated caseins. The increased particle mass (Table 2-2) and the reduced dimension (Figure 2-1b) contribute to similar or slightly increased turbidity during acidification (Figure 2-1a). The drastic difference in particle mass (~3 times) after alkalization to pH 10.0 and pH 11.0 (Table 2-2) may have contributed to the differences between our findings and Huppertz et al. (Huppertz, et al., 2008). Physically, CM are dissociated to a greater extent at pH 11.0, which, analogous to the nucleation theory, provides more sites for attracting the dissociated caseins during acidification. This can increase the population and reduce the dimension of rCM and therefore turbidity for treatments at pH 11.0 when compared to those at pH 10.0. The following part focuses on samples at acidic and neutral conditions after processing using different routes in Table 2-1.

### 2.4.2 Occurrence of covalent bonding during treatments

Alkaline treatment of milk dispersions can potentially cause covalent bonding and Maillard-type reactions between protein and protein, and between protein and lactose (Hemar, Law, Horne, & Leaver, 2000). The impacts of different routes in Table 2-1 were studied for SDS-PAGE.
and Ab$_{s420}$, with the former examining $M_w$ changes of proteins and the latter the occurrence of Maillard reaction. SDS-PAGE data in supplementary Figure 2-S1 did not show apparent difference in $M_w$ of proteins. For Ab$_{s420}$, samples treated with Routes C (pH10/citric), D (pH11/HCl), and E (pH11/citric) had a significantly lower magnitude than those of A and B (Figure 2-S2A). As presented below, treatments with Routes A and B had much higher turbidity and bigger particle dimension, which corresponded to greater turbidity of supernatants after centrifugation of samples adjusted to pH 4.6 (Figure 2-S2B). Therefore, there is no indication of chemical reactions at the studied conditions.

2.4.3 Properties of alkaline-treated dispersions acidified to pH 5.5-7.0

2.4.3.1 Sample appearance and turbidity

Visual appearance and normalized turbidity of samples acidified to pH 5.5-7.0 are shown in Figure 2-2 and Figure 2-3, respectively. At pH 3.0-5.0, precipitation was observed for all samples and the precipitated proteins separated into a bottom phase below the transparent serum (not shown). At pH 5.5-7.0, sample turbidity varied significantly with methods of acidification and pH. Overall, samples were more turbid at a lower pH, which is expected because the reduced number of net negative charges and thus weakened electrostatic repulsion between caseins favor protein aggregation as pH approaches isoelectric point (pI) of ~4.6 (Walstra, et al., 1984). Samples acidified with Routes B (pH10/HCl) –E (pH11/citric) generally had lower turbidity than those directly acidified using HCl (Route A - control). Route B (pH10/HCl) was the least effective in reducing sample turbidity, while Route E (pH11/citric) was the most effective. Routes C (pH10/citric) and D (pH11/HCl) appeared to have had similar impacts on sample turbidity when pH was 7.0. At pH 5.5-6.5, citric acid was more effective than HCl in reducing turbidity. Results
in Figures 2-2 and 2-3 indicate that the dissociated CM reformed to different structures upon acidification using the studied routes.

Route B (pH10/HCl) was used by Huppertz et al. (Huppertz, et al., 2008), and our results verified the properties of rCM upon acidification from pH 10.0. By substituting HCl (Route B - pH10/HCl) with citric acid during acidification (Route C - pH10/citric), calcium ions are partially chelated by titrating citrate (Morr, 1967; Odagiri, et al., 1964) and have reduced availability for bridging calcium-sensitive caseins (Schmidt, 1982), corresponding to theoretically smaller rCM and reduced turbidity. For samples acidified from pH 11.0 using HCl (Route D - pH11/HCl), the effectiveness in lowering turbidity was similar to Route C (pH10/citric) (but without using citrate) at pH 7.0 but was less significant than Route C (pH10/citric) at pH 5.5-6.5 (Figures 2-2 and 2-3). The combined chelating effects of citric acid and extensive dissociation of CM at pH 11.0 enabled the greatest reduction in turbidity for Route E (pH11/citric).

2.4.3.2 Concentration of ionic calcium during acidification

To study the role of calcium in formation of rCM during acidification, concentrations of ionic calcium in samples prepared from Routes A (control), D (pH11/HCl), and E (pH11/citric) were determined (Figure 2-4). Direct acidification of dispersions from pH 7.0 using HCl (Route A - control) liberated calcium from CM, corresponding to a higher ionic calcium concentration at a lower pH. When dispersion pH was increased to 11.0, ionic calcium concentration was only 2.2 ppm. This can be caused by two effects: precipitation of calcium phosphate at alkaline pH and binding of calcium to highly negatively charged dairy proteins. Upon acidification from pH 11.0 using HCl, samples from Route D (pH11/HCl) had higher concentrations of ionic calcium than those from Route A (control), possibly because calcium phosphate initially liberated during the extensive dissociation of CM at pH 11.0 was gradually dissolved as ionic calcium during
acidification. As for samples from Route E (pH11/citric), citrate ions effectively chelated the liberated calcium ions during acidification, corresponding to a concentration not higher than the dispersion at pH 7.0 without derivitization.

Additionally, there was no difference in ionic calcium concentration between Routes D (pH11/HCl) and E (pH11/citric) at pH 9 and above (Figure 2-4), which agrees with no difference in turbidity and $D_{4,3}$ of the two treatments (Figure 2-1). At pH 7.0 and below, the higher ionic strength (ionic calcium) of treatments in Route D (pH11/HCl) than Route E (pH11/citric) weakens electrostatic repulsion due to the shortened Debye length and increases the possibility of particle aggregation, corresponding to higher turbidity and bigger $D_{4,3}$ for Route D (pH11/HCl) (Figures 2-1, 2-2 and 2-3).

**2.4.3.3 Particle size and morphology of dispersions**

The $D_{4,3}$ of dispersions adjusted to pH 7.0 is shown in Figure 2-5. Samples from Routes C (pH10/citric), D (pH11/HCl) and E (pH11/citric) have much smaller $D_{4,3}$ than those from Routes A (control) & B (pH10/HCl), with that from Route E (pH11/citric) being the smallest. The DLS results are in agreement with turbidity data in Figure 2-3. The correlation between turbidity and particle size of a colloidal dispersion suggests that, unlike at alkaline pH (Figure 2-1), particle size is the major contributor of turbidity at neutral pH when intra-particle repulsion is not as strong.

AFM was conducted to study the morphology of structures after acidification to pH 7.0 and 5.5 using Routes A (control) and E (pH11/citric) (Figure 2-6). At pH 7.0, spherical particles were observed for the sample from Route A (without derivitization - control), and the mean diameter of particles marked in azure was 84.4 nm. Although the particle dimension from AFM is smaller than the DLS result (~170 nm) because of the drying effect during AFM sample preparation, the particle mean diameter is in the 50-250 nm range of CM diameters reported in the
literature (Udabage, et al., 2003). When the dispersion was acidified to pH 5.5 using HCl directly (Route A - control), irregularly-shaped aggregates were observed, with a much bigger mean diameter of 172 nm (Figure 2-6a vs. 2-6b), and could have been caused by localized low pH when 4 M HCl was used in acidification. The coarse aggregate was evident as visible precipitates in Figure 2-2.

For samples prepared using Route E, discrete particles were observed at both pH (Figure 2-6c and 2-6d). At pH 7.0, mean particle diameter was about one-half of the sample prepared from Route A (control) (Figure 2-6c vs. 2-6a). This indicates fewer casein molecules in rCM when processed using Route E (pH11/citric) than Route A (control). The dissociation and reformation of CM also agree with the less spherical structures in Figure 2-6c than those in Figure 2-6a. At pH 5.5, the sample from Route E (pH 11/citric) showed individual particles with mean particle diameters similar to those in the sample at pH 7.0 (Figure 2-6d vs. 2-6c). AFM results support the turbidity data in Figures 2-2 and 2-3 and different stability against aggregation is due to structure differences of rCM processed with various routes.

**2.4.3.4 Zeta-potential of dispersions at pH 7.0**

The zeta-potentials of dispersions adjusted to pH 7.0 using different routes are shown in Figure 2-7. The data followed the opposite trend from the particle size (Figure 2-5). The pI of $\alpha_s$-1, $\alpha_s$-2, $\beta_s$, and $\kappa$-casein is 4.94, 5.23, 5.14 and 5.90, respectively, with net charges of -21.9, -17.1, -13.3, and -2.0 at pH 6.6, respectively (John E O'Connell & Flynn, 2007). Theoretically, smaller rCM correspond to a smaller quantity of $\kappa$-casein per particle and therefore the highest zeta-potential magnitude is expected for the sample processed with Route E (pH11/citric), which is opposite to results in Figure 2-7. Conversely, zeta-potentials in Figure 2-7 are in line with the increase of electrolytes associated with pH treatments. As a greater amount of NaOH is needed to
increase pH to 11.0 than 10.0 and a greater amount of citric acid (weak acid, with trivalent citrate) is used in acidification, the treatment processed with Route E (pH11/citric) to pH 7.0 has the highest ionic strength, which reduces the Debye length, electrophoretic mobility, and thus the measured magnitude of zeta potential (Walstra, 2002). The treatments of Routes A (control) and B (pH10/HCl) had similar zeta-potentials, which agrees with the results of Huppertz et al. (Huppertz, et al., 2008). It is likely that the above phenomena due to the alkalization to pH 10.0 and acidification to pH 7.0 with HCl are unable to cause statistical significance under the studied conditions.

Dephosphorylation of caseins by alkaline phosphase (Bingham, Carroll, & Farrell, 1972) or alkaline pH (Freimuth & Krause, 1980) can change pI and therefore surface charge and solubility characteristics of caseins. However, Freimuth and Krause (Freimuth, et al., 1980) observed no more than 0.5% dephosphorylation after 24-h treatment at a temperature below 30 °C and a pH no higher than 11. Because samples were treated at 21 °C and pH 11 for 1 h in the present study, dephosphorylation was not quantified.

2.4.4 Differences between chelating effects and structural reformation

Because CM can be dissociated by simply adding citrate (Morr, 1967; Odagiri, et al., 1964; Udabage, et al., 2000), the last set of experiments was used to study skim milk dispersions supplemented with 0-60 mM sodium citrate and adjusted to pH 7.0 using 1 M HCl, where 15 mM citrate was equivalent to the treatment using Route E (pH11/citric). The turbidity and $D_{4,3}$ of this group of treatments are shown in Figure 2-8. There was a negative correlation between the turbidity and the citrate concentration (Figure 2-8a), verifying that chelating of calcium by citrate directly leads to the dissociation of CM (Morr, 1967; Odagiri, et al., 1964). Smaller $D_{4,3}$ at a higher citrate concentration (Figure 2-8b) further verified the dissociation properties of the chelating agent. The
treatment with 15 mM citrate had a normalized turbidity of 0.64 and $D_{4,3}$ of 119.9 nm, bigger than the normalized turbidity of 0.14 and $D_{4,3}$ of 47.3 nm for the comparable treatment from Route E (pH11/citric). Therefore, Route E (pH11/citric) used in this study is more effective in improving dispersibility of skim milk powder than simply supplementing citrate, resulting from the controlled formation of rCM as discussed above.

2.5 Conclusion

In conclusion, CM in skim milk dispersions dissociated to a greater extent at more alkaline conditions, which allowed the reformation to smaller particles that were further assisted by the calcium-chelating citrate during acidification. The combination of alkaline treatment at pH 11.0 and acidification with citric acid corresponded to the smallest rCM, the lowest dispersion turbidity, and the best stability against acid coagulation, especially at acidic conditions down to pH 5.5. The smaller rCM in Route E (pH11/citric) resulted from a lower concentration of ionic calcium due to the chelating effect by citrate. The structural reformation in Route E (pH11/citric) was also observed to be different from the simple chelating function of citrate. This work provides a novel approach to reduce the dimension of casein particles, which can be directly used in aqueous dispersions such as transparent beverages requiring stability and clarity and further explored for other applications such as encapsulation.

2.6 Acknowledgements

Funding of this work was provided by the University of Tennessee, Dairy Research Institute (Rosemont, IL) and United States Department of Agriculture. The authors are grateful to the Bioanalytical Resources Facility at the University of Tennessee for use of the analytical ultracentrifuge and Dr. Edward Wright for assisting the experiments.
References


## Appendix

Table 2-1. Codes representing different routes used to dissociate casein micelles at alkaline pH and reform casein micelles by acidification.

<table>
<thead>
<tr>
<th>Route code</th>
<th>Dissociation pH</th>
<th>Acid used to reform casein micelles</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (control)</td>
<td>None (control)</td>
<td>4.0 M HCl</td>
</tr>
<tr>
<td>B (pH10/HCl)</td>
<td>10.0</td>
<td>4.0 M HCl</td>
</tr>
<tr>
<td>C (pH10/citric)</td>
<td>10.0</td>
<td>2.0 M citric acid</td>
</tr>
<tr>
<td>D (pH11/HCl)</td>
<td>11.0</td>
<td>4.0 M HCl</td>
</tr>
<tr>
<td>E (pH11/citric)</td>
<td>11.0</td>
<td>2.0 M citric acid</td>
</tr>
</tbody>
</table>
Table 2-2. AUC molecular weight (Mw) characteristics of skim milk dispersions when increasing pH from 7.0 to 11.0 (↑) using 4 M NaOH and subsequent acidification from pH 11.0 to 7.0 (↓) using 4 M HCl.

<table>
<thead>
<tr>
<th>Sample pH</th>
<th>Mw (× 10^6 Da) and percentage (in parentheses) of each fitted peak</th>
<th>Number average Mw (× 10^6 Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7↑</td>
<td>0.926 (8.66%), 1.20 (8.26%), 1.68 (13.0%), 2.94 (22.8%), 6.87 (47.4%)</td>
<td>4.32</td>
</tr>
<tr>
<td>9↑</td>
<td>0.162 (13.4%), 0.348 (22.0%), 1.07 (9.42%), 1.50 (11.4%), 2.36 (17.5%), 4.74 (26.3%)</td>
<td>2.15</td>
</tr>
<tr>
<td>10↑</td>
<td>0.162 (13.8%), 0.418 (29.3%), - (11.7%), 1.50 (19.0%), 2.41 (26.2%), 4.83 (26.2%)</td>
<td>2.01</td>
</tr>
<tr>
<td>11</td>
<td>0.340 (79.7%), 1.13 (6.06%), 1.58 (6.21%), 2.66 (8.03%)</td>
<td>0.65</td>
</tr>
<tr>
<td>10↓</td>
<td>0.170 (17.1%), 0.481 (60.5%), - (13.5%), 2.50 (8.85%)</td>
<td>1.08</td>
</tr>
<tr>
<td>9↓</td>
<td>0.347 (46.4%), - (17.6%), 1.34 (17.9%), 2.26 (18.1%)</td>
<td>1.69</td>
</tr>
<tr>
<td>7↓</td>
<td>0.263 (18.9%), 0.790 (21.0%), 1.32 (17.2%), 2.02 (17.2%), 3.53 (13.1%), 7.63 (12.6%)</td>
<td>2.21</td>
</tr>
</tbody>
</table>
Figure 2-1. Turbidity (a) and D4,3 (b) of samples when increasing pH from 7.0 to 9.0 – 11.0 (black squares) and subsequent acidification with citric acid (red circles) or HCl (blue triangles). Error bars are standard deviations from triplicate samples.
Figure 2-2. Appearance of dispersions constituted with 9% w/w skim milk powder after acidification to pH 5.5-7.0, as labeled on vials, using (a) Route A (control), (b) Route B (pH10/HCl), (c) Route C (pH10/citric), (d) Route D (pH11/HCl), and (e) Route E (pH11/citric), as detailed in Table 2-1.
Figure 2-3. Normalized turbidity of dispersions constituted with 9% w/w skim milk powder after acidification to pH 5.5-7.0 using Routes A-E as described in Table 2-1. Error bars are standard deviations from 3 replicates. Different letters above bars indicate statistical difference among samples at the same pH ($p < 0.05$).
Figure 2-4. Concentrations of ionic calcium in skim milk dispersions after acidification using Routes A (control), D (pH11/HCl), and E (pH11/citric), normalized by that at pH 7.0 adjusted using Route A (100.5 ppm). Error bars are standard deviations from 3 replicates.
Figure 2-5. Volume-length mean particle diameter ($D_{4,3}$) (a), and the protein concentration measured using the Bradford method (b) of the dispersions constituted with 9% w/w skim milk powder after adjusting to pH 7.0 using Routes A-E (as labeled on x-axis) as detailed in Table 2-1. Error bars are standard deviations from 3 replicates. Different letters above bars indicate statistical difference ($p < 0.05$).
Figure 2-6. Atomic force microscopy topography images (left) of skim milk dispersions after adjusting pH to (a) 7.0 and (b) 5.5 using Route A (control), in comparison to pH adjustment to (c) 7.0 and (d) 5.5 using Route E (pH11/citric). Particles picked by the software and the analyzed mean particle diameter are shown on the right. The scanned area is 2.0×2.0 μm in each image.
Figure 2-7. Zeta-potential of dispersions constituted with 9% w/w skim milk powder after adjusting to pH 7.0 using Routes A–E as detailed in Table 2-1. Different letters below bars indicate statistical difference (p < 0.05).
Figure 2-8. Normalized turbidity (a) and volume-length mean particle diameter ($D_{4,3}$) (b) of skim milk dispersions supplemented with different concentrations of sodium citrate after direct adjustment of pH to 7.0 using 1 M NaOH or HCl. Error bars are standard deviations from 3 replicates. Different letters above bars indicate statistical difference ($p < 0.05$).
Figure 2-S1. SDS-PAGE of milk dispersions. Letters A – E indicate samples treated with Routes A-E, M indicates molecular weight marker, and α, β, and κ indicate purified α-, β- and κ-casein samples from Sigma-Aldrich Corp. (St. Louis, MO).
Figure 2-S2. Absorbance at 420 nm ($Abs_{420}$, a) and 600 nm ($Abs_{600}$, b) of the supernatants obtained from skim milk dispersions acidified to pH 4.6 using Routes A – E (as labeled on x-axis) in Table 1. Different letters above the bars indicate statistical difference ($p < 0.05$).
Chapter 3. Thymol nanoencapsulated by sodium caseinate: Physical and anti-listerial properties
A version of this chapter was originally published by Kang Pan, Huaiqiong Chen, P. Michael Davidson, and Qixin Zhong:

Pan, K., Chen, H., Davidson, P. M., & Zhong, Q. (2014). Thymol nanoencapsulated by sodium caseinate: physical and antilisterial properties. *Journal of Agricultural and Food Chemistry, 62*(7), 1649-1657. My primary contributions to this paper include sample preparation, data collection and analysis, results interpretation and writing except the antimicrobial parts, which were conducted, analyzed, and wrote by Huaiqiong Chen
3.1 Abstract

In this work, thymol was encapsulated in sodium caseinate using high shear homogenization. The transparent dispersion at neutral pH was stable for 30 days at room temperature as determined by dynamic light scattering and atomic force microscopy, which agreed with high zeta-potential of nanoparticles. The slightly decreased particle dimension during storage indicates the absence of Ostwald ripening. When molecular binding was studied by fluorescence spectroscopy, thymol was observed to bind with tyrosine and possibly other amino acid residues away from tryptophan of caseins. At pH 4.6 (isoelectric point of caseins), the stabilization of thymol nanoparticles against aggregation was enabled by soluble soybean polysaccharide, resulting from the combined electrostatic and steric repulsions. The encapsulated thymol showed the significantly improved anti-listerial activity in milk with different fat levels when compared to thymol crystals, resulting from the quicker mixing and increased solubility in the milk serum. The transparent thymol nanodispersions have promising applications to improve microbiological safety and quality of foods.

Key words: Sodium caseinate, thymol, nanoencapsulation, stability, antimicrobial, Listeria monocytogenes
3.2 Introduction

The food processing industry faces constant concerns about microbial contamination of food products with potential consequences including transmission of food-borne pathogens and reduction in product shelf life. *Listeria monocytogenes* (*L. monocytogenes*) is a Gram positive bacterium transmitted via contaminated food and responsible for food-borne illnesses. The disease *L. monocytogenes* causes, listeriosis, has a high mortality rate of 30-40% (Datta, 2003). The most frequently reported products linked to listeriosis are ready-to-eat foods, such as processed meats, salads, and dairy products (Garcia, et al., 2004). To enhance microbiological food safety, various preservation technologies have been used including the use of antimicrobial compounds. Some naturally-occurring compounds (e.g., spice essential oils) have potential for use as antimicrobial preservatives and are particularly attractive for consumers as they are perceived to be “natural” components (Tippayatum & Chonhenchob, 2007).

Thymol is the major antimicrobial component of the essential oil prepared from the herb thyme (*Thymus vulgaris*) which is classified as generally recognized as safe (GRAS) by the US Food and Drug Administration (Johny, Darre, Donoghue, Donoghue, & Venkitanarayanan, 2010). The antimicrobial effectiveness of thymol has been known for decades (Myers & Thienes, 1925). However, there are some limitations concerning the use of thymol in food products. Because of its low water solubility, it is difficult to disperse thymol evenly in food matrices (Griffin, Wyllie, Markham, & Leach, 1999) when a dose above the solubility is required. The uneven dispersion of thymol may affect its function throughout the food matrix. Furthermore, the antimicrobial activity of thymol is reported to be related to depolarization of the bacterial cytoplasmic membrane (Xu, Zhou, Ji, Pei, & Xu, 2008) and a sufficiently high thymol concentration is required to access and modify the structure of that membrane. A common approach to overcoming the low solubility of
bioactive compounds is to dissolve them in a less polar solvent or disperse them in delivery systems, such as emulsions or biopolymer particles (Bhavini, Davidson, & Qixin, 2012).

Caseins make up about 80% of the protein in bovine milk, are good sources of calcium and essential amino acids, and have excellent surface activities. (Brugman, et al., 2004) There are four types of caseins (\(\alpha_s1\), \(\alpha_s2\), \(\beta\), and \(\kappa\)) that exist in bovine milk in the “micellar” form, with a diameter of 50-250 nm (Pan & Zhong, 2013; Udabage, McKinnon, & Augustin, 2003). Sodium caseinate (NaCas) is produced by precipitation from milk at acidity nearby isoelectric point (pI, at pH 4.6) followed by neutralization of the precipitate using sodium hydroxide (P. Walstra & Jenness, 1984). The emulsifying property of NaCas is related to its high content of hydrophobic amino acids such as proline, tyrosine, and tryptophan, which makes it a naturally-occurring amphiphilic block copolymer (P. Walstra, et al., 1984). This property can be utilized to encapsulate hydrophobic bioactive compounds (Pan, Zhong, & Baek, 2013). However, nanoencapsulation of lipophilic essential oils and their antimicrobial components like thymol has not been studied for caseins.

The first objective of this research was to characterize physical and antimicrobial properties of thymol dispersions prepared with NaCas by high shear homogenization. Structures of nanodispersions were characterized with dynamic light scattering, zeta-potential and atomic force microscopy (AFM). Interactions between thymol and NaCas were studied using fluorescence spectroscopy. Because caseins precipitate at acidity below about pH 5.5 and soluble soybean polysaccharide (SSPS) can stabilize caseins at acidic conditions (Nakamura, Furuta, Kato, Maeda, & Nagamatsu, 2003), the stabilization of thymol nanoparticles by SSPS at pH 4.6 (pI of caseins) and during thermal treatment was studied. Antimicrobial properties of the nanoencapsulated thymol were characterized and compared with free thymol using \(L.\ monocyctogenes\) as a target bacterium in microbial growth media and milk. Milk with different fat contents was studied as
model food systems to investigate the impacts of binding with proteins and lipids on anti-listerial properties of thymol. To further understand the binding between thymol and milk components, the second objective was to characterize thymol content in the milk serum to correlate antimicrobial properties observed in growth media and milk.

3.3 Materials and methods

3.3.1 Chemicals

NaCas was purchased from Sigma-Aldrich Corp. (St Louis, MO). The product had a purity of over 90% w/w according to the vendor. Thymol (>99% purity), pyrene, and sodium azide were purchased from Sigma-Aldrich Corp. SSPS was a commercial product from Fuji Oil Corp. (Osaka, Japan). Ultrahigh temperature-processed skim milk, 2% reduced-fat milk and full (3.3%) fat milk were products of Simple Truth Organic™ (San Diego, CA).

3.3.2 Encapsulation

NaCas was hydrated at 5% w/w in deionized water overnight at room temperature (21°C). The pH of the solution was 6.8. Thymol was mixed in the NaCas solution at 0, 5, 10, 15, and 20 mg/mL. The mixture was homogenized at 15,000 rpm for 2 min using a Cyclone I.Q. microprocessor homogenizer (VirTis Co., Gardiner, NY).

3.3.3 Determination of encapsulation efficiency in transparent dispersions

The concentration of thymol encapsulated by NaCas was determined by UV-Vis. spectroscopy. The dispersions as prepared above were centrifuged at 6,000g for 10 min (Minispin plus, Eppendorff, Hamburg, Germany). The upper transparent supernatant was transferred and diluted 100 (for dispersions prepared with 5 and 10 mg/mL thymol) or 200 (for dispersions prepared with 15 and 20 mg/mL thymol) times in volume using hexane. After vortexing for 5 min,
the mixture was allowed to stand until two separate transparent phases formed. The upper organic phase was taken to measure the absorbance at 275 nm to determine thymol concentration based on a calibration curve established previously using standard solutions with 0.01 – 0.1 mg/mL thymol dissolved in hexane. Three independent emulsion replicates were measured. The encapsulation efficiency was calculated based on the total thymol (g) used in encapsulation, the mass in the transparent dispersion, and the amount truly dissolved (eq. 1). To quantify the amount of thymol truly dissolved in water (not encapsulated in casein nanoparticles), the dispersion prepared with 10 mg/mL thymol was acidified to pH 4.6 using 6 M HCl to precipitate casein nanoparticles (with encapsulated thymol) and was centrifuged at 10,000 g for 5 min. The supernatant was filtered through a 0.22 µm polyvinyl difluoride filter (Fisher Scientific, Co., Pittsburgh, PA), then 300 µL of the permeate was mixed with 1.5 mL hexane and the thymol concentration determined as above.

\[
\text{Encapsulation efficiency}\% = \frac{\text{Dispersed thymol} - \text{dissolved thymol}}{\text{Total thymol} - \text{dissolved thymol}} \times 100\%
\]  
(1)

### 3.3.4 Particle size and zeta-potential

The hydrodynamic diameter \(D_h\) was measured using a Delsa Nano analyzer (Beckman Coulter, Atlanta, GA). Samples were diluted 30 times with 10 mM phosphate buffer at pH 7.0. The scattering angle was fixed at 165°. The zeta-potential of thymol dispersion, SSPS and their mixture at pH 3.0-7.0 was also measured using the Delsa Nano analyzer. The dispersion, prepared with 10 mg/mL thymol, was diluted 100 times to a casein concentration of 0.05% using deionized water and adjusted to pH 3.0-7.0 using 1 and 0.25 M HCl. Solutions with 0.05% w/v SSPS and the mixture of the thymol dispersion and SSPS with same biopolymer concentrations at pH 3.0-7.0 were prepared similarly for zeta potential measurements.
3.3.5 *Atomic force microscopy (AFM)*

The topography of NaCas nanoparticles before and after encapsulation of thymol was characterized using AFM. A 5% w/w NaCas dispersion and the dispersion prepared with 10 mg/mL thymol were diluted to a NaCas concentration of 10 mg/L using deionized water. 10 μL of each diluted sample was spread evenly onto a freshly cleaved mica sheet mounted on a sample disk (Bruker Corp., Santa Barbara, CA). A rectangular cantilever having an aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m (FESPA, Bruker Corp.) were used on a Multimode VIII microscope (Bruker AXS, Billerica, MA). The tapping mode was used to scan the sample, and topography images were generated with a preset scan area of 2.0×2.0 μm at a scanning speed of 1 Hz.

3.3.6 *Storage stability*

The storage stability of dispersions prepared with 0, 5, and 10 mg/mL thymol was determined by measuring the $D_h$ during storage at room temperature (21 °C) for 30 d. The dispersion without thymol was added with 0.02% w/w sodium azide to prevent microbial spoilage.

3.3.7 *Stabilization of dispersions at pH 4.6 using SSPS*

The dispersion prepared with 5% NaCas and 10 mg/mL thymol was diluted to a NaCas concentration of 0.25% and mixed with 0-0.5% SSPS. The pH was adjusted to 4.6 with 0.25 M HCl. Samples were heated at 88 °C for 2 min and the absorbance was measured at 400 nm as an indicator of turbidity before and after heating, with deionized water as the blank.
3.3.8 Fluorescence spectroscopy

The interactions between thymol and NaCas were studied by fluorescence spectroscopy using a spectrofluorometer (Model RF-1501, Shimadzu Corp., Kyoto, Japan). Briefly, thymol stock solution was prepared at 1.0% w/v in 95% aqueous ethanol and was diluted to 0-7.5 mg/L (10-50 µM) in 0.01 mg/mL aqueous NaCas (pH 7.0) and incubated at room temperature (21°C) for at least 2 h before measurement. Fluorescent spectroscopy was carried out with excitation wavelength of 280 or 295 nm and emission spectra were recorded from 290 to 550 nm. The slit width for excitation and emission was set at 10 nm. The fluorescence intensity of thymol dissolved in 10% aqueous ethanol was used as background fluorescence for calibrating the spectra. Three replicates were performed at each condition.

3.3.9 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of encapsulated thymol

An antimicrobial microbroth dilution assay in 96 well microtiter plates was used to determine the MIC and MBC of encapsulated and free thymol against *L. monocytogenes* Scott A (Basile, et al., 2006; Dung, Kim, & Kang, 2008). *L. monocytogenes* strain was from the culture collection of the Department of Food Science and Technology at the University of Tennessee. The stock *L. monocytogenes* culture, stored in glycerol at -20 °C, was transferred in tryptic soy broth (TSB) at 32 °C for two consecutive days to activate bacteria. *L. monocytogenes* was grown for 18 h in TSB at the same temperature before use and diluted in TSB to ca. 7 log CFU/mL as a working culture. Free thymol stock solution was prepared in 95% ethanol at a concentration of 100 mg/mL, which was then diluted to 4 mg/mL with TSB as the working solution. The 4 mg/mL working solution was further diluted in TSB to 0.1-2 mg/mL thymol with 0.1 mg/mL increments. Thymol (10 mg/mL) encapsulated in NaCas was prepared and diluted to the same range as free thymol.
The diluted thymol or thymol-NaCas samples (120 μL) were mixed with 120 μL of the working culture in the 300 μL wells of the microtiter plate, resulting in overall thymol concentrations of 0.05-1 mg/mL. Microtiter plates were then incubated at 32 °C or 21 °C for 24 h. The MIC was determined as the lowest thymol concentration that produced no visible growth (Basile, et al., 2006; Dung, et al., 2008). To determine the MBC, 20 μL of the culture broth from each well showing no visible growth was transferred onto tryptic soy agar plates and incubated for another 48 h at 32 °C or 21 °C. The lowest concentration of thymol corresponding to the complete absence of *L. monocytogenes* growth on the agar surface was defined as the MBC. Experiments were performed in triplicate using three independent fresh working cultures.

### 3.3.10 Anti-listerial activity of thymol in milk with different fat levels

Fat content is a dominant parameter impacting the effectiveness of lipophilic antimicrobials in milk (Bhavini, Davidson, & Zhong, 2013). Skim, 2% reduced fat, or whole milk were mixed with different volumes of the 10 mg/mL aqueous NaCas-thymol dispersion and sterile water to a total volume of 10 mL to obtain different fat levels (Table 3-1), so that the findings can be applied not only in fluid milk but also other dairy and relevant products with varying fat contents. Because liquid thymol dispersion was used to mix milk, it was not possible to simulate full fat (3.3%) milk. The overall concentration of thymol was 1 mg/mL in skim milk and 4.5 mg/mL in other milk treatments. These two concentrations were chosen because preliminary experiments showed complete inhibition of *L. monocytogenes* in skim milk within 4 h by 4.5 mg/mL of both free and encapsulated thymol, with no recovery in 7 days, while 1 mg/mL thymol in milk with 1% or higher fat corresponded to no inhibition of *L. monocytogenes*. For free thymol, thymol crystals were weighed and added directly into the milk at the same concentrations as the encapsulated thymol (Table 3-1). Sterile water was used to make up the total volume of 10 mL, followed by mixing
using an end-to-end shaker for 30 min at room temperature. The 100 µL working culture with ca. 8 log CFU/mL \textit{L. monocytogenes} was inoculated in the final milk mixture to an overall population of ca. 6 log CFU/mL using the method described previously (Xiao, Davidson, & Zhong, 2011). After incubation at room temperature (~21 °C) for up to 7 d, \textit{L. monocytogenes} was enumerated using the tryptic soy agar pour plating method with a detection limit of 1 log CFU/mL. Experiments were performed in triplicate using three independent fresh working cultures.

\subsection*{3.3.11 Quantification of thymol dissolved in the aqueous phase of milk}

The amount of thymol dissolved in the aqueous phase of milk was quantified as follows. Free thymol and encapsulated thymol samples were prepared in milk as described above except that the 0.1 mL of bacterial culture was replaced with 0.1 mL of deionized water. After incubation at room temperature (21°C) for 0 and 48 h, samples were adjusted to pH 4.6 using 6 M HCl to precipitate caseins, followed by centrifugation at 10,000g for 5 min and filtration of the supernatant through a 0.22 µm polyvinyl difluoride membrane filter. The 300 µL of permeate was mixed with 1.5 mL hexane to determine thymol concentration using the procedures described previously. Duplicate samples were measured.

\subsection*{3.3.12 Solubility of thymol in water at room temperature}

The solubility of thymol in water was measured by adding an excess amount of thymol crystals to deionized water (2 mg/mL). After stirring overnight at room temperature (21 °C), the aqueous phase with dissolved thymol was mixed with hexane to determine thymol concentration, as described above. Two independent sample replicates were tested.
3.3.13 Statistical analysis

Statistical analyses were performed using the JMP program (JMP Statistical Software, SAS Institute, Cary, NC). One-way analysis of variance was carried out. Differences between means were determined using a Fisher’s least significant difference test. The significance level was set at 0.05.

3.4 Results and discussion

3.4.1 Encapsulation efficiency

Transparent samples were observed after homogenizing thymol at overall concentrations of 5 and 10 mg/mL, which was well above its water solubility at 21 °C (0.75 mg/mL). Conversely, thymol crystals were visible after homogenization at overall thymol concentrations of 15 and 20 mg/mL (not shown), which indicated that 5% w/v NaCas reached its encapsulation capacity under the conditions studied. The dissolved thymol concentration determined in the transparent dispersion with 10 mg/mL thymol was 0.74 mg/mL, similar to the water solubility of thymol crystals. The efficiency of encapsulating thymol in transparent dispersions was determined according to eq. 1 and listed in Table 3-2. There was no significant difference between samples with 5 and 10 mg/mL thymol, with encapsulation efficiency of 89% and 92.3%. The encapsulation efficiency as determined decreased to around 72% when thymol was emulsified at overall concentrations of 15 and 20 mg/mL.

3.4.2 Physical properties of transparent dispersions

Because thymol was not completely encapsulated at an overall concentration of 15 and 20 mg/mL, only samples with 5 and 10 mg/mL thymol were characterized for storage stability. The $D_h$ of transparent dispersions prepared with 5 and 10 mg/mL thymol during storage at room
temperature for 30 days is show in Figure 3-1. There was no significant increase of $D_h$ during storage. The $D_h$ of NaCas, also sheared at homogenization conditions, was around 130 nm, and the dispersion after encapsulating 5 or 10 mg/mL thymol had a $D_h$ of about 110 nm. The smaller dimension of casein particles after encapsulation was also observed in AFM (Figure 3-2). The smaller dimension of particles measured in AFM than $D_h$ may be the result of drying of particles during sample preparation for AFM. The reduction of casein nanoparticle dimension after encapsulation with thymol may be the result of enhanced packing of casein molecules due to hydrophobic attraction by thymol. Furthermore, the stable $D_h$ during storage indicates the insignificance of Ostwald ripening as has been observed by others for dispersions prepared with small molecule surfactants like Tween 80 (Chang, McLandsborough, & McClements, 2012).

NaCas dispersions with thymol had a much higher magnitude of zeta-potential when compared with NaCas alone, and the thymol concentration did not affect the zeta-potential significantly (Figure 3-3). The pIs of $\alpha_s1$-, $\alpha_s2$-, $\beta$-, and $\kappa$-casein are 4.94, 5.23, 5.14 and 5.90, respectively, with respective net charges of -21.9, -17.1, -13.3, and -2.0 at pH 6.6 (O'Connell & Flynn, 2007). Theoretically, a more negative zeta-potential is expected for smaller casein particles because of a smaller quantity of $\kappa$-casein per particle. The zeta-potential results in Figure 3-3 therefore agree with this expectation based on particle dimension (Figures 3-1 and 3-2). The high magnitude of zeta-potential can prevent particle aggregation during storage (Figure 3-1).

3.4.3 Interactions between thymol and NaCas

Binding between proteins and small molecules in aqueous solutions can be conveniently studied using intrinsic fluorescence properties of proteins (Pan, Zhong, et al., 2013). Figure 3-4 shows the emission spectra of NaCas with different concentrations of thymol. Figure 3-4a shows little change of the fluorescence intensity of NaCas after interacting with various amounts of
thymol at the excitation wavelength of 295 nm which reflects the binding near tryptophan (Trp) residues (Liang, Tajmir-Riahi, & Subirade, 2008). Conversely, the fluorescence intensity of NaCas excited at 280 nm, due to both Trp and tyrosine (Tyr) residues (Liang, et al., 2008), decreased with an increase in thymol concentration (Figure 3-4b). Therefore, thymol binds with caseins at Tyr or other residues away from Trp of caseins, effectively quenching the fluorescence excited at 280 nm.

The intrinsic fluorescence was further analyzed using the Stern-Volmer equation (Zhang, et al., 2011):

\[
\frac{F_0}{F} = 1 + k_q \times \tau_0 \times [Q] = 1 + K_{sv} \times [\text{Thymol}] \tag{2}
\]

where \(F_0\) and \(F\) are fluorescence intensities in the absence and presence of a quencher; \([\text{Thymol}]\) is the concentration of thymol (M); \(k_q\) is the fluorescence quenching rate constant; \(\tau_0\) is the lifetime of fluorophore fluorescence in absence of quenchers and equals \(10^{-8}\) s; and \(K_{sv}\) is the Stern-Volmer quenching constant which can be determined by linear fit of \(\frac{F_0}{F}\) plot against \([\text{Thymol}]\) (Lakowicz, 2009). The linear plots of \(\frac{F_0}{F}\) as a function of \([\text{Thymol}]\) excited at 295 nm and 280 nm are given in the inset of Figure 3-4a and Figure 3-4b, respectively. The good linear fit of data to eq. 2 (\(R^2 > 0.99\)) showed the applicability of the Stern-Volmer model, and the obtained \(K_{sv}\) values were 100 ± 212 M\(^{-1}\) and 1900 ± 141 M\(^{-1}\) at the excitation wavelength of 295 nm and 280 nm, respectively. The \(K_{sv}\) at excitation wavelength of 295 nm is not statistically different from 0 (\(P > 0.05\)), which indicates no interactions between thymol and Trp. Fluorescence quenching can result from collision (dynamic quenching) or complex formation (static quenching) between biopolymers and quenchers (Zhang, et al., 2011). The \(k_q\) at excitation wavelength of 280 nm is \(1.9 \times 10^{11}\) M\(^{-1}\) s\(^{-1}\), which is about ten-fold higher than the maximum dynamic quenching constant (\(2 \times 10^{10}\) M\(^{-1}\) s\(^{-1}\)) for quenchers interacting with biopolymers (Lange, Kothari, Patel, & Patel, 1998). The results
suggest the fluorescence quenching is static and therefore complexes are formed when thymol binds with caseins at low NaCas concentrations.

### 3.4.4 Stabilization of thymol dispersions at pH 4.6 using SSPS

The dispersion prepared with 10 mg/mL thymol was diluted to an overall NaCas concentration of 0.25% w/v and mixed with 0-0.5% w/v SSPS. After adjusting to pH 4.6, samples were heated at 88 °C for 2 min, equivalent thermal processing conditions for acidic beverages (pH < 4.6) (Pontius, Rushing, & Foegeding, 1998), and the absorbance at 400 nm before and after heating was measured to indicate turbidity, (presented in Figure 3-5). Without SSPS, the dispersion showed precipitation both before and after heating. With 0.1-0.5% SSPS, dispersions were stable before and after heating. The dispersion with 0.1% SSPS had higher absorbance than the other two SSPS treatments (Figure 3-5c), possibly because of insufficient SSPS to prevent particle aggregation. There is no significant difference ($P > 0.05$) between the dispersions with 0.25% and 0.5% SSPS, which indicates SSPS is able to stabilize equal mass of NaCas at pH 4.6.

The forces stabilizing colloidal particles from aggregation include electrostatic and steric repulsions (Pieter Walstra, 2002; P. Walstra, et al., 1984). At neutral pH, caseins are highly negatively charged, and electrostatic repulsion itself is strong enough to prevent particle aggregation, as indicated in Figure 3-1 for stable $D_h$ during storage. To understand the stabilization of dispersions by SSPS at pH 4.6, the zeta-potential of SSPS, the thymol dispersion, and their mixture at pH 3.0-7.0 was measured and is presented in Figure 3-6. The lowest zeta-potential magnitude of thymol dispersion was observed at around pH 4.6, the pI of caseins, and the weakened electrostatic repulsion caused the aggregation and precipitation of casein capsules, as discussed above. The zeta-potential of SSPS alone was negative at pH 3.0-7.0, with a smaller magnitude at a lower pH. After mixing thymol dispersion with SSPS, the zeta-potentials were in
between the individual components, with the magnitude being close to zero at pH 3.5, suggesting complex formation between SSPS and casein particles (Nakamura, et al., 2003). Because dispersions were stable at pH 3.0-7.0, even after heating at 88 °C for 2 min (Figure 3-5d and 3-5e), both electrostatic and steric interactions contributed to the stability of mixture system, with steric interactions being dominant at acidity near the pI of caseins. Therefore, SSPS stabilized thymol dispersions similarly to acidified milk, by providing steric repulsion after adsorbing on free casein or casein-coated thymol particles (Nakamura, et al., 2003), as discussed previously. This enables the application of casein-based encapsulation systems in various acidic conditions.

3.4.5 Anti-listerial properties and correlation to solubility

The MICs and MBCs of free and encapsulated thymol against L. monocytogenes were 0.2 and 0.3 mg/L, respectively, at both 21 and 32°C. These concentrations are below the water solubility limit of 0.75 mg/mL, and the similar MIC and MBC of encapsulated and free thymol suggest that the binding between thymol and caseins does not affect the activity of thymol interacting with bacteria. Similar results were previously reported for thymol against Staphylococcus aureus ATCC 25923 and Escherichia coli ATCC 25922, after encapsulation in methylcellulose/ethylcellulose (Wattanasatcha, Rengpipat, & Wanichwecharungruang, 2012).

Figure 3-7 presents the growth activity of L. monocytogenes in milk with different fat levels in the presence of free and encapsulated thymol. The L. monocytogenes growth curves were similar for the control (no treatment) and NaCas control indicating that NaCas alone has no antimicrobial activity. In skim milk (Figure 3-7a), encapsulated thymol has slightly better anti-listerial activity (ca. 1 log CFU/ml) than free thymol in the first 48 h while no difference was observed at 72 or 168 h. The concentration of thymol in the aqueous phase of milk at 0 h was significantly higher for encapsulated thymol than for free thymol (Figure 3-8a). The delay in anti-listerial activity of free
thymol may be due to the slow dissolution process of thymol from crystals compared to the encapsulated form that enabled faster distribution into the milk matrix (Gaysinsky, Taylor, Davidson, Bruce, & Weiss, 2007). The concentration of thymol (1 mg/mL) required to inhibit *L. monocytogenes* in skim milk is higher than the MIC and MBC of free and encapsulated thymol in TSB. Furthermore, the respective thymol concentration in the aqueous phase of skim milk at 0 h was 0.26 to 0.33 mg/mL for free and encapsulated thymol, which was much lower than the water solubility of 0.75 mg/mL, and reduced after 48 h (Figure 3-8a). This suggests that the reduced thymol activity in skim milk is due to binding by proteins and fat globules (<0.5% in skim milk).

To study effects of fat content on anti-listerial properties, 4.5 mg/mL thymol was added to milk adjusted to 1.1-1.8% fat (Table 3-1). As shown in Figure 3-7b-e, the inactivation of *L. monocytogenes* was slower at a higher fat level, and the encapsulated thymol consistently reduced the *L. monocytogenes* population to a lower level in a shorter time than free thymol. With 1.14% (Figure 3-7b) and 1.33% (Figure 3-7c) fat, both free and encapsulated thymol reduced *L. monocytogenes* to below the detection limit of 1 log CFU/mL in 168 h. With 1.49% fat (Figure 3-7d), *L. monocytogenes* was reduced to about 2.5 log CFU/mL in 168 h by free and encapsulated thymol. The greater than 3 log reduction after 48 h in milk with 1.14-1.49% fat is in agreement with thymol concentrations in the milk serum higher than the MBC (0.3 mg/mL) at both 0 and 48 h (Figure 3-8). At 1.83% fat (Figure 3-7e), the encapsulated and free thymol used at an overall concentration of 4.5 mg/mL was detected at 0.28 and 0.25 mg/mL in the aqueous phase of milk (Figure 3-8b), respectively, at 48 h. Because these concentrations were above the MIC (0.2 mg/mL) but below the MBC (0.3 mg/mL), inactivation of *L. monocytogenes* to a lesser extent was observed than in milk with lower fat levels, with the encapsulated thymol being more effective (Figure 3-7e).
Figures 3-7 and 3-8 indicate the significant effects of fat on the activity of hydrophobic antimicrobials like thymol. The antimicrobial activity of hydrophobic essential oil components is enabled after dissolution in phospholipids of the bacterial cell membrane to a sufficient quantity to result in substantial changes in membrane structures and cell metabolism (Burt, 2004). The cell membrane of bacteria is covered by structures including peptidoglycan that can prevent the direct access of caseins and thymol enclosed in nanocapsules. Therefore, only the truly dissolved thymol may be able to diffuse into the cell membrane. For thymol binding with/encapsulated in caseins, the partitioning to the continuous water phase is expected, which is expected to further partition with bacterial cells. Because bacterial cells have phospholipids that are more hydrophobic than water-soluble caseins, the partitioning of lipophilic thymol to bacteria may be more favorable than binding with caseins and may become irreversible because of the much higher polarity of the continuous water phase. This may have resulted in the same MIC (0.2 mg/mL) and MBC (0.3 mg/mL) of free and encapsulated thymol, both of which are well below the water solubility of thymol (0.75 mg/mL). For the dispersion with 10 mg/mL thymol, the amount of truly dissolved thymol (after precipitation of casein capsules at pH 4.6, eq 1) is 0.74 mg/mL, which is close to the water solubility, while the rest is being dissolved/dispersed in casein nanocapsules. When the thymol dispersion is mixed with milk, the truly dissolved thymol is expected to decrease, and the encapsulated thymol is again expected to be partitioning with the continuous water phase. The dissolved thymol also can diffuse into porous casein micelles (Slattery & Evard, 1973) and bind with fat globules in milk. Analogous to bacterial cells, fat globules in milk are covered by membranes composed of phospholipids and proteins (Salton, 1953), may be more favorable than caseins and water phase, and therefore reduce the amount of thymol that can potentially be dissolved in the continuous water phase and subsequently the bacterial membrane. For skim milk
(with <0.5% fat), the impacts of binding by fat globules are not as significant, as 1 mg/mL thymol, ~3 times higher than the MBC (0.3 mg/mL), is sufficient to inhibit *L. monocytogenes* (Figure 3-7a). As fat concentration increases in milk, the binding by fat globules becomes more and more significant, reducing the amount of truly dissolved thymol (Figure 3-8) and therefore anti-listerial activity (Figure 3-7), despite the higher overall thymol usage level (4.5 mg/mL). Findings from the present study agree with the speculations based on physics and mass transport (Gaysinsky, et al., 2007; Terjung, Loffler, Gibis, Hinrichs, & Weiss, 2012).

### 3.5 Conclusions

In conclusion, 5% w/v NaCas enabled the encapsulation of 1% w/v thymol as transparent dispersions that were stable at neutral pH during 30-day storage at room temperature. The encapsulation reduced the dimension of NaCas and increased the zeta-potential. Thymol selectively binds with Tyr and possibly other amino acids not close to Trp of NaCas based on fluorescence spectroscopy. Under acidic conditions, the adsorption of SSPS on thymol-loaded casein particles provided steric repulsion and effectively prevented the aggregation and precipitation of casein capsules, even at pH 4.6. Lastly, encapsulation of thymol in casein nanocapsules enabled the fast and even distribution and increased the solubility and anti-listerial activity of lipophilic antimicrobials in milk with different fat levels. Findings from the present study indicate the potential of the studied antimicrobial delivery system to improve the microbiological safety of complex food matrices such as Spanish cheeses.

### 3.6 Acknowledgement

The present study was supported by the University of Tennessee, the US Department of Agriculture, and Dairy Research Institute (Rosemont, IL).
References


Appendix

Table 3-1. Composition of mixtures prepared from milk and 10 mg/mL NaCas-thymol dispersions used in anti-listerial experiments.

<table>
<thead>
<tr>
<th>Volume of constituents (mL)</th>
<th>Fat content of mixture</th>
<th>Overall thymol concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk volume and type</td>
<td>Thymol dispersion</td>
<td>Sterile water</td>
</tr>
<tr>
<td>A 9, skim</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>B 5.5, 2% reduced-fat</td>
<td>4.5</td>
<td>-</td>
</tr>
<tr>
<td>C 4, full fat</td>
<td>4.5</td>
<td>1.5</td>
</tr>
<tr>
<td>D 4.5, full fat</td>
<td>4.5</td>
<td>1</td>
</tr>
<tr>
<td>E 5.5, full fat</td>
<td>4.5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>Same volume and type of milk as in A-E</td>
<td>10 minus milk volume</td>
</tr>
</tbody>
</table>
Table 3-2. Encapsulation efficiency of dispersions prepared with 5% w/v NaCas and different thymol concentrations.

<table>
<thead>
<tr>
<th>Overall thymol concentration (mg/mL)</th>
<th>Encapsulation efficiency (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>89.0 ± 3.1^a</td>
</tr>
<tr>
<td>10</td>
<td>92.3 ± 2.2^a</td>
</tr>
<tr>
<td>15</td>
<td>72.1 ± 1.4^b</td>
</tr>
<tr>
<td>20</td>
<td>72.4 ± 1.3^b</td>
</tr>
</tbody>
</table>

*Numbers are mean ± standard deviation from triplicate samples. Different superscripts indicate differences in the means ($P < 0.05$).
Figure 3-1. Effective hydrodynamic diameter of 5% w/v sodium caseinate dispersions homogenized with 0, 5, and 10 mg/mL thymol during storage at room temperature (21 °C).
Figure 3-2. AFM topography images of NaCas (a) without or (b) with thymol. The dispersion was prepared with 10 mg/mL thymol and 5% NaCas before dilution for AFM. The NaCas only dispersion was also homogenized before dilution for AFM. Mean diameter of the particles was measured using the instrument software. Image dimension is 2 μm × 2 μm. The scale on the right is for particle height.
Figure 3-3. Zeta-potential of the dispersions prepared with 5% NaCas and different concentrations of thymol at pH 7.0. The dispersions were centrifuged and the supernatant was collected and diluted before measurement.
Figure 3-4. Fluorescence emission spectra of NaCas at excitation wavelength of (a) 295 nm and (b) 280 nm in 10 mM phosphate buffer at pH 7.0, after interacting with 0-7.5 mg/L (0-50 µM) thymol. The plot of $F_0/F$ versus thymol concentration is shown in the inset, with the lines showing the linear fit of data with an intercept of 1.
Figure 3-5. Appearance of thymol-containing dispersions containing 0.25% NaCas, diluted from the dispersion prepared with 5% NaCas and 10 mg/mL thymol, with 0%, 0.1%, 0.25%, and 0.5% SSPS (as labeled on vial caps) at pH 4.6, (a) before and (b) after being heated at 88°C for 2 min. The absorbance at 400 nm is shown in panel (c), where different letters above bars indicate significant difference ($P < 0.05$) in the mean. Photos (d) and (e) show the respective samples, with 0.25% NaCas and 0.25% SSPS adjusted to pH 3.0-7.0, before and after heating at 88 °C for 2 min.
Figure 3-6. Zeta-potential of thymol dispersion, SSPS, and their mixture (with equal mass of NaCas and SSPS) at pH 3.0-7.0.
Figure 3-7. Growth of *Listeria monocytogenes* Scott A in milk prepared to different fat levels at room temperature (~21°C) treated by different concentrations of free or capsulated thymol: (a) 1 mg/mL thymol in skim milk; 4.5 mg/mL thymol in milk with (b) 1.14%, (c) 1.33%, (d) 1.49%, and (e) 1.83% fat. Milk compositions are listed in Table 3-1. The control was the treatment with milk added with sterile water and culture only. The 5% NaCas treatment was conducted by adding 5% NaCas solution at the same volume as encapsulated thymol into milk. Error bars are standard deviations (*n* = 3).
Figure 3-8. Concentration of thymol in the continuous phase of model milk systems with different fat contents after (a) 0 h and (b) 48 h at room temperature (21°C). Different letters above bars indicate significant difference ($P < 0.05$) in the mean.
Chapter 4. Enhanced dispersibility and bioactivity of curcumin by encapsulation in casein nanocapsules
A version of this chapter was originally published by Kang Pan, Qixin Zhong, and Seung Joon Baek:

4.1 Abstract

In this work, a novel encapsulation method was studied by spray-drying warm aqueous ethanol solution with co-dissolved sodium caseinate (NaCas) and lipophilic food components, using curcumin as a model compound. The encapsulation caused the loss of crystallinity. After hydration of spray-dried powder and centrifugation, 137 μg/mL curcumin was dispersed in the transparent dispersion, which was 4 decades higher than its water solubility. Dynamic light scattering and atomic force microscopy results showed that curcumin-loaded casein nanoparticles were bigger than those of NaCas processed at encapsulation conditions but were smaller than those of the native NaCas. The increased nanoparticle dimension, together with fluorescence and FTIR spectroscopy results, suggested that curcumin was entrapped in the nanoparticle core through hydrophobic interactions. The curcumin encapsulated in casein nanoparticles had higher biological activity, as assessed by antioxidant and cell proliferation assays, than pristine curcumin, likely due to the improved dispersibility. Our simple approach may be applied to encapsulate various lipophilic bioactive compounds.

Key words: Sodium caseinate, curcumin, encapsulation, dispersibility, antioxidant activity, activity against cancer cell growth
4.2 Introduction

Curcuminoids, including curcumin I (diferuloylmethane), curcumin II (monodemethoxycurcumin), and curcumin III (bisdemethoxycurcumin), are natural polyphenolic compounds isolated from the rhizome of turmeric (*Curcuma longa*) (Ramsewak, DeWitt, & Nair, 2000). Due to the phenolic groups and conjugated double bonds, curcumin has been observed for high cytotoxic activity against tumor cells, strong antioxidant properties, and anti-carcinogenesis against a wide range of cell lines (Babu, Shylesh, & Padikkala, 2002; Ruby, Kuttan, Dinesh Babu, Rajasekharan, & Kuttan, 1995; Soudamini & Kuttan, 1988). Curcumin III has a higher activity as a cytotoxic agent than curcumin I and curcumin II, which may be ascribed to the demethylation (Ruby, et al., 1995). Curcumin exhibits the potential to interrupt the NF-κB and AP-1 pathways, thereby inhibiting the expression of certain genes that are critical regulators of inflammation (Chen & Tan, 1998; Singh & Aggarwal, 1995). When tested as a cancer prevention compound, human clinical trials showed some benefits without significant side effects, when curcumin was orally admitted up to 8 g daily for 3 months (Cheng, et al., 2001). The promise of curcumin as a therapeutic agent against cancer, Alzheimer’s disease, inflammatory-related diseases, however, requires strategies overcoming its limited bioavailability (Kawamori, et al., 1999; Ringman, Frautschy, Cole, Masterman, & Cummings, 2005). A critical factor limiting the bioavailability is the extremely low water solubility that is estimated to be 11 ng/mL (Kaminaga, et al., 2003).

Several techniques have been studied to increase the solubility and bioavailability of curcumin. Glucosylation of curcumin to curcumin-4',4''-O-β-D-digentiobioside by *Catharanthus roseus* increased the solubility to around 240 mg/mL (Kaminaga, et al., 2003). However, the bioactivity and toxicity of the glucosylated curcumin were not evaluated. Dissolving curcumin in the oil body of emulsions improved dispersibility, bioavailability and anti-inflammation activity.
Nanoformulations utilizing synthesized or natural polymers such as zein nanoparticles, poly(ethylene glycol)-poly(ε-caprolactone), alginate-chitosan-pluronic composite, and Pluronic block copolymer also increased the therapeutic effectiveness (Gomez-Estaca, Balaguer, Gavara, & Hernandez-Munoz, 2012; Gou, et al., 2011; Sahu, Kasoju, Goswami, & Bora, 2011). Pharmaceutical-grade cyclodextrins have been used to load curcumin to a solubility up to ~2 mg/mL (Tønnesen, Másson, & Loftsson, 2002) and hydrophobically-modified starch was observed to disperse 18.4 μg/mL curcumin after high pressure homogenization and centrifugation (H. Yu & Huang, 2010). Solid lipid nanoparticles involving polysorbate 80 (Kakkar, Singh, Singla, & Kaur, 2011) and organogel-based nanoemulsions utilizing Tween 20 were also studied to enhance the bioavailability of curcumin (H. L. Yu & Huang, 2012). Despite these significant developments, much work is needed to prepare nanodispersions for transparent functional beverage applications using generally recognized as safe ingredients like naturally occurring food biopolymers.

Caseins have unique properties for fabricating delivery systems of food and pharmaceutical compounds, in addition to being a very important source of calcium and essential amino acids (Brugman, et al., 2004). As relevant to this study (Esmaili & Ghaffari, 2011), purified camel β-casein was mixed with curcumin in ethanol, followed by evaporation to remove the solvent and dilution with distilled water. After centrifugation, the supernatant contained 28 μg/mL curcumin. The β-casein can self-associate to form micelles that are capable of dissolving lipophilic compounds but is too costly for food applications. Casein micelles in bovine milk can be dissociated into nanoclusters by high pressure homogenization (Orlien, Boserup, & Olsen, 2010), which was used to encapsulate vitamin D2 in nanoparticles of sodium caseinate formed upon depressurization (Semo, Kesselman, Danino, & Livney, 2007). High pressure homogenization
however is currently expensive for food applications. Casein micelles can also be dissociated by calcium chelating agents such as citrate and ethylenediaminetetraacetate (Udabage, McKinnon, & Augustin, 2000) or at mild acidic conditions (McMahon, Du, McManus, & Larsen, 2009). These dissociation conditions however may not be applicable to encapsulate curcumin. Conversely, dissociation of casein micelles has been reported by heating to above 60 °C in aqueous ethanol with more than 35% ethanol, because of the improved solvent quality and the shifting of pKa values of phosphoserine (O'Connell, Kelly, Fox, & de Kruif, 2001). This important property of casein micelles has not been utilized to encapsulate lipophilic bioactive compounds, like curcumin, that have a much higher solubility in aqueous ethanol than in water. Curcumin and casein in warm aqueous ethanol may form nanoscale complexes that can be prepared to the powdered form by spray drying for the convenience of transportation, storage and application. Spray drying is a low-cost and scalable technique that has been extensively used for solvent removal and encapsulation (Fuchs, et al., 2006; Rosenberg, Kopelman, & Talmon, 1990). If spray-dried powder can be hydrated to prepare transparent dispersions with encapsulated lipophilic compounds such as curcumin, a simple encapsulation method can then be developed for the production of transparent functional beverages.

The primary objective of this work was to study the possibility of preparing transparent dispersions of curcumin by hydrating powders spray-dried from warm aqueous ethanol solution with curcumin and caseins. This was supported by physicochemical properties of powders and the prepared aqueous dispersions, including nanoscale structural changes of caseins. The secondary objective was to evaluate the potential of encapsulation in improving the bioactivity of curcumin, which was tested for anti-oxidant properties and in vitro activity against the growth of cancer cells, as studied by Yu and Huang (H. Yu, et al., 2010). To simplify sample preparation, sodium
caseinate (NaCas), a commercially available ingredient produced by acid precipitation of caseins from bovine milk followed by neutralization using sodium hydroxide for spray drying, was used in this work. This enables a safe, low-cost, and scalable approach to incorporate lipophilic bioactive compounds in products such as functional beverages.

4.3 Materials and methods

4.3.1 Chemicals

Curcumin was purchased from Sigma-Aldrich Corp. (St Louis, MO). The product had a purity of over 90% w/w according to the vendor. NaCas was from American Casein Co. (Burlington, NJ). Other chemicals were obtained from either Sigma-Aldrich or Thermo Fisher Scientific (Pittsburgh, PA).

4.3.2 Encapsulation of curcumin by spray drying

Four g NaCas was hydrated in 200 mL of 40% v/v aqueous ethanol. After being heated at 60 °C in a water bath for 5 min, an excess amount (1.0085 g) of curcumin was mixed with the NaCas solution by blending at 10,000 rpm for 4 min using a Cyclone I.Q. microprocessor homogenizer (VirTis, Gardine, NY). The homogenizer was equipped with a 20 mm diameter rotor–stator shaft assembly with openings of 1 mm in width and 10 mm in height. Centrifugation at 290g (model 4540 R, Eppendorff, Hamburg, Germany) for 5 min was carried out to remove the excess amount of curcumin. The supernatant was transferred and spray dried at an inlet temperature of 105 °C and an outlet temperature of 68 °C using a B-290 mini spray-dryer (Büchi Labortechnik AG, Flawil, Switzerland). A NaCas sample was processed at the same conditions without curcumin, named as processed NaCas hereafter.
4.3.3 Estimation of curcumin loading in spray-dried powder

Five mg of spray-dried powder was suspended in 10 mL chloroform and was stirred overnight at room temperature (21 °C). After centrifugation at 6,000 g for 10 min (Minispin plus, Eppendoff, Hamburg, Germany), the supernatant was transferred and filtered through a PTFE syringe filter with 0.45 μm pore size (Fisher Scientific, Pittsburgh, PA). The permeate was diluted 20 times in chloroform, and the absorbance at 419 nm was measured using a UV-Vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA) (H. Yu, et al., 2010) to determine curcumin concentration based on a calibration curve previously established using standard solutions with different amounts of free curcumin dissolved in chloroform.

4.3.4 Preparation of transparent dispersions and determination of curcumin concentration

To prepare transparent dispersions, 0.25 g spray dried powder was hydrated in 20 mL deionized water for 6 h at room temperature (21 °C) using a stirring plate, followed by centrifugation at 12,800 g for 15 min (model 4540 R, Eppendoff, Hamburg, Germany) to obtain the transparent supernatant. The concentration of casein in the transparent dispersion was determined using the Commassie blue reagent from Thermo Fisher Scientific (Pittsburgh, PA), with bovine serum albumin as a reference protein. To determine curcumin content using the literature method (H. Yu, et al., 2010), the transparent dispersion was mixed with a certain amount of chloroform by vortexing for 1 min and subsequent stirring overnight. The bottom organic phase was transferred after phase separation and the absorbance at 419 nm was determined to obtain curcumin concentration as above. A control sample estimating the complexation ability of NaCas was prepared by blending curcumin and NaCas using a Cyclone I.Q. microprocessor homogenizer (VirTis, Gardine, NY) operated at 10,000 rpm for 4 min in deionized water, at the same mass ratio.
as in the preparation of spray-dried powder, followed by centrifugation and quantification of curcumin as above.

4.3.5 Atomic force microscopy (AFM)

The transparent curcumin dispersion and native NaCas dispersion were diluted in deionized water to an overall solute concentration of 10 ppm. Two μL of each diluted sample was spread evenly onto freshly cleaved mica sheets that were mounted on sample disks (Bruker Corp., Santa Barbara, CA) for AFM. A rectangular cantilever having an aluminum reflective coating on the backside and a quoted force constant of 2.80 N/m (FESPA, Bruker Corp., Santa Barbara, CA) and a Multimode microscope (Bruker AXS, Billerica, MA) operated in the tapping mode were used to scan the sample. Images were generated with a preset scan area of 2.0×2.0 μm at a scanning speed of 1 Hz.

4.3.6 Differential scanning calorimetry (DSC)

The thermal stability and crystallinity of powder samples were characterized using a model Q2000 calorimeter (TA Instrument, New Castle, DE). Ten mg powdered sample was sealed in hermetic aluminum pans and heated from 30 to 250 °C at a rate of 5 °C/min. Nitrogen was used as the transfer gas at a flow rate of 50 mL/min.

4.3.7 X-ray diffraction (XRD)

The XRD patterns of samples were characterized using a XRD-6000 powder X-ray diffractometer (Shimadzu Corp., Tokyo, Japan) with Cu Kα radiation at a wavelength (λ) of 1.5418 Å. Measurements were performed at a voltage of 30 kV and 10 mA. The 2θ angle was set from 5° to 55° and the scanning rate was 2°/min.
4.3.8 Dynamic light scattering (DLS) and zeta-potential

In addition to the transparent dispersion with curcumin, two other dispersions were measured in DLS: native NaCas and processed NaCas hydrated in deionized water for 6 h. The dispersion pH was 6.8. Experiments were conducted with a Delsa Nano analyzer (Beckman coulter, Atlanta, GA) at a scattering angle of 165°. Samples were filtered through PTFE syringe filters with 0.45 μm pore size (Fisher Scientific, Pittsburgh, PA). The time correlation functions were analyzed with a Laplace inversion program (CONTIN) to obtain the hydrodynamic diameter. Average hydrodynamic diameters, polydispersibility, and zeta-potential were reported based on 3 replicates.

4.3.9 Fluorescence spectroscopy

The fluorescence spectra were recorded using a RF-1501 spectrofluorometer (Shimadzu Corp., Tokyo, Japan). The excitation wavelength was 419 nm and the emission spectra from 450 to 700 nm were recorded. The slit width was set at 10 nm for both excitation and emission. The transparent curcumin dispersion was diluted to proper concentrations using deionized water to reach the instrument sensitivity range. For free curcumin, it was dissolved in ethanol before dilution in deionized water to the same final concentration as in the encapsulated curcumin. Dispersions of native and processed NaCas were prepared in deionized water and measured for fluorescence after being diluted to NaCas concentrations identical to the curcumin dispersion.

4.3.10 Fourier transform infrared (FTIR) spectroscopy

FTIR spectra were compared for curcumin crystals, native NaCas, processed NaCas, and curcumin capsules prepared as above. The powdered sample was mixed and milled with KBr powder, and the mixture was pressed into a thin pellet. The FTIR spectra were measured by using a Nicolet Nexus 670 FT-IR spectrometer (Thermo Nicolet Corp., Madison, MI) equipped with a
Germanium attenuated total reflection (ATR) accessory, a DTGS KBr detector and a KBr beam splitter. All spectra were taken via the ATR method with a resolution of 4 cm$^{-1}$ using 64 scans.

4.3.11 Quantification of total antioxidant activity (ABTS assay)

A literature assay protocol was adopted to quantify total antioxidant activity (Re, et al., 1999). The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was dissolved in deionized water to a 7 mM concentration. Potassium persulfate was dissolved at an overall concentration of 2.45 mM in the ABTS solution and was allowed to stand in dark at room temperature for 12-16 h before use. The ABTS radical solution was diluted with 10 mM phosphate buffer saline (PBS) to an absorbance of 0.70 ($\pm$ 0.02) at 734 nm and equilibrated at 30 °C. Curcumin crystal was first dissolved in 95% ethanol. Ethanol-dissolved and encapsulated curcumin were diluted 10 times with a 10 mM PBS buffer to achieve a 20%–80% inhibition of the blank absorbance. Ten $\mu$L samples or Trolox standards (at 0.5, 1.0, 1.5, 2.0, and 2.5 mM concentrations) were dissolved in 1 mL ABTS radical solution, and the absorbance reading was taken at 30 °C exactly 1 min after initial mixing for up to 6 min. The 10 mM PBS was used as a blank in each run. Each sample was measured in triplicate, and the mean and standard deviation were calculated. The scavenging capability of test compounds was determined using Equation 1 and converted to Trolox equivalent antioxidant capacity (TEAC) using the standard curve from Trolox solutions.

\[
\text{ABTS}^+ \text{ scavenging (\%)} = (1 - \frac{A_s}{A_c}) \times 100
\]  

where $A_s$ and $A_c$ are the absorbance of test and control (PBS) solutions, respectively.
4.3.12 In vitro cell proliferation assay

Human colon cancer cell line HCT-116 was purchased from American Type Culture Collection (Manassas, VA) and was cultured in McCoy’s 5A medium (Mediatech, Manassas, VA). The culture media contained 10% fetal bovine serum (Hyclone), 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were cultured at 37 °C under a humidified atmosphere of 5% CO₂. Anti-cancer activity of curcumin was tested by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, WI). Briefly, HCT-116 cells were seeded in 96-well microliter plates at a density of 1,000 cells per well in a final volume of 100 μL medium. After 24 h, the cells were treated with a medium containing dimethyl sulfoxide (DMSO)-dissolved or casein-encapsulated curcumin. Other cells were untreated (negative controls) or treated only with DMSO or NaCas at the concentrations as in the dispersions with encapsulated curcumin (positive controls). After 48 h, the cell culture medium was replaced with 20 μL of CellTiter 96 Aqueous One Solution and was incubated for 1 h at 37 °C. Absorbance at 490 nm was compared with a microplate reader (Bio-Tek Instruments, Winooski, VT). Relative cell viability was expressed as the absorbance normalized by that of the untreated wells. The mean and standard deviation from four-well replicates were calculated. The cell viability was calculated according to Equation 2. The normalized cell viability was obtained after normalizing the viability of a treatment by the viability of control cells treated with DMSO only.

\[
\text{Cell viability (\%) } = (A_{\text{treated}}/A_{\text{untreated}}) \times 100
\]

where \(A_{\text{treated}}\) and \(A_{\text{untreated}}\) are the absorbance of the treated and untreated cells, respectively.
4.3.13 Statistical analysis

Statistical analyses were performed using the JMP program (JMP Statistical Software, SAS Institute, Cary, NC, USA). One-way analysis of variance was carried out. Differences between pairs of means were compared using a Tukey test. The significance level was set at 0.05.

4.4 Results and discussion

4.4.1 Encapsulation properties

The spray-dried powder contained 16.7% (dry basis) curcumin. Compared to the theoretical loading before spray drying (1.0085 g curcumin and 4 g NaCas, or 20.1% curcumin in total non-solvent mass), the encapsulation efficiency (EE) was about 83.1%. The EE and loading level (LL) are higher than the 25.7-70.2% EE and 1.4-4.3% LL when encapsulated in Ploronic F68 block copolymers at different curcumin:polymer ratios (Sahu, et al., 2011). The EE is also comparable to the EE of 85-90% for curcumin encapsulated in zein nanoparticles by electrohydrodynamic atomization (Gomez-Estaca, et al., 2012) and 71.1-86.8% for curcumin encapsulated in zein nanoparticles by antisolvent precipitation (Patel, Hu, Tiwari, & Velikov, 2010).

4.4.2 Impacts of encapsulation on crystallinity and thermal stability of curcumin

To study the effects of encapsulation on the crystallinity and thermal stability of curcumin, XRD and DSC were performed. The XRD pattern of pristine curcumin showed several characteristic peaks resulting from its crystalline structure, while the typical amorphous XRD pattern was observed for NaCas (Figure 4-1A). After encapsulation, the characteristic peaks of pristine curcumin disappeared (Figure 4-1A), while the simple physical mixture of pristine curcumin and NaCas powder at the same ratio still maintained certain peaks of pristine curcumin
(Figure 4-1B). The loss of crystalline structure of curcumin indicates successful encapsulation. Similar results have been reported after encapsulating curcumin in zein nanoparticles, which corresponded to turbid dispersions after hydrating freeze-dried powder in water (Patel, et al., 2010).

DSC results are shown in Figure 4-2. The smooth curve of NaCas indicates amorphous structure of caseins and the thermal stability up to 250 °C. The sharp peak of pristine curcumin at 177.3 °C corresponds to the melting of curcumin crystals, which is consistent with the reported value of 176.4-177.5 °C (Jasim & Talib, 1992). The absence of the endothermic peak for curcumin capsules further confirms the loss of crystalline structure as observed in XRD.

**4.4.3 Physical properties of transparent dispersions**

The transparent dispersion obtained by centrifuging the sample reconstituted with spray-dried capsules was determined to have 136.7±1.3 μg/mL curcumin and 7.2 mg/mL casein. At the same curcumin concentration, free curcumin appeared as insoluble particulates (Figure 4-3), because of its low solubility of 11 ng/mL (Kaminaga, et al., 2003). The encapsulation approach in this work thus dispersed curcumin more than 4 decades above the solubility, while maintaining the transparent appearance.

It was previously reported that native casein micelles are capable of binding with curcumin in aqueous solution through hydrophobic interactions (Sahu, Kasoju, & Bora, 2008). NaCas also contains four types of caseins, with $\alpha_{s1}$-, $\alpha_{s2}$-, and $\beta$- caseins being more hydrophobic than $\kappa$-casein. The curcumin concentration in the supernatant obtained by centrifuging the dispersion prepared by homogenizing hydrated NaCas and curcumin in water was determined to be 4.6±0.1 μg/mL, which was about 30 folds lower than the 136.7 μg/mL obtained by spray-drying warm aqueous ethanol solution, hydration of spray-dried powder, and centrifugation. Our results also
were much higher than the 28 µg/mL curcumin dissolved in micelles of purified β-casein (Esmaili, et al., 2011).

The dispersion with curcumin encapsulated in NaCas had a zeta-potential of -31.1±0.9 mV at pH 6.8. The hydrodynamic diameters of dispersions prepared from native NaCas, processed NaCas and curcumin capsules are presented in Table 4-1. The dispersion of the processed NaCas had a significantly smaller hydrodynamic diameter than that of native NaCas, and the hydrodynamic diameter of the dispersion with curcumin nanocapsules was significantly larger than that of the processed NaCas but smaller than that of the native NaCas. Particles in dispersions of native NaCas and curcumin nanocapsules were further imaged using AFM, with the topography images presented in Figure 4-4 that also shows the heights of selected particles. Native NaCas particles were mostly spherical and had a relatively narrow height distribution from 8 to 15 nm (Figure 4-4a). In comparison, curcumin-containing nanoparticles were less regular in shape and had a wider height distribution, ranging from 5 to 20 nm (Figure 4-4b), than those of native NaCas (Figure 4-4a).

There are considerable differences between NaCas and casein micelles. Compositionally, the two materials have different amounts of calcium phosphate (Panouille, Durand, Nicolai, Larquet, & Boisset, 2005). Functionally, NaCas is more soluble than casein micelles (Walstra, Wouters, & Geurts, 2006). Dispersions of NaCas have been reported for a much smaller hydrodynamic radius, from 10 nm to 100 nm (Lucey, Srinivasan, Singh, & Munro, 2000) than that of casein micelles. However, there are other studies reporting a hydrodynamic diameter bigger than 200 nm in NaCas dispersions (Liu & Guo, 2008), which agreed with transmission electron microscopy results in a study (Semo, et al., 2007). Sample preparation conditions including the
source and concentration (HadjSadok, Pitkowski, Nicolai, Benyahia, & Moulai-Mostefa, 2008) of NaCas may have resulted in the discrepancies in the literature.

Nevertheless, structural changes of caseins in warm aqueous ethanol are expected to be driven by polarity that impacts solvent quality and thus dissociation and reassociation of casein molecules (O’Connell, Kelly, Auty, Fox, & de Kruif, 2001). The hypothesized mechanism of structural changes in the present study is then presented in Figure 4-5. When heated in aqueous ethanol, NaCas dissociates to smaller structures due to the improved solvent quality (O’Connell, Kelly, Auty, et al., 2001), which was in agreement with our observation of the much reduced turbidity at 60 °C than at 21 °C (not shown). After spray drying and rehydration, the dissociated caseins reformed into nanoparticles smaller than those of native NaCas (Table 4-1), likely due to the irreversibility of disrupted physical forces (hydrophobic, hydrogen bonds, and calcium phosphate bridges). The dissociation of NaCas in warm aqueous ethanol increases the availability of hydrophobic sites of caseins for contacting with curcumin during mixing. When spray-dried powder is hydrated, caseins reorganize into nanoparticles with curcumin, which increases the hydrodynamic diameter and broadens the particle size distribution of capsules when compared to the processed NaCas. For spray-dried particles with abundant curcumin, local precipitation of curcumin results in irregularly-shaped nanoparticles with the surface being adsorbed by caseins (Figure 4-4b).

4.4.4 Interactions between curcumin and sodium caseinate

The FTIR spectra of native NaCas, processed NaCas, pristine curcumin, and NaCas encapsulated with curcumin are shown in Figure 4-6 to study the interactions between curcumin and caseins. The characteristic absorbance peak around 3507 cm⁻¹, which corresponds to the –OH stretching vibration of curcumin, disappeared after encapsulation. There was a shift of the
absorbance peak from 1529 cm\(^{-1}\) to 1544 cm\(^{-1}\) after encapsulation of curcumin in NaCas, when compared with the native NaCas. This peak shift is due to structural changes of NaCas at the conditions used in encapsulation, as shown for the spectrum of the processed NaCas. The peak of native NaCas at 1658 cm\(^{-1}\) shifted to 1631 cm\(^{-1}\) after encapsulation of curcumin. Because this shift was not observed for processed NaCas, this change in FTIR spectrum after encapsulation is caused by the binding between curcumin and NaCas, possibly with the amide I group.

The binding between curcumin and NaCas was further confirmed by fluorescence spectroscopy (Figure 4-7). The fluorescence spectrum of proteins can be affected by interactions with hydrophobic compounds and can be applied to study the microenvironment changes in aqueous systems (Barik, Priyadarsini, & Mohan, 2003). It has been reported previously that curcumin binds with casein micelles through hydrophobic interactions (Levine, Mosoni, Berlett, & Stadtman, 1996), which is also the case when curcumin binds with bovine serum albumin (Barik, et al., 2003). The blue shift of curcumin peak at 543 nm, to 495 nm, after encapsulation and the sharp increase in fluorescence intensity (Figure 4-7) agree with the results reported previously (Sahu, et al., 2008; Sahu, et al., 2011). This indicates the environmental change of curcumin, from a more polar environment when dissolved in aqueous ethanol to the hydrophobic regions of caseins after encapsulation. This also suggests that curcumin is present at the core of nanocapsules in aqueous dispersions.

**4.4.5 Bioactivity of encapsulated curcumin**

The TEAC of curcumin before and after encapsulation is shown in Figure 4-8. The anti-oxidant properties have also been studied for caseins and casein-derived peptides (Cervato, Cazzola, & Cestaro, 1999; Kitts, 2005), which are ascribed to amino acid residues such as tryptophan and methionine cysteine that can be oxidized by free radicals (Garrison, 1987; Levine,
et al., 1996; Stadtman & Levine, 2003). Therefore, the TEAC of the processed NaCas was also determined, which was 300 μM (Figure 4-8). For curcumin, it potentially has a high antioxidant activity because its phenolic –OH groups play a major role in the oxidation reactions (Khopde, Priyadarsini, Venkatesan, & Rao, 1999). However, pristine curcumin has a low antioxidant activity (240 μM TEAC) when dissolved in 95% ethanol followed by dilution in water for testing, because the low solubility limits the amount of dissolved curcumin molecules to react with free radicals in the aqueous phase. The encapsulated curcumin had a much higher TEAC (8.87 mM) than those of pristine curcumin, the processed NaCas, and their summation (p < 0.05). By dispersing in casein nanoparticles, the encapsulated curcumin is evenly distributed in the reaction medium, and the big surface area due to the nanometer-sized structures facilitates the kinetics of reaction with free radicals in the aqueous phase. The much improved antioxidant activity of curcumin after dispersion in colloidal particles such as beta-casein and lecithins also has been reported (Esmaili, et al., 2011; Takahashi, Uechi, Takara, Asikin, & Wada, 2009).

Figure 4-9 shows the normalized viability of human colon cancer HCT-116 cells, after being treated with DMSO-dissolved or encapsulated curcumin at different concentrations. DMSO or NaCas alone did not show any significant inhibition of cell growth. However, the encapsulated curcumin exhibited a higher activity of cell growth arrest than curcumin dissolved in DMSO. The significant difference was noticed when the curcumin concentration was higher than 20 μg/mL (p < 0.05). The improved anti-cancer activity might have resulted from the much improved dispersibility after encapsulation in casein nanoparticles that provide a large surface area to contact with cancer cells. Similar improvements in anti-cancer activity have been reported for curcumin after binding with casein micelle (Sahu, et al., 2008) or complexing with hydrophobically-modified starch (H. Yu, et al., 2010).
The approach in the present study does not require high pressure homogenization and structural modification of food biopolymer, and the encapsulated curcumin is in the powdered form. These features are advantageous to encapsulate lipophilic bioactive compounds in industrial settings, particularly in the production of transparent beverages. It however should be noted that the eventual application of nanoencapsulated curcumin will require further testing of in vivo bioavailability and in vivo biological activities, as well as in vivo and in vitro toxicology studies to ensure the safe application in foods and beverages. The allergenicity of caseins (Wal, 2002) is another limitation to some consumers.

4.5 Conclusions

In conclusion, dissociation of NaCas in warm aqueous ethanol exposed more hydrophobic regions of caseins for contacting curcumin. Upon hydration of spray-dried powder, the amount of curcumin in transparent dispersions improved 4 folds when comparing to simply mixing NaCas and curcumin and was 4 decades higher than the water solubility of curcumin. Encapsulation reduced the crystallinity and improved the thermal stability of curcumin. The bigger particle size of curcumin nanocapsules than that of the processed NaCas and the fluorescence spectroscopy together with the FTIR results suggested that curcumin was present in the capsule core due to hydrophobic interactions with caseins. The much improved dispersibility after encapsulation improved the reactivity of curcumin, resulting in enhanced biological activity as assessed by antioxidant and cell growth assays. The simple approach used in this work may be used to deliver a variety of lipophilic bioactive compounds to improve the health and wellness of consumers.
4.6 Acknowledgements

The authors sincerely thank Dr. Xiaobo Zhang from Department of Biomedical and Diagnostic Sciences in College of Veterinary Medicine at The University of Tennessee in Knoxville for his instruction and technical assistance.
References


Esmaili, M., & Ghaffari, S. M. (2011). Beta casein-micelle as a nano vehicle for solubility enhancement of curcumin; food industry application. LWT-Food Science and Technology, 44(10), 2166-2172.


Appendix

Table 4-1. Average hydrodynamic diameter of native sodium caseinate (NaCas), processed NaCas, and curcumin capsules after hydration in deionized water.

<table>
<thead>
<tr>
<th>Dispersion</th>
<th>Mean diameter (nm)*</th>
<th>Polidispersity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native NaCas</td>
<td>216.0 ± 6.0a</td>
<td>0.222 ± 0.010a</td>
</tr>
<tr>
<td>Processed NaCas</td>
<td>134.1 ± 3.5b</td>
<td>0.224 ± 0.005a</td>
</tr>
<tr>
<td>NaCas encapsulated with curcumin</td>
<td>168.7 ± 10.2c</td>
<td>0.280 ± 0.011b</td>
</tr>
</tbody>
</table>

*Numbers are mean ± standard deviation. Different superscript letters represent significant differences of mean.
Figure 4-1. (A) XRD patterns of the processed sodium caseinate (NaCas, black), curcumin crystals (red), and curcumin encapsulated in NaCas (blue). (B) A simple mixture of curcumin crystals and NaCas at the same ratio as in the capsules in (A).
Figure 4-2. DSC thermograms of the processed sodium caseinate (NaCas, red), curcumin crystals (black), and curcumin encapsulated in NaCas (blue) during heating from 30 to 250 °C at a rate of 5 °C/min.
Figure 4-3. Free (a) and encapsulated (b) curcumin dispersed in deionized water at the concentration of 137 µg/mL.
Figure 4-4. AFM topography images of the native sodium caseinate (a) and curcumin capsules (b). The plot indicates the height distribution along the measurement line.
Figure 4-5. Schematic mechanism of the structural changes of sodium caseinate (NaCas) and encapsulation of curcumin.
Figure 4-6. FT-IR spectra of the native sodium caseinate (NaCas, red), the processed NaCas (blue), curcumin crystals (black), and encapsulated curcumin (green).
Figure 4-7. Fluorescence emission spectra of the native sodium caseinate (NaCas, red), the processed NaCas (blue), free curcumin (black) and encapsulated curcumin (green).
Figure 4-8. Trolox equivalent antioxidant capacity (TEAC) of the processed sodium casein (NaCas), free curcumin, and encapsulated curcumin at 30 °C. Different letters above the bars denote significant difference in mean.
Figure 4-9. Normalized cell viability of DMSO-dissolved (hatched bars) and encapsulated (open bars) curcumin against HCT-116 cell line at different overall concentrations in media. The blanks were controls by using DMSO and sodium caseinate alone for free curcumin and encapsulated curcumin, respectively. The results were normalized by the viability of control group treated with DMSO only. Error bars are standard deviations from four replicates. The **denotes that the sample is significantly different from other treatments (p < 0.05).
Chapter 5. pH-Driven encapsulation of curcumin in self-assembled casein nanoparticles for enhanced dispersibility and bioactivity
A version of this chapter was originally published by Kang Pan, Yangchao Luo, Yundi Gan, Seung Joon Baek, and Qixin Zhong:

5.1 Abstract

The poor water solubility and bioactivity of lipophilic phytochemicals can be potentially improved by delivery systems. In this study, a low-cost, low-energy, and organic solvent-free encapsulation technology was studied by utilizing pH-dependent solubility properties of curcumin and self-assembly properties of sodium caseinate (NaCas). Curcumin was deprotonated and dissolved, while NaCas was dissociated at pH 12 and 21 °C for 30 min. The subsequent neutralization enabled the encapsulation of curcumin in self-assembled casein nanoparticles. The degradation of curcumin at encapsulation conditions was negligible based on visible light and nuclear magnetic resonance spectroscopy. The dissociation of NaCas at pH 12 and reassociation after neutralization were confirmed using dynamic light scattering and analytical ultracentrifugation. The curcumin encapsulated in casein nanoparticles showed significantly improved anti-proliferation activity against human colorectal and pancreatic cancer cells. The studied encapsulation method is promising to utilize lipophilic compounds in the food or pharmaceutical industries.

Keywords: curcumin, casein, encapsulation, pH, self-assembly, degradation, anti-cancer potential
5.2 Introduction

Phytochemicals are important constituents of secondary metabolites produced by plants. Numerous food-sourced phytochemicals have been recognized for the significance to human health with anti-oxidant, anti-cancer, and anti-inflammatory activities (Scalbert, et al., 2011). More than one-half of food-derived phytochemicals are lipophilic, and their poor solubility in aqueous solutions is an obstacle of applying them in food and pharmaceutical industries as anti-cancer and chemopreventive agents (Ting, Li, Ho, & Huang, 2013). The solubility is one of the critical determinants of their bioactivity and bioavailability. Therefore, strategies are needed to improve the solubility, bioactivity and bioavailability of lipophilic phytochemicals.

Chemical and biological approaches have been studied to improve the solubility of phytochemicals, e.g., by glycosylation of curcumin in cell cultures of Catharanthus roseus (Kaminaga, et al., 2003). Structurally-modified phytochemicals however require extensive toxicity studies before their applications. Physical methods, particularly, through encapsulation in appropriately designed delivery systems have been the most dominant research approach to overcome the aforementioned limitations of lipophilic phytochemicals. This has led to numerous studies synthesizing molecules and developing processes to encapsulate phytochemicals to improve the dispersibility in water and in vivo bioactivity and bioavailability (Kohane & Langer, 2010). This calls for the study of biocompatibility issues due to the toxicity of fabricated materials (synthetic polymers or surfactants), residual organic solvents and acid, as well as any products that are degraded during preparation (Kohane, et al., 2010). The possible side effects or complications may be especially more problematic for cancer patients with compromised immune systems. The side effects and complications also negate the advantages of phytochemicals being naturally-occurring cancer curing and prevention agents. As prevention strategies, phytochemicals fall in
the category of functional foods (Johnson, 2003). For functional foods applications, only generally-recognized-as-safe (GRAS) ingredients can be used, and residual organic solvents, if used in encapsulation as processing aids, are to be below the regulated levels. In addition, the costs of encapsulation materials and processes have to be sufficiently low for such applications.

Food proteins have received increasing attention for use as GRAS materials to fabricate delivery vehicles for nutraceuticals in forms such as hydrogels, emulsions, microparticles, and nanoparticles (Chen, Remondetto, & Subirade, 2006). The biodegradability, biocompatibility, non-toxicity, abundance, sustainability, and inexpensiveness make food proteins viable materials to develop delivery systems of bioactive compounds. The demonstrated benefits of protein-based delivery vehicles include the improved stability and extended shelf life of labile nutraceuticals, enhanced intestinal absorption and cellular uptake, prolonged blood residence time, and reinforced anti-cancer efficacy (Elzoghby, Samy, & Elgindy, 2012). During the preparation of protein-based delivery systems, some procedures may still involve chemical crosslinking or organic solvents (mostly, ethanol or methanol), which are potentially harmful to human being. Therefore, much work is needed to study physical methods to fabricate protein-based delivery systems without organic solvent.

Caseins consist of about ~80% of proteins in bovine milk. There are four types (\(\alpha_{s1}\), \(\alpha_{s2}\), \(\beta\), and \(\kappa\)) of caseins present in the supramolecular structures known as “casein micelles” in bovine milk, with about \(10^4\) casein molecules and a diameter ranging from 40 to 300 nm (Walstra, Wouters, & Geurts, 2006). In addition to hydrophobic interactions, calcium phosphate, in the form of \(\text{Ca}_9(\text{PO}_4)_6\) clusters, “glues” phosphoproteins (\(\alpha\) and \(\beta\) caseins), resulting in the casein micelle structure with the center being mostly \(\alpha\) and \(\beta\) caseins (Walstra, et al., 2006). The presence of hydrophobic and hydrophilic amino acids makes caseins excellent naturally occurring block
copolymers feasible for encapsulation of hydrophobic compounds to enhance stability and improve cellular uptake (Luo, Teng, Wang, & Wang, 2013). Physical forces in casein micelles have been utilized to dissociate and re-assemble caseins. Under high hydrostatic pressure, casein micelles are dissociated (Huppertz & De Kruif, 2006), which has been used in high pressure homogenization to encapsulate vitamin D$_2$ in casein particles with similar dimensions to native casein micelles (with a mean diameter of ~200 nm) (Semo, Kesselman, Danino, & Livney, 2007). Casein micelles are also dissociated above 60 °C in aqueous ethanol with more than 35% ethanol (O’Connell, Kelly, Auty, Fox, & de Kruif, 2001; O’Connell, Kelly, Fox, & de Kruif, 2001; Pan, Zhong, & Baek, 2013). The aqueous ethanol provides a solvent for lipophilic phytochemicals, which was used in our recent study to dissolve curcumin for spray drying (Pan, Zhong, et al., 2013). The activity of encapsulated curcumin against HCT-116 colorectal cancer cell line was higher than that pre-dissolved in dimethylsulfoxide (DMSO). Casein micelles can also be dissociated at alkaline conditions (Pan, Zhong, et al., 2013; Vaia, Smiddy, Kelly, & Huppertz, 2006). Alkaline dissociation at pH 11.0 followed by acidification was used in our other recent study to produce casein nanoparticles with diameters of <60 nm (Pan & Zhong, 2013). The molecular mass of casein particles decreased during alkalization and increased during the subsequent acidification using HCl, indicating the dissociation and re-association, respectively. Since phytochemicals like curcumin are insoluble at neutral acidity but can be dissolved at alkaline pH due to deprotonation (Scheme 1) (Leung, Colangelo, & Kee, 2008), they could be co-dissolved with alkaline-dissociated caseins and be encapsulated in self-assembled casein nanoparticles during acidification. These properties enable the innovation of a low-cost, low-energy, and organic solvent-free nanoencapsulation method to improve anti-cancer activity of lipophilic phytochemicals based on caseins.
In the present study, curcumin was studied as a model phytochemical for encapsulation in casein nanoparticles by first increasing pH to dissolve curcumin and dissociate caseins and subsequent neutralization to reassociate caseins with in situ precipitated curcumin. Curcumin is extracted from turmeric *Curcuma longa* and has high activity to suppress the nuclear factor-κB (NF-κB), enabling their anticancer activity (Kunnumakkara, et al., 2007). Sodium caseinate (NaCas) was used as casein ingredients. NaCas is produced by precipitation of casein micelles at its isoelectric point (pI, around pH 4.6) from bovine milk, followed by neutralization using NaOH before spray drying (Walstra, et al., 2006). The structure of NaCas during alkaline treatment was studied by dynamic light scattering (DLS), analytical ultracentrifugation (AUC), and reducing protein electrophoresis. Because curcumin is known to be degraded at alkaline conditions (Leung, et al., 2008; Tonnesen, 2002), the structure of curcumin before and after treatment was characterized by UV-vis spectroscopy and nuclear resonance magnetic (NMR) spectroscopy. The bioactivity of curcumin after encapsulation was evaluated for anti-oxidant capacity and the activity against the proliferation of human colorectal and pancreatic cancer cells.

Scheme 1. Structure of curcumin, with deprotonable hydroxyl groups highlighted in red.
5.3 Experimental methods

5.3.1 Chemicals

Curcumin and NaCas were purchased from Sigma-Aldrich Corp. (St Louis, MO). The products had a purity of over 90% w/w according to the vendor. Other chemicals were obtained from either Sigma-Aldrich or Thermo Fisher Scientific (Pittsburgh, PA).

5.3.2 Encapsulation protocol

To prepare the samples, 2% w/w of NaCas was hydrated in deionized water for more than 6 h at room temperature (21 °C) and was adjusted to pH 12 using 4 M NaOH. After incubation for 30 min, various amounts of curcumin crystals were mixed with NaCas dispersion at pH 12. After stirring on a magnetic stirring plate operating at 300 rpm for 10, 30 and 60 min, the mixture was acidified to pH 7 using 4 M HCl. Because of relatively high loading content and encapsulation efficiency, the sample prepared by mixing 1 mg/mL curcumin in the 2% NaCas solution at pH 12 for 30 min followed by neutralization and centrifugation at 10000 g for 10 min to remove big particulates was also lyophilized (model 12 EL freeze drier, VirTis Company, Inc., Gardiner, NY) for below differential scanning calorimetry (DSC) and cell proliferation assays. The lyophilization at 26.6 kPa under pressure was conducted at subsequent steps of -40 °C for 600 min, -30 °C for 610 min, -20 °C for 610 min, -10 °C for 350 min, 0 °C for 350 min, 5 °C for 240 min, and 10 °C for 240 min.

5.3.3 Encapsulation Efficiency (EE) and Loading Content

The above dispersion after neutralization was centrifuged at 10000 g for 10 min. The supernatant was transferred and mixed with different volumes of chloroform (to the range of calibration curve). After stirring overnight at room temperature (21 °C) and allowing the mixture
to separate to two phases, the bottom chloroform phase was transferred and measured for absorbance at 419 nm ($Abs_{419}$) using a UV-Vis spectrophotometer (Evolution 201, Thermo Scientific, Waltham, MA). The $Abs_{419}$ was used to determine curcumin concentration based on a calibration curve previously established using standard solutions with different amounts of free curcumin dissolved in chloroform (Pan, Zhong, et al., 2013). EE was then calculated as the percentage of curcumin in the transparent supernatant after centrifugation with respect to the total curcumin used in encapsulation. Loading content was calculated as the percentage of curcumin mass with respect to the total mass of curcumin and NaCas. Three independent replicates were measured.

5.3.4 UV-vis Spectroscopy

Degradation of curcumin at pH 12 (Leung, et al., 2008; Tonnesen, 2002) was studied with and without NaCas. Curcumin was pre-dissolved at 5 mg/mL in methanol, and 200 μL of the curcumin stock solution was mixed with 40 mL of water or aqueous solutions with 1 mg/mL NaCas or 5 mg/mL sodium dodecyl sulfate (SDS), all adjusted to pH 12 using 4 M NaOH. The absorbance spectra of curcumin solutions were acquired from 350 to 700 nm every 5 min using the above UV-vis spectrophotometer, with deionized water being a blank.

5.3.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

The encapsulation was conducted as above, by mixing 1 mg/mL curcumin in the 2% NaCas solution at pH 12 and incubating at room temperature for 30 min before being adjusted to pH 7.0. After centrifugation to remove the precipitate, the supernatant was transferred and added with 10-fold volume of chloroform to extract curcumin as above. The chloroform was removed in a rotator evaporator at 40 °C and was vacuum dried for 30 min. Pristine curcumin crystals, as a control,
were dissolved in chloroform and vacuum-dried at the same conditions as the encapsulated curcumin. The obtained curcumin powders were dissolved in dimethyl sulfoxide-$d_6$ (DMSO-$d_6$) for $^1$H and $^{13}$C NMR using a VARIAN VNMRS 500 MHz spectrometer (Varian, Inc., Palo Alto, CA). $^1$H NMR of curcumin (500 MHz, DMSO-$d_6$) was identified as chemical shift ($\delta$) of 3.90 (6H, s, OCH$_3$), 6.06 (1H, s, C(OH)=CH), 6.76 (2H, d, 2,6-H), 6.82 (2H, d), 7.15 (2H, d), 7.32 (2H, s), 7.55 (2H, d, 1,7-H), and 9.70 (2H, s, phenolic OH). (Anderson, Mitchell, & Mohan, 2000) $^{13}$C NMR of curcumin (125 MHz, DMSO-$d_6$) was identified as $\delta$ 56.21, 101.48, 111.84, 116.26, 121.62, 123.65, 126.89, 141.26, 148.54, 149.88, and 183.74 (Anderson, et al., 2000).

5.3.6 Dynamic Light Scattering (DLS) and Zeta-potential

The dispersions were diluted 20 times using deionized water or deionized water previously adjusted to pH 12.0 using 4 M NaOH and were measured with a Delsa Nano analyzer (Beckman Coulter, Atlanta, GA, USA) at a scattering angle of 165°. The time correlation functions were analyzed with a Laplace inversion program (CONTIN) to obtain the distribution of hydrodynamic diameter. The diluted samples at pH 7.0 were also measured for zeta-potential (model Zetasizer, Malvern Instruments, Worcestershire, UK).

5.3.7 Analytical Ultracentrifugation (AUC)

AUC (model XL-I, Beckman Coulter, Inc., Palo Alto, CA) experiments were conducted according to the procedures in our earlier work (Pan & Zhong, 2013). Briefly, the dispersion with 2% w/w NaCas only at pH 7.0 was adjusted to pH 12.0 and incubated at room temperature for 30 min, followed by neutralization to pH 7.0 as above. The pH 12.0 sample was diluted to a protein concentration of 1.2 mg/mL in deionized water and readjusted to pH 12. The pH 7.0 samples before and after the treatment were also diluted to the same protein concentration. The centrifugation was
performed at 50000 rpm and 25 °C. The SEDFIT software was used to analyze data using the continuous c(s) distribution model, with the anhydrous frictional ratio (f/f₀) set at 1.2.

5.3.8 Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed under the reducing conditions using a Mini Protein Tetra cell (Bio-Rad Laboratories, Inc., Hercules, CA) at a constant voltage of 200 V. The 15% Tris-HCl gel (Ready Gel Precast Gel) was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). The gel was stained with Coomassie blue.

5.3.9 Fluorescence spectroscopy

The interactions between curcumin and casein at pH 12 were studied by fluorescence spectroscopy using a spectrofluorometer (model RF-1501, Shimadzu Corp., Kyoto, Japan). Briefly, curcumin stock solution was prepared at 2.5 mg/mL in 50% v/v methanol and was adjusted to pH 12 before being diluted to 4.5 – 40.5 µM in 0.2 mg/mL aqueous NaCas in 20 mM pH 12 disodium hydrogen phosphate buffer. Fluorescent spectroscopy was carried out with an excitation wavelength of 280 nm and emission spectra were recorded from 300 to 500 nm. The slit width for excitation and emission was set at 20 nm. The fluorescence intensity of pristine curcumin dissolved in the 20 mM pH 12 buffer at concentrations identical to those with NaCas was used as background fluorescence for calibrating the spectra.

5.3.10 Transmission Electron Microscopy (TEM)

The morphology of NaCas only before and after treatment at encapsulated conditions and the encapsulation treatment with an initial curcumin concentration of 1 mg/mL was studied using TEM. The freshly prepared sample was diluted 100 times with deionized water. One drop of a diluted sample was placed on a freshly glow-discharged carbon film on a 400-mesh copper grid
and then stained with 1% uranyl acetate. Samples were imaged with a Zeiss Libra TEM operating at 200 kV (Carl Zeiss Microscopy, Oberkochen, Germany).

5.3.11 Free Radical Scavenging Activity

Free radical scavenging activity of pristine and nanoencapsulated curcumin was compared in two methods where the assay solvent was either a solvent (ethanol/methanol mixture) or non-solvent (water) of curcumin. Fresh curcumin dispersions were prepared by mixing 1 mg/mL curcumin in the 2% NaCas solution at pH 12 for 30 min followed by neutralization. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities of free and nanoencapsulated curcumin were determined according to the method described by Zhang et al. (Zhang, Luo, & Wang, 2011) with some modifications. Briefly, curcumin dispersion or pristine curcumin predissolved in methanol was mixed with 2 mL of a 0.1 mM DPPH solution that was previously prepared by dissolving in ethanol, corresponding to final curcumin concentrations of 3.5 and 7 µg/mL, respectively. The mixture was vortexed and allowed to stand in dark for 30 min at room temperature (21 °C) and the absorbance was measured at 517 nm using the aforementioned UV-vis spectrophotometer. Controls were prepared by replacing the test sample with deionized water or methanol. The DPPH radical scavenging activity was calculated according to Equation 1:

\[
\text{Scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \quad (1)
\]

where \(A_{\text{sample}}\) and \(A_{\text{control}}\) are the absorbance of test sample and control, respectively.

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities of pristine and nanoencapsulated curcumin were determined according to the method described previously (Pan, Zhong, et al., 2013), with modification. Briefly, ABTS was dissolved at 7 mM in deionized water. Potassium persulfate was dissolved at an overall concentration of 2.45
mM in the ABTS solution and was allowed to stand in dark at room temperature (21 °C) for 12-16 h before use. The ABTS radical solution was diluted with 10 mM phosphate-buffered saline (PBS) at pH 7 to an absorbance value of 0.70 ± 0.02 at 734 nm and equilibrated at room temperature. Ten μL of a curcumin sample was dissolved in 1 mL ABTS radical solution to an overall concentration of 40 or 80 μg/mL and the absorbance reading was taken exactly 1 min after initial mixing for up to 6 min. The 10 mM PBS was used as a blank in each run. Each sample was measured in triplicate, and the mean and standard deviation were calculated. The scavenging capability of test compounds was determined using Equation 2:

$$Scavenging\ activity\ (\%) = \frac{A_{control} - A_{sample}}{A_{control}}$$ (2)

where $A_{sample}$ and $A_{control}$ are the equilibrium absorbance of test sample and control, respectively.

**5.3.12 In Vitro Cell Proliferation Assay**

Human colon cancer cell line HCT-116 and human pancreatic cancer cell line BxPC3 were purchased from American Type Culture Collection (Manassas, VA) and were cultured in McCoy’s 5A and RPMI1640 media (Mediatech, Herdon, VA). Both cells were cultured in media supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/mL streptomycin under a humidified atmosphere with 5% CO$_2$ at 37 °C. Anticancer activity of curcumin was tested using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corp., Madison, WI). Briefly, HCT-116 cells and BxPC3 cells were seeded in 96-well microtiter plates at a respective density of 1,000 and 10,000 cells per well in a final volume of 100 μL of medium. After 24 h, the cells were treated with a medium containing DMSO-dissolved or NaCas-encapsulated curcumin. For the treatment, 10% serum containing media were changed to 2% serum to minimize the interactions between curcumin and serum. Other cells were untreated (negative controls) or treated
only with DMSO or NaCas at the concentrations as in the dispersions with encapsulated curcumin (positive controls). After 48 h treatment, 20 μL of CellTiter 96 Aqueous One Solution (Promega, WI) was added into each well and then incubated for 1 h at 37 °C. Absorbance at 490 nm was measured with a microplate reader (Bio-Tek Instruments, Winooski, VT). Relative cell viability was expressed as the absorbance normalized by that of the control cells treated with same amounts of DMSO and casein as in DMSO-dissolved and encapsulated curcumin samples (equation 3). The mean and standard deviation from four-well replicates were calculated. The normalized cell viability was obtained after normalizing the viability of a treatment by the viability of control cells treated with DMSO only.

\[
Cell \text{ Viability} \, (\%) = \frac{A_{\text{treated}}}{A_{\text{control}}} \times 100 \tag{3}
\]

where \(A_{\text{treated}}\) and \(A_{\text{control}}\) are the absorbance of the wells with cells treated by curcumin and control, respectively.

5.3.13 Cell uptake studied by flow cytometry

Cell uptake of free and encapsulated curcumin was evaluated by using the method of Yallapu et al. (Yallapu, Jaggi, & Chauhan, 2010b) with modifications. Briefly, HCT-116 and BxPC3 cells were seeded in a 6-well plate at a density of 50,000 cells/well and were incubated under a humidified atmosphere with 5% CO\textsubscript{2} at 37 °C for 24 h for attachment. Free curcumin prepared at 10 mg/mL in DMSO and the fresh dispersion containing 40 μg/mL of encapsulated curcumin were used as working solutions. The cells were then treated with 0.4, 4, and 40 μg/mL of free or encapsulated curcumin diluted from the working solution in the cell media, followed by incubation for another 12 h at the above conditions. The cells were then washed twice with 1 mL PBS, trypsinized, centrifuged, and re-suspended in 1 mL PBS. The final cell suspensions were
injected into a flow cytometer to determine the fluorescence (model Accuri C6, Accuri Cytometers, Inc., Ann Arbor, MI, USA) at excitation and emulsion wavelengths of 488 and 530 nm, respectively.

5.3.14 Statistical Analysis

Statistical analyses were performed using the SAS program (version 9.3, SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was carried out. Differences between pairs of means were compared using a Tukey’s test. The significance level was set at 0.05.

5.4 Results and discussions

5.4.1 Encapsulation properties

Preliminary experiments by dissolving curcumin in NaCas dispersions at pH 11.0 for up to 60 min before neutralization demonstrated EE of less than 10%. After mixing 0.2-2.0 mg/mL curcumin in 2.0% NaCas dispersions at pH 12.0 for 10, 30 and 60 min followed by neutralization and centrifugation, the supernatants were transparent and had a darker color at a higher curcumin content (Figure 5-1a). The encapsulation efficiency (EE) of treatments, the percentage of curcumin in the transparent supernatant, is presented in Figure 5-1b. A lower EE was observed when a larger amount of curcumin was mixed with the same amount of NaCas. When curcumin was used at 0.2 mg/mL, the incubation time at pH 12.0 did not affect the encapsulation efficiency ($P > 0.05$). At higher curcumin usage levels, the EE increased significantly ($P < 0.05$) when the incubation time at pH 12.0 increased from 10 to 30 min, followed by insignificant increase ($P > 0.05$) after extending incubation to 60 min. The results indicate that it takes about 30 min at pH 12.0 and 21 °C to obtain a sufficient extent of curcumin deprotonation to enable the dissolution and subsequent encapsulation in caseins. The transparent dispersion obtained from the treatment using 1.0 mg/mL
curcumin and 30 min incubation at pH 12.0 was lyophilized after centrifugation and the powder was analyzed using DSC. Compared to the sharp endothermic peak of pristine curcumin corresponding to the melting of crystals, the lyophilized powder showed a smooth thermal curve and therefore amorphous structures (supplementary Figure 5-S1). The DSC results verify the encapsulation of curcumin in NaCas, as elaborated in our earlier study where curcumin was encapsulated by spray-drying warm aqueous ethanol solutions with co-dissolved curcumin and NaCas (Pan, Zhong, et al., 2013). Because DSC results agreed with X-ray diffraction data in our earlier study, (Pan, Zhong, et al., 2013) the lyophilized powder was not studied using X-ray diffraction in the present study.

The loadings of curcumin (1% to ~7% w/w, Figure 5-2) and EEs (70% to ~100%, Figure 5-1b) are compared well with several studies. Encapsulation of curcumin in zein nanoparticles based on anti-solvent precipitation was reported to have resulted in a loading of 1.6-4.1% and EE of 71.1-86.8% (Patel, Hu, Tiwari, & Velikov, 2010). EEs of 78.1-90% were reported in another study where curcumin was encapsulated in zein or synthetic polymers such as polycaprolactone or poly(lactic-co-glycolic acid) (PLGA) through the electrohydrodynamic atomization technique (Gomez-Estaca, Balaguer, Gavara, & Hernandez-Munoz, 2012; Xie, Marijnissen, & Wang, 2006).

The hydrodynamic diameter ($D_h$) of the transparent supernatants from the treatments incubated at pH 12.0 for 30 min is presented in Figure 5-2 that also shows the treatment with NaCas only. The $D_h$ of NaCas was 213 nm after the pH treatments and decreased when a larger amount of curcumin was used in encapsulation, to about 104 nm for the treatment with 2 mg/mL curcumin. Although the EE decreased (Figure 5-1b), the concentration of curcumin in transparent dispersions was higher when a larger amount of curcumin was mixed with 2% w/w NaCas (Figure 5-2), corresponding to a higher loading level. Our results are quite different from the increased
particle dimension when a higher amount of curcumin was loaded in PLGA nanoparticles (Shaikh, Ankola, Beniwal, Singh, & Kumar, 2009). In their study, PLGA and curcumin dissolved in a water-miscible organic solvent (ethyl acetate) was dropped in an aqueous phase, followed by homogenization and dilution in water to cause the precipitation of PLGA and curcumin. In the present study, caseins are soluble throughout the encapsulation process and are assembled as porous particles (Liu & Guo, 2008). Additionally, when curcumin was directly mixed with the NaCas dispersion at pH 7.0, no changes in $D_h$ were observed, indicating no encapsulation. The possible mechanism causing the reduced $D_h$ after encapsulation is discussed below.

5.4.2 Chemical structure changes of curcumin due to encapsulation

Alkaline degradation of curcumin has been observed and studied by many researchers (Price & Buescher, 1997; Tonnesen, Masson, & Loftsson, 2002). The structural changes of curcumin in the present study, due to exposure at pH 12.0 for up to 60 min at 21 °C, were studied using visible light spectroscopy and NMR. Figure 5-3 shows visible light spectra of curcumin during incubation in pH 12 aqueous solutions with and without NaCas or SDS for up to 60 min. The reduction of absorbance centered on 468 nm ($Abs_{468}$) indicates the possible degradation of curcumin at alkaline conditions (Leung, et al., 2008). The $Abs_{468}$ of 0.025 mg/mL curcumin at pH 12 without additives reduced by 4.3% after 60 min. In contrast, the reduction of $Abs_{468}$ after 60 min was only 2.9% with the presence of 1 mg/mL NaCas. The protection of curcumin degradation at pH 12 by NaCas was more apparent after 12 h storage at room temperature, showing <30% and >45% reduction in $Abs_{468}$ for treatments with and without NaCas, respectively.

Leung et al. (Leung, et al., 2008) encapsulated curcumin in cationic and anionic surfactant micelles at neutral pH and observed the degradation of curcumin after increasing the system pH to 13. Cationic surfactants cetyl trimethylammonium bromide and dodecyl trimethylammonium
bromide effectively suppressed the degradation of curcumin at pH 13.0 when compared to the treatment without surfactants. Conversely, anionic surfactant SDS facilitated the degradation at pH 13.0. At pH 13.0, curcumin is negatively charged after deprotonation and is repelled from SDS micelles but remains entrapped in cationic surfactant micelles, which results in opposite effects in stabilizing curcumin. Since caseins are negatively-charged at pH 12, the degradation of 0.025 mg/mL curcumin with 5 mg/mL SDS was compared in Figure 5-3c. As at pH 13.0 (Leung, et al., 2008), SDS caused a greater extent of curcumin degradation.

Hydrolysis to smaller molecules has been proposed for the degradation of curcumin at alkaline pH (Tonnesen, Karlsen, & Vanhenegouwen, 1986; Wang, et al., 1997). Since 30 min treatment at pH 12.0 was sufficient to encapsulate curumin at the studied conditions (Figure 5-1b), curcumin was extracted from the supernatant for the treatment using 1.0 mg/mL curcumin in encapsulation. The extracted curcumin and pristine curcumin showed identical absorption spectra at 250-600 nm (Figure 5-S2). The extracted curcumin and pristine curcumin were also dissolved in DMSO for $^1$H and $^{13}$C NMR. Both $^1$H and $^{13}$C spectra showed sharp peaks corresponding to individual H and C atoms and peaks were observed at identical positions for pristine curcumin and that extracted from capsules (Figure 5-4). The NMR results indicate the reversal deprotonation and protonation processes after incubating at pH 12.0 for 30 min and acidifying to pH 7.0 at 21 °C, as reported previously (Payton, Sandusky, & Alworth, 2007). Furthermore, the $^1$H NMR signal of the extracted curcumin, at $\delta$ 6.06 (hydroxyl group) indicates a keto-enol tautomer rather than a $\beta$-diketone (Huang, et al., 1997; Surh, 2003). Thus, it can be concluded that the hydrolysis of curcumin at pH 12 for 30 min at 21 °C is negligible.
5.4.3 Binding between casein and curcumin at pH 12 studied by fluorescence spectroscopy

Intrinsic fluorescence of proteins is a convenient way to study properties of proteins in aqueous solutions as affected by binding with small molecules. Figure 5-5 shows the emission spectra of casein with different concentrations of curcumin at pH 12 after excitation at 280 nm, which reflects the binding near tryptophan (Trp) and tyrosine (Tyr) residues. (Liang, Tajmir-Riahi, & Subirade, 2008) The greater reduction in fluorescence with an increase in curcumin concentration (Figure 5-5) indicates the binding between casein and curcumin. The intrinsic fluorescence was further analyzed using the Stern-Volmer equation: (Y. Zhang, et al., 2011)

\[
F_0/F = 1 + k_q \times \tau_o \times [Q] = 1 + K_{sv} \times [Q]
\] (4)

where \(F_0\) and \(F\) are fluorescence intensities in the absence and presence of a quencher \([Q]\); \([Q]\) is curcumin concentration (M); \(k_q\) is the fluorescence quenching rate constant; \(\tau_o\) is the lifetime of fluorophore fluorescence at the absence of quenchers and equals \(10^{-8}\) s; and \(K_{sv}\) is the Stern-Volmer quenching constant which can be determined by linear fit of \(F_0/F\) plot against \([Q]\) (Lakowicz, 2009).

The linear plot of \(F_0/F\) as a function of \([Q]\) excited at 280 nm is given in the inset of Figure 5-5. The obtained average \(K_{sv}\) value was 7700 M\(^{-1}\) at pH 12. Fluorescence quenching can result from collision (dynamic quenching) or complex formation (static quenching) between biopolymers and quenchers (Y. Zhang, et al., 2011). The \(k_q\) at excitation wavelength of 280 nm was \(7.7 \times 10^{11}\) M\(^{-1}\) s\(^{-1}\), which is about forty-fold higher than the maximum dynamic quenching constant (\(2 \times 10^{10}\) M\(^{-1}\) s\(^{-1}\)) for quenchers interacting with biopolymers (Lange, Kothari, Patel, & Patel, 1998). The results suggest the fluorescence quenching is static and therefore complexes are
formed when curcumin binds with caseins at pH 12, which may have been effective in reducing curcumin degradation at alkaline conditions (Figures 5-3 and 5-4).

5.4.4 Physical structural changes related to encapsulation

To further understand the encapsulation process, supramolecular structures of NaCas were studied at pH 7.0, after alkalization to pH 12.0, and the subsequent acidification to pH 7.0 using DLS and AUC. Increasing pH from 7 to 12 broadened the $D_h$ distribution of NaCas, and the subsequent acidification to pH 7 resulted in the $D_h$ distribution narrower than the pH 7 dispersion before pH adjustment, with the center shifting to a smaller dimension (Figure 5-6). The AUC results showed the reduced mass of casein particles after increasing pH from 7 to 12, and the casein particle mass increased when the pH was adjusted back to 7 but was smaller than the sample without alkaline treatment (Figure 5-7a). Since proteins can be hydrolyzed at alkaline pH, especially at high temperatures (Kinsella, 1979), SDS-PAGE was used to compare the molecular weight changes of caseins before and after 30-min incubation at pH 12 and 21 °C, showing no detectable differences at the studied conditions (Figure 5-7b).

Therefore, the $D_h$ and AUC data can be interpreted by the supramolecular changes of casein particles. The AUC data in Figure 5-7a suggest the dissociation of caseins when pH increases from 7 to 12, due to the enhanced intra-particle electrostatic repulsion, and re-associate to particles with fewer casein molecules after neutralization. The reduced casein population in the particles agrees with the reduced $D_h$ after alkaline treatment (Figure 5-6), while the enhanced intra-particle repulsion at pH 12, although with fewer caseins molecules associated loosely, can increase $D_h$ and broaden its distribution (Liu, et al., 2008; B. C. Zhang, et al., 2011).
TEM images of NaCas before and after treatment at encapsulation conditions are shown in Figure 5-8, with comparison to the encapsulation treatment with an initial curcumin concentration of 1.0 mg/mL. Native NaCas had irregular and heterogeneous structures (Figure 8a) that became smaller after treatment at encapsulation conditions (Figure 5-8b), which agreed with the DLS (Figure 5-6) and AUC (Figure 5-7) results. The flocculated structures accounted for the measured \(D_h\) being greater than 200 nm (Figure 5-2). After encapsulating curcumin, nanoparticles became more uniform and compact (Figure 5-8c). Structures in Figure 8c were smaller than 100 nm and were smaller than \(D_h\) (Figure 5-2). This again indicates some extent of flocculation of nanoparticles resulting the bigger dimension measured in DLS.

Zeta-potentials of dispersions prepared with different initial curcumin concentrations are presented Table 5-1. The zeta-potential of NaCas increased after treatment at encapsulation conditions. As presented above and discussed previously, (Pan, Chen, Davidson, & Zhong, 2014) the supramolecular structures of casein nanoparticles reduced after the pH cycle treatment, and the reassembled particles on average had a smaller number of less negatively-charged \(\kappa\)-casein that increased the overall magnitude of negative zeta-potential. After encapsulation of curcumin, the zeta-potential was similar to that of NaCas treated at identical conditions (Table 5-1), which indicates the supramolecular structural changes of caseins determine the surface properties of nanocapsules. Previously, (Pan, et al., 2014) when NaCas was used to directly emulsify thymol using high shear homogenization at neutral pH, the reduction of \(D_h\) and increase in the magnitude of negative zeta-potential were also observed after encapsulation, which was also interpreted by the re-distribution of different types of caseins.

Combining above results, the principle of encapsulating curcumin in NaCas at the studied conditions may be presented in Figure 5-9. At pH 7, curcumin is not soluble. When pH is increased
to 12, the deprotonation of curcumin makes it negatively charged to become solubilized, while casein particles lose some mass and become highly porous because of strong intra-particle electrostatic repulsion. The open structure of casein particles allows the inner diffusion and static binding of curcumin, which effectively protects curcumin from degradation at pH 12. When the alkaline dispersion is being neutralized, curcumin loses charges and solubility due to protonation and remains encapsulated in casein particles, which can also be added with other caseins (Figure 5-7) and curcumin. The increased hydrophobicity of curcumin during neutralization attracts surrounding caseins to a greater extent, which enhances the packing of caseins in nanoparticles and effectively reduces the $D_h$ (Figure 5-2). When the amount of caseins is insufficient, the initially dissolved curcumin becomes precipitated, causing the lowered EE at increased usage amounts of curcumin (Figure 5-1b).

5.4.5 Effects of nanoencapsulation on anti-oxidant and anti-proliferative activity of curcumin

The bioactivity of nanoencapsulated curcumin was first evaluated for antioxidant activity using the DPPH free radical scavenging activity test where curcumin is soluble in the assay solvent (methanol/ethanol mixture) and ABTS free radical scavenging activity test with aqueous system (pH 7 buffer). There was no significant difference between pristine and encapsulated curcumin (Figure 5-10), verifying that the maintained structure of curcumin (Figures 5-3 and 5-4) did not change the potential bioactivity of encapsulated curcumin.

The bioactivity of curcumin was further evaluated for the activity against proliferation of human colorectal cancer cells HCT-116 (Figure 5-11a) and human pancreatic cancer cells BxPC3 (Figure 5-11b). Curcumin encapsulated in casein nanoparticles showed the improved anti-proliferation activity against both HCT-116 and BxPC3 cells when compared to curcumin pre-dissolved in DMSO, which is similar to our earlier study using HCT-116 cells (Pan, Zhong, et al.,
2013). Interestingly, the two cell lines exhibited different responses to curcumin. Although free curcumin at 0.4 µg/mL did not show significant anti-proliferation effect on both HCT-116 and BxPC3 cells, with mean cell viability of 92.8% and 96.8%, respectively, the encapsulated curcumin at 0.4 µg/mL significantly lowered the viability of HCT-116 and BxPC3 cells to 73.2% and 82.7%, respectively. Similar improvement in anti-proliferation activity of encapsulated curcumin was observed at a dose of 4 µg/mL. At the highest concentration of 40 µg/mL tested in the present study, no significant difference between encapsulated and free curcumin was observed against BxPC3 cells, which could be ascribed to the saturation of anti-proliferation effect of curcumin against BxPC3 cancer cells (Li, Braiteh, & Kurzrock, 2005), but the difference was significant when tested against HCT-116 cells. The IC50 values of free and encapsulated curcumin against HCT-116 cells were estimated to be 37.4 µg/mL and 15.5 µg/mL respectively, and were 29.4 µg/mL and 25.3 µg/mL, respectively, against BxPC3 cells. Flow cytometry data (Figure 5-12) showed higher intracellular fluorescence of the encapsulated curcumin than free curcumin for both types of cancer cells, indicating the enhanced cellular uptake of curcumin after encapsulation. This agrees with several studies showing the improved cellular uptake after encapsulating curcumin in various carrier materials. (Mohanty & Sahoo, 2010; Yallapu, Jaggi, & Chauhan, 2010a; Yallapu, et al., 2010b) Because nanocapsules were stable during incubation (supplementary Table 5-S1) and nanoencapsulated curcumin had a smaller release rate than (supplementary Figure S3) but similar radical scavenging activity (Figure 5-10) as DMSO-dissolved curcumin, the enhanced cell anti-proliferation activity after nanoencapsulation in NaCas can be concluded to have resulted from the enhanced cell uptake. The significantly increased anti-proliferation activity of encapsulated curcumin further indicates the potential of the present encapsulation technology for utilizing phytochemicals as anti-cancer agents in functional food and pharmaceutical products.
5.5 Conclusions

In conclusion, curcumin has been successfully encapsulated in casein nanoparticles based on pH-dependent solubility characteristics of curcumin and dissociation and re-association properties of NaCas. Degradation of curcumin at alkaline conditions was suppressed by NaCas and no structural changes of curcumin before and after encapsulation were detected. At pH 12.0, casein particles lost some mass due to dissociation and became porous, allowing the binding with deprotonated curcumin. Upon neutralization, caseins re-associated into smaller particles that were packed more efficiently by the bound curcumin. The encapsulation of curcumin in casein particles showed significant improvement in cell uptake by and thus anti-proliferation activity against human colorectal and pancreatic cancer cells under the studied conditions. The low-cost, low-energy, and organic solvent free encapsulation technology utilizing GRAS food proteins can be used to incorporate lipophilic bioactive compounds such as phytochemicals in the functional food and pharmaceutical products.

5.6 Acknowledgements

The authors are grateful to the Bioanalytical Resources Facility at the University of Tennessee for use of the analytical ultracentrifuge and Dr. Edward Wright for assisting with the experiments.
References


Appendix

Table 5-1. Zeta-potential of dispersions after encapsulation with various initial amounts of curcumin, with comparison to NaCas alone before and after treatment at encapsulation conditions.

<table>
<thead>
<tr>
<th>Initial curcumin concentration (mg/mL)</th>
<th>Zeta-potential (mv)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Native NaCas)</td>
<td>-21.77 ± 0.89&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0 (NaCas after pH treatments)</td>
<td>-38.40 ± 0.95&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.2</td>
<td>-39.07 ± 1.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.4</td>
<td>-37.70 ± 1.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.0</td>
<td>-37.63 ± 2.51&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.0</td>
<td>-38.70 ± 1.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Numbers are mean ± standard deviation (n = 3). Different superscript letters indicate significant differences (P < 0.05).
Figure 5-1. Examples of transparent dispersions containing 0.2-0.8 mg/mL curcumin encapsulated by 2% w/w NaCas (a). The encapsulation efficiency (b) is shown for treatments mixing 2.0% w/v NaCas dispersion at pH 12.0 with 0.2-2.0 mg/mL curcumin at 21 °C for 10, 30, and 60 min followed by neutralization. Error bars are standard deviations (n = 3). Different letters above bars indicate significant differences in the mean (P < 0.05).
Figure 5-2. Hydrodynamic diameter (squares) of and the loading content of curcumin (circles) in the transparent supernatants prepared from treatments mixing 2.0% w/v NaCas and 0-2.0 mg/mL curcumin at pH 12.0 for 30 min at 21 °C followed by neutralization to pH 7.0 and centrifugation. Error bars are standard deviations (n = 3).
Figure 5-3. Absorbance spectra of 0.025 mg/mL curcumin dissolved in aqueous solutions at pH 12 without (a) and with 1 mg/mL NaCas (b) or 5 mg/mL SDS (c). The spectra were collected every 5 min for up to 60 min at 21 °C.
Figure 5-4. $^1$H (a and b) and $^{13}$C (c and d) NMR spectra of pristine curcumin (a, c) and that extracted from nanodispersion (b, d). Chloroform corresponds to the chemical shift ($\delta$) of 8.32 in the $^1$H spectrum and 79.16 in the $^{13}$C spectrum.
Figure 5-5. Fluorescence emission spectra at excitation wavelength of 280 nm of 0.2 mg/mL casein in 20 mM phosphate buffer at pH 12.0, after interacting with 0 – 40.5 µM curcumin (i to x, with 4.5 µM increase successively). The plot of $F_0/F$ versus curcumin concentration is shown in the inset, with the line showing the linear fit of data with an intercept of 1.
Figure 5-6. Hydrodynamic diameter distributions of NaCas at pH 7.0 (pH 7 up, black), after adjusting pH to 12 (red) and subsequent neutralization to pH 7 (pH 7 down, blue).
Figure 5-7. NaCas dispersions at pH 7 (pH 7 up), after adjusting to pH 12, and subsequent neutralization to pH 7 (pH 7 down) as studied for AUC sedimentation profile (a) and SDS-PAGE (b), with lanes (Left to Right) being samples before and after pH treatments and markers.
Figure 5-8. TEM topography images of (a) native NaCas, (b) NaCas processed at encapsulation conditions, and (c) NaCas encapsulated with curcumin at an initial curcumin concentration of 1 mg/mL.
Figure 5-9. The proposed principle of the pH-driven encapsulation of curcumin in self-assembled casein nanoparticles.
Figure 5-10. DPPH (a) and ABTS (b) radical scavenging activity of methanol- or DMSO-dissolved curcumin, with comparison to the encapsulated curcumin. Error bars are standard deviations (n = 3). No difference was observed for free and encapsulated curcumin at a same concentration ($P > 0.05$), and the activity was significantly higher at a higher curcumin concentration ($P < 0.05$).
Figure 5-11. Anti-proliferation activity of 0.4, 4, and 40 μg/mL DMSO-dissolved and encapsulated curcumin against human colorectal HCT-116 (a) and pancreatic BxPC3 (b) cancer cells. NaCas alone (control) did not show inhibition against both cells. Error bars are standard deviations (n = 4). The encapsulated curcumin treatments labeled with asterisks above bars represent significant differences (P < 0.05) from the same concentration of DMSO-dissolved curcumin.
Figure 5-12. Flow cytometry fluorescence intensity of (a) HCT-116 and (b) BxPC3 cells after treatment by 0.4, 4, and 40 μg/mL (left to right plots in each panel) of free and encapsulated curcumin.
Chapter 6. Low energy, organic solvent-free co-
assembly of zein and caseinate to prepare stable
dispersions
6.1 Abstract

Water-insoluble zein (corn prolamins) has great potential for use as delivery systems and packaging materials. In this study, stable zein nanoparticle dispersions were produced using a pH-cycle method to form complexes with sodium caseinate (NaCas) without using an organic solvent. NaCas was mixed with zein at pH 11.5 and the subsequent neutralization to pH 7 enabled the co-assembly of zein and NaCas. The mean hydrodynamic diameter of spherical nanoparticles was smaller than 100 nm based on dynamic light scattering, scanning electron microscopy, and atomic force microscopy. The nanodispersions were stable during 30-day storage at 4 °C. The freeze-dried sample showed excellent re-dispersibility. Films prepared from nanoparticles had a Young’s modulus of 1.5–1.6 GPa and homogeneous nanostructures with a surface roughness of 19.2 nm. The current study showed the promise of this novel method to prepare zein nanoparticles for applications such as delivery systems and edible packaging films.

Keywords: zein nanoparticle; casein; pH-driven self-assembly; dispersibility, Young’s modulus
6.2 Introduction

Zein is a group of alcohol-soluble (prolamine) proteins extracted from maize and has more than 50% hydrophobic amino acid residues (Argos, Pedersen, Marks, & Larkins, 1982; Luo & Wang, 2014). There are four types of zein (α-, β-, γ-, and δ-) that differ in solubility and amino acid sequences. α-Zein is the most abundant, contributing to 75 – 80% of the total protein, and has two fractions of Z19 and Z22 with a respective molecular weight of 19 and 22 kDa (Tatham, et al., 1993). Because of its high hydrophobicity, biodegradability, and generally-recognized-as-safe regulatory status, zein has been studied extensively as a coating material or a carrier in delivery systems (Chen, Zhang, & Zhong, 2015; Chen & Zhong, 2015; Luo, et al., 2014; Paliwal & Palakurthi, 2014; Zhong & Jin, 2009b). For the latter, micro- or nanoparticles of zein have been studied for encapsulation of colorant and bioactive compounds such as curcumin and lysozyme using methods such as supercritical anti-solvent precipitation, spray drying, and electrohydrodynamic atomization (Gomez-Estaca, Balaguer, Gavara, & Hernandez-Munoz, 2012; Zhong & Jin, 2009a; Zhong, Jin, Davidson, & Zivanovic, 2009). However, the insolubility in water limits the application of zein, and methods are to be developed to improve the dispersibility of zein in aqueous systems.

Liquid-liquid dispersion (LLD) is a commonly used method to prepare zein nanoparticles, with the possibility to simultaneously encapsulate lipophilic compounds. This is enabled by first dissolving zein (and a lipophilic compound) in aqueous ethanol followed by dispersing into water to precipitate zein as nanoparticles when the overall ethanol concentration becomes a non-solvent of zein (Zhong & Jin, 2009b). To stabilize hydrophobic zein nanoparticles, several biopolymers such as chitosan and sodium caseinate (NaCas) have been found to form stable complexes with zein (Luo, et al., 2014; Luo, Zhang, Whent, Yu, & Wang, 2011). NaCas is a good source of
essential amino acids and has good surface activity due to a balanced composition of hydrophilic and lipophilic amino acids, which enables its application in food and pharmaceutical delivery systems (Hogan, McNamee, O'Reiordan, & O'Sullivan, 2001; Pan, Chen, Davidson, & Zhong, 2014; Pan, Zhong, & Baek, 2013; Y. Zhang & Zhong, 2013). Formation of NaCas-zein complexes improves the re-dispersibility of freeze- or spray-dried zein nanoparticle powder at neutral pH (Chen & Zhong, 2014, 2015; Luo, Teng, Wang, & Wang, 2013; Patel, Bouwens, & Velikov, 2010). Despite the efficacy of the LLD method in producing zein nanoparticles, ethanol presents the flammability hazard and is not permitted in alcohol-free foods.

Previously, we showed casein micelles in skim-milk powder were dissociated in alkaline pH and resembled as smaller particles after neutralization of pH (Pan & Zhong, 2013). This pH-cycle method, by controlling dissociation and reassociation of caseins, also enables the encapsulation of curcumin that is dissolved at alkaline pH to interact with dissociated caseins and becomes precipitated and in situ entrapped in re-assembled casein particles during acidification back to neutral pH (Pan, Luo, Gan, Baek, & Zhong, 2014). This enables the fabrication of nanoparticles with encapsulated hydrophobic compounds without using an organic solvent and sophisticated equipment. Because zein was observed to be soluble at alkaline pH in preliminary studies, we hypothesize the pH-cycle method can be used to fabricate zein-NaCas complex nanoparticles with excellent stability. This can eliminate the use of an organic solvent that is required in producing zein nanoparticles with the LLD method.

The objective of the present study was to fabricate and characterize zein-NaCas nanoparticles produced with the pH-cycle method by first increasing aqueous dispersions with different NaCas:zein mass ratios to pH 11.5, followed by acidification to pH 7.0. The re-dispersibility, particle size, stability, hydrophobicity, zeta-potential, and morphology of zein-
NaCas particles produced from the pH-cycle method were compared with those produced by the LLD method. Thermal properties of zein-NaCas particles were studied to understand the interaction and structure of particles. Finally, to study the potential of nanoparticles for use as a packaging material, the Young’s modulus and surface roughness of dried films were quantified using atomic force microscopy.

6.3 Materials and methods

6.3.1 Materials

Purified α-zein and ethanol (200 proof) were purchased from Acros Organics (Morris Plains, NJ, USA). NaCas was purchased from American Casein Company (Burlington, NJ, USA). Other chemicals were purchased from Fisher Scientific Inc. (Pittsburgh, PA, USA).

6.3.2 Sample preparation

Preparation of dispersions was conducted at room temperature (21 °C). Zein at 2% w/w and NaCas at 0.5, 1.0, 2.0, and 4.0% w/w were suspended separately in deionized (DI) water. The pH of the suspensions was brought to 11.5 using 4.0 N NaOH, which was determined in preliminary trials to be the lowest pH to completely solubilize 2% w/w zein with visual absence of large particulates. The alkaline solutions of zein and NaCas were mixed at equal volumes to prepare samples with NaCas:zein mass ratios of 0:1, 1:4, 1:2, 1:1, and 2:1. After incubation for 30 min, mixtures were acidified to pH 7.0 using 4.0 N HCl while being stirred at 600 rpm on a magnetic stir-plate. Dispersions with equal amounts of zein and NaCas were also produced using the LLD method with modifications (Chen, et al., 2014). Briefly, 2% w/w of zein was dissolved in 80% v/v aqueous ethanol and was mixed drop-wise with the same volume of 2% w/w NaCas
aqueous solution on the same stir-plate running at 600 rpm, and the pH was adjusted to 7.0. Samples were freeze-dried for further analysis.

6.3.3 Turbidity, particle size, and zeta-potential measurement

The absorbance at 500 nm ($A_{500}$) of fresh samples produced with the pH-cycle method and those rehydrated with freeze-dried samples produced with both methods was measured using a UV-Vis spectrophotometer (model Evolution 201, Thermo Scientific, Waltham, MA, USA) with a 1 cm light path-length. The hydrodynamic diameter ($D_h$) of samples was measured with a Delsa Nano analyzer (Beckman Coulter, Atlanta, GA, USA) at a scattering angle of 165° after centrifugation at 4000g for 10 min (Minispin plus, Eppendorf, Hamburg, Germany) to remove large particulates and proper dilution to the instrumental range using DI water. The zeta-potential of diluted samples at pH 7.0 was measured using a Zetasizer ZS analyzer (Malvern Instruments, Worcestershire, UK).

6.3.4 Scanning electron microscopy (SEM)

The morphology of zein-NaCas nanoparticles produced with the pH-cycle method at NaCas:zein mass ratios of 1:0, 4:1, 2:1, and 1:1 was studied using SEM. Briefly, fresh dispersion samples were centrifuged at 4000g for 10 min (Minispin plus, Eppendorf, Hamburg, Germany) and one drop of the supernatant was cast-dried on a 5×5 mm piece of silicon wafer. A thin layer (<10 nm) of gold coating was applied with a sputter coater (Structure Probe Inc., West Chester, PA, USA) and samples were observed with a Zeiss Auriga microscope operating at 5 kV (Carl Ziess Microscopy, Germany).
6.3.5 Storage stability

The storage stability of particles produced with the pH-cycle method using equal masses of zein and NaCas was evaluated by measuring $D_h$. Freshly prepared samples were dissolved with 0.02% w/v sodium azide and stored at 4 °C to prevent microbial spoilage. The $D_h$ of samples was measured during storage for up to 30 days.

6.3.6 Re-dispersibility of freeze-dried zein nanoparticles

To quantify and compare the re-dispersibility of zein nanoparticles produced through the pH-cycle and LLD methods, freeze-dried samples were re-hydrated at 0.5% w/v in DI water for 1 h. After centrifugation at 4000g for 10 min, supernatants were transferred to measure protein concentrations using the Coomassie protein assay. Samples were mixed with the Coomassie reagent (Thermo Fisher Scientific, Rockford, IL, USA) and were incubated at room temperature (21 °C) for 15 min before absorbance measurement at 595 nm. Absorbance was corrected with the blank of Coomassie reagent only, and protein concentrations were calculated based on a pre-established standard curve using bovine serum albumin (Thermo Fisher Scientific, Rockford, IL, USA). The re-dispersibility was then calculated using the following equation:

$$\text{Re-dispersibility} \ (% \%) = \left( \frac{\text{Protein content after centrifugation}}{\text{Protein content before centrifugation}} \right) \times 100\%$$  \hspace{1cm} (1)

6.3.7 Surface hydrophobicity ($H_o$) measurement

Surface hydrophobicity of dispersions was measured according to a literature method with modifications (Cardamone & Puri, 1992; Liu & Zhong, 2013). Briefly, 4 mL samples with different concentrations (0.01% to 0.1% w/w) of protein dissolved in 10 mM phosphate-buffered saline (PBS) at pH 7.0 were mixed with 20 μL of a working solution with 8 mM 8-anilinonaphthalene-1-sulfonic acid (ANS) in 10 mM PBS. The emission spectra were recorded
from 400 to 500 nm using a RF-1501 fluorescence spectrophotometer (Shimadzu Corp., Tokyo, Japan) with the excitation wavelength set at 365 nm. The slope of peak fluorescence intensity against protein concentrations was used to calculate the $H_0$.

### 6.3.8 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed under the reducing condition using a Mini Protein Tetra cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a constant voltage of 200 V. The 12% Tris-HCl gel (Precast TGX Gel) was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). The gel was stained with Coomassie blue.

### 6.3.9 Atomic force microscopy (AFM) analysis of nanoparticle topography

The topography of nanoparticles produced with equal masses of zein and NaCas using the pH-cycle and LLD methods was characterized using AFM. Dispersions rehydrated with freeze-dried samples were diluted to 100 ppm of total biopolymer mass using DI water and 20 μL of each diluted sample was spread evenly onto a freshly cleaved mica sheet mounted on a sample disk (Bruker Corp., Santa Barbara, CA, USA). A triangular cantilever having an aluminum reflective coating on the backside and a quoted force constant of 0.4 N/m (SCANASYST-AIR, Bruker Corp.) was used on a Multimode VIII microscope (Bruker AXS, Billerica, MA, USA). The ScanAsyst tapping mode was used to scan the sample, and topography images were generated with a preset scan area of 2.0×2.0 μm at a scanning speed of 1 Hz.

### 6.3.10 Quantitative AFM nanomechanical mapping

To quantify the Young’s modulus of films formed with zein-NaCas particles, 50 μL of a dispersion hydrated with 2% w/v freeze-dried sample was spread evenly onto a freshly cleaved mica sheet mounted on a sample disk (Bruker Corp., Santa Barbara, CA, USA). A rectangular
cantilever having an aluminum reflective coating on the backside and a quoted force constant of 40 N/m (RTESPA, Bruker Corp.) was used on a Multimode VIII microscope (Bruker AXS, Billerica, MA, USA). The Peakforce quantitative nanomechanical mode (PF-QNM) was used to scan the sample, and images were generated with a preset scan area of 2.0×2.0 μm at a scanning speed of 1 Hz. A relative method was used to calibrate the cantilever using a polystyrene film with a nominal modulus of 2.7 GPa. The Derjaguin–Mueller–Toporov (DMT) modulus was analyzed using the Nanoscope Analysis software of the instrument.

6.3.11 Differential scanning calorimetry (DSC)

Thermal properties of powder samples were characterized using a model Q2000 calorimeter (TA Instrument, New Castle, DE). About 5 mg powdered sample was sealed in a hermetic aluminum pan and the lid was pinned with a 0.5 mm hole to exclude the interference of moisture (Aso, Yoshioka, & Kojima, 2000; Farritor & Tao, 1970; Hall & Larsen, 1991). Samples were heated to 100 °C and were equilibrated for 30 min before cooled down to 25 °C, followed by a second heating from 25 to 230 °C. The heating and cooling rates were all 10 °C/min. Nitrogen was used as the transfer gas at a flow rate of 50 mL/min.

6.3.12 Statistical Analysis

Statistical analyses were performed using the SAS program (version 9.3, SAS Institute, Cary, NC, USA). One-way analysis of variance (ANOVA) was carried out. Differences between pairs of means were compared using a Tukey’s test. The significance level was set at 0.05.
6.4 Results and discussion

6.4.1 Physical and chemical properties of zein-NaCas particles

The pH-dependent properties of zein and casein have been studied previously (Pan & Zhong, 2013; B. Zhang, Luo, & Wang, 2011). A higher extent of deprotonation at a higher pH increases the magnitude of net negative charges to cause the solubilization of zein and dissociation of casein micelles. This enables the molecular interactions between zein and NaCas at alkaline conditions. In the present study, zein suspension was translucent at pH 11.5 but became precipitated without dispersibility as defined in Eq. 1 after pH was adjusted back to 7.0 (Figure 6-1). This is expected because zein has an isoelectric point of around pH 6.8 (Cabra, et al., 2005) and has a large percentage of hydrophobic amino acids (Argos, et al., 1982; Luo, et al., 2014).

The mass ratio of NaCas:zein was studied from 1:4 to 2:1. With an increased amount of NaCas, the $A_{500}$ (Figure 6-2) and $D_h$ (Figure 6-3) decreased significantly. Suspensions with NaCas:zein mass ratios of 1:4 and 1:2 were turbid and had visible precipitates, indicating the insufficient amount of NaCas to stabilize zein. When the mass ratio reached 1:1, no visible precipitate was observed after overnight storage at room temperature (Figure 6-1), and the $D_h$ was about 100 nm (Table 6-1). The $A_{500}$ further decreased from 1.47 to 1.24 ($P < 0.05$) when the NaCas:zein mass ratio increased from 1:1 to 2:1 (Figure 6-2), but the $D_h$ did not decrease significantly (Figure 6-3). Turbidity can be affected by both particle population, dimension, and density (Giroux, Houde, & Britten, 2010). Because the sample with a NaCas:zein mass ratio of 2:1 has a greater amount of NaCas (particle population) than that of 1:1, the higher $A_{500}$ of the dispersion with a NaCas:zein mass ratio of 1:1 than that of 2:1 possibly resulted from the greater particle density. It appeared that a NaCas:zein mass ratio of 1:1 was sufficient to produce stable
colloidal particles. This mass ratio was used for the rest of this study, with the goal to reduce the amount of NaCas to stabilize zein.

The morphology of particles formed with 0:1-1:1 mass ratios of NaCas:zein was studied using SEM after centrifugation to remove large aggregates, shown in Figure 6-4. Zein alone was not stable after the pH-cycle and formed large aggregates due to its insolubility in water at neutral pH. As the NaCas:zein mass ratio increased, particle dimension and heterogeneity decreased, which agreed with the $D_h$ results (Figure 6-3). When the mass ratio reached 1:1, particles were smaller than ~100 nm and the surface of dried particles was smooth and had fewer areas depleted of particles, which indicates the potential to form films with uniform properties.

The ability of colloidal particles remaining dispersed in aqueous systems during shelf-life storage is an important property. The mean $D_h$ of zein-NaCas particles prepared at 1:1 mass ratio remained stable ($P > 0.05$) during 30-day storage at 4 °C (Figure 6-5), and no visual changes of the dispersion was observed. Therefore, the zein-NaCas particles produced with the pH-cycle method had excellent stability against aggregation and precipitation at neutral pH.

Hydrolysis of peptide bonds and deamidation of amino acids are possible reactions at alkaline pH and during heating (Cabra, Arreguin, Vazquez-Duhalt, & Farres, 2007). SDS-PAGE was used to study the molecular weight of zein in aqueous ethanol solution before and after incubation at pH 11.5 for 30 min and neutralization of acidity. As shown in Figure 6-6, the identical band intensity and migration distances suggested the absence of protein hydrolysis and the maintained protein primary structure, which likely resulted from mild temperature (21 °C) and short duration (30 min) of the alkaline treatment. Previously, the shifting of isoelectric point caused by deamidation was reported when zein was incubated in 0.5 N NaOH at 70°C for more than 12 h, but mild treatments as those in the present study resulted in negligible deamidation (Cabra, et
al., 2007). Similarly, no structural changes of NaCas after incubation at pH 12 for 30 min were observed in our previous study (Pan, Luo, et al., 2014). Therefore, the pH-cycle method at mild treatment conditions can be used to produce protein-based colloidal particles.

6.4.2 Comparisons of zein-NaCas colloidal particles produced by the pH-cycle and liquid-liquid dispersion (LLD) methods

Physicochemical properties of zein-NaCas (1:1) particles produced by the LLD and pH-cycle methods were compared for samples produced at same stirring conditions. The re-dispersibility of powder was determined according to Eq. 1 because it is important for practical applications (Luo, et al., 2014). NaCas has previously been used to stabilize zein particles because the adsorbed NaCas on hydrophobic zein particles provides repulsive electrostatic and steric forces to prevent particle aggregation and improves re-hydration properties of freeze- and spray-dried powder (Chen, et al., 2014; Patel, et al., 2010). The freeze-dried powder prepared from the dispersion produced by the pH-cycle method had a re-dispersibility of greater than 99%, which is significantly higher than the 92% of the sample produced with the LLD method (Table 6-1). The re-dispersibility was in agreement with the lower $A_{500}$ (0.22 vs. 1.99) and smaller $D_h$ (96.9 vs. 197.3 nm) of the treatment prepared with the pH-cycle method. The low turbidity and consistent $D_h$ (Table 6-1 vs. Figure 6-3) before and after drying indicated that the particles produced by the pH-cycle method had an excellent stability against dehydration.

Surface properties of particles produced by the two methods were compared for $H_o$ and zeta-potential. As shown in Table 6-1, $H_o$ of particles produced with the pH-cycle method was 15% higher than that of the LLD method. The higher $H_o$ indicates the accessibility of the probe (ANS) to hydrophobic zein. This can result from the reduced density of NaCas on particle surface and increased surface area. The latter is evident because the $D_h$ of particles in the pH-cycle method
is smaller than that from the LLD method (Table 6-1). The zeta-potential of particles produced with the pH-cycle method was significantly less negative than those produced with the LLD method (Table 6-1). Because zein, with an isoelectric point of around pH 6.8 (Cabra, et al., 2005), is expected to have a low zeta-potential, data in Table 6-1 suggest a greater quantity of NaCas adsorbed on zein nanoparticles produced with the LLD method, which can additionally reduce $H_0$.

The morphology of re-hydrated particles was studied using AFM (Figure 6-7). Particles produced from both methods had a spherical shape and the dimension of these particles agreed with $D_h$ (Table 6-1). To study and compare the potential of zein-NaCas colloidal particles as film forming materials, dispersions rehydrated with 2% freeze-dried powder were dried as films on mica sheets. Surface morphology and nanomechanical properties studied in the PF-QNM mode (Adamcik, et al., 2012) are shown in Figure 6-8. The surface roughness of the film for the sample prepared by the pH-cycle method (19.1 nm) was significantly smaller than the 34.3 nm for the sample prepared with the LLD method. The reduced surface roughness indicates the greater ability of nanoparticles prepared with the pH-cycle method to fabricate nanoscale properties of films that can impact macroscopic properties. The Young’s moduli were comparable, around 1.5 – 1.6 GPa, for films prepared from dispersions produced by both methods, but the film dried from a 1% w/v zein solution in 80% aqueous ethanol had a significantly higher Young’s modulus (2.1 GPa; Table 6-2). The Young’s modulus of zein-NaCas films was in the similar range of zein films prepared with glycerol used at 0.7-1.0 g/g zein as a plasticizer (Ghanbarzadeh, et al., 2007). These properties show the potential of Zein-NaCas nanoparticles forming films without using a plasticizer.

6.4.3 Possible mechanism of forming zein-NaCas particles

There are two mechanisms proposed for the stabilization of zein nanoparticles produced using the LLD method by NaCas (Patel, et al., 2010). The first mechanism proposes physical
adsorption of NaCas on zein nanoparticle surface to provide electrostatic and steric repulsions, while the second mechanism proposes the formation of zein-NaCas complex nanoparticles with a portion of NaCas being embedded in nanoparticles (Patel, et al., 2010).

DSC can be used to study miscibility of biopolymer mixtures using glass transition temperature \( (T_g) \) as an indicator (Herrera, et al., 2005; Sakurai, Maegawa, & Takahashi, 2000). DSC thermograms of zein, NaCas, their simple mixture, and zein-NaCas particles produced using the LLD and pH-cycle methods are shown in Figure 6-9. NaCas and zein had a \( T_g \) of around 202 °C and 160 °C, respectively, which agreed with other studies (Khwaldia, Banon, Perez, & Desobry, 2004; Magoshi, Nakamura, & Murakami, 1992). For the simple mixture of NaCas and zein powders, two distinct \( T_g \) similar to the individual powders were observed, indicating no interactions between the two biopolymers by simply mixing powders. The Zein-NaCas sample produced with the LLD method also showed two \( T_g \), with one at 199 °C corresponding to NaCas and the other at 179 °C. The shifting of zein \( T_g \) from 160 °C to 179 °C may be caused by the packing of zein in nanoparticles (quick precipitation during LLD) being different from bulk powder (slow drying process). The attachment of NaCas on zein nanoparticles, as proposed previously (Patel, et al., 2010), may also further change \( T_g \). For zein-NaCas particles produced with the pH-cycle method, there was only one \( T_g \) at 184 °C, indicating the complete mixing between zein and NaCas (Herrera, et al., 2005; Sakurai, et al., 2000). The improved miscibility between zein and NaCas prepared by the pH-cycle method may explain the improved re-dispersibility (Table 6-1), because the overall improvement of hydrophilicity of nanoparticles enhances the hydration of freeze-dried powder, in addition to the smaller particle dimension (Table 6-1) allowing better interaction with water.
The mechanism of forming zein-NaCas nanoparticles during the pH cycle is proposed in Figure 6-10. At pH 11.5, the dissolved zein and NaCas are well-blended to allow interactions with each other. As pH decreases during slow acidification back to 7.0, zein becomes protonated and gradually loses the solubility, and the nearby NaCas, with its amphiphilic property, can co-assemble with zein through hydrophobic attraction and potentially electrostatic attraction. This results in the formation of stable zein-NaCas complex nanoparticles, with the hydrophilic segments of NaCas providing repulsive electrostatic and steric interactions to stabilize colloidal particles against aggregation.

6.5 Conclusions

In conclusion, co-assembled zein-NaCas complex nanoparticles were produced using a low energy method and pH as a fabrication mechanism, without the need of an organic solvent as in the LLD method. Nanoparticles produced with equal amounts of NaCas and zein were spherical and had a mean $D_h$ smaller than 100 nm that was stable during storage for 30 days. The freeze-dried particles produced with the pH-cycle method had much improved rehydration properties than those produced by the LLD method. Nanoparticles prepared with the pH-cycle method also formed films with reduced surface roughness and had a Young’s modulus smaller than the zein film without a plasticizer. The current study shows the promise of the pH-cycle method to dispersible zein nanoparticles for developments of delivery systems and packaging materials.
References


Appendix

Table 6-1. Comparisons of re-dispersibility, absorbance at 500 nm ($A_{500}$), hydrodynamic diameter ($D_h$), surface hydrophobicity ($H_o$), and zeta-potential of dispersions hydrated with freeze-dried powder of colloidal particles produced with equal masses of zein and NaCas using the pH-cycle and liquid-liquid dispersion methods.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH-cycle</th>
<th>Liquid-liquid dispersion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re-dispersibility (%)</td>
<td>$99.4 \pm 0.4%^a$</td>
<td>$91.7 \pm 3.7%^b$</td>
</tr>
<tr>
<td>$A_{500}^#$</td>
<td>$0.22 \pm 0.04^b$</td>
<td>$1.99 \pm 0.06^a$</td>
</tr>
<tr>
<td>$D_h$ (nm)</td>
<td>$96.9 \pm 6.6^b$</td>
<td>$197.3 \pm 12.1^a$</td>
</tr>
<tr>
<td>$H_o$ ($\times 10^3$)</td>
<td>$8.3 \pm 0.71^a$</td>
<td>$7.2 \pm 0.33^b$</td>
</tr>
<tr>
<td>Zeta-potential (mV)</td>
<td>$-36.5 \pm 0.85^b$</td>
<td>$-50.2 \pm 0.51^a$</td>
</tr>
</tbody>
</table>

* Numbers are mean ± standard deviation (n = 3). Different superscript letters in the same row indicate significant differences (P < 0.05).

# The dispersions were diluted to 0.25% w/w powder.
Table 6-2. Young’s moduli of films formed of dispersions with 1% w/v zein and 1% w/v NaCas using the liquid-liquid dispersion (LLD) and pH-cycle methods, in comparison to films prepared with an aqueous ethanol solution with 1% w/v zein.

<table>
<thead>
<tr>
<th>Sample used to prepare Films</th>
<th>Young’s Modulus (GPa)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zein solution</td>
<td>2.09 ± 0.18 a</td>
</tr>
<tr>
<td>Dispersion prepared with the LLD method</td>
<td>1.50 ± 0.34 b</td>
</tr>
<tr>
<td>Dispersion prepared with the pH–cycle method</td>
<td>1.58 ± 0.31 b</td>
</tr>
</tbody>
</table>

* Numbers are mean ± standard deviation from the frame average modulus of 3 films. Different superscript letters indicate significant differences (P < 0.05).
Figure 6-1. Visual appearance of 1% w/w zein without (Left) or with (Right) 1% w/w NaCas after incubation at pH 11.5 for 30 min followed by acidification to pH 7.0 and storage overnight at room temperature.
Figure 6-2. Absorbance at 500 nm ($A_{500}$) of pH 7 dispersions prepared with the pH-cycle method using 1% w/w zein and different mass ratios of NaCas:zein. Error bars are standard deviations (n = 3). Different letters above bars indicate significant differences (P < 0.05).
Figure 6-3. Mean hydrodynamic diameter of pH 7.0 dispersions prepared with the pH-cycle method using 1% w/w zein and different mass ratios of NaCas:zein and after centrifugation. Error bars are standard deviations (n = 3). Different letters above bars indicate significant differences (P < 0.05).
Figure 6-4. SEM images of particles produced using the pH-cycle method and NaCas:zein mass ratios of (a) 0:1, (b) 1:4, (c) 1:2, and (d) 1:1.
Figure 6-5. Hydrodynamic diameter of zein-NaCas (1:1) particles produced using the pH-cycle method during storage at 4°C for 30 days. Error bars are standard deviations (n = 3).
Figure 6-6. SDS-PAGE of protein markers (left lane) and zein dissolved in 80% ethanol before (middle lane) and after (right lane) incubation at pH 11.5 for 30 min at room temperature and acidification to pH 7.0.
Figure 6-7. AFM of zein-NaCas (1:1) particles produced by the liquid-liquid dispersion method (upper panel) or pH-cycle method (lower panel) in height (left), peak force error (middle), and 3-D (right) channels.
Figure 6-8. Peakforce Quantitative Nano Mechanical mapping of films formed from 2% w/v zein-NaCas (1:1) particles produced by the liquid-liquid dispersion method (upper panel) or pH-cycle method (lower panel) in height (left), DMT modulus (middle), and 3-D (right) channels.
Figure 6-9. Differential scanning calorimetry analysis of NaCas, zein, physical mixture of NaCas and zein powders, and freeze-dried NaCas-zein (1:1) particles prepared using the liquid-liquid dispersion or pH-cycle method. The curves were shifted vertically for visualization.
Figure 6-10. Proposed mechanism for co-assembling of NaCas and Zein during acidification from pH 11.5 to pH 7.0.
Chapter 7. Amyloid-like fibrils formed from intrinsically disordered caseins: physicochemical and nanomechanical properties
A version of this chapter was originally published by Kang Pan and Qixin Zhong:

7.1 Abstract

Amyloid-like fibrils are studied because of their significance in understanding pathogenesis and creating functional materials. Amyloid-like fibrils have been studied by heating globular proteins at acidic conditions. In the present study, intrinsically disordered α-, β-, and κ-caseins were studied to form amyloid-like fibrils at pH 2.0 and 90 ºC. No fibrils were observed for α-caseins, and acid hydrolysis was found to be the rate-limiting step of fibrillation of β- and κ-caseins. An increase of β-sheet structure was observed after fibrillation. Nanomechanic analysis of long amyloid-like fibrils using peak-force quantitative nanomechanical atomic force microscopy showed the lowest and highest Young’s modulus for β-casein (2.35±0.29 GPa) and κ-casein (4.14±0.66 GPa), respectively. The dispersion with β-casein fibrils had a viscosity more than 10 and 5 times higher than those of κ-casein and β-lactoglobulin, respectively, at 0.1 s⁻¹ at comparable concentration. The current findings may assist not only the understanding of amyloid fibril formation but also the development of novel functional materials from disordered proteins.

**Keywords:** amyloid-like fibrils, intrinsically disordered protein, casein, nanomechanical properties, viscosity
7.2 Introduction

Amyloid fibrils formed in human tissues such as brain are significant to the understanding of pathogenesis of diseases related to neurodegenerative disorders and amyloidosis like Alzheimer’s and Parkinson’s diseases (Uversky, 2008). The formation mechanism of amyloid fibrils has been investigated extensively in vivo and in vitro with the hope to find a preventive therapy or revert fibril structures. Although it has not been identified conclusively, there are also some amyloid-like fibrils found in the mammary tissue of animals such as bovine, which cause the reduction of milk secretion (Nickerson, Sordillo, Boddie, & Saxton, 1985). It is generally believed that the formation of amyloid or amyloid-like fibrils involves the nucleation of partially unfolded proteins or peptides, followed by extension reactions to form beta-sheets through anti-parallel stacking of beta-strands by inter-strand hydrogen bonds (Harrison, Sharpe, Singh, & Fairlie, 2007; Stefani, 2004).

Besides proteins related to pathogenesis, several globular proteins with a significant portion of β-sheet structures have been observed to form amyloid-like fibrils in vitro, such as β-lactoglobulin, yeast prions, α-lactalbumin, ovalbumin, and lysozyme (Goers, Permyakov, Permyakov, Uversky, & Fink, 2002; Jones & Mezzenga, 2012; Lara, Adamcik, Jordens, & Mezzenga, 2011; Lara, Handschin, & Mezzenga, 2013; Liu & Zhong, 2013; Reynolds, Charnley, Mezzenga, & Hartley, 2014; Serio, et al., 2000). Instead of applying physiological conditions, heating these globular proteins at acidic conditions is commonly used to form amyloid-like fibrils that can be used to unveil the fibrillation process. Studies have shown that the in vitro fibrillation involves steps of acid hydrolysis, nucleation and growth of acid-hydrolyzed peptides by β-sheet alignment (Dave, Loveday, Anema, Jameson, & Singh, 2014; Lara, et al., 2011). β-lactoglobulin is among the most extensively studied globular proteins and can form amyloid-like fibrils at a wide
pH range below its isoelectric point (pI) of around pH 5.3 by heating (Gosal, Clark, Pudney, & Ross-Murphy, 2002). Several parameters affect the rate and type of fibril formation. Longer fibrils are formed at a higher rate when heated at a lower ionic strength, a higher temperature, and without co-solutes such as polysaccharides and polyols (Bolisetty, Harnau, Jung, & Mezzenga, 2012; Dave, et al., 2014; Jones, Adamcik, Handschin, Bolisetty, & Mezzenga, 2010; Lara, Gourdin-Bertin, Adamcik, Bolisetty, & Mezzenga, 2012; Loveday, Su, Rao, Anema, & Singh, 2011). These amyloid-like fibrils also have potential for use as functional materials to provide unique rheology, encapsulate compounds, and serve as scaffolds in tissue engineering (Graveland-Bikker & de Kruif, 2006; Humblet-Hua, Scheltens, van der Linden, & Sagis, 2011; Reynolds, et al., 2014).

In vitro fibril formation from amorphous proteins is relatively scarce (Uversky, 2008). Caseins abundant in mammalian milk lack of defined three-dimensional structures and are classified as intrinsically disordered proteins (van der Lee, et al., 2014). Several studies have characterized the properties of bovine caseins in forming fibrils. In bovine milk, caseins consist of about 80% of proteins (Fox, 2003) and four types of caseins (\(\alpha_1\), \(\alpha_2\), \(\beta\), and \(\kappa\)) assemble as mostly spherical colloidal particles with a diameter of 50-250 nm (Udabage, McKinnon, & Augustin, 2003). Caseins have less secondary or tertiary structures than globular proteins under physiological conditions and can function as molecular chaperones to stabilize proteins through electrostatic and hydrophobic interactions (Carver, Rekas, Thorn, & Wilson, 2003; Uversky, 2002). Caseins also have numerous applications based on their balanced hydrophilicity/hydrophobicity and self-assembling properties (Esmaili & Ghaffari, 2011; Pan, Chen, Davidson, & Zhong, 2014; Pan, Luo, Gan, Baek, & Zhong, 2014; Pan, Zhong, & Baek, 2013). \(\kappa\)-casein can form amyloid-like fibrils under physiological conditions with or without a reducing agent (Thorn, et al., 2005). The fibrillation involves the formation of multimers from individual \(\kappa\)-casein molecules and
conformational rearrangement to form more organized species, which subsequently undergo micellar assembling and further growing to form amyloid-like fibrils (Leonil, et al., 2008; Thorn, et al., 2005). The fibrillation of κ-casein can be inhibited by αs- and β-caseins, which results in the absence of amyloid-like fibrils when different types of caseins are present under physiological conditions (Thorn, et al., 2005). The fibrillation of αs2-casein at physiological conditions was also observed but was inhibited by β- or αs1-casein (Thorn, Ecroyd, Sunde, Poon, & Carver, 2008; Thorn, et al., 2005). These findings collectively suggest the prevention of amyloidosis accumulation in mammary tissues and the development of corpora amylacea (Thorn, et al., 2008; Thorn, et al., 2005). It was also observed that αs1-casein inhibits fibrillogenesis of Aβ1-40, a peptide involved in Alzheimer's disease, by increasing the nucleation lag-time and slowing down the overall fibrillation (Carrotta, et al., 2012). Currently, formation of amyloid-like fibrils by heating caseins at acidic conditions has not been studied.

In the present study, the objective was to study amyloid-like fibrils produced from bovine caseins by heating at 90 °C and pH 2.0, and compare their physicochemical and nanomechanical properties with fibrils formed from β-lactoglobulin. The fibrillation kinetics was monitored using Thioflavin T (Th-T) fluorescence spectroscopy for up to 48 h. The morphology of fibrils was characterized using transmission electron microscopy (TEM) and atomic force microscopy (AFM). The hydrolysis kinetics was quantified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and changes of secondary structures were studied using Fourier transform infrared (FTIR) spectroscopy and circular dichroism (CD) spectroscopy. Finally, rheological properties of fibril dispersions formed from different proteins were characterized.
7.3 Materials and methods

7.3.1 Materials

α-, β-, and κ-caseins and β-lactoglobulin were purchased from Sigma Aldrich Corp. (St. Louis, MO) and were used without further purification. Polyacrylamide gels and protein markers were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Dialysis tubing with a nominal molecular weight cutoff (MWCO) of 6000-8000 Da and β-mercaptoethanol were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Other chemicals were of analytical grade and were purchased from Fisher Scientific, Inc. (Pittsburgh, PA).

7.3.2 Preparation of fibrils

Each protein was dissolved at 10 mg/mL in deionized water, and the pH was adjusted to 2.0 using 1M HCl. Samples were heated at 90 °C for up to 48 h by incubating in the glycerol bath heated on a hot magnetic stir pate operating at 300 rpm. Samples were transferred and quenched in an ice bath at pre-determined time intervals.

7.3.3 Thioflavin T (ThT) fluorescence spectroscopy

The ThT fluorescent properties of samples were characterized using spectrofluorometry (model RF-1501, Shimadzu Corp., Tokyo, Japan) as an indicator of fibril formation (Ban, Hamada, Hasegawa, Naiki, & Goto, 2003; Torok, et al., 2002). 50 μL of a protein sample was mixed with 5 mL of the ThT working solution (0.8 mg/mL). The emission spectra at a range of 460–700 nm were acquired using an excitation wavelength of 440 nm. The fluorescence of protein samples was calibrated by subtracting the background of the ThT working solution without protein. The maximum fluorescence intensity of a sample ($F_m$) was used to calculate the normalized intensity
by that of the same protein sample produced after 24-h heating ($F_{24}$) that showed the highest intensity among the time points:

\[
\text{Normalized intensity} = \frac{F_m - F_0}{F_{24} - F_0}
\]

where $F_0$ is the maximum fluorescence intensity of a sample before heating.

The ThT fluorescence data were fitted with the following model (Dave, et al., 2014; Morris, Watzky, Agar, & Finke, 2008):

\[
f_t = \alpha - \frac{\beta + \alpha}{1 + \frac{\beta}{\alpha \gamma} \exp[t(\beta + \alpha \gamma)]}
\]

where $f_t$ is the normalized fluorescence intensity at time $t$, and $\alpha$, $\beta$, and $\gamma$ are arbitrary constants.

The lag phase length ($t_{\text{lag}}$) and the time required to reach one-half of the maximum fluorescence ($t_{1/2\text{max}}$) were calculated using Eq. (3) and (4), respectively.

\[
 t_{\text{lag}} = \frac{1}{\beta + \alpha \gamma} \left( \ln \left( \frac{\alpha \gamma}{\beta} \right) - 4 \frac{\alpha \gamma}{\beta + \alpha \gamma} + 2 \right)
\]

\[
 t_{1/2\text{max}} = \frac{\ln(2 + \alpha \gamma)}{\beta + \alpha \gamma}
\]

7.3.4 Transmission electron microscopy (TEM)

The morphology of fibrils produced after 24-h heating was studied using TEM. The freshly prepared sample was diluted 100 times with deionized water. One drop of a diluted sample was placed on a freshly glow-discharged carbon film on a 400-mesh copper grid and then stained with 1% uranyl acetate. Samples were imaged with a Zeiss Libra TEM operating at 200 kV (Carl Zeiss Microscopy, Oberkochen, Germany).
7.3.5 *Atomic force microscopy (AFM)*

The topography and nano-mechanical properties of fibrils were characterized using AFM. 10 μL of a 200-fold diluted sample was spread evenly onto a freshly cleaved mica sheet mounted on a sample disk (Bruker Corp., Santa Barbara, CA), and was rinsed with 1 mL deionized water previously adjusted to pH 2.0 after incubation at room temperature for 1 minute. A rectangular cantilever having an aluminum reflective coating on the backside and a quoted force constant of 40 N/m (RTESPA, Bruker Corp.) was used on a Multimode VIII microscope (Bruker AXS, Billerica, MA). The peak-force quantitative nanomechanical mode was used to scan the sample, (Adamcik, Lara, et al., 2012), and topography images were generated with a preset scan area of 2.0×2.0 μm at a scanning speed of 1 Hz. A relative method was used to calibrate the cantilever on the polystyrene film with a 2.7 GPa nominal modulus.

7.3.6 *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

A precast 15% gradient polyacrylamide gel from Bio-Rad Laboratories, Inc. (Hercules, CA) was used in SDS-PAGE. 40 μL of a protein sample was mixed with 200 μL of a SDS-PAGE sample buffer (catalog number MB01015, GenScript Corp., Piscataway, NJ). After heating at 95 °C for 5 min, 10 μL of each sample was loaded to the gel well for electrophoresis at 200 V till the indicator dye reached the gel bottom. The intensity of the band corresponding to each protein after heating \(I\) for a duration of \(t\) was quantified using ImageJ software (NIH, Bethesda, MD) and was normalized by the intensity of unheated protein \(I_o\) run on the same gel. The data were analyzed using the first-order kinetics: (Dave, et al., 2014)

\[
I = I_o \cdot e^{-k_h \cdot t} \\
\]

where \(k_h\) is the hydrolysis rate constant.
7.3.7 Circular dichroism (CD) spectroscopy.

The instrument used in the far-UV CD spectroscopy was a Jasco model J-815 CD spectrometer (Jasco, Inc., Easton, MD). Fibrils were dialyzed using a membrane with a MWCO of 6000-8000 Da at 4 °C for 4 days with mild stirring against water acidified to pH 2.0. The concentrations of proteins and fibrils were measured using the bichinconinic acid assay method after 10-fold dilution in deionized water previously adjusted to pH 2. The diluted sample was scanned 10 times at a rate of 50 nm/min from 192 to 260 nm with a bandwidth of 1 nm.

7.3.8 Fourier-transform infrared spectroscopy (FTIR)

The structural changes of caseins before and after heating at 90 °C for 24 h were studied for FTIR following previously reported methods with modifications (Farrell, Wickham, Unruh, Qi, & Hoagland, 2001; Hiramatsu & Kitagawa, 2005). Briefly, the liquid samples after dialysis as above were dried on a mica surface and the spectra were acquired using an ATR-FTIR Nicolet Nexus 670 IR spectrometer (Thermo Nicolet Corp., Madison, MI) which was equipped with a Germanium attenuated total reflection (ATR) accessory. The IR transmittance was acquired from 1600 to 1700 cm\(^{-1}\) with a resolution of 2 cm\(^{-1}\). 100 repeated scans were undertaken for each sample. All signals were collected against a background spectrum recorded from the mica substrate only. The IR spectra were further subjected to Fourier self-deconvolution (FSD) using the OMNIC software (Thermo Nicolet Corp., Madison, MI), and the FSD spectra were then curve-fitted assuming a Gaussian band profile. Assignment of peaks on the FSD spectra followed the literature (Curley, Kumasinski, Unruh, & Farrell, 1998), and the content of each secondary structure was expressed as the percentage of total peak areas representing secondary structures.
7.3.9 Rheology

The viscosity of samples before and after heating was measured using a stress-controlled rheometer (model AR2000, TA Instruments Inc., New Castle, DE). The cone-plate geometry with a cone angle of 1° and a cone diameter of 40 mm was used in shear rate ramps from 0.1 to 100 s$^{-1}$ at 25 °C.

7.3.10 Statistical analysis

Statistical analyses were performed using the SAS program (version 9.3, SAS Institute, Cary, NC). One-way analysis of variance (ANOVA) was carried out. Differences between pairs of means were compared using a Tukey’s test. The significance level was set at 0.05.

7.4 Results and discussion

7.4.1 Amyloid-like fibril formation kinetics

Amyloid-like fibrils are formed by sequentially stacking of beta-strands to beta-sheets perpendicularly to the main axil of the fibril (Harrison, et al., 2007; Stefani, 2004). Because the selective binding between ThT and fibrils strengthens the emitted fluorescence intensity, the quantified fluorescence intensity can be used to monitor the growth of amyloid-like fibrils (Ban, et al., 2003; Torok, et al., 2002). Figure 7-1 shows the normalized ThT fluorescence intensity of samples after heating α-, β-, and κ-caseins and β-lactoglobulin for different durations. Heating α-casein at 90 °C and pH 2.0 did not change ThT fluorescence, indicating the absence of fibrils. The α-casein in the current study is a mixture of αs1- and αs2-caseins. The mixed α-casein does not form fibrils at physiological conditions, but the purified αs2-casein does, which indicates the suppression of fibril formation from αs2-casein by αs1-casein (Thorn, et al., 2008; Thorn, et al., 2005).
Unfortunately, we were not able to find purified $\alpha_s$- and $\alpha_{s2}$-caseins to verify similar inhibition occurred during heating at pH 2.0.

$\kappa$-Casein and $\beta$-lactoglobulin showed similar and typical sigmoidal increase in ThT fluorescence intensity at extended heating durations (Figure 7-1). Both proteins reached a plateau after heating at 90 °C for 12 h, which is similar to previous studies (Liu, et al., 2013; Loveday, et al., 2010). Different from $\kappa$-casein and $\beta$-lactoglobulin, $\beta$-casein showed a delayed increase in ThT fluorescence in the first 12 h and did not reach an equilibrium after 24 h. Therefore, each casein showed distinctly different properties in forming amyloid-like fibrils during heating at pH 2.0.

### 7.4.2 Acid hydrolysis kinetics of proteins

The fibril formation kinetics can be affected by several parameters such as pH and temperature that determine the kinetics of acid hydrolysis, as well as nucleation and growth which are a function of both hydrolysate structures and environment conditions (Jones, et al., 2010; Krebs, Devlin, & Donald, 2009). To understand the rate-determining step during fibril formation, SDS-PAGE was used to monitor acid hydrolysis during heating (Figure 7-2). Hydrolysis of proteins to peptides smaller than 10 kDa mostly completed in the first 12 h, with $\beta$-casein being the slowest. The hydrolysis kinetics quantified from SDS-PAGE after fitting the model in Eq. 2 is shown in Figure 7-3. $\alpha$-Casein was hydrolyzed the fastest, with a $k_h$ of $0.332 \pm 0.008$ h$^{-1}$. $\kappa$-Casein and $\beta$-lactoglobulin showed similar hydrolysis kinetics with $k_h$ of $0.301 \pm 0.022$ h$^{-1}$ and $0.310 \pm 0.015$ h$^{-1}$, respectively. The $k_h$ of $\beta$-casein hydrolysis ($0.228 \pm 0.018$ h$^{-1}$) was significantly smaller than the other three proteins. The calculated hydrophobicity of proteins follows the decreasing order of $\beta$-casein > $\kappa$-casein = $\beta$-lactoglobulin > $\alpha_{s1}$-casein > $\alpha_{s2}$-casein (Chaplin, 1986). Therefore, a more hydrophobic protein is hydrolyzed more slowly during heating at acidic conditions. This is expected because a more hydrophobic protein reduces the exposure of amide bonds for acid
hydrolysis, which also is in agreement with the ThT fluorescence intensity changes (Figure 7-1). With the exception of α-casein (no fibril formation), acid hydrolysis is thus the rate-limiting step in fibrillation of these proteins during heating at acidic conditions.

7.4.3 Morphology and nano-mechanical properties of fibrils

The morphology of proteins after heating at 90 °C and pH 2.0 for 24 h was studied using TEM, shown in Figure 7-4. As indicated for absence of fibrils based on ThT fluorescence (Figure 7-1), individual particles and aggregates without amyloid-like fibrils were observed for α-casein (Figure 7-4). In contrast, amyloid-like fibrils were evident for β-casein, κ-casein, and β-lactoglobulin treatments.

AFM is also commonly used to characterize the structure of amyloid-like fibrils, and the nanoscale structures facilitate the understanding of fibrillation mechanism (Adamcik & Mezzenga, 2012; Gosal, et al., 2002). Characterization of nanoscale mechanical properties of materials is a unique feature of AFM, and the peak force value in the force-distance curves can be used to calculate Young’s modulus in real time by fitting data in the Derjaguin–Mueller–Toporov model (Adamcik & Mezzenga, 2012; de Abajo & Aizpurua, 1997). Figures 7-5 to Figure 7-7 show morphological and mechanical properties of β-casein, κ-casein, and β-lactoglobulin before and after incubation at pH 2.0 and 90 °C for 24 h. All three proteins showed spherical structures before heating. After heating, amyloid-like fibrils with a length greater than 1 μm were observed. General observations showed that fibrils of β-lactoglobulin were overall longer than those of β-casein and κ-casein.

The average Young’s moduli measured from different fibrils are summarized in Table 1. The Young’s modulus of β-lactoglobulin fibrils (3.45 GPa) was close to the previously reported value of 3.7 GPa (Adamcik, Lara, et al., 2012). The Young’s modulus of β-casein fibrils (2.35
GPa) was lower than that of β-lactoglobulin but was close to that of α-synulein and end-capped heptapeptide CH$_3$CONH-βAβAKLVFF-CONH$_2$ fibrils (Adamcik, Lara, et al., 2012; Sweers, van der Werf, Bennink, & Subramaniam, 2011). κ-Casein fibrils had the highest modulus (4.14 GPa) among the three protein fibrils. The difference in fibril modulus can be caused by the strength of intermolecular forces and can be correlated to material stiffness (Knowles & Buehler, 2011). These fibrils may be used to design functional materials with different mechanical properties.

### 7.4.4 Structural changes of proteins analyzed using FTIR and CD spectroscopy

The secondary structural changes of caseins before and after heating for 24 h were analyzed using ATR-FTIR, with the spectra presented in supplementary Figure 7-S1 and the percentages of secondary structures de-convoluted around the Amide I region of the FTIR spectra summarized in supplementary Table 7-S1. Contrasting with no changes of α-casein, IR peaks of β- and κ-caseins shifted to a lower wavenumber after heating (Figure 7-S1), indicating the increased contents of ordered structures (Hiramatsu, et al., 2005; Leonil, et al., 2008). There was an increase in β-sheet content by 6.6% and 32.3% for β-casein and κ-casein, respectively, at the expense of both helix and disordered structures (Table 7-S1), indicating a transition from an amorphous state to a more organized state corresponding to the formation of amyloid-like fibrils verified in ThT fluorescence (Figure 7-1).

To confirm with ATR-FTIR results, Far-UV CD spectra of β-casein and κ-casein were measured before and after heating for 24 h (Figure 7-6). The large negative ellipticity centered on 200 nm and 201 nm for β-casein and κ-casein, respectively, before heating and the low ellipticity at 220 nm are characteristics of disordered structures (Uversky, 2002). After heating, there were redshifts of the minimum in ellipticity for β-casein and κ-casein to 202 nm and 210 nm, respectively. Coupled by the increase of ellipticity at 192-200 nm region and decrease at 210-200
nm region, the spectra indicated an increase in β-sheet and a reduced content of irregular structure after heating (Thorn, et al., 2008). The greater extent of redshift observed for the CD spectrum of κ-casein after heating indicates the greater increase of contents of regular structures than β-casein, which agreed with the higher content of β-sheet structures in κ-casein sample studied in FTIR (Table 7-S1). The greater content of β-sheet structures in κ-casein (Table 7-S1) is in agreement with the higher Young’s modulus of its fibrils than those of β-casein (Table 7-3).

### 7.4.5 Rheological property of fibril dispersions

Viscosity is an important property to assess the potential of materials in applications such as texture modifiers or adhesives (Sun, et al., 2014). The viscosity of all protein samples increased after heating for 24 h, which has also been observed in other fibril systems (Liu, et al., 2013; Loveday, et al., 2011). The increased viscosity can be explained by the increased mass and hydrodynamic diameter of amyloid-like fibrils after acid hydrolysis and structure assembly (Liu, et al., 2013; Robinson, Rossmurphy, & Morris, 1982; Smidsrød & Haug, 1971). The apparent viscosity of κ-casein fibril dispersion at 0.1 s⁻¹ (~0.1 Pa-s) was about one-half of the β-lactoglobulin fibril sample. The β-casein fibril dispersion showed the highest viscosity and was more than one decade higher than the viscosity of κ-casein fibril dispersion at 0.1 s⁻¹. There was a negative correlation between Young’s modulus and viscosity among β-casein, κ-casein, and β-lactoglobulin ($p < 0.05$). This correlation may be interpreted by the flexibility of fibrils that is negatively correlated to Young’s modulus (Table 7-3).

### 7.5 Conclusions

In conclusion, the present study suggests for the first time that amyloid-like fibrils can be formed from β- and κ-caseins in a controlled manner during heating at acidic pH, especially β-casein that has long been used as a molecular chaperone to inhibit the formation of amyloid-like
fibrils in vitro. The fibrillation of caseins was limited by the acid hydrolysis rate and resulted in more organized structures with increased contents of β-sheet. Fibrils of β- and κ-caseins differed in nanomechanical properties and bulk viscosity, suggesting the potential of κ-casein fibrils as novel biomaterials to provide mechanical strength and β-casein fibrils as an effective texture modifier or adhesive. The current findings may be important to not only the enhanced understanding of amyloid formation but also the exploration of novel biomaterials.

7.6 Acknowledgements

This work was supported by the University of Tennessee and the USDA National Institute of Food and Agriculture Hatch Project 223984.
References


Esmaili, M., & Ghaffari, S. M. (2011). Beta casein-micelle as a nano vehicle for solubility enhancement of curcumin; food industry application. *LWT-Food Science and Technology, 44*(10), 2166-2172.


Appendix

Table 7-1. Fibrillization parameters estimated from ThT fluorescence based on Eqs. 2-4.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$t_{\text{lag}}$ (h)</th>
<th>$t_{1/2\text{max}}$ (h)</th>
<th>Adjusted $r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\beta$-casein</td>
<td>2.96</td>
<td>9.74</td>
<td>0.9983</td>
</tr>
<tr>
<td>$\kappa$-casein</td>
<td>1.51</td>
<td>3.45</td>
<td>0.9924</td>
</tr>
<tr>
<td>$\beta$-lactoglobulin</td>
<td>0.05</td>
<td>2.21</td>
<td>0.9994</td>
</tr>
</tbody>
</table>
Table 7-2. Protein hydrolysis rate constant (kh) estimated from SDS-PAGE (Figure 7-2).

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_h$ (h$^{-1}$)*</th>
<th>Adjusted $r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-casein</td>
<td>0.332 ± 0.008</td>
<td>0.9970</td>
</tr>
<tr>
<td>β-casein</td>
<td>0.228 ± 0.018</td>
<td>0.9683</td>
</tr>
<tr>
<td>κ-casein</td>
<td>0.301 ± 0.022</td>
<td>0.9748</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>0.310 ± 0.015</td>
<td>0.9889</td>
</tr>
</tbody>
</table>

*Data are mean ± standard error from fitting.
Table 7-3. Average Young’s modulus of β-casein, κ-casein, and β-lactoglobulin fibrils produced by heating at 90 °C and pH 2.0 for 24 h measured using atomic force microscopy operated in quantitative nanomechanics mode.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Young’s Modulus (GPa)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-casein</td>
<td>2.35 ± 0.29c</td>
</tr>
<tr>
<td>κ-casein</td>
<td>4.14 ± 0.66a</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>3.45 ± 0.67b</td>
</tr>
</tbody>
</table>

*Data are mean ± standard deviation from 100 fibrils.
Figure 7-1. Normalized ThT fluorescence of α-casein, β-casein, κ-casein, and β-lactoglobulin solutions after heating at 90 °C and pH 2.0 for 0-48 h at a protein concentration of 10 mg/mL. Error bars are standard deviations from triplicates. Solid lines indicate the fitting of data to Eq. 1.
Figure 7-2. SDS-PAGE analysis of α-casein, β-casein, κ-casein, and β-lactoglobulin after heating at 90 °C and pH 2.0 for 0-48 h.
Figure 7.3. Normalized SDS-PAGE band intensity showing hydrolysis kinetics of α-casein, β-casein, κ-casein, and β-lactoglobulin after heating at 90 °C and pH 2.0 up to 12 h.
Figure 7-4. TEM images showing structures of α-casein, β-casein, κ-casein, and β-lactoglobulin after heating at pH 2.0 and 90 °C for 24 h.
Figure 7-5. AFM images of β-casein (a) before treatment in height channel and after treatment in (b) height, (c) peak force error, and (d) Derjaguin-Muller-Toporov (DMT) modulus channels.
Figure 7-6. AFM images of κ-casein (a) before treatment in height channel and after treatment in (b) height, (c) peak force error, and (d) Derjaguin-Muller-Toporov (DMT) modulus channels.
Figure 7-7. AFM images of β-lactoglobulin (a) before treatment in height channel and after treatment in (b) height, (c) peak force error, and (d) Derjaguin-Muller-Toporov (DMT) modulus channels.
Figure 7-8. CD spectra of (A) β-casein and (B) κ-casein before and after heating at 90 °C and pH 2.0 for 24 h.
Figure 7-9. Viscosity of dispersions with 10 mg/mL β-casein, κ-casein, and β-lactoglobulin before and after heating at 90 °C and pH 2.0 for 24 h. The inset shows the partial data up to 0.10 Pa-s.
Figure 7-S1. FTIR spectra of α-, β-, and κ-casein before (0h) and after (24h) fibrillation by heating at 90 °C and pH 2.0 for 24 h. The curves were shifted vertically for visualization.
Table 7-S1. Percentages of secondary structures measured around the Amide I bond from the FTIR spectra of proteins before and after heating at 90 °C and pH 2.0 for 24 h.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Structure (wavenumber, cm(^{-1}))</th>
<th>Before heating</th>
<th>After heating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-casein</td>
<td>Sheet (1612-1638)</td>
<td>26.0%</td>
<td>25.8%</td>
</tr>
<tr>
<td></td>
<td>Irregular (1642-1648)</td>
<td>13.2%</td>
<td>12.9%</td>
</tr>
<tr>
<td></td>
<td>Helix (1650-1660)</td>
<td>31.4%</td>
<td>26.0%</td>
</tr>
<tr>
<td></td>
<td>Turns (1670-1695)</td>
<td>29.5%</td>
<td>35.4%</td>
</tr>
<tr>
<td>Beta-casein</td>
<td>Sheet (1612-1638)</td>
<td>33.3%</td>
<td>35.5%</td>
</tr>
<tr>
<td></td>
<td>Irregular (1642-1648)</td>
<td>10.6%</td>
<td>9.4%</td>
</tr>
<tr>
<td></td>
<td>Helix (1650-1660)</td>
<td>21.6%</td>
<td>19.9%</td>
</tr>
<tr>
<td></td>
<td>Turns (1670-1695)</td>
<td>34.4%</td>
<td>35.1%</td>
</tr>
<tr>
<td>Kappa-casein</td>
<td>Sheet (1612-1638)</td>
<td>32.2%</td>
<td>42.6%</td>
</tr>
<tr>
<td></td>
<td>Irregular (1642-1648)</td>
<td>11.8%</td>
<td>9.4%</td>
</tr>
<tr>
<td></td>
<td>Helix (1650-1660)</td>
<td>15.8%</td>
<td>10.6%</td>
</tr>
<tr>
<td></td>
<td>Turns (1670-1695)</td>
<td>40.3%</td>
<td>37.3%</td>
</tr>
</tbody>
</table>
Chapter 8. Concluding remarks and future work
8.1 Conclusions

This dissertation demonstrated that the nanostructures of caseins can be utilized to delivery various functionalities by controlling their self-assembly properties. When skim milk powder dispersions were subjected to alkalinization to pH 10.0 or 11.0, the particle mass was reduced during the following acidification with hydrochloric or citric acid, the reformation of casein particles was observed. The combination of treatment at pH 11.0 and acidification with citric acid resulted in translucent dispersions at pH 5.5-7.0 with the lowest turbidity and smallest particles at the studied conditions.

The development of a food grade delivery system was studied to encapsulate thymol with NaCas using high speed homogenization. The transparent nanodispersion was stable in a wide pH range, even around the isoelectric point of NaCas at ~ pH 4.6 by forming complexes with soluble soybean polysaccharide that provided electrostatic and steric repulsions. The encapsulated thymol showed significantly improved anti-listerial activity in model milk systems with different fat levels when compared to thymol crystals.

The nanostructure of NaCas was further controlled to develop a novel encapsulation method by spray-drying warm aqueous ethanol solution with co-dissolved NaCas and curcumin. The curcumin encapsulated in casein nanoparticles had higher biological activity, as assessed by antioxidant and cell proliferation assays, than pristine curcumin, and the simple approach may be applied to encapsulate various lipophilic bioactive compounds.

A scalable, low-cost, low-energy, and organic solvent-free encapsulation technology was then studied by utilizing pH-dependent solubility properties of curcumin and self-assembly properties of NaCas. Curcumin was deprotonated and dissolved, while NaCas was dissociated at pH 12 and 21 °C for 30 min. The subsequent neutralization enabled the encapsulation of curcumin
in self-assembled casein nanoparticles. The curcumin encapsulated in casein nanoparticles showed significantly improved anti-proliferation activity against human colorectal and pancreatic cancer cells.

The potential applications of the pH-cycle method to stabilize macromolecules were then studied by mixing NaCas with zein at pH 11.5, and the subsequent neutralization to pH 7 enabled the co-assembly of zein and NaCas. The freeze-dried sample showed excellent re-dispersibility. Physicochemical properties of the zein-NaCas complex nanoparticles and films showed the promise of this novel method to prepare zein nanoparticles for applications such as delivery systems and edible packaging films.

Studies on amyloid-like fibrils provide insights to understand pathogenesis and create functional materials. Intrinsically disordered α-, β-, and κ-caseins were studied to form amyloid-like fibrils at pH 2.0 and 90 ºC. No fibrils were observed for α-caseins, while β- and κ-caseins formed amyloid-like nanofibrils with increase β-sheet secondary structure. The nanomechanical analysis of long amyloid-like fibrils using PF-QNM AFM showed the lowest and highest Young’s modulus for β-casein and κ-casein, respectively. The dispersion with β-casein fibrils had a significantly higher viscosity than those of κ-casein and β-lactoglobulin at comparable concentrations. The current findings may assist not only the understanding of amyloid fibril formation but also the development of novel functional materials from disordered proteins.
8.2 Future work

The results presented in this dissertation indicate that novel functional ingredients can be developed by controlling the nanostructures of caseins. Although various *in vitro* physicochemical and biological characterizations have been conducted to understand the underlying mechanism and functionalities, it will be necessary to study the fate of these nanostructures *in vivo*. These studies can include physicochemical stabilities, digestibility, and absorption of the nanostructures in alimentary tract. Since proteins are easily hydrolyzed by enzymes in the gastrointestinal environment, applying another layer of indigestible polysaccharides or formation of hydrogel beads with indigestible polysaccharides can be studied to develop colon-specific delivery systems for treatment of colon cancer. For the nanofibrils produced from NaCas, preliminary studies showed that surface properties of these nanostructure are different from NaCas and the increased hydrophobicity at neutral pH may be produce stable foams for applications such as ice creams.
VITA

Kang Pan was born in a small village in China during the Lantern Festival in February 1989. He enjoyed the country life very much before his family moved to downtown Xuzhou city, Jiangsu province in China when he was ready for his second year in elementary school. After graduating from high school in 2007, he decided to pursue a Bachelor of Engineering degree in Food Science and Engineering at Jiangnan University in China, preparing for his entrepreneurship after graduation. He lived a very active life and tried to learn as much as he could in various areas during his college life. Later, he became fascinated with science and research during a national scientific competition, when he was working with his partner to develop biodegradable antimicrobial films from corn straw to extend the shelf-life of fresh meat. Also being deeply touched by Philosophy, he decided to pursue a Doctor of Philosophy (PhD) degree, to fulfill his strong curiosity towards both Science and Philosophy. He was fortunate to have Dr. Qixin Zhong as his advisor for his PhD program in Food Science and Technology at University of Tennessee, Knoxville, TN, USA in 2011. To make it more challenging and to better prepare for the “big data” era, he decided to pursue a concurrent Master of Science degree in Statistics with the support from Dr. Zhong. He still remains his strong curiosity toward the world and is ready for something interesting and exciting!