Design and Functionality of Dense Protein Particles

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Hep eksiklikler yaşayacaksın-ve hep fazlalıklar.

Yaşamın bu olacak işte: eksik-fazla.

DE Kİ İŞTE, ORUÇ ARUOBA

Table of contents

Chapter 1

General Introduction

Food products that contain high levels of protein can help to control food intake and to maintain a healthy body weight due to their strong satiating properties [1-5]. Preparation of such high protein food products with desired sensory properties is difficult. This is mainly due to protein aggregation during processing and storage. This aggregation becomes more prevalent at higher protein concentrations and leads to undesired sensory properties and decreased product stability. Aggregation of proteins can be reduced or eliminated through addition of other components to the system. Another possible route can be prevention of such aggregation on a length scale that is most relevant to the properties of the product. This can be achieved by using pre-fabricated protein structures, such as protein particles with controlled internal and surface properties, which are stable against aggregation. A high protein concentration within such particles will yield an overall high protein concentration in the product. The aim of this thesis is to explore the design of dense protein particles and to study their functionality in systems at high protein concentrations, including the maximal protein concentration for a given system thickness. In this chapter we first address the importance of proteins as nutrients and a more detailed account of challenges that exist in the formulation of high protein products. After this we underline the approaches to control the stability of proteins. In the final part we present the outline of the thesis.

Importance of proteins in diet

Proteins are major and important macronutrients of food products. They provide amino acids that are required as building blocks of body proteins. Several studies have elucidated physiological properties of proteins that contributes to a healthier diet. It has been reported that proteins have stronger satiating effect in comparison to carbohydrates and fats [1-5]. Protein-induced satiety was investigated in some studies performed under controlled environmental conditions. It was found that satiety and fullness were higher and hunger, appetite and the desire to eat were lower on a high-protein diet compared to a normal protein diet [4, 6]. Similar results were also observed when a protein-high diet was compared to a carbohydrate-high diet: proteins resulted in decreased hunger and food intake compared to carbohydrates [7]. The greater thermic effect of proteins upon consumption is another suggested benefit [8]. The thermic effect of a food product is the increase in the energy consumption above the baseline, which is required for the digestion,

absorption and disposal of metabolized nutrients. It is known that protein has the highest and longest thermic effect among the macronutrients and a high-protein diet results in increased energy expenditure (thermogenesis) following the consumption compared to a low protein diet [3-5, 8]. This effect strongly depends on the protein source and it is reported that proteins from animal sources show higher thermogenesis than proteins from vegetable sources [9, 10]. It is also proposed that the higher thermogenesis during protein metabolism might play a role in protein-induced satiety [3]. These findings strongly suggest that a high protein diet can help in the regulation of food intake and to maintain a healthy body weight.

Protein-rich products play also a significant role in medical nutrition. Protein malnutrition is a common problem among hospitalized patients [11-14]. Continuation of a balanced oral diet is often not possible for patients suffering from severe illnesses, such as cancer or AIDS, due to development of anorexia, eating difficulty and reduced intestinal function [6, 12]. Those patients are at risk of losing weight and developing impaired function in organs and muscles. There is a need for a dietary supplement, such as a small volume of liquid with a high content of nutrients, especially proteins and minerals [6, 12, 13]. High-protein, high-energy liquid supplements are suggested to be beneficial for those patients. For example, regular consumption of a high-protein energy sip feed supplement (containing approximately 6.25% (w/v) protein) resulted in an improved energy intake and reduced body weight loss in elderly patients [13]. Similar findings were also reported in a study that focused on a patient group infected with HIV [6]. Therefore development of stable liquid foods at high protein concentrations can be useful in medical nutrition.

Several studies have also emphasized the importance of proteins in diets of elderly. Higher protein intake may have positive effects on the bone and muscle health of the elderly [15-17]. Due to chewing difficulties and loss of appetite, elderly people may consume less protein than required amounts. It was proposed that the recommended dietary allowance (RDA) of protein (0.8 grams/kg body weight) may not be enough for protein needs of elderly people [16, 18, 19] and protein intake larger than the RDA may help against sarcopenia (progressive loss of muscle mass), an important aging-related disease [20, 21]. This suggests that to maintain and restore the muscle mass in the elderly, nutritional supplements rich in protein might be helpful. In the simulation of muscle synthesis, essential amino acids, particularly leucine, is reported to be the most efficient amino acid [21-23]. Thus, in the

case of a nutritional supplement for the elderly people, a formulation containing specific proteins that are rich in leucine would be beneficial.

Protein enriched foods: challenges

Due to their strong satiating effect and other health related properties, protein-rich foods are important elements of a healthy diet. Thus, development of food products with substantially higher protein concentration is an important subject.

Whey proteins and caseins are abundant dairy proteins that are readily digested and have a balanced amino acid composition. Especially whey proteins are a good source of branched chain amino acids, i.e. leucine, isoleucine and valine, which are reported to stimulate muscle protein synthesis [24, 25]. They are commonly used in the formulation of protein-containing foods, such as dairy drinks, infant formulas, protein bars, etc. It is known that both whey proteins and caseins have limited structural stability. They are sensitive to changes in environmental parameters, such as ionic strength, mineral composition, pH and temperature [26-29].

During processing, such as thermal treatment and storage, whey proteins are susceptible to denaturation followed by aggregation. Thermal treatment of whey protein solutions will results in aggregate formation and this will lead to changes in the product properties. Increased turbidity, viscosity and precipitation are some problems reported for relatively dilute systems. Upon increasing the protein concentrations, these changes will become more significant. A gel may be formed upon heat treatment and possibly during prolonged storage.

These changes, such as viscosity increase, will make the consumption of the product more difficult. This is undesired, particularly for the liquid medical formulations, which requires a high protein concentration (6% w/w proteins) with an acceptable product viscosity [30]. Furthermore, other sensory properties of the product, such as taste and mouthfeel, may also be negatively influenced. This was, for example, investigated for dairy drinks enriched with whey proteins [31]. During heat treatment, proteins coagulated leading to sedimentation. Whey protein enriched drinks were reported to have a chalky mouthfeel and an off-flavor. These negative sensory attributes became more articulated with an increasing concentration of whey proteins in the drinks. In another study, whey protein concentrates and caseinates were used for the fortification of yoghurt [32]. A high graininess for whey protein-fortified and low water holding capacity for caseinate-

fortified yoghurts were reported as sensory defects. As an alternative to dairy proteins, soy proteins were also used for protein enrichment of yoghurts [33]. Likewise, sensory attributes of the yoghurts were reported to be negatively influenced: thickness, chalkiness and off-flavor taste increased with increasing soy protein concentrations.

High protein content may also introduce other stability problems, such as slow hardening of the texture, which is reported for highly concentrated systems, particularly for high protein bars. The hardening of the bars may start directly after the production. During storage the product becomes harder and may become unstable [34]. It has been suggested that this hardening, observed in the nutritional bars containing relatively high concentration of protein, is a result of strong protein-protein interactions, eventually leading to protein aggregation and phase separation [35]. In summary, since protein-containing processed foods are only marginally stable, the task to increase protein concentrations without imparting the product characteristics becomes a challenging task.

Approaches in controlling stability of proteins

Among several parameters that might influence stability of the proteins, thereby changing the product characteristics, stability at elevated temperatures is important, especially for the products requiring pasteurization or sterilization.

To improve thermal stability of proteins several approaches have been investigated. One of the common methods investigated to improve thermal stability of proteins is addition of solutes or co-solvents. It is reported that sugars can increase the thermal denaturation temperature of globular proteins [36-39]. It has been shown that this increase in the denaturation temperature of proteins resulted in an increased gelation temperature for whey proteins [40] and bovine serum albumin (BSA) [41]. In both studies the influence of sucrose concentrations (0-40% w/w) on the denaturation and gelation temperature was investigated. The results showed that both the denaturation and gelation temperature were increased with increasing sucrose concentration, due to the favored native state of the proteins. Addition of sorbitol [42, 43] and glycerol [43] was also shown to increase denaturation and gelation temperature of globular proteins. However, addition of glycerol in concentrations above 10% resulted in gelation of a beta-lactoglobulin solution (10% w/w) after heating, which is not observed in the case of sorbitol addition [43]. Enzymatic cross-linking of proteins was also studied to improve the heat stability

of proteins. Treatment of milk proteins by transglutaminase results in formation of protein polymers through intermolecular cross-linking of proteins [44, 45]. It is reported that protein polymers produced by transglutaminase cross-linking have a large influence on the properties of proteins, such as water holding capacity, gelation, rheological properties and heat stability [45-48]. Transglutaminase treatment of whey protein concentrate (WPC) was studied by Soeda and Hokazono [49]. These authors have shown that water holding capacity and viscosity of the enzyme treated WPC increased, whereas heat-coagulation was suppressed. Similarly, Lorenzen [50] has shown improved heat stability of WPC and sodium caseinate after transglutaminase treatment. By increasing transglutaminase to substrate ratio, extensive cross-linking of whey proteins was obtained [51]. The cross-linked whey proteins showed gelation at lower temperatures in comparison to non-treated whey proteins and a significant decrease in the gel strength was observed.

Applying new processing techniques can also be an alternative way of improving stability of proteins. Recently, it was shown that ultrasound treatment of whey proteins enhanced heat stability of proteins, particularly of whey proteins [52]. The ultrasound treatment involved a pre-heating of whey protein solution at 80 ^{o}C for 1 min, followed by a short sonication step. After the ultrasound treatment, samples were heated a second time to assess the changes in the viscosity. Aggregates formed during pre-heating were broken down by the ultrasound treatment and prevented from reforming in the second heating step. As a result, the viscosity of ultrasound-treated whey protein concentrate at a protein content of 6.4% (w/w) remained low upon heat treatment. The low viscosity of the aqueous protein solutions was also preserved upon freeze- or spray-drying of the samples.

Using protein particles that could have special advantages in food formulations has been investigated by several authors. O' Kennedy et al. [53] studied the formation of whey protein particles by using calcium phosphate. Here, whey protein pre-aggregates were first produced by heating a 7.5% (w/w) whey protein isolate solution. Subsequently, calcium and phosphate were introduced to this pre-heated dispersion, which resulted in the formation of WPI particles in the range of a few hundred nanometers. The authors showed that this dispersion of whey protein/calcium phosphate was stable against heating at neutral pH. In addition, a combination of heat and high-pressure shearing was used to modify whey proteins [54]. This process resulted in formation of whey protein particles having increased heat stability compared to native whey proteins. An alternative way to prepare

protein particles to enhance heat stability of liquid protein formulations was recently reported [55, 56]. Here whey protein nano-particles were prepared (diameter smaller than 100 nm) through addition of a whey protein isolate solution to a w/o micro-emulsion containing reverse micelles of a surfactant, followed by a heating step. The dispersion of those whey protein nano-particles was transparent and liquid-like after thermal treatment, while WPI at the same protein concentration gelled after heat treatment. The emphasis of this work was on the formation of small particles to be used in transparent beverages. Therefore the protein concentrations studied was rather low (5% w/v).

Most of the described studies focused on improving stability of proteins by using different strategies. Although some of these methods have shown promising results, the protein concentrations studied were rather low and the emphasis was not given to increasing the total protein concentration.

Objective and Outline of the thesis

Current knowledge on how to control and prevent protein aggregation in concentrated protein systems is limited, thereby hampering the development of high protein food products. Ideally, one would like to be able to control the protein concentration in foods, without influencing the other properties of the product, like sensory properties and stability.

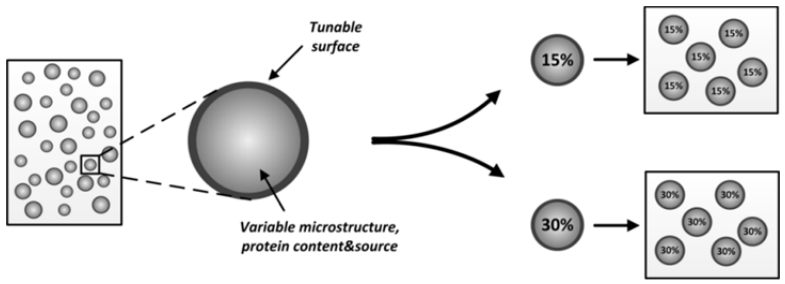

Figure 1.1 Schematic overview of dense protein particles with controlled size, internal protein and surface properties as a possible tool to uncouple protein concentration from the effects on structure.

A possible route to achieve such an uncoupling between protein concentration and structure is to use pre-fabricated protein structure elements. Dense protein particles with controlled size, internal protein concentration and surface properties

can be an example of such structure elements. As illustrated in figure 1.1, the underlying assumption is composed of two parts. Firstly, when the particles are small enough, their effect on the food texture is independent of their internal protein concentration and also protein source. Secondly, the interaction of the particles with the food matrix or with each other is controlled by their surface properties. In this way, the protein concentration of a product can be increased without changing the textural properties of a product, while the textural properties can be tuned by the surface properties of the particles.

The aim of this thesis is to design dense protein particles and study their functional properties. Special attention is given to their behavior at high particle volume fractions, corresponding to systems at high protein concentration. The health benefits of high protein foods and the challenges in developing products with relatively higher protein content were discussed in this chapter (Chapter 1). In Chapter 2, a robust method to prepare dense protein particles with controlled size, internal density and surface properties is presented. In Chapter 3, rheological properties of protein particle dispersions at high volume fractions are studied and the effect of heat treatment at varying dispersing media on the rheological properties is addressed. In Chapter 4, the influence of preparing the protein particles at different gelling conditions, such as different pH and salt concentrations, is studied. The change in the microstructure and internal protein density of the particles are characterized. To investigate if the particle properties can be predicted from the bulk whey protein gels, the microstructure of the protein particles and bulk whey protein isolate gels are also compared in Chapter 4. The results from Chapter 3 and 4 suggest that swelling of the particles has a significant effect on the rheological properties and heat stability of the particle dispersions, especially at high particle volume fractions. Therefore, the swelling and the protein leakage of the particles, under various solvent conditions, are investigated in Chapter 5. In Chapter 6, the heat stability of a liquid model system is studied as a function of protein concentration. Different type of protein particles are used to investigate the influence of particle type on heat stability. To examine the behavior of protein particles dispersed in a continuous phase, that also contains protein, mixed systems are studied (Chapter 7). The influence of particle incorporation on the mechanical properties of whey protein isolate gels is investigated. In the final chapter (Chapter 8), the findings of this thesis are discussed in detail and protein

particles, as possible ingredients for the development of novel high protein foods, are reviewed.

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Chapter 2

Preparation of dense protein particles using two-step emulsification

In this chapter we present a robust procedure for preparing protein particles with a high internal protein content. Protein particles were formed through emulsification of a whey protein isolate (WPI) solution in sunflower oil containing Polyglycerol Polyricinoleate (PGPR) as an oil-soluble emulsifier. This emulsion (w/o) was heated to induce gelation of the whey proteins inside the emulsion droplets and oil was removed through centrifugation. The average diameter of the particles was in the order of a few micrometers, depending on the initially applied mixing conditions. Confocal laser scanning microscopy (CLSM) analysis of protein particles revealed that protein is homogeneously distributed throughout the particles and there is oil associated with the particles, either surrounding the particles and/or distributed throughout the particles. NMR analysis showed that this amount of oil does not exceed 1.8% (w/w).

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Introduction

Recent literature suggests that food products with an increased protein content have important health benefits. Several nutritional studies showed that proteins have stronger satiating effect as compared to carbohydrates and fats [1-5]. Therefore high protein foods are considered to be a potential candidate for body-weight control and treatment of obesity.

Besides having high nutritional value in foods, proteins are also important because of their contribution to food structure and texture. Mainly as a result of heat-induced protein aggregation, several problems related to food texture may occur in protein enriched foods. Singh and Nath studied enrichment of an acidified dairy drink with whey proteins [6]. The authors showed that during the heat treatment of the drinks whey proteins coagulated, which led to sediment formation. As a result, sensory properties of protein enriched drinks were negatively affected; drinks were reported to have a chalky mouthfeel and an off-flavor taste which became more pronounced with increased protein concentration. Similar observations were also reported in a study on fortification of yoghurts with soy protein [7]. Negative textural attributes of high protein foods are a strong function of the actual protein content. For example, Kangli et al. [8] studied the effect of protein concentration on the texture of soy protein gels formed through heating, and found a very strong protein concentration dependence of the 'hardness' and 'toughness'. Gels formed at higher protein concentrations were described as firm, tough and unfracturable. Similar dependencies of gel properties on protein concentration have also been reported in other studies [9-12].

These examples show that the development of stable and consumer-appealing protein-rich foods is a challenging topic, in which unwanted protein aggregation plays a prominent role. To be able to control the protein content of foods independently from the other important attributes, such as sensory properties and stability, use of protein particles having controlled internal and surface properties can be a possible route. The effects on food texture can be controlled largely through the surface properties of the particles and their interactions with the food matrix. As a result, the effects of type and concentration of the protein on texture can be minimized.

Most of the previous work on the preparation of protein particles has focused on using proteins for encapsulation, controlled delivery, double emulsification or food

structuring at relatively low protein concentrations. To produce whey protein gel beads, Beaulieu et al. [13] used a combination of emulsification and cold gelation. An oil-in-water emulsion, in which a WPI solution formed the aqueous phase, was added dropwise into a $CaCl_2$ solution to induce bead formation. The resulting protein beads were spherical in shape and the internal structure of the beads consisted of multiple oil droplets entrapped in a whey protein gel matrix. The protein concentration inside the beads was lower than 5.6% (w/w). Also, the size of the particles was rather large (average diameter ~ 2 mm), which might be a disadvantage in several applications. In a more recent study, somewhat denser protein beads were formed by phase separation in a protein-polysaccharide mixed system [14]. In this study, protein-protein interactions were increased by lowering the pH to the isoelectric point of the protein, resulting in the formation of protein-rich spherical domains. The size of those domains varied from 2 μm to 50 μm depending on the type of polysaccharide used. In another study protein particles were prepared by lyophilization of a protein-polyethylene glycol (PEG) aqueous mixture [15, 16]. In this study co-lyophilization of bovine serum albumin or gelatin in the presence of PEG led to the formation of micron-sized, spherical protein particles, again as a consequence of phase separation. Recently, Surh et al.[17] have studied the gelation of the internal aqueous phase of a water-in-oil-in-water (w/o/w) emulsion in order to improve emulsion stability. The internal aqueous phase used in this study was a 15% (w/w) WPI solution. The final emulsion consisted of oil droplets (average diameter ~ 10 μm) containing several gelled protein particles as the internal phase. As the droplets contained a significant amount of oil, the average protein content of the composite droplets was much lower than 15% (w/w).

The idea of using prefabricated protein structure elements that could have special advantages in formulations has been investigated by other authors. For example, O' Kennedy et al. [18] studied the formation of whey protein particles by using calcium phosphate. In this work, first whey protein pre-aggregates were produced by heating a 7.5% (w/w) whey protein isolate solution. Subsequently, calcium and phosphate was introduced to this pre-heated dispersion, which resulted in formation of WPI particles with a size of a few hundred nanometers. The authors showed that this dispersion of whey protein/calcium phosphate has a sufficient stability against heating at neutral pH. In a more recent study Zhang and Zhong showed that whey protein nano-particles can improve heat stability as compared

to native whey protein isolate [19]. Their whey protein nano-particles were prepared through addition of a whey protein isolate solution in a w/o micro-emulsion containing reverse micelles of surfactant and subsequent heating at 90 °C for 20 min. The method resulted in whey protein particles having an average diameter smaller than 100 nm.

Unfortunately, in none of these studies emphasis was given to adequate characterization and increasing the protein concentration of the protein particles. An internal protein content of at least 20% (w/w) would be ideal for use as a "protein structure element" in high protein foods. In this chapter, we investigate a possible method for producing dense protein particles. The method is robust in the sense that it allows the use of various protein sources and concentrations for the particle inside, and various stabilizers, for use on the particle surface. Our work extends the work of Surh et al. [17] and is based on an early study describing the preparation of double emulsions [20]. Besides describing the procedure, we also report on extensive characterization of the protein particles, both in their final state, and at various intermediate steps of the procedure.

Experimental

Materials

Whey Protein Isolate (WPI, BiPro JE 034-7-440-1) was obtained from Davisco Foods International Inc. (Le Sueur, MN). The composition of WPI as stated by the manufacturer was 97.9% protein , 0.3% fat, 1.8% ash (dry weight basis) and 4.9% moisture (wet weight basis). Polyglycerol Polyricinoleate (Grindsted PGPR 90, Denmark) was purchased from Danisco and consisted of polyglycerol ester of poly-condensed ricinoleic acid (E476) with added antioxidants: Alpha-tocopherol (E 307) and Citric acid as stated by the manufacturer. Sodium caseinate (EM 7) was obtained from DMV international (Veghel, the Netherlands). Sunflower oil (Reddy, NV Vandemoortele, Breda) was purchased from a local supermarket. FITC (Fluorescein isothiocyanate isomer I) and Nile red were purchased from SIGMA-ALDRICH (Steinheim, Germany).

Preparation of solutions

Whey protein isolate (WPI) solutions were prepared by dispersing WPI powder in Millipore water (Millipore Corp., Billerica, MA). The solutions were stirred overnight, and refrigerated before usage. PGPR was dissolved in sunflower oil by

stirring for at least 2 h at room temperature. Sodium caseinate (Na-caseinate) powder was dissolved in Millipore water in the same way as described for WPI solutions. The pH values of the WPI and sodium caseinate solutions were left unadjusted.

Preparation of whey protein particles

Whey protein particles were prepared by modifying a known two-step emulsification technique [20]. A water in oil (w/o) emulsion was prepared by adding 25 % (w/w) WPI solution into sunflower oil containing 2.5 % (w/w) PGPR as an hydrophobic emulsifier. The WPI solution was slowly added into the oil, while mixing with a high speed mixer (Ultra-turrax T 25, IKA Werke, Germany) either at 6500, 9500 or 13500 RPM. The total mixing time was kept at 5 min. The ratio of the aqueous phase to the oil phase in the primary emulsion was 30:70 (w/w). This primary emulsion was heated at 80 ^{o}C for 20 min in a temperature-controlled water bath and then centrifuged (at 33768 RCF, Avanti J-26 XP, Beckman Coulter, U.S.A) for 1 h to remove the excess of the oil. The pellet, which mainly consisted of protein particles, was dispersed in a 4 % (w/w) Na-caseinate solution by mixing at 6500 RPM for 5 min. In the next step, this dispersion was homogenized with a lab-scale homogenizer (Delta Instruments, Drachten, The Netherlands) at 150 Bar (6 passes). Centrifugation and washing with Na-caseinate solution were repeated several times, until most of the oil was removed from the dispersion, each time with a ratio of pellet to Na-caseinate solution of 1:2 (w/w).

Optical microscopy

Samples taken from different steps during the preparation of protein particles were analyzed using an optical microscope (Axioskop plus, Zeiss, Germany) equipped with a CCD video camera (Hitachi KP-D20B, Tokyo, Japan). To record the microscopic images, first each sample was gently diluted (100x) with its continuous phase. A drop of diluted sample was transferred onto the microscope slide and then covered with a cover slip prior to analysis. An oil immersion objective (100x magnification) was used to obtain microscopy images.

Determination of particle size distribution

The particle size distribution of the protein particles was determined using light scattering (Mastersizer 2000, Malvern Instruments, Worcestershire, U.K.). The light scattering equipment measures the angular intensity of the scattered light using a series of photosensitive detectors and predicts the particle size distribution using a

Mie scattering model. For the measurements, samples were diluted in Millipore water to avoid multiple light scattering. The size distribution of each sample was measured at least five times. Average sizes reported are number averaged diameters.

Determination of hydrodynamic volume fraction

The Einstein expression for the effective viscosity of an emulsion was used to calculate the hydrodynamic volume fraction (Φ) of the protein particles:

$$\eta_{eff} = \eta_c \left(1 + \frac{5}{2}\Phi\right) \tag{2.1}$$

where η_{eff} and η_c are the dynamic viscosities of the dispersion and the continuous phase, respectively. The final particle dispersions were diluted 5, 10, 20 and 50 times in order to ensure that the dispersions are sufficiently diluted to be able to use Einstein equation. Dynamic viscosities of the diluted samples were determined using a capillary viscometer (Ubbelohde) placed in a water bath at 25°C. Each dilution was measured three times and an average viscosity was used for further calculations. The experimental error, when determining the volume fraction of the particles, was found to be around 5%.

Determination of protein content

Protein content of the particle dispersion was determined by DUMAS. A Flash EA 1112 N/protein analyzer (Thermo Scientific, Waltham, US) was used to determine the nitrogen content of the samples. Nitrogen values were multiplied by 6.38 to calculate the protein concentration of the dispersion. Internal protein content of the particles ($C_{p,w/w}$) was calculated from:

$$C_{p,w/w} = \frac{C_{eff,w/v} - C_{c,w/v}(1-\Phi)}{\Phi\ \rho_p} \tag{2.2}$$

where $C_{eff,w/v}$ and $C_{c,w/v}$ are the protein concentration of the dispersion and continuous phase, respectively and ρ_p is the density of the protein particles. Densities of particle dispersions and protein solutions were measured using an oscillating U-tube density meter (Anton Paar DMA 5000, Graz, Austria). Density of the protein particles was calculated from:

$$\rho_p = \frac{\rho_{eff} - \rho_c (1 - \Phi)}{\Phi} \tag{2.3}$$

where ρ_{eff} and ρ_c are the densities of the particle dispersion and continuous phase, respectively.

Confocal laser scanning microscopy (CLSM)

For CLSM analysis, protein particles were prepared with FITC labeled (covalently) WPI solution and Nile red stained oil. For labeling, a 1% (w/w) WPI solution was prepared in 0.1 M carbonate buffer (pH:9.1). FITC was dissolved in DMSO at 2 mg/ml. For each ml of protein solution, 50 µl of FITC solution was slowly added into the protein solution, while gently stirring. The sample was incubated in the dark for 5 h. After incubation, the protein solution was transferred into dialysis membranes (MWCO 12-14 kDa, Medicell International Ltd.) and the excess of FITC was removed by dialyzing this solution against buffer solution. Dialysis was performed in the dark at 4 ^{o}C for at least 60 h and buffer solutions were refreshed every 12 h. The pH of the labeled protein solution was adjusted to 6.8 by adding 6M HCl. Staining of the oil was done by dissolving Nile red in the oil at 0.2 g/l. As an alternative fluorescent die, to monitor the presence of protein and oil in the particles, we have also used Nile blue. For this analysis, a dispersion of protein particles (containing no dye) was stained with few drops of 1 % (w/v) Nile Blue A (Janssen Chimica Beerse, Belgium) and directly analyzed with CLSM. All the samples were diluted 10x prior to analysis.

Imaging of the samples was performed using a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems B.V., Rijswijk, Netherlands) equipped with a DMI6000 inverted microscope. The system was equipped with an Argon laser. Samples containing FITC and Nile red were excited at 488 nm and detected sequentially at 500-550 (FITC channel) and 600-700 nm (Nile red channel). For Nile

Blue stained samples, to detect the oil phase, samples were excited at 488 nm and detected at 520-602 nm. To detect the protein phase, excitation and detection was performed at 633 nm and 661-749 nm wavelengths, respectively.

Cryo-SEM

A small droplet of the final dispersion of protein particles was put on top of an aluminum rivet. Subsequently, this sample was plunge frozen in melting ethane (-182 °C) and transferred into the Cryo-preparation chamber (Oxford CT 1500HF), by using nitrogen slush and vacuum to prevent water vapor condensation. In the Cryo-preparation chamber, the sample was cut by a cooled knife and the newly created surface of the sample was etched, using sublimation of ice at -90 °C, thus removing the water to a depth of a few microns and revealing the structures just below the surface. After sublimation, the sample was sputter coated with a thin layer of gold/palladium and transferred into the SEM (Jeol 6340). The sample was analyzed at -125 °C, with an acceleration voltage of 3 kV.

Determination of oil content

Protein particles were prepared according to the procedure described above. After the last centrifugation step, 5 g of sample was taken from the pellet and freeze dried. The freeze-dried sample was used for NMR analysis. A small amount of sample (±0.1 mg) was dissolved in $CDCI_3$ containing 0.01% DMSO and the precipitate was removed by centrifugation, before being loaded in the NMR tube. 1H/13C-NMR spectra were recorded at 20 °C on a Bruker 500 MHz Ultrashield Avance III system equipped with ATM/TCI probe. For the calculation of the oil content in the sample, a calibration curve using a known quantity of sunflower oil in Chloroform-d was prepared. The calibration curve was derived from the measurements of 9 solutions of known oil content. 1H-NMR spectra were processed and the integral of the CH_3 and DMSO signals were measured. The oil content was then derived from the obtained calibration curve. The analysis was performed 4 times and the oil content of the sample was expressed as the average of these analyses.

Results and discussion

Formation of protein particles

Dense protein particles were prepared using a two-step emulsification method. A schematic overview of the preparation procedure is shown in figure 2.1. The

procedure consists of two main parts; in the first part, gelled protein particles suspended in oil were formed and the excess of the oil was removed from the system, and in the second part protein particles were washed and dispersed in an aqueous phase. The primary w/o emulsion was prepared by slowly adding a 25% (w/w) WPI solution into the sunflower oil, containing 2.5% (w/w) PGPR, while mixing at 6500 RPM with a high speed mixer. The main objective of this study is the preparation of individual protein particles. Therefore, in this initial step it is crucial that we produce only w/o droplets and no higher order droplets, such as o/w/o droplets, are formed.

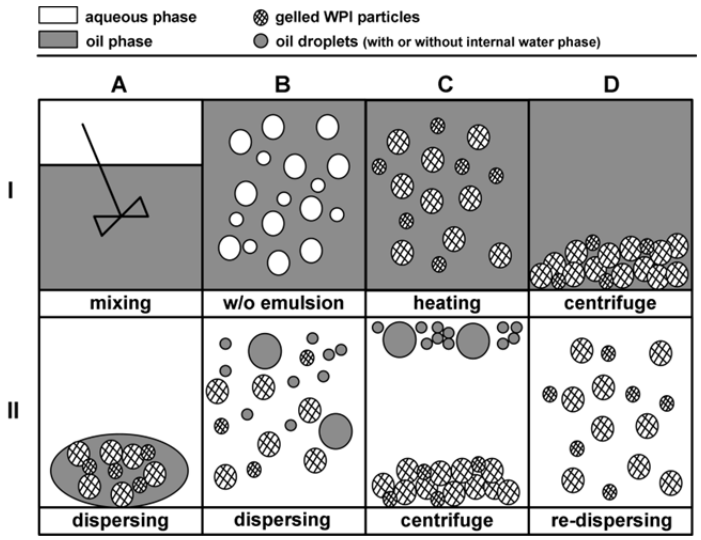

Figure 2.1 Schematic representation of the procedure for the preparation of protein particles. First row; aqueous phase: 25% (w/w) WPI solution, oil phase: 2.5% (w/w) PGPR in sunflower oil, mixing: high speed mixer, heating: 20 min at 80 ^{o}C with gentle stirring, centrifuge: 1 h at 33.768 RCF. Second row; aqueous phase: 4% (w/w) Na-caseinate solution, dispersing: high speed mixer + homogenization at 150Bar, centrifuge: 1 h at 33.768 RCF.

Also inversion of the emulsion from w/o to o/w during this step is undesirable. This sensitively depends on variables, such as the volume fraction of the phases and the concentrations of the surfactants. For preparation of a w/o emulsion, PGPR was chosen as the oil-soluble emulsifier. It is reported by several authors that PGPR can form stable w/o emulsions [17, 21-24]. By using a 2.5 % (w/w) PGPR in the primary emulsification step, we have obtained stable w/o emulsions. We did not observe inversion of the emulsion, until a weight fraction of 60% aqueous phase

(WPI solution). For preparing protein particles, we have used primary emulsions, for which the fraction of WPI solution in oil did not exceed 30% (w/w). We have confirmed that under these conditions, we almost exclusively form WPI containing water droplets dispersed in an oil continuous phase (Fig. 2.1-IB). Droplets were a few microns in size. The size distribution was polydisperse, as can be seen from the CLSM micrographs (Fig. 2.2-A). The actual protein particles are formed when the primary emulsion is heated in a temperature-controlled water bath at 80 °C for 20 min. These conditions proved to be sufficient to induce gelation of the WPI inside the particles. To obtain a more homogeneous heating and to minimize aggregation of the particles, slight stirring of the emulsion during heating was found to be helpful. Figure 2.2-B shows a CLSM micrograph of the w/o emulsion after the heating step. The microstructure of the w/o emulsion after heating was very similar to that prior to heating. No flocculation or changes in the size or shape of the particles were observed.

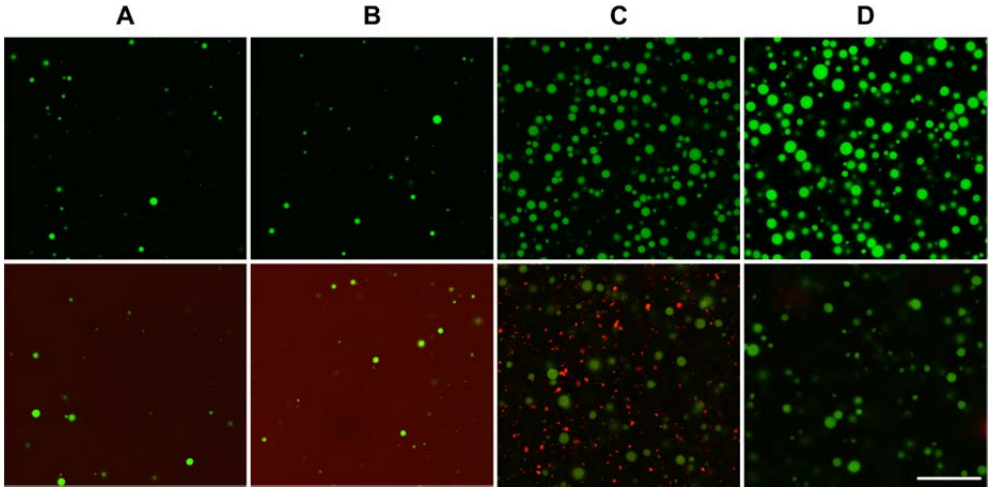

Figure 2.2 CLSM micrographs of protein particles taken after different steps during the preparation. A: w/o emulsion, B: w/o emulsion after heating step, C: protein particles after first dispersing step, D: protein particles after washing/centrifugation steps. First row; FITC labeled samples. Second row; FITC labeled and Nile red stained samples. Scale bar: 25 μm.

The heating step simply results in formation of internally gelled particles, and does not cause any other significant changes in the emulsion microstructure, as was also reported by Surh et al. [17] under slightly different conditions. To isolate protein

particles, the w/o emulsion was centrifuged (1 h at 33.768 RCF) after the heating step. This resulted in formation of a dense, white colored pellet, consisting of mainly gelled protein particles. The supernatant obtained after the first centrifugation step consisted mainly of sunflower oil, and had a turbid appearance. This suggests that some protein particles are also present in the supernatant. The protein content of the supernatant was determined to be lower than 1% (w/w). Apparently, the conditions that we have used are such that not all protein particles have sedimented during the first centrifugation step. In the first centrifugation step approximately 90% (w/w) of the total oil was separated from the protein particles, which means that there was still a significant amount of oil present in the pellet, distributed between and/or inside the particles.

In the next step, the pellet was re-dispersed in an aqueous phase. The aqueous phase should contain a stabilizer or surfactant, since the protein particles may still have oil associated with them after the first centrifugation step. The pellet was first dispersed in a 4% (w/w) sodium caseinate (Na-caseinate) solution by high speed mixing (5 min at 6500 RPM). The resulting dispersion still contained aggregates/lumps of particles visible to the eye. Next, the sample was homogenized at 150 Bar (6 passes) to obtain complete separation of the gelled particles from each other. The micrograph of the sample after homogenization is shown in figure 2.2-C (second row). Beside the protein particles (in green), also several oil droplets/lumps/aggregates (in red) are still present in this sample. During the preparation of double emulsions, applying severe mixing conditions by using high speed mixers or a homogenizer in the secondary emulsification may result in the rupture of the oil layer around the water droplets [24, 25]. Similarly, we expect that the homogenization used to disperse the protein particles may also cause the rupturing of some part of the oil covering the protein particles. Therefore centrifugation/dispersing steps were repeated and it is expected that most of the remaining oil was removed from the system. Indeed, as can be seen from figure 2.2-D (a micrograph of the final dispersion of the particles), no significant amount of oil droplets was observed in this sample.

The size distribution of the particles was determined using light scattering (Fig. 2.3). The rather polydisperse size distribution corresponds to a surface mean diameter, D [3,2], of the particles of 2.7 μm. We have also tested the effect of applying different mixing rates on the particle size. The applied mixing rates in the first emulsification step were adjusted to 6500, 9500 and 13500 RPM, while the rest

of the preparation was kept the same. From figure 2.3, it is clear that the size of the final particles could be decreased by increasing the mixing rate during the primary emulsification step.

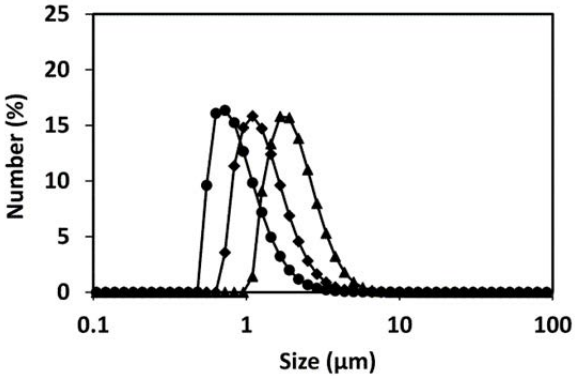

Figure 2.3 Particle size distribution of protein particles prepared at different mixing rate of the primary emulsion (w/o). Mixing rates were: 6500 RPM (▲), 9500 (♦) and 13.500 (●).

The microstructure of the particles at various stages of the procedure was monitored by optical microscopy (Fig. 2.4). The micrographs in figure 2.4-A represent the samples obtained after the first homogenization step; after re-dispersing the pellet in the Na-caseinate solution (see Fig. 2.1-IIA). From those pictures, it is clear that at the higher mixing rates applied in the primary emulsification step (9500 and 13500 RPM), a fraction of multiple emulsion droplets was formed at some stage in the procedure. The sample prepared at 6500 RPM contained almost no multiple droplets: it only had single or flocculated oil droplets containing no inner aqueous phase.

In the classical two-step emulsification technique for preparation of double emulsions, high-shear conditions are applied in the primary emulsification step to obtain small droplets, whereas the secondary emulsification step is deliberately kept very gentle to avoid break-up of multiple droplets and rupture of the internal phase [25-27]. In our procedure, when the mixing rate in the primary emulsification step was increased from 6500 to either 9500 or 13500 RPM, smaller water droplets were formed. As mentioned, after the first centrifugation step, a significant amount of oil was still present in the sample. Presumably, in the re-dispersion and homogenization step that follows, these smaller particles more easily form

multiple emulsion droplets containing several protein particles as internal phase. In the washing/centrifugation steps that follow, the multiple emulsion droplets are removed at the expense of a lower overall yield.

Figure 2.4-B shows the micrographs of the final dispersion of protein particles after repeated washing and centrifugation steps (see Fig. 2.1-IID). It is also clear from the micrographs that multiple emulsion droplets, formed in the previous steps, were removed and particle sizes do indeed decrease with increasing mixing rate.

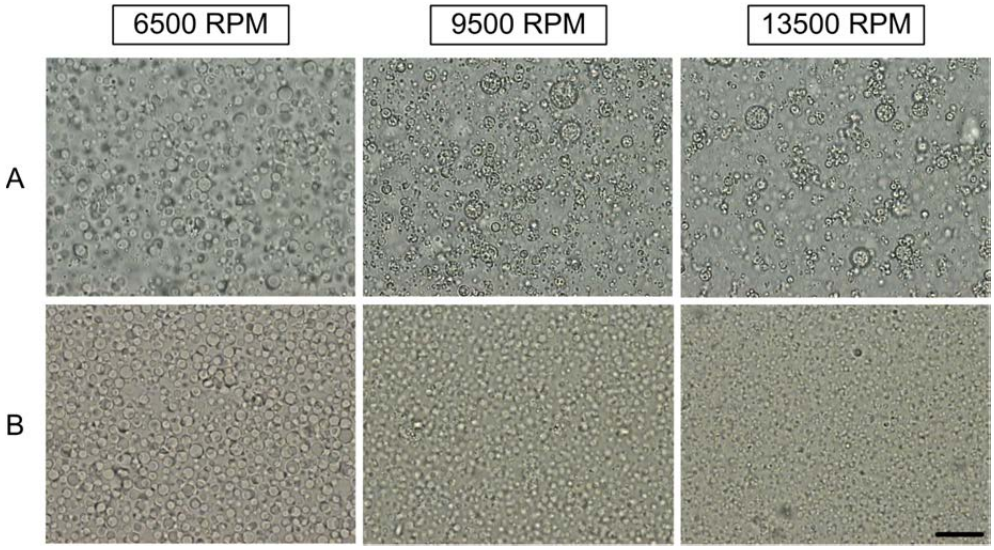

Figure 2.4 Optical microscopy images of protein particles prepared at different mixing rates. (A): protein particles after first dispersing step. (B): protein particles after several washing/centrifugation steps. Scale bar: 20 μm.

Microstructure of protein particles

Cryo-SEM analysis

Cryo-SEM micrographs of the particles are presented in figure 2.5. Figure 2.5-A shows the freeze-dried specimen. Micrographs were taken at the edge of the sample (as pointed out by an arrow), where the size of the ice crystals and damage to the particles are expected to be minimal.

The spherical domains in the micrographs (Fig. 2.5-B and -C) are the protein particles. In figure 2.5-D a higher magnification SEM image of one single particle is shown. According to this micrograph, protein particles contain an isotropic dense

protein network. No evidence of multiple droplets or any oil associated with the particles was observed in the SEM micrographs.

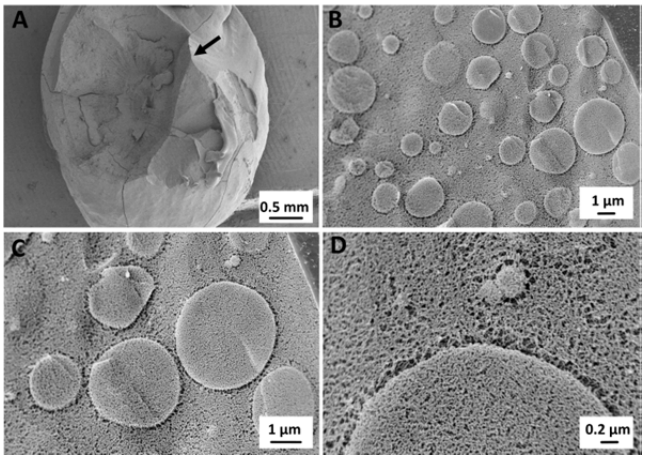

Figure 2.5 Cryo-SEM images of protein particles dispersed in 4% (w/w) Na-caseinate solution. Panel A (top left): low magnification micrograph of the freeze-dried sample. Micrographs at higher magnifications were taken at the edge of the sample as indicated by an arrow.

CLSM analysis

Protein particles were also analyzed using CLSM. For this purpose, FITC and Nile red were chosen as fluorescence dyes for monitoring the protein and oil, respectively. In several studies the combination FITC-Nile red has been successfully used for double labeling of protein and oil in different systems [28-32]. In our analysis labeling of protein with FITC was done covalently, while Nile red was used to stain the oil. To detect the protein and oil compartments, a sequential scan was performed. In figure 2.6 the CLSM micrographs of protein particles are shown. In the micrographs, green areas (FITC signal) represent protein while red areas (Nile red) represent the oil. As can be seen, spherical particles were detected both in the FITC and Nile red channel. The signal observed in the Nile red channel was rather weak.

According to these micrographs both protein and oil are homogenously distributed throughout the particles. However, this observation should be interpreted with caution. Although double labeling to monitor oil and protein with a combination of FITC and Nile red may have given good results in some studies, it also has been

reported that the complete spectroscopic separation of FITC and Nile red signals is not possible and some fluorescence of protein might be visible in the lipid (or oil) image [32]. Therefore we have also analyzed protein particles prepared using Nile red stained oil, containing no FITC. In micrographs (data not shown) of these samples, spherical particles were still visible with a weak fluorescence signal, indicating that overlap of FITC and Nile red signals is not what gives rise to this observation.

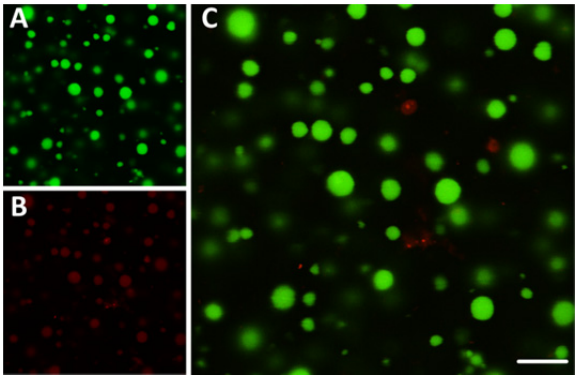

Figure 2.6 CLSM micrographs of protein particles. (A): FITC signal, (B): Nile red signal, (C): overlay of FITC and Nile red signals. Scale bar: 10 μm.

We have also used Nile blue as an alternative fluorescent dye to monitor both the protein and the oil distribution in the sample. For this, protein particles containing no dye were stained with Nile blue and directly analyzed with CLSM. The sample was excited at 488 nm and emission spectra were recorded between 520-602 nm wavelength for the detection of apolar parts (oil channel, Fig 2.7-A), while the excitation at 633 nm followed by detection between 661-749 nm was done to monitor polar parts (protein channel, Fig 2.7-B). CLSM micrographs of protein particles stained with Nile blue (Fig. 2.7) are similar to those of the doubly labeled FITC/Nile red samples. Spherical particles were visible both in the protein and in the oil channel. For the Nile blue labeled samples, we observed that the oil signal on the surface of some particles was much stronger than the oil signal from the inside of the particles. Overlays (Fig. 2.7-C and -D) clearly show an oil ring surrounding some of the protein particles and oil patches attached to the particles. The results for Nile red and Nile blue are somewhat unexpected and preclude a definitive conclusion as to the localization of the oil. Nile red is a fluorescent probe

which can be used for the detection of lipids in combination with CLSM [33-35]. However it has also been reported that Nile red can strongly bind to native proteins such as β-lactoglobulin and κ-casein having non-polar domains [36]. These authors have also shown that exposure of hydrophobic groups (for example after partial denaturation of proteins) can enhance the binding and as a result cause an increased fluorescence intensity. Later work has also demonstrated that Nile red binds to other proteins [37, 38]. Therefore, the Nile red fluorescent observed from the inside of the particles may, at least in part, be due to staining of protein with Nile red.

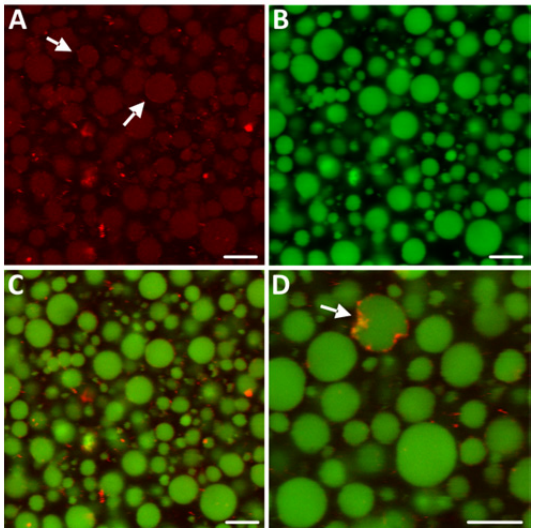

Figure 2.7 CLSM micrographs of protein particles stained with Nile blue. (A): oil signal, (B): protein signal, (C): overlay of both signals. (D): overlay of both signals at higher magnification. Arrows indicate oil layer around protein particle. Scale bar: 5 μm.

Since Cryo-SEM analysis did not clearly point to the presence of oil inside the particles either, we conclude that the continuous oil distribution throughout the particles in CLSM micrographs may be a labeling artifact, although we cannot exclude the presence of a small amount of oil distributed throughout the particles. On the other hand, the stronger fluorescent signal around some of the particles suggests that particles may be covered with a very thin oil layer.

Composition of protein particles

Protein concentration

The internal protein content of the particles was determined using the method described in detail in the experimental section. In brief, first the total protein content of the final dispersion was determined using DUMAS. The internal protein content was then calculated using the known total amount of protein and the volume fraction and density of the particles (which are determined independently using viscosimetry and a density meter). The calculations assume an average composition for all of the particles: Cryo-SEM and CLSM analysis of protein particles shows that the microstructure of the particles is quite homogeneous. The internal protein content of the particles was found to be approximately 18% (w/w), based on these calculations. Consistently, an analysis of the protein content of the pellet obtained before the final dispersing step also suggests that protein particles contain around 20% (w/w) protein.

If it is assumed that particles do not undergo any changes during the preparation, such as swelling, syneresis or diffusion of whey proteins from the particles, the expected internal protein content would be around 24% (w/w), which is the total protein content of the protein solution used in this study. However the calculated protein content is significantly (25%) lower than this value. One of the possible explanations for this observation might be that protein particles indeed do take up water during preparation and/or storage, due to the osmotic pressure difference in the continuous phase and inside the particles. Swelling of the particles due to water uptake would result in an increase in the hydrodynamic radius of the particles and a decrease in the internal protein concentration. Another possibility are the errors in the method used for estimating the internal protein concentration. The calculations are based on measured hydrodynamic volume fraction and densities of the particles. Variations in the measured hydrodynamic volume fraction of the particles (using Ubbelohde capillary viscometers) were quite large. In addition, depending on the structure of the surface layer, the hydrodynamic volume fraction may be significantly larger than the real volume of the particles. An underestimation of the internal protein concentration of the particles is consistent with a somewhat higher estimate based on the protein concentration of the pellet. In any case, we conclude that the internal protein concentration of the particles is around 20% (w/w).

Oil content

The oil content was determined using NMR. For this purpose, an aliquot of the same dispersion of the protein particles that was also used for the other analyses was freeze-dried. The oil content of the freeze-dried sample was determined to be 8.3 (±0.3)% (w/w). The protein content of the freeze-dried sample was determined to be approximately 90% (w/w). Since the protein content of particles before freeze drying is (~ 20% w/w), the weight of water inside the particles is about 4.5 times the weight of protein. Assuming the oil is exclusively associated with the particles, this would imply an oil content of 1.8% (w/w) of the water swollen protein particles. However, as was shown in CLSM micrographs (Fig. 2.2-D and Fig. 2.7-C), there is a small amount of oil still present in the continuous phase of the dispersion, not associated with the particles. Therefore the figure of 1.8% (w/w) represents the total oil, both associated and non-associated with the particles, and the actual oil associated with the particles is less than 1.8% (w/w).

Conclusions

Using prefabricated structure elements can be a possible way to create products with desired properties and to overcome problems when formulating novel products, such as high protein foods. In this study, we have shown that dense protein particles, as a possible structure element, could be prepared using a process based on two-step emulsification. Protein particles prepared through this method were spherical in shape and had an average diameter of few micrometers. The size distribution of particles could be controlled to some extent by changing the applied shear rate in the primary emulsification step. The structural analyses indicated that the particles consist of a homogeneous protein network formed through heat gelation of whey proteins. Although the results suggested presence of oil associated with the particles, complete elucidation on this issue could not be achieved with the techniques used in this study.

The method that we have developed to prepare internally dense protein particles is versatile and robust: we expect that it should be possible to extend it in various ways. Extensions that are expected to be important for future use include different types of proteins and different types of stabilizers to tune the physical-chemical properties of the particles, either in solution, or embedded in a matrix. Besides the use as a food ingredient, these particles could be useful also for other applications such as encapsulation and controlled release.

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Chapter 3

Concentrated whey protein particle dispersions: heat stability and rheological properties

This chapter investigates the heat stability and rheological properties of concentrated whey protein particle dispersions in different dispersing media. Particles were dispersed (volume fraction of particles ~ 0.35) in solutions of sodium caseinate, whey protein isolate or gum arabic and heat treated at 90 ^{o}C for 30 min. All dispersions were liquid-like and no significant change in the microstructure was observed after heat treatment. When 1% (w/w) gum arabic was used as stabilizer, no change in the viscosity was observed after heat treatment, whereas when sodium caseinate or whey protein isolate was used, viscosity increased in the low-shear regime and shear-thickening was observed in the high shear regime. Heat treatment did not significantly alter the zeta potential of the particles, whereas the size of the particles increased after heating due to swelling. The results show that swelling of the particles plays a significant role in the heat stability and rheological properties of these dispersions.

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Introduction

Whey proteins are widely used as food ingredients because of their techno-functional properties and high nutritional value. Although whey proteins are included in many food formulations, the limited heat stability of beta-lactoglobulin, in particular, may give rise to problems [1-4]. Whey proteins denature and aggregate upon heating and may form a gel, depending on the conditions. While the ability of whey proteins to thicken and form a gel upon heating can be an advantage for some applications, it may be a disadvantage for some others. Several problems related to food structure and texture may occur for example in protein-enriched foods. In whey protein-enriched drinks undesirable changes, such as a turbid appearance or thick, undrinkable texture may form during the thermal processing due to aggregation [5]. However the development of novel food products with high protein content is of great interest, because of health benefits and satiating properties reported for high protein foods [6-10].

To eliminate these problems and modify the functional properties of whey proteins, several approaches have been investigated. Some authors have focused on modification of whey proteins through cross-linking by transglutaminase [11, 12]. These authors report that cross-linked whey protein concentrates have improved heat stability. Recently, it was shown in another study that ultrasound treatment of whey proteins after a pre-heating step enhanced the heat stability of whey proteins in subsequent heating steps [13]. The viscosity of whey protein concentrate remained low upon heat treatment, because aggregates formed during pre-heating were broken down by the ultrasound treatment and prevented from reforming in the second heating step.

Heat-stable protein particles with controlled size, surface properties and internal density are interesting candidates for the development of novel high protein foods. Formation of heat-stable whey protein particles was investigated in a few other studies. A combination of heat and high-pressure shearing was used to modify whey proteins [4]. This process resulted in formation of whey protein particles having increased heat stability compared to native whey proteins. An alternative way to prepare protein particles to enhance the heat stability of liquid protein formulations was recently reported [14, 15]. Here, whey protein nano-particles were prepared (average diameter smaller than 100 nm) through addition of a whey protein isolate solution in a w/o micro-emulsion, containing reverse micelles of surfactant and subsequent heating at 90 °C for 20 min or transglutaminase

treatment. The dispersion of those whey protein nano-particles was transparent and liquid-like after thermal treatment, while WPI formed a gel at the same protein concentration after heat treatment. The emphasis in this work was given to formation of very small particles to be used in clear beverage applications, therefore the protein content studied was rather low (5% w/w).

Design of protein particles is also an important subject in other fields than the food industry, like, the pharmaceutical industry: processes, such as spray-drying and jet-milling are used to prepare fine protein particles [16, 17]. Aqueous phase separation was extensively studied by Morita et al. [18], for the formation of spherical micron-sized protein particles that might be suitable for encapsulation and delivery of protein drugs. Whey proteins are reported to have good micro-encapsulation properties and several studies focused on preparation of microspheres from whey proteins through emulsification, heat gelation or chemical cross-linking [19, 20].

The aim of this chapter was to investigate the heat stability and rheological behavior of whey protein particle dispersions. For this purpose, whey protein particles were prepared as described in Chapter 2 and dispersed at a volume fraction (Φ) of approximately 0.35 in different media. The physical properties of the dispersions were characterized before and after heat treatment. Changes in the surface properties or in the volume fraction of the particles during heat treatment may lead to differences in the physical properties of the dispersions. Swelling of whey protein gels [21-23] and particles prepared from whey proteins [19] was already reported. Here we will also show that swelling of whey protein particles dominates the viscosity changes observed in the dispersions after heating and this extensively alters the rheological properties of the protein particle dispersions.

Experimental

Preparation of solutions

Whey protein isolate (WPI, Davisco Foods International Inc., Le Sueur, MN), Sodium caseinate (Na-caseinate, EM 7, DMV international, Veghel, the Netherlands) and gum arabic (Merck, Darmstadt, Germany) were dissolved at desired amounts in Millipore water (Millipore Corp., Billerica, MA). The solutions were stirred overnight, and refrigerated before usage. The pH of the solutions was left unadjusted. The pH values of 1% (w/w) WPI, Na-caseinate and gum arabic solutions were 6.8, 6.9 and 5.8 respectively. PGPR (Danisco, Grindsted PGPR 90,

Denmark) was dissolved in sunflower oil at 2.5% (w/w), by stirring for at least 2 h at room temperature and stored in a dark cabinet.

Preparation of whey protein particles

Protein particles were prepared according to the method described in Chapter 2. In brief, first a water in oil (w/o) emulsion was prepared by mixing a 25% (w/w) WPI solution in sunflower oil (containing PGPR at 2.5% w/w) with the help of a high speed mixer (Ultra-turrax T 25, IKA Werke, Germany). The total mixing time was kept at 5 min and the mixing speed was fixed at 6500 RPM. Directly after preparation, the w/o emulsion was heated at 80 ^{o}C for 20 min and subsequently centrifuged (33768g, Avanti J-26 XP, Beckman Coulter, U.S.A) for 1 h to remove the excess oil. The following washing and dispersing steps were done using solutions of either Na-caseinate, WPI or gum arabic at different concentrations. All the dispersions had a pH value close to 7.0 ±0.2 after preparation and no further adjustment of the pH was done.

Optical microscopy

Microstructural analysis of protein particles by optical microscopy was performed according to the method described in Chapter 2.

Confocal laser scanning microscopy (CLSM)

A dispersion of protein particles was stained with a few drops of BODIPY™ 665/676 (5 mg/ml in DMF, Molecular Probes Inc., Eugene, OR, USA) and directly analyzed with CLSM. All the samples were diluted 10x prior to analysis. Imaging of the samples was done using a Leica TCS-SP5 confocal laser scanning microscope (Leica Microsystems B.V., Rijswijk, Netherlands) equipped with a DMI6000 inverted microscope. The system was equipped with an Argon laser. The excitation wavelength and emission maxima of BODIPY™ was 647 nm and 675 nm, respectively.

Determination of particle size distribution and zeta-potential

The particle size distribution of the protein particles was determined using a Mastersizer 2000 (Malvern Instruments, Worcestershire, U.K.). The Mastersizer 2000 measures the angular intensity of the scattered light using a series of photosensitive detectors and determines the particle size distribution using the Mie scattering theory. For the measurements, samples were diluted in Millipore water

to avoid multiple scattering. The size distribution of each sample was measured at least five times. Average sizes reported are volume-averaged diameters.

To determine the zeta potential of the particles, a Zetasizer Nano (Malvern Instruments, Worcestershire, U.K.) was used. Samples were diluted in Millipore water (1000x) and the Smoluchowski model was used to calculate the zeta potential from the mobility values.

Determination of protein concentration

The protein concentration of the dispersions was determined by DUMAS. A Flash EA 1112 N/protein analyzer (Thermo Scientific, Waltham, US) was used to determine the total nitrogen content of the samples. Nitrogen values were multiplied by 6.38 to calculate the total protein content of the samples (% w/w).

Determination of hydrodynamic volume fraction

Hydrodynamic volume fraction (Φ) of the protein particles was determined according to the method described in Chapter 2.

Heating of the dispersions

Approximately 20 ml from each sample was transferred into a glass tube and closed tightly. Samples were heated at either 70, 80 or 90 ^{o}C for 30 min, in a temperature-controlled heating plate (RT15, IKA Werke, Germany). Samples were mildly stirred by a magnetic stirrer during heat treatment to avoid particle sedimentation and to facilitate heat transfer. The experiments were performed in duplicate.

Viscosity measurements

The viscosity of the samples was measured by using a Physica MCR 501 Rheometer (Anton Paar, Graz, Austria). The measuring geometry was a concentric cylinder (CC17/T200/SS, cup diameter: 18.08 mm, bob diameter: 16.66 mm). 5 ml of each sample was placed in the measuring cell and the surface of the sample was covered with paraffin oil to avoid evaporation of water. The shear viscosity of samples was measured over the shear rate range 1-1000 s^{-1}. All measurements were performed in duplicate and at 25 ^{o}C.

Swelling experiments

Gels were prepared by heating a 25% (w/w) WPI solution in a water bath at 80 ^{o}C for 20 min. The solutions were gelled in glass tubes coated with Sigmacote (Sigma-Aldrich, St. Louis, USA). After the gels were cooled to room temperature, they

were removed from the glass tubes and cut into identical pieces (~ 5 g) and placed into 75 ml of different aqueous solutions to observe swelling. After 2, 24 and 48 h, gel pieces were removed from the swelling media and the excess of solution was drained. The weight change of the gels was recorded and the swelling ratio (%) was calculated by:

$$Swelling\ \ ratio\ \ (\%) = \frac{w_s - w_o}{w_o} \times 100\ (\%) \qquad (3.1)$$

where w_o is the weight of WPI gel prior to swelling and w_s is the weight of WPI gel after swelling. Five measurements were done for each aqueous solution and the average of those measurements was presented in the results.

Results and discussion

Effect of heat treatment on the properties of whey protein particle dispersions

Heating temperature

After whey protein particles were separated from the oil phase and washed, they were dispersed in a 4% (w/w) Na-caseinate solution at a volume fraction (Φ) of ~ 0.35 and heated either at 70, 80 or 90 ^{o}C for 30 min. The total protein concentration of the dispersions was approximately 9% (w/w). The macroscopic appearance of all dispersions was similar before and after heat treatment and no gelation was observed after heat treatment at different temperatures. In figure 3.1-A, particle size distribution of protein particles before and after heat treatment is shown. There was a small shift in the particle size distribution towards larger particle sizes after heat treatment. Microscopic analysis (data not shown) has shown that, protein particles did not undergo any significant changes upon heat treatment; particles remained spherical in shape, no clusters or flocculated particles were detected after the heat treatment.

In figure 3.1-B viscosities of the dispersions, before and after heat treatment at different temperatures are shown. Before heat treatment, a Newtonian viscosity profile was observed in most of the shear regime; the viscosity was independent of the shear rate. At a shear rate close to 1000 s^{-1}, a small increase in the viscosity was

observed. After heat treatment, the viscosity of dispersions increased with increasing heating temperature; the viscosity of dispersions heated at 70 °C increased less than 2-fold, while the viscosity of dispersions heated at 90 °C increased 4-fold, compared to the viscosity of the non-heated dispersion. The shear viscosity profiles of all heated dispersions were similar; first a Newtonian behavior was observed in the low shear regime and viscosity started to increase (shear-thickening) at a critical shear rate. At higher shear rates, viscosity decreased (shear-thinning) again. The critical shear rate, at which the onset of shear-thickening observed was lower for samples that were heated at higher temperatures.

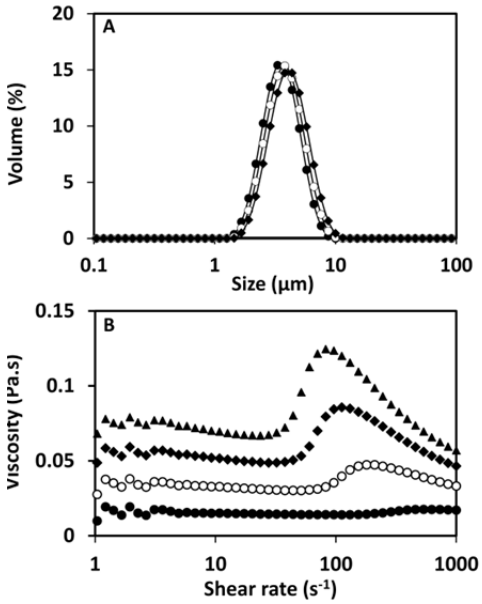

Figure 3.1 Particle size distribution (A) and viscosity (B) of whey protein particle dispersions before and after heat treatment. Protein particles were prepared from a 25% (w/w) WPI solution at pH 6.8 and dispersed in 4% (w/w) Na-caseinate solution at a volume fraction (Φ) of ~ 0.35. Samples were analyzed before (●) and after heat treatment at 70 °C (○), 80 °C (♦) and 90 °C (▲) for 30 min. The curves for (♦) and (▲) coincide in Graph A.

Overall, the viscosities of the dispersions increased but the dispersions were still liquid-like after heat treatment. These results indicate that the heat treatment changes one or more properties of the whey protein particle dispersion, which influence the low-shear viscosity as well as shear-thickening behavior.

Mleko and Foegeding [24] studied modification of WPI through a two-step heating of WPI solutions (2 to 4% w/v) at different pH values. Although the difference in the viscosity after first and second heating step was not characterized in their work, a shear rate specific increase in the viscosity was reported for some of the whey protein aggregate dispersions, which is similar to our findings.

It is well known that colloidal dispersions of stabilized solid particles may show reversible shear-thickening [25-29]. In these shear-thickening systems, above a critical value of shear rate, the viscosity begins to increase. After shear-thickening, the viscosity may level out to a new plateau value and decrease again. The viscosity profiles of whey protein particle dispersions, especially after heat treatment, are similar to those reported for the systems containing solid colloidal particles.

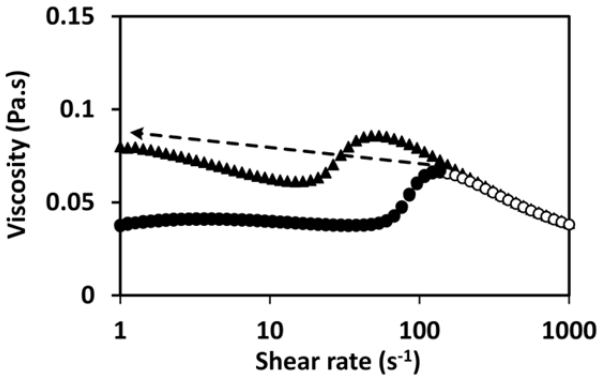

Figure 3.2 Reversibility of shear-thickening in whey protein particle dispersions (Φ ~ 0.35) after heat treatment at 90 °C for 30 min. Shear viscosity of the heated dispersion was shown by circles: shear rate between 1-1000 s^{-1} (○) and 1-137 s^{-1} (●). When the viscosity reached a maximum value (at 137 s^{-1}), measurement was stopped and the sample was hold at rest for 2 h. Triangles show the viscosity of the sample after this resting period, at a shear rate between 1-1000 s^{-1}. Arrow indicates the change in the viscosity after the rest period.

Repeated shear experiments were performed to test the reversibility of shear-thickening in heat-treated whey protein particle dispersions. For this purpose viscosities of heat-treated dispersions were recorded until the shear rate at which the viscosity reached the maximum value. Next, the sample was kept at rest for approximately 2 h and the viscosity of the same sample was measured for a second time at shear rates 1-1000 s^{-1}. This experiment was performed twice and the results were reproducible. Results of this experiment are shown in figure 3.2. The viscosity

of the sample after a rest period of 2 h was higher than the same sample sheared for the first time (as indicated by an arrow), which shows that shear-thickening observed in heated protein particle dispersions is irreversible on the time scale of at least a few hours. During the second shear rate sweep, the viscosity of the dispersion first decreased and started to increase at a specific shear rate, but the onset of shear-thickening was found at a lower shear rate, compared to the sample sheared the first time. With a further increase of shear rate, the viscosity of the sample decreased and finally reached the same viscosity value as during the first shear-rate scan.

The irreversibility (or at least history-dependence) of the shear-thickening that was observed for whey protein particles (Fig. 3.2) differs from the typical reports of shear-thickening for dispersions of solid particles: for that case it is usually reported that when shear rate increases, the viscosity increases, but as soon as the shear rate is zero, the viscosity returns to its original zero-shear value [27]. The irreversible increase in viscosity observed in the dispersions of protein particles leads us to conclude that some attractive interaction between the protein particles might have caused long-lived clusters. The attraction cannot be very strong, since at very high shear rates, the return to (history-independent) low values of viscosity suggests that the clusters are broken down again.

Volume fraction of protein particles

The effect of volume fraction of particles on the heat stability and rheological behavior of the dispersions was tested. For this purpose, dispersions containing protein particles with a volume fraction (Φ) of approximately 0.05, 0.15, 0.25 and 0.35 were prepared. 4% (w/w) Na-caseinate was used as the washing and dispersing medium for all the dispersions. In figure 3.3, the shear viscosity of the dispersions before and after heating at 90 ^{o}C for 30 min are presented. All non-heated dispersions behaved Newtonian before heat treatment and viscosity increased with increasing volume fraction of particles. After heat treatment, no changes were recorded in the viscosity of dispersion containing particles at volume fraction of 0.05. The viscosity of the dispersions, however increased after heating, when the volume fraction of particles was higher. Shear-thickening was observed after heating when the volume fraction of particles was increased either to 0.25 or 0.35. As can be seen in figure 3.3, shear-thickening was more pronounced at Φ ~ 0.35 and it shifted to lower shear rates: the onset of shear-thickening was at 150 s^{-1}

for Φ ~ 0.25, whereas it dropped to 50 s^{-1} for Φ ~ 0.35. It can be concluded that, in addition to the viscosity increase, the volume fraction of particles also has a strong influence on the onset and magnitude of shear-thickening, that is observed after heat treatment.

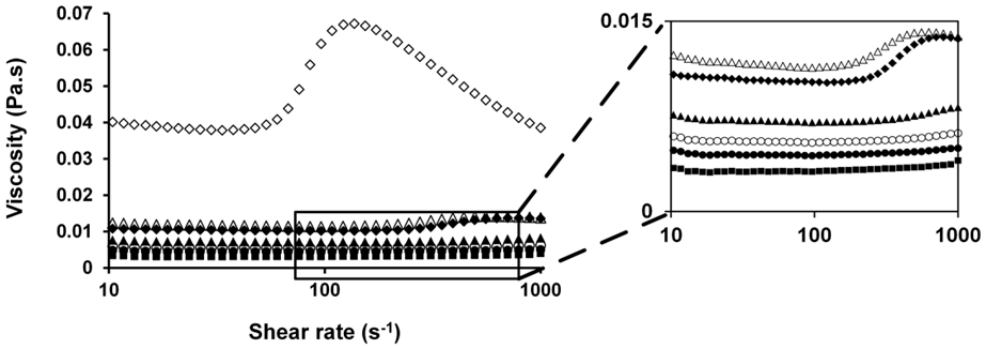

Figure 3.3 Viscosity of whey protein particle dispersions at different volume fractions before and after heating at 90 °C for 30 min. Solid symbols: before heat treatment, open symbols: after heat treatment. Volume fraction (Φ) of particles were: 0.05 (■), 0.15 (●), 0.25 (▲) and 0.35 (♦). The curves for (■) and (□) coincide. Continuous phase: 4% (w/w) Na-caseinate. Insert: Close up of the lower viscosity curves.

It is reported for colloidal dispersions that the shear-thickening is sensitive to the volume fraction of the particles [27]. The critical shear rate, where the onset of shear-thickening is observed, decreases with increasing volume fraction of particles, if the phase volume is less than 50%. Other work on reversible shear-thickening in concentrated dispersions of silica particles also showed that the magnitude of shear-thickening was significantly suppressed when the volume fraction of the dispersed phase was reduced [30]. In this respect, our findings implies that the dispersions of whey protein particles at different particle volumes behave as typical colloidal dispersions.

Effect of stabilizer on the heat stability and rheological properties

Stabilizer concentration

Whey protein particles were washed with, and subsequently dispersed in 0.5, 1 or 4% (w/w) Na-caseinate solutions. After heat treatment at 90 °C for 30 min, the changes in the particle size distribution and shear viscosity of the dispersions were determined. The particle size distribution of whey protein particles increased slightly after heat treatment (data not shown) and the increase became more

noticeable at lower concentrations of Na-caseinate in the continuous phase. In figure 3.4, the shear viscosity of the dispersions before and after heat treatment is shown. The shear viscosity values of all dispersions before heat treatment was around 0.01 Pa.s. In the dispersion having 4% (w/w) Na-caseinate as a stabilizer, the viscosity after heating increased 4-fold and the shear-thickening was observed at 50 s^{-1} (as shown in the insert). When the concentration of Na-caseinate in the continuous phase was decreased to 1% (w/w), the viscosity increased approximately 30-fold after heat treatment and the onset of shear-thickening shifted to a lower shear rate (~ 6 s^{-1}) compared to the dispersion having 4% (w/w) Na-caseinate in the continuous phase. A further decrease in the concentration of Na-caseinate (0.5% w/w) resulted in a larger viscosity increase and a further shift in the onset of shear-thickening to the lower shear rates.

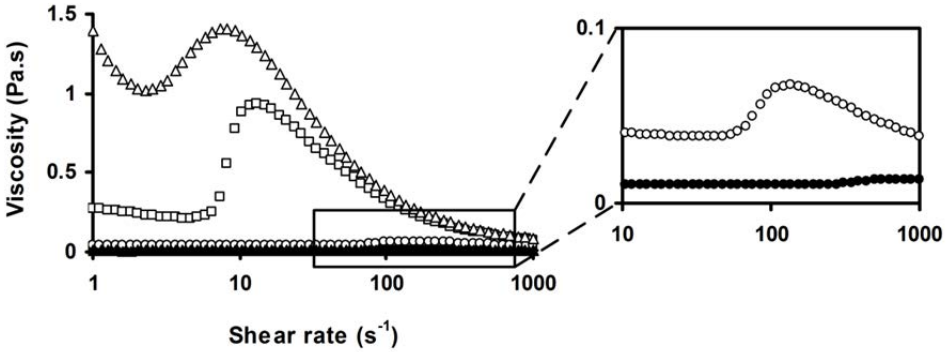

Figure 3.4 Viscosity of whey protein particle dispersions at a volume fraction (Φ) of~ 0.35, before and after heating at 90 °C for 30 min. Solid symbols: before heat treatment (the curves coincide), open symbols: after heat treatment. Concentration of Na-caseinate (w/w) in the continuous phase was; 4% (●), 1% (■), 0.5% (▲). Insert: enlargement of the viscosity of dispersion in 4% (w/w) Na-caseinate.

In the shear regime until the onset of shear-thickening, protein particles dispersed in 4% (w/w) Na-caseinate had a Newtonian viscosity profile after heating. However, in the same shear regime, shear-thinning was observed when 1% or 0.5% (w/w) Na-caseinate was used as stabilizer (Fig. 3.4). The shear-thinning was more pronounced for 0.5% (w/w) Na-caseinate, as can be seen from the slopes of the viscosity curves in this shear-regime. This behavior suggests that particle aggregation might have occurred in these dispersions after heat treatment.

Stabilizer type

The role of the stabilizer type on the heat stability and flow properties of particle dispersions was studied after whey protein particles were washed with and subsequently dispersed in 1% (w/w) solutions of Na-caseinate, WPI or gum Arabic (GA). In the microscopy images of dispersions prepared by either 1% (w/w) Na-caseinate or 1% (w/w) WPI, protein particles seemed somewhat larger after heat treatment (Fig. 3.5-A and -B). For 1% (w/w) Na-caseinate few clusters of particles could be observed after heat treatment. This further confirms particle aggregation on heating. For the same samples, as can be seen in figure 3.5-C, there was also a shift in the particle size distribution towards the larger sizes. When 1% (w/w) gum arabic was used as stabilizer, no change was measured in the particle size distribution.

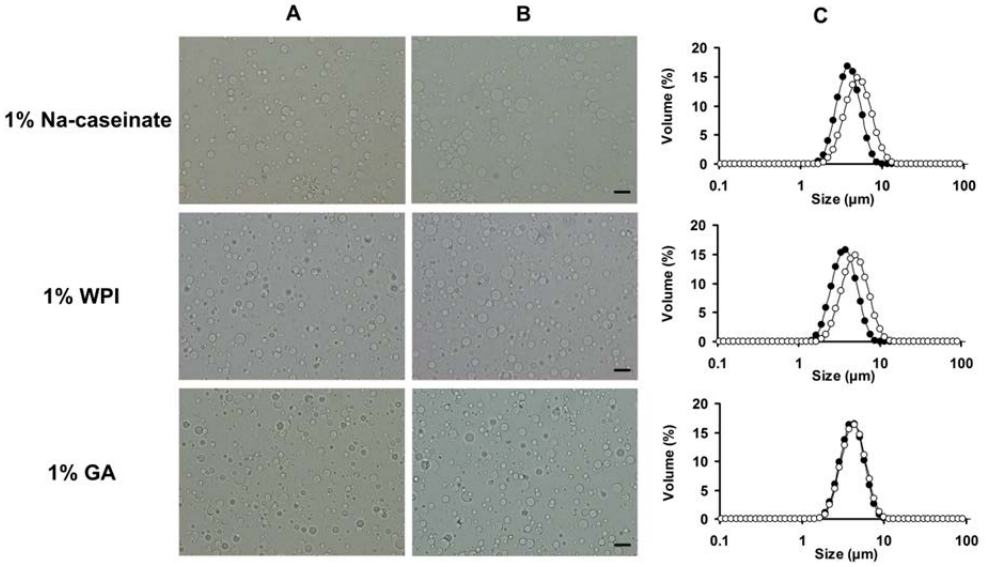

Figure 3.5 Effect of stabilizer type on the heat stability of protein particle dispersions. Microscopy images of dispersions before (A) and after heating (B) at 90 °C for 30 min are shown. Scale bar: 10 μm. The change in the size distribution of the particles (C) was determined by light scattering. Solid symbols: before heat treatment, open symbols: after heat treatment.

Figure 3.6 shows the viscosity of these dispersions before and after heat treatment. Similar to previous observations, all dispersions of protein particles behaved Newtonian before heat treatment and only at very high shear rates a slight increase

in the viscosity was recorded. After heat treatment the shear viscosity of the 1% (w/w) gum arabic stabilized dispersion did not change, whereas the viscosities of dispersions increased when 1% (w/w) Na-caseinate or 1% (w/w) WPI was present in the continuous phase.

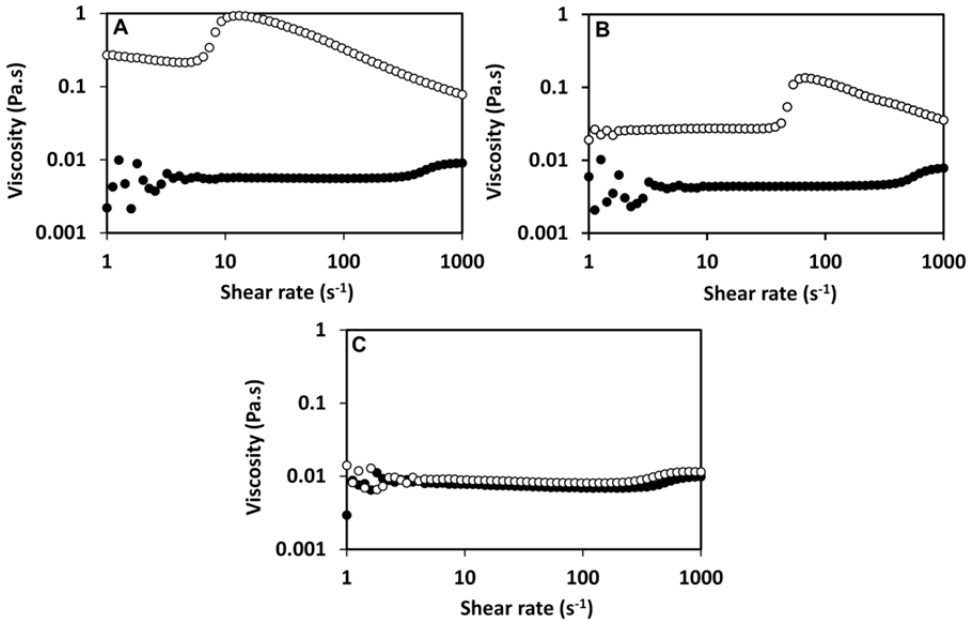

Figure 3.6 Viscosity of protein particle dispersions at volume fraction (Φ) of ~ 0.35 before (solid symbols) and after heating (open symbols) at 90 °C for 30 min. Protein particles were dispersed in 1% (w/w) solutions of Na-caseinate (A), WPI (B) and gum arabic (C).

The increase in the viscosity was an order of magnitude larger for the 1% (w/w) Na-caseinate-stabilized dispersion in comparison to the 1% (w/w) WPI-stabilized dispersion. Both samples showed shear-thickening and the onset of shear-thickening was around 40 s^{-1} for the 1% (w/w) WPI-stabilized dispersion, while it was around 6 s^{-1} for the 1% (w/w) Na-caseinate-stabilized dispersion. To determine whether the continuous phase had any significant effect on the viscosity changes observed after heat treatment, 1% (w/w) solutions of Na-caseinate, WPI and gum arabic, containing no whey protein particles were heated in the same way as dispersions and the shear viscosity before and after heating was measured. No significant change in the viscosities of these solutions was observed (data not shown). Therefore, the changes in the viscosity profile of the dispersions can be

solely attributed to the changes in the particle properties during/after heat treatment.

Interactions between particles play an important role in determining rheological properties of colloidal dispersions. Barnes [27] reported that shear-thickening observed in colloidal dispersions was reduced, when the particles have the least tendency to adhere to one another. Maranzano and Wagner [31] showed that the viscosity of silica particle dispersions were strongly affected in both low- and high-shear regime by short range electrostatic forces; charge neutralization of the particles significantly altered the rheological behavior of the particles. In the same study, it was also shown that the shear stress required for a shear-thickening to be observed decreased upon reduction of the repulsive forces, through neutralization. The authors concluded that repulsive forces act to suppress the onset of shear-thickening in concentrated dispersions. In a more recent study a predictive model has been developed for shear-thickening in polymer-coated colloidal dispersions and similarly it was suggested that the onset of shear-thickening was sensitive to the repulsive forces arising from the polymer coat [29]. In our study, dispersions of protein particles were prepared by using continuous phases containing different stabilizers.

Table 3.1 Zeta potential of protein particles before and after being heated at 90 °C for 30 min.

	Zeta-potential	
Continuous phase (1% w/w)	Before heating	After heating
Na-caseinate	-29.45 mV	-29.20 mV
WPI	-25.45 mV	-25.44 mV
gum arabic	-20.40 mV	-19.90 mV

To understand the role of electrostatic interactions, zeta potential of the particles dispersed in different stabilizer solutions was measured before and after heat treatment. The results are presented in table 3.1. The net charge of Na-caseinate, WPI and gum arabic is close to zero around the pH values of 4.6 [32] , 5.1 [33] and 2.2 [34], respectively. All solutions used in our study were at significantly higher pH values than these and all dispersions reached a similar final pH (~7.0), due to the buffering effect of the protein particles. Therefore, it may be assumed that the dispersed protein particles are negatively charged. As can be seen from table 3.1,

heat treatment did not significantly affect the zeta potential, hence heat-induced changes of the zeta potential can be ruled out as a major contributor to the changes that are seen after heating the whey protein particle dispersions.

As shown in figure 3.3, the changes in the volume fraction of the particles is another important parameter which can alter the rheological properties of whey protein particles dispersions. This implies that, a probable increase of the particle volume during heat treatment, as a result of for example particle swelling, may be responsible for the changes observed in the viscosity after heating. To investigate this further, we have compared the viscosity of a heated dispersion with the viscosity of a non-heated dispersion at higher volume fraction (Fig. 3.7).

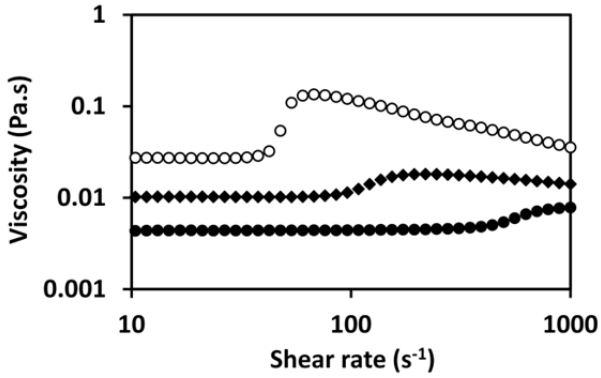

Figure 3.7 Comparison of the viscosity of protein particle dispersions at volume fraction (Φ) of ~ 0.35 before (●) and after (○) heating at 90 °C for 30 min, with the viscosity of a non-heated dispersions at particle volume fraction (Φ) of ~ 0.43 (♦). For this measurement, a 1% (w/w) WPI solution was used in the continuous phase.

Indeed the change in the viscosity when the volume fraction of the particles were increased from Φ ~ 0.35 to Φ ~ 0.43 was similar to the change in the viscosity of the dispersion (Φ ~ 0.35) after heating. The higher viscosity and more pronounced shear-thickening in the heated sample must be due to higher final particle volumes after heating. This observation indicates that protein particles might have swollen after heat treatment.

We see a very significant difference in the behavior of particles, when 1% (w/w) gum arabic is used as stabilizer. In order to determine the influence of the continuous phase on the swelling behavior of the particles, swelling of 25% (w/w) bulk WPI gels was studied. For this purpose, first WPI gels were prepared by

heating a 25% (w/w) WPI solution at 80 °C for 20 min. The gels were cut into cylindrical pieces and swelling of the gel pieces was analyzed in different swelling media with or without a heating step. The results presented in figure 3.8-A show that 25% (w/w) WPI gels swelled in all dispersing media, when no heating step was applied. The swelling was already 20% after 2 h and increased significantly with time. The highest swelling percentage after 48 h was observed in 1% (w/w) WPI medium. The swelling of the gels in 1% (w/w) gum arabic solutions was lower in comparison to the swelling ratio obtained in other swelling media. An increase in the concentration of Na-caseinate in the swelling medium suppressed the swelling of the gels. In figure 3.8-B, the swelling percentage after heating the gel pieces in the swelling media at 90 °C for 30 min is shown.

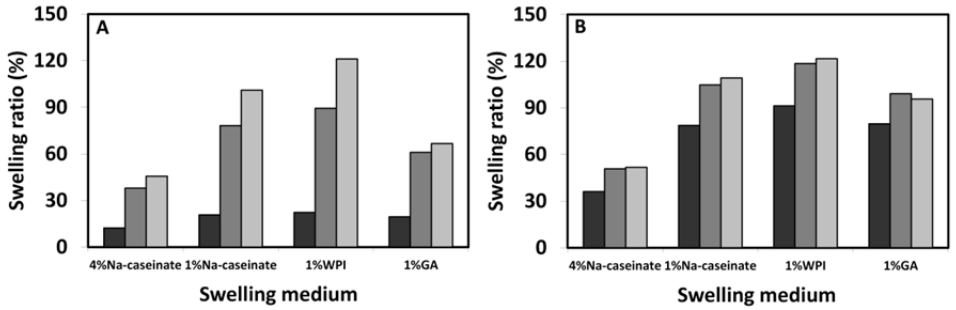

Figure 3.8 Swelling of WPI gels, without (A) and with (B) a heating step (90 °C/20 min) in the dispersing media. WPI gels were prepared by heating a 25% (w/w) WPI solution (pH 6.8) at 80 °C for 20 min. Swelling ratio of the gels was measured after 2 h (black), 24 h (gray), 48 h (light gray).

The swelling percentage after 2 h was approximately 3 times more in the heated gels than non-heated gels. However, the total swelling percentage after 48 h was not significantly altered by the heating step, except when the swelling medium was 1% (w/w) gum arabic solution, where an increase in the swelling ratio was observed. It can be concluded that 25% (w/w) WPI gels swell in all the aqueous environments tested and heating of the gels in the aqueous environment results in a faster swelling of the gels. According to these results, swelling of the protein particles is expected to occur in all the dispersing media during or after heating. We can see that the swelling ratio was suppressed, when the concentration of Na-caseinate increased, which could partially explain the less pronounced viscosity changes observed in the protein particles dispersions at increasing Na-caseinate concentration (See Fig.3.4). This may be a result of an increased osmotic pressure

due to the presence of higher amount of Na-caseinate molecules in the continuous phase. However, when 1% (w/w) gum arabic solution was used, no change in the average particle size and viscosity was observed (Fig. 3.5-C and 3.6-C), suggesting no significant particle swelling.

During the preparation of the particles, repeated washing steps were applied to remove the excess of oil from the system (Chapter 2). In this study, we have kept the washing and the dispersing medium the same, which means, if 1% (w/w) gum arabic was used as the dispersing medium, it is also used as the washing medium during the formation of the particles. This may result in changes in the surface properties of whey protein particles. In Chapter 2, it was shown that particles might have oil layer/oil patches on the surface. In figure 3.9, CLSM images of protein particles prepared using either 1% (w/w) Na-caseinate (Fig. 3.9-A) or 1% gum arabic (Fig. 3.9-B) are compared.

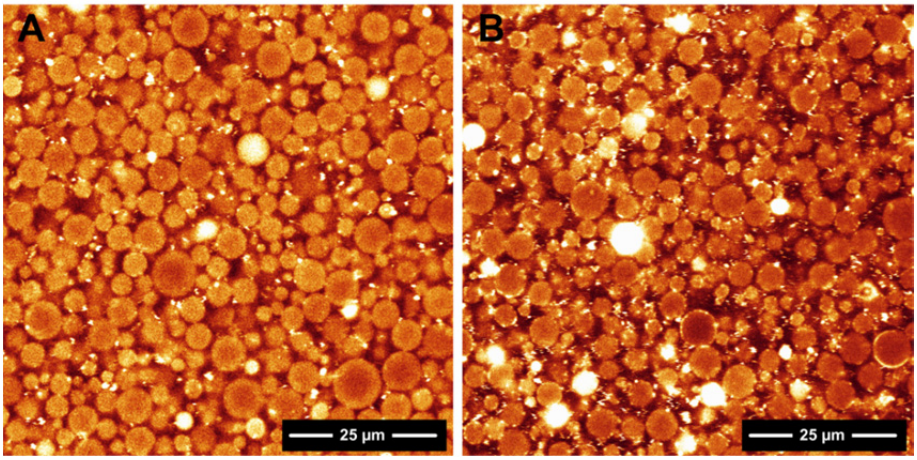

Figure 3.9 CLSM images of protein particles stained by BODIPY™ 665/676. Particles were prepared from a 25% (w/w) WPI solution and washed/dispersed either in a 1% (w/w) Na-caseinate (A) or 1% (w/w) gum arabic (B) solution.

Protein particles were stained with BODIPY™ 665/676, which is an lipophilic dye, to observe the oil signal on the surface of the particles. A stronger BODIPY™ 665/676 signal on the surface of the particles could be detected when 1% gum arabic was used as washing medium and some of the particles had an oil ring surrounding them. No strong fluorescent signal was detected on the surface of the

protein particles when they were prepared by using 1% (w/w) Na-caseinate (Fig. 3.9-A).

Although it is difficult to reach a final conclusion based on these micrographs, it is likely that the type of the stabilizer present in the washing medium have influenced the surface properties of the particles, i.e. thickness or homogeneity of the oil layer around the particles. This possible difference in the surface of the particles might be one of the factors effecting the heat stability and rheological behavior of the particles, i.e. presence of a thicker oil layer around the particles might suppress particle swelling.

Conclusions

This study focused on understanding the functionality of dense whey protein particles formed through two-step emulsification, followed by heat gelation. The results show that heat stability and flow behavior of whey protein particle dispersions are considerably influenced by the type and concentration of the stabilizer used in the continuous phase. Using gum arabic as a stabilizer resulted in dispersions that are most stable after heat treatment. In gum arabic-stabilized dispersions no change in the microstructure, particle size and shear viscosity after heat treatment was observed. This observation is in sharp contrast with the results obtained for the dispersions of whey protein particles prepared with Na-caseinate or whey protein isolate as stabilizers in the continuous phase. For these dispersions particle size increased and the flow behavior changed after heating; the viscosity increased in the low shear regime and shear-thickening was observed in the high-shear regime. The results suggest that increased volume of the particles due to swelling during heat treatment might be the main cause for the changes observed upon heating. However it is not possible to exclude the interactions between the particles as an additional reason, since these are also likely to be influenced by the stabilizer type and concentration used in the continuous phase.

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Chapter 4

Relation between gelation conditions and the physical properties of whey protein particles

This chapter studies formation of whey protein particles at different pH (6.8 or 5.5) and NaCl concentrations (50, 200 or 400 mM). Particles formed at pH 6.8 were spherical whereas those formed at pH 5.5 were irregular and had a cauliflower-like appearance. Particles formed at pH 5.5 had substantially higher internal protein content (~ 39% w/v) than the particles formed at pH 6.8 (~ 18% w/v). Similarly particle morphology and protein density were also affected by initial NaCl concentration: particles formed at 50 mM NaCl (pH 6.8) were spherical whereas particles formed at either 200 mM NaCl (pH 6.7) or 400 mM NaCl (pH 6.6) were irregular and protein density of the particles increased with increasing initial NaCl concentration. Whey protein particles formed at pH 5.5 showed an excellent heat stability: viscosity of the dispersions containing approximately 30% of protein particles formed at pH 5.5 did not show any change after heating at 90 ^{o}C for 30 min while the viscosity of dispersions containing protein particles prepared at other conditions increased after heating.

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Introduction

Several studies have shown that whey proteins are suitable ingredients for the design of protein containing particles with different purposes. Microspheres with an average diameter between 15 to 75 μm were produced through w/o emulsification and glutaraldehyde crosslinking of whey proteins for rapid release of drugs [1]. Calcium-induced gelation of a pre-denatured WPI solution was used to produce whey protein gel beads for encapsulation of retinol [2]. The resulting protein beads were spherical with an average diameter of 2 mm and were shown to yield a good protection against enzymatic attack during the stomach digestion and oxidation of fat-soluble biomaterials. In a recent study extrusion of a pre-denatured whey protein isolate solution into a cold gelling medium was used for the formation of whey protein beads (diameter ~ 200 μm) [3]. These beads were loaded with probiotic bacteria, and resulted in good targeted delivery characteristics. In another study o/w/o emulsification combined with heat-induced gelation of whey proteins is used for the formation of microcapsules (diameter 10-100 μm) containing fat as core material and gelled whey proteins as a wall solid [4]. These papers focus on the preparation of protein particles that can be used for encapsulation, controlled delivery, double emulsification or food structuring. The preparation methods used in these studies are quite specific and hard to generalize relative to protein source, while the obtained particles were low in protein content with a rather large diameter which might be a disadvantage in several applications.

In Chapter 2, we have presented a robust method, to prepare spherical, few micron-sized whey protein particles with high internal protein density that might be useful for the development of novel food products with a high protein content. The development of high protein foods is of great interest because of health benefits for the elderly, and for the resulting high level of satiation upon consumption [5-9]. However, when developing high protein foods maintaining a desired food structure is difficult, mainly as a result of heat-induced protein aggregation. This can result in, e.g. undesired viscosity increase in protein drinks upon heat treatment. Therefore, heat-stable protein particles with high internal protein content, controlled size and surface properties can be useful to reduce or even eliminate such effects of increased protein content on food structure. An alternative way to prepare protein particles was recently reported by Zhang and Zhong [10, 11]. Here whey protein nano-particles were prepared (average diameter

smaller than 100 nm) through incorporation of whey protein isolate solutions in micro-emulsions and subsequent heating at 90 °C for 20 min. The dispersion of these nano-particles was shown to have an improved heat stability relative to a whey protein solution of the same protein concentration. The emphasis in this work was given to formation of very small particles to be used in clear beverage applications, therefore authors did not focus on increasing protein density of the particles and the protein contents studied was rather low (5% w/v).

In this Chapter, we are focused on formation of highly dense protein particles with a good heat stability, i.e. particles do not aggregate or swell upon heating. It is not straightforward to increase the particle density by increasing the protein concentration of the protein solution with the method described in Chapter 2. First, the protein particles are polyampholyte gels that tend to swell when dispersed in an aqueous phase [12]. Due to this swelling, increasing the initial protein concentration does not simply result in a similar gain in protein concentration inside the particles. Second, due to the changed viscosity ratio between the oil and water phases at higher concentration of WPI solution (the viscosity of the WPI solution increases dramatically at concentrations approaching ~ 30-40%), formation of multiple droplets may occur during the emulsification step which also results in lower internal protein contents.

The method described in Chapter 2 to prepare whey protein particles includes a heating step to gel the proteins inside the particles. When whey proteins are heated in aqueous solutions they aggregate and at high enough protein concentrations gels are formed through interaction of those aggregates. The aggregation kinetics of whey proteins and the amount of aggregated material at the gel point determine the gel microstructure and are sensitive to the changes in the pH and ionic strength [13, 14]. Studies on the formation of heat-induced WPI gels showed that rheological, optical and water holding properties of the gels are also influenced by pH and NaCl concentration of the solution [13-18]. Langton and Hermansson [16] showed that whey protein gels formed between pH 4.0 and 6.0 were opaque and had a particulate network, which can be described as a network consisting of aggregated particles. It has also been reported that the size of those aggregates changed with pH, i.e. around pH 4.5 the microstructure is composed of spherical particles of 200-300 nm in diameter and when the pH was increased to 5.4, denser and well-packed aggregates were formed [16, 19]. Microstructure of whey protein gels consist of finer protein structures when pH values are far from the isoelectric point (<pH 4.0 and

>pH 6.0) [16, 18]. Similar effects on whey protein gel network were also reported when gelation occurred in the presence of NaCl. When NaCl concentration was increased from 25 to 150 mM, the size of the protein strands and pores of the protein gel increased and above 200 mM an aggregated network with large pores and locally denser structures were formed [17]. A strong decrease in the gel strength was also reported when the microstructure of the gels transformed from a fine stranded to an aggregated network at high NaCl concentrations (around ~ 200 mM) [17, 18, 20].

Those studies have clearly shown that small changes in the gelation conditions can allow manipulation of the gel microstructure and the final physical properties of whey protein gels. This feature of whey proteins can be used in the design of protein particles with different functionalities. The present study aims at understanding the effects of gelation conditions on the physical properties of whey protein particles; particularly internal protein density of the particles. Protein particles were prepared from a 25% (w/w) whey protein isolate solution at different pH (6.8 or 5.5) and at different ionic strength (NaCl concentrations of 50, 200 or 400 mM). The morphology and internal protein density of whey protein particles were characterized and the influence of different gelling conditions on the rheological properties and heat stability were assessed. From a practical point of view, it would be convenient to be able to predict the physical properties of protein particles based on the properties of macroscopic gels. For that reason we have also compared the morphology of the particles with the macroscopic WPI gels prepared using the same gelling conditions.

Experimental

Preparation of solutions

WPI solutions (25% w/w and 1% w/w) were prepared by dissolving protein powder in Millipore water (Millipore Corp., Billerica, MA). The solutions were first stirred at room temperature for at least 2 h. The protein solutions were kept overnight at 4 °C, while slowly stirring to allow complete hydration. The pH values of WPI solutions were either left unadjusted (pH 6.8) or adjusted to pH 5.5 using 6M HCl. For the experiments with added NaCl, after the preparation of protein solution, NaCl was added in the crystal form and the solution was stirred at least for 30 min until the salt was completely dissolved.

Preparation of whey protein particles

Protein particles were prepared according to the method described in Chapter 2. In brief, first a water-in-oil (w/o) emulsion was prepared by mixing a 25% (w/w) WPI solution in sunflower oil (containing PGPR 2.5% w/w) with the help of a high speed mixer (Ultra-turrax T 25, IKA Werke, Germany). The weight ratio of WPI solution to sunflower oil was 1:9. The total mixing time was kept at 5 min and the mixing speed was fixed at 6500 RPM. Directly after preparation, the w/o emulsion was heated at 80 ^{o}C for 20 min and then centrifuged (33768xg, Avanti J-26 XP, Beckman Coulter, U.S.A) for 1 h to remove the excess oil. The centrifugation step was repeated three times and subsequent washing and dispersing steps were performed using solutions of 1% (w/w) WPI to remove the residual oil from the dispersion.

Determination of particle size distribution

The particle size distribution of the protein particles was determined using a Mastersizer 2000 (Malvern Instruments, Worcestershire, U.K.). The Mastersizer 2000 measures the angular intensity of the scattered light using a series of photosensitive detectors and determines the particle size distribution. Since the typical size of inhomogeneities of the particle are smaller than the wavelength of the light (as evidenced by SEM), we have used Mie scattering theory in the particle size calculations which supposes homogeneous spherical particles. For the measurements, samples were diluted in Millipore water to avoid multiple scattering and refractive index of the particles was set to 1.59. The size distribution of each sample was measured at least five times. The average sizes reported are volume-averaged diameters.

Determination of hydrodynamic volume fraction

Hydrodynamic volume fraction (Φ) of the protein particles was determined according to the method explained in Chapter 2.

Determination of internal protein content of particles

The method described in Chapter 2 was used for the calculation of the internal protein content of the protein particles.

Optical microscopy

Samples taken from different steps during the preparation of protein micro-particles were analyzed using an optical microscope (Axioskop plus, Zeiss,

Germany) equipped with a CCD camera (DCU 224 M, THORLABS, Germany). To record the microscopic images, first each sample was gently diluted (100x) with its continuous phase. A drop of diluted sample was transferred onto the microscope slide and then covered with a cover slip prior to analysis. An oil immersion objective (100x magnification) was used to obtain microscopy images.

Scanning electron microscopy (SEM)

The microstructure of protein particles was analyzed by SEM. Samples were either air- or critical-point dried. For critical point drying (CPD) of the samples clean circular cover slips of 8 mm (Menzel, Brauschweig, Germany) were coated with 0.2% Poly-L-lysine hydrobromide in water (Sigma-Aldrich, Inc., USA) and dried for 1 h. A droplet of sample was transferred to the coated cover slips. After waiting 30 min, cover slips were gently rinsed in water and dehydrated in a series of acetone (30, 50, 70, 100% acetone in water solutions, 10 min per step). The samples were subsequently critical point dried with carbon dioxide (CPD 030, BalTec, Liechtenstein). To perform air drying a drop of sample was put on a silicon nitride sieve and dried. Finally both critical- point and air-dried samples were sputter-coated with 2 nm Tungsten (MED 020, Leica, Vienna, Austria).

For SEM analysis of macroscopic WPI gels, a 25% (w/w) WPI solution was filled into the cylindrical glass tubes that were coated with Sigmacote (Sigma-Aldrich, St. Louis, USA). The glass tubes were placed in a water bath and samples were gelled at 80 ±0.5 ^{o}C for 20 min. After the gels were brought to room temperature, they were removed from the glass tubes and cut into 5 mm thick cylindrical pieces. The gel pieces were first placed into a 50% DMSO in water solution (30 min) and then put into liquid nitrogen. While being kept in liquid nitrogen, frozen gel pieces were fractured into smaller gel fragments with the help of a razor blade. After being thawed in the 50% DMSO in water solution, these gel fragments were washed with acetone solutions and critical point dried by following the procedure described above. After critical point drying, gel fragments were glued on a sample holder by Carbon glue (Leit- C, Neubauer Chemicalien, Germany), air dried for 2 h and subsequently stored overnight under vacuum. Finally the samples were sputter coated with 10 nm Iridium in SCD 500 (Leica, Vienna, Austria) prior to analysis. Due to limited availability, Iridium was used instead of Tungsten for this analysis. Since both metals are considered equally suitable coating materials for high resolution SEM imaging, one may expect that the difference in coating material still gives comparable SEM results. All samples were analyzed with a field emission

scanning electron microscope (Magellan 400, FEI, Eindhoven, the Netherlands) at room temperature at a working distance of 4 mm with SE detection at 2 kV. Images were digitally recorded.

Heating experiments

Approximately 20 ml from each sample was transferred into a glass tube and closed tightly. Samples were heated at 90 °C for 30 min in a temperature-controlled heating plate (RT15, IKA Werke, Germany). Samples were stirred mildly by a magnetic stirrer during heat treatment to avoid particle sedimentation and to facilitate the heat transfer. The experiments were performed in duplicate.

Rheological measurements

Viscosity of the samples was measured by using a Physica MCR 501 Rheometer (Anton Paar, Graz, Austria). The measuring geometry was a concentric cylinder (CC17/T200/SS, cup diameter: 18.08 mm, bob diameter: 16.66 mm). 5 ml of each sample was placed in the measuring cell and surface of the sample was covered with paraffin oil to avoid evaporation of water. For shear rate ramps, the shear viscosity of the dispersions before and after heat treatment was measured over the shear rate range 1-1000 s^{-1} at 25 °C. During the temperature ramps the shear viscosity of a non-heated dispersion was measured at a constant shear rate of 45 s^{-1}. The temperature was ramped from 25 to 90 °C, kept at 90 °C for 30 min and cooled back from 90 °C to 25 °C. The heating and cooling steps were performed at a rate of 3.25 °C /min. All measurements were performed in duplicate.

Results and discussion

Formation and microstructure

Whey protein particles prepared at different pH

A 25% (w/w) WPI solution at either pH 6.8 or pH 5.5 was used to prepare protein particles. In figure 4.1, optical microscopy images of whey protein particles at different steps during preparation are shown. When a 25% (w/w) WPI solution at pH 6.8 was used, a w/o emulsion containing particles of a few micrometers was formed after initial mixing (Fig. 4.1-A, first row). After heating the w/o emulsion to gel the proteins inside the particles, no coalescence or aggregation of the particles was observed (Fig. 4.1-B, first row) and a dispersion of spherical particles was obtained after repeated centrifugation and washing in a 1% (w/w) WPI solution

(Fig. 4.1-C, first row). Likewise, when the pH of the 25% (w/w) WPI was adjusted to 5.5, spherical particles with a few micrometers of average diameter were formed after the initial mixing step (Fig. 4.1-A, second row). After heating this w/o emulsion to gel the whey proteins, a mixture of spherical and irregular particles was observed (Fig. 4.1-B, second row). In the microscopy pictures taken after centrifugation and washing steps (Fig. 4.1-C, second row) the irregularity in the shape of particles can be more clearly seen and no spherical particles were present in this sample.

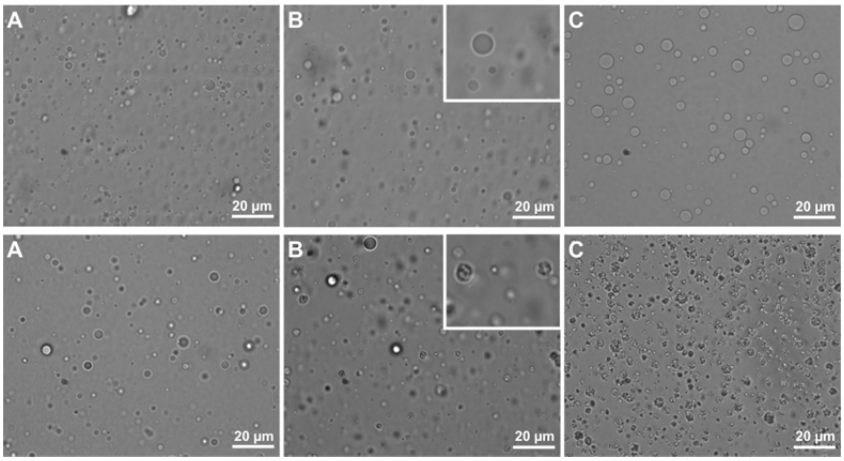

Figure 4.1 Influence of initial pH of WPI solution on formation of particles as monitored by optical microscopy. Particles were prepared from a 25% (w/w) WPI solutions at pH 6.8 (first row) or at pH 5.5 (second row). A: protein particles in oil containing 2.5% (w/w) PGPR (w/o emulsion), B: protein particles in oil containing 2.5% (w/w) PGPR after heating at 80 °C for 20 min to gel the whey proteins inside the particles, C: protein particles dispersed in a 1% (w/w) WPI solution at pH 6.9 after repeated centrifuge and washing steps. The inserts in B show regular and irregular shaped particles formed at pH 6.8 (first row) and pH 5.5 (second row) respectively.

The particle size distribution of protein particles revealed that protein particles prepared at pH 5.5 were slightly smaller than the particles prepared at pH 6.8 (data not shown). Most likely shrinkage of the particles during the gelation step at pH 5.5 is the main reason for the irregularity in shape and the decrease in the size of the particles. Spherical particles observed after heating by optical microscopy are probably the water droplets formed due to expelled water from the protein particles. This is further more corroborated by their absence in the final particle

dispersion, which can be explained by the fact that water droplets were removed through centrifugation.

More detailed structural analysis of protein particles was done by scanning electron microscopy (SEM) after critical point drying (CPD) with CO_2 and the microstructure of the particles at different magnifications are presented in figure 4.2. Particles that are formed at pH 6.8 (Fig. 4.2, first row) are spherical and have a homogeneous surface structure with small protein strands in the size of 20 to 40 nm. The surface of those particles is rough and a few randomly distributed pores in the size of 50 to 100 nm can be seen on the surface of the particles. The structure of protein particles prepared from a WPI solution at pH 5.5 (Fig. 4.2, second row) was considerably different from the protein particles prepared from a WPI solution at pH 6.8. Particles formed at pH 5.5 had a cauliflower-like appearance: spherical domains in the size of a few hundred nanometers were formed through aggregation of much smaller particles and the structure exhibited large pores compared to the particles prepared at pH 6.8.

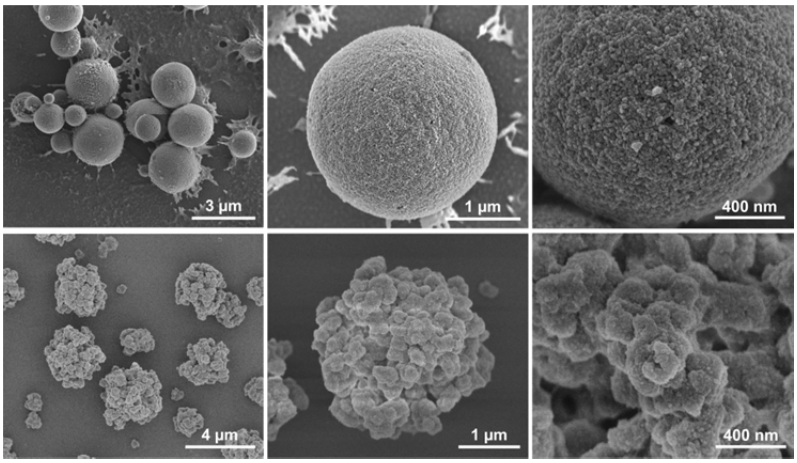

Figure 4.2 Influence of initial pH of WPI solution on particle morphology as analyzed by SEM after CPD. Particles were prepared from a 25% (w/w) WPI solutions at pH 6.8 (first row) or at pH 5.5 (second row). Samples were diluted 100x in Millipore water and subsequently critical-point dried with CO_2 prior to analysis. Images were recorded at room temperature at a working distance of 4 mm with SE detection at 2 kV.

Other studies in which gelation of whey proteins is used for microcapsule formation also reported on the effects of pH on the particle microstructure. For

example, Lee and Rosenberg [4] studied the formation of WPI microcapsules; having milk fat droplets as core material and whey proteins as an encapsulating matrix. Those microcapsules were spherical and had a very smooth surface when prepared at pH 7.2, whereas they had a more wrinkled and porous surface structure when prepared at either pH 4.5 or pH 5.5. Although those microcapsules did not have a cauliflower-like morphology and looked different than the whey protein particles prepared in our study, the inner structure of those microcapsules consisted of large protein aggregates. Recently Doherty et al. [3] also reported on formation of whey protein micro-beads with a similar wrinkled and porous structure at pH 4.6 by using cold gelling of whey proteins.

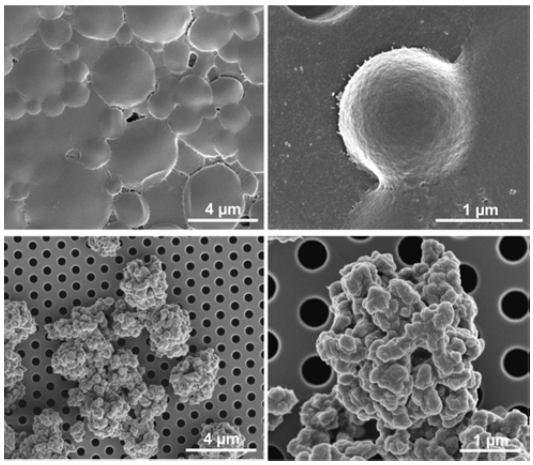

Figure 4.3 Influence of air-drying on the structure of protein particles as monitored by SEM. Protein particles were prepared from a 25% (w/w) WPI solution. The samples were diluted 100 times in Millipore water and subsequently air dried prior to analysis. Images were recorded at room temperature at a working distance of 4 mm with SE detection at 2 kV. Protein particles prepared from WPI solution at pH 6.8 (first row) were collapsed while protein particles prepared from WPI solution at pH 5.5 (second row) stayed intact after air-drying.

CPD of the samples was chosen for SEM analysis because the majority of the protein particles formed at pH 6.8 collapsed after being dried by air (Fig. 4.3, first row). However protein particles formed at pH 5.5 were still intact after air drying (Fig. 4.3, second row) and had a similar morphology to the protein particles dried by CPD. During air drying samples are subject to the interfacial tension in the liquid-vapor phase boundary as the liquid evaporates and this interfacial tension may result in the collapse of the structure within the sample. Because a liquid-

vapor interface is avoided in CPD method, samples dried by CPD are not exposed to interfacial tension effects. The fact that pH 5.5 particles survive the interfacial stresses present during air-drying suggests that they must be conceivably stronger and more compact than protein particles formed at pH 6.8.

A noticeable difference between the SEM micrographs after CPD (Fig. 4.2) and air drying (Fig. 4.3) is in the surface appearance of the particles: when protein particles were dried by air, the surface was smoother whereas when protein particles were dried by CPD, the surface of the particles looked much rougher. Although CPD has the advantage of preserving the structure, it requires intensive washing steps in series of acetone solutions in order to remove water and this presumably results in removal of acetone-soluble components from the particle surface and interior. In contrast, air-drying allows analysis of the samples directly without any washing step. In previous chapters, confocal scanning laser microscopy (CSLM) analysis of the protein particles formed at pH 6.8 showed that protein particles might be covered by a very thin oil layer or residual oil patches on the surface. Therefore much smoother surface observed in the air-dried particles can be attributed to the oil components attached to the surface of the particles.

Whey protein particles prepared at different NaCl concentrations

NaCl was added into a 25% (w/w) WPI solution at concentrations of 50, 200 or 400 mM NaCl and solutions were stirred at least for 30 min to allow dissolution of the salt. The pH of the solution slightly dropped after addition of salt from pH 6.8 to pH 6.7 for 50 and 200 mM and to pH 6.6 for 400 mM NaCl. The microstructure of the particles was analyzed by SEM, and the micrographs are shown in figure 4.4

Increased NaCl concentration resulted in significant change in the morphology of the particles. Particles formed at 50mM NaCl had a spherical shape similar to those formed without NaCl. However the microstructure of the particles formed at 50 mM NaCl looked coarser and the size of the strands forming the protein network was 60 to 80 nm, which is somewhat larger than the strand sizes of particles formed in the absence of NaCl. With increasing NaCl concentration, the microstructure became even coarser and the shape of the particles was irregular when they were formed at either 200 or 400 mM NaCl concentrations. Similar to what is observed when the particles were formed at pH 5.5, transformation from a spherical to an irregular shape occurred after the gelling step (optical microscopy, data not shown). However the microstructure of protein particles formed at 200 or

400 mM NaCl concentrations did not have spherical domains that are clearly separated from each other as observed in the particles formed at pH 5.5. Their morphology appears much more irregular and less dense than the protein particles formed at pH 5.5.

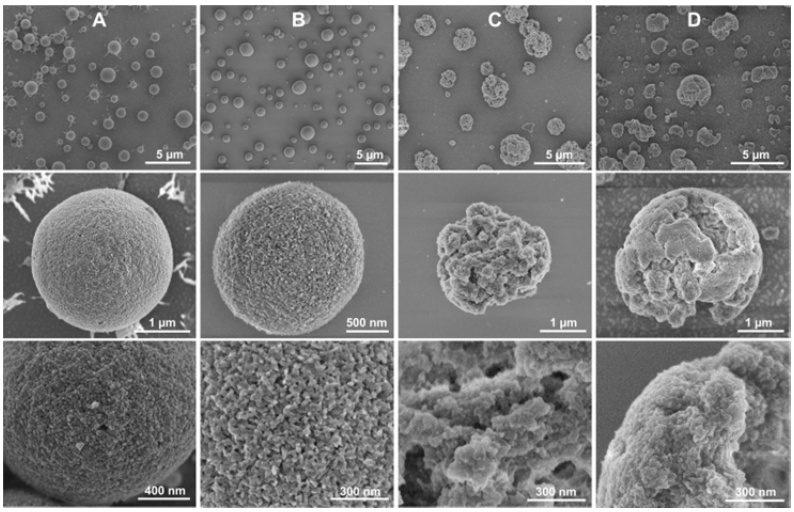

Figure 4.4 Influence of NaCl content of WPI solution on particle morphology as monitored by SEM. Particles were prepared from a 25% (w/w) WPI solutions with or without NaCl. A: no NaCl, B: 50mM NaCl, C: 200 mM NaCl and D: 400mM NaCl. Samples were critical-point dried prior to analysis. Images were recorded at room temperature at a working distance of 4 mm with SE detection at 2 kV. The shape of protein particles became irregular with increased NaCl concentration.

Several studies on heat-induced aggregation of whey proteins have shown formation of spherical aggregates in the size of a few hundred nanometers when the pH is close to the isoelectric point of proteins, whereas aggregate sizes became smaller when the pH was set above or below the isoelectric point [16, 21-25]. Although high concentrations of NaCl have led to formation of larger aggregates, the pH value of the protein solutions (closer to neutral pH) when particles were prepared at either 200 mM or 400 mM NaCl might have resulted in this microstructural difference observed between the particles, prepared at pH 5.5 or at high NaCl concentrations.

Several broken particle fragments were observed when protein particles were prepared at 400 mM NaCl concentration (Fig. 4.4-D, first row). This indicates that particles formed at 400 mM NaCl are quite brittle and fracture of particles possibly

occurred during re-dispersing the particles in the aqueous phase, which requires the use of a homogenizer operating at 150 bar.

Microstructure of whey protein particles vs macroscopic WPI gels

From a practical point of view, it would be convenient to be able to predict the physical properties of protein particles based on the properties of macroscopic gels. However, the microstructure of the gels might be influenced by the typical length scales involved during gelation. Therefore microstructure of whey protein particles and macroscopic WPI gels were compared (Fig. 4.5).

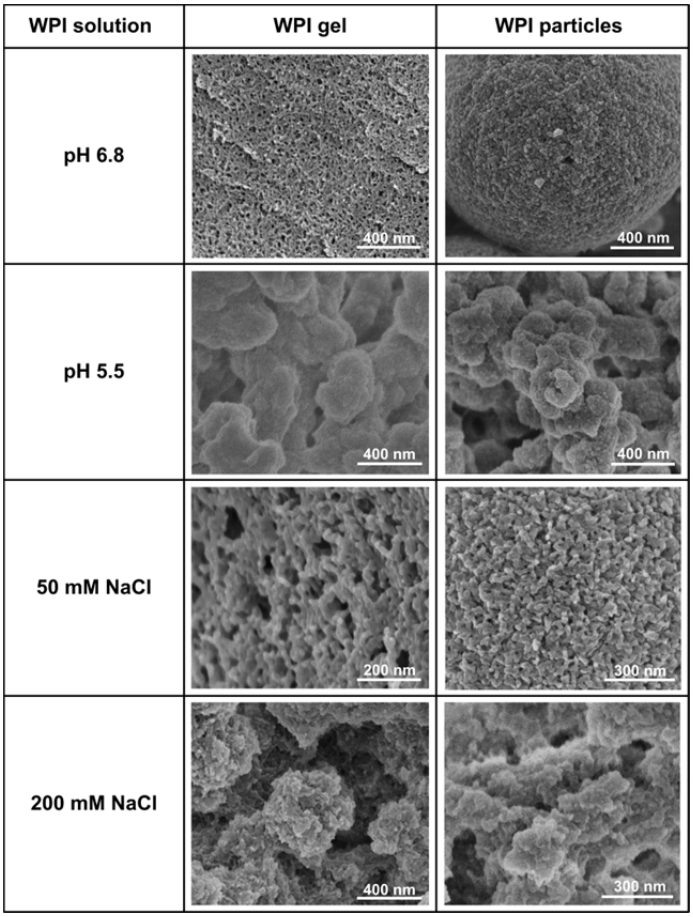

Figure 4.5 Comparison of microstructure (as monitored by SEM) of whey protein particles and macroscopic 25% (w/w) WPI gels prepared at different pH and NaCl concentrations.

Macroscopic WPI gels were prepared by heating a 25% (w/w) WPI solution at 80 °C for 20 min in cylindrical glass tubes. Formation of a water layer on the top of the gels was observed when the pH of WPI solution was at pH 5.5 or the NaCl concentrations were high (200 mM or 400 mM). This shows that syneresis occurs during the gelation of whey proteins at different gelling conditions. Similar phenomena can be also expected to occur during the formation of whey protein particles and this further confirms the shrinkage of the particles during the heat gelling step. In figure 4.5, the microstructure of macroscopic WPI gels and microstructure of whey protein particles are compared. Macroscopic gels of 25% (w/w) WPI formed at pH 6.8 have a homogeneous microstructure with a relatively small pore size (between 10-30 nm) and the pores are homogeneously distributed through the gel network. When the pH of the WPI solution was adjusted to 5.5, a coarser microstructure consisting of spherical and denser domains in the size of a few hundred nanometers was formed. The microstructure contained much larger pores than the microstructure of the gels formed at pH 6.8.

Formation of the WPI gels in the presence of NaCl similarly resulted in a much coarser and porous gel network, as can be seen in figure 4.5. Although addition of 50 mM NaCl resulted in larger pore size (between 60-100 nm) the microstructure of this gel was similar to the microstructure of the gel formed at pH 6.8 without added NaCl. Increasing the NaCl concentration from 50 to 200 mM (Fig. 4.5) or 400 mM (data not shown) altered the gel microstructure significantly; the microstructure was much coarser and the pore size increased significantly. These observations are in good agreement with the previous studies which reported on the effects of varying pH and NaCl concentrations on the microstructure of macroscopic WPI gels [14, 16, 17, 22]. The microstructure of whey protein particles also followed a similar pattern as macroscopic gels of WPI when the pH or the NaCl concentration of the initial WPI solution was altered. Despite the small differences in the size of the building blocks and pores, in general it can be concluded that the microstructure of whey protein particles is similar to the microstructure of macroscopic WPI gels formed under similar gelling conditions. This suggests that gelation of whey proteins on different length scales did not have a significant influence on the resulting microstructure.

Influence on internal protein content of protein particles

Internal protein content of protein particles is estimated based on the hydrodynamic volume fraction of particles when they are dispersed in an aqueous

medium and the total protein content of this dispersion. For the calculation of the hydrodynamic volume fraction, the viscosity of highly diluted particle dispersions was measured to allow usage of Einstein's equation.

Table 4.1 Difference in the internal protein content of whey protein particles prepared from a 25% WPI solution at different gelling conditions

Initial protein solution	Internal protein content of particles (% w/v)
25% WPI pH 6.8	18.5 ±1.1
25% WPI pH 5.5	39.2 ±1.1
25% WPI NaCl 50 mM	21.2 ±1.1
25% WPI NaCl 200 mM	24.5 ±1.1
25% WPI NaCl 400 mM	23.6 ±1.1

Protein content of protein particles are presented in table 4.1. Protein particles prepared at pH 6.8 had an internal protein content of around 18.5% (w/v) which is significantly lower than the initial protein content of the WPI solution (~ 23.5% w/v). As we have shown in previous chapters, protein particles might contain oil patches on their surface which can be one of the factors that decreases the protein content of the particles. However the amount of oil is rather small (~ 1.8% w/v) and it is not expected to observe this significant decrease in the total protein content due to the oil associated with the particles. This implies that particles might have already swollen after being dispersed in the aqueous phase.

Protein particles prepared from a 25% (w/w) WPI solution at pH 5.5 had the highest internal protein content which was approximately 39.2% (w/v). This is in line with the results presented in the previous section and confirms particle shrinkage during the heating step to gel the proteins inside the particles. If particles would not have shrunk (or swell) the maximum protein content of the particles would be around 23.5% (w/v) which is the initial protein content of the WPI solution. As can be seen in table 4.1., the internal protein content of the particles increased with increasing NaCl concentration of initial WPI solution and above 200 mM NaCl concentration no further increase in the protein content was measured. These results imply that pH and ionic strength of the initial WPI solution used for the formation of the particles is a very important factor in determining the protein density of the particles and obviously pH values close to

the isoelectric point of the proteins and high NaCl concentrations (above 200 mM) can be used to obtain denser protein particles.

Influence on heat stability

Effect of gelation conditions on the heat stability of whey protein particles was assessed. After repeated centrifugation and washing, a dense pellet containing protein particles was obtained. The pellet was dispersed in the same aqueous phase as the washing solution (1% (w/w) WPI solution, pH 6.8) with a weight ratio of 1:2. Due to the differences in the density of the particles, the final volume fraction of the particles and the total protein content differed between the resulting dispersions. Volume fractions were approximately $\Phi \sim 0.36$ for particles prepared at pH 6.8, $\Phi \sim 0.31$ for particles prepared at pH 5.5, $\Phi \sim 0.34$ for particles prepared at 50 mM, $\Phi \sim 0.38$ for particles prepared at 200 mM and $\Phi \sim 0.40$ for particles prepared at 400 mM NaCl concentrations.

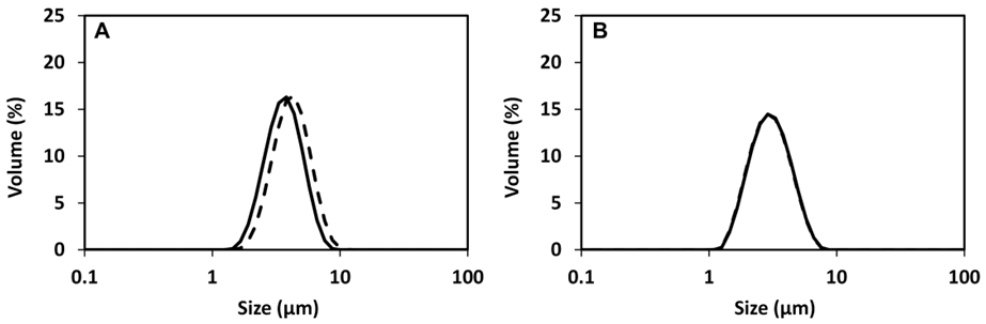

Figure 4.6 Influence of heat treatment (90 °C/30 min) on the size (diameter) distribution of protein particles prepared from a 25% (w/w) WPI solutions at pH 6.8 (A) and pH 5.5 (B). Solid line: before heating, dashed line: after heating. The lines in graph B coincide.

To assess the heat stability, dispersions of protein particles were heated at 90 °C for 30 min while being slightly stirred by magnetic stirrer. In figure 4.6, the particle size distribution before and after heating is shown. The size distribution of the particles prepared at pH 6.8 slightly shifted towards larger sizes while no change in the size distribution was observed when particles are prepared at pH 5.5 (the size distribution before and after heating coincide). Even though the size of protein particles prepared at pH 6.8 was increased after heating, no cluster formation or aggregation was observed in these samples (optical microscopy, data not shown). The viscosity of the dispersion containing protein particles prepared at pH 6.8

increased after heating and shear-thickening was observed at high shear regime (Fig. 4.7-A), as we have already shown in Chapter 3. Remarkably, no change in the viscosity of the dispersion containing protein particles prepared at pH 5.5 was observed after heating at 90 °C for 30 min (Fig. 4.7-B).

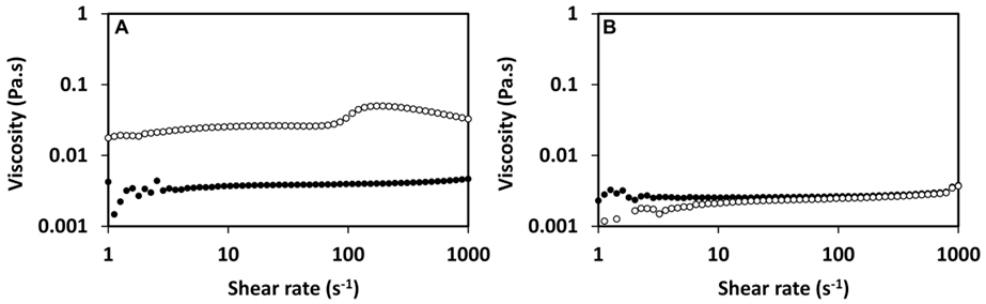

Figure 4.7 Influence of heat treatment (90 °C/30 min) on the shear viscosity of protein particle dispersions. Protein particles were prepared from a 25% WPI solutions at pH 6.8 (A) and pH 5.5 (B). Solid symbols: before heating, open symbols: after heating. Estimated volume fraction (Φ) is ~ 0.36 for the particles prepared at pH 6.8 (A) and ~ 0.31 for the particles prepared at pH 5.5 (B).

Viscosity measurements during temperature ramps were performed for the same samples at a constant shear rate of 45 s^{-1}. After the temperature was ramped from 25 °C to 90 °C, the sample was kept at 90 °C for 30 min. In figure 4.8, the viscosity of the dispersions at 90 °C is shown.

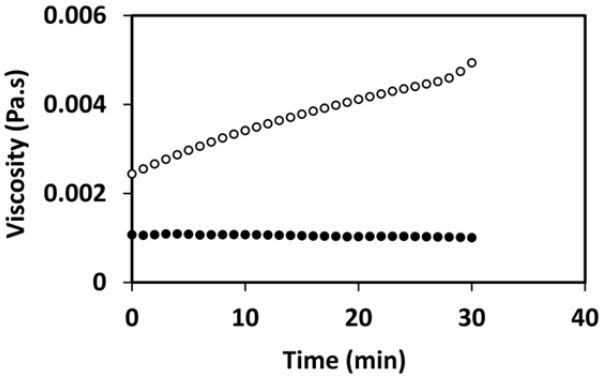

Figure 4.8 Viscosity of protein particle dispersions at 90 °C at a constant shear rate of 45 s^{-1}. Temperature was first ramped from 25 °C to 90 °C at a rate of 3.25 °C/min. Protein particles were prepared from a 25% WPI solutions at pH 6.8 (open symbols) and pH 5.5 (solid symbols). Estimated volume fraction (Φ) is ~ 0.36 for the particles prepared at pH 6.8 and ~ 0.31 for the particles prepared at pH 5.5.

The viscosity of the dispersion containing protein particles prepared at pH 5.5 was constant whereas the viscosity of the dispersion containing protein particles prepared at pH 6.8 increased continuously when kept at 90 °C.

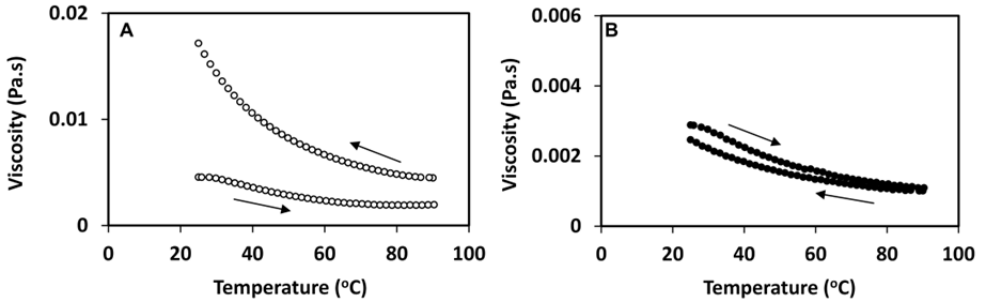

Figure 4.9 Temperature ramps for the dispersion of protein particles prepared at pH 6.8 (A) and at pH 5.5 (B). Temperature was ramped from 25 to 90 °C (20 min) and back to 25 °C (20 min) at a rate of 3.25 °C/min. Before cooling down samples were kept at 90 °C (30 min). Estimated volume fraction (Φ) is ~ 0.36 for the particles prepared at pH 6.8 (A) and ~ 0.31 for the particles prepared at pH 5.5 (B).

When the temperature was ramped from 25 °C to 90 °C (Fig. 4.9), a small decrease in the apparent viscosity of both samples was observed which can be attributed to the decreasing viscosity of the water (continuous phase) due to the increasing temperature. The viscosity of the dispersion containing particles prepared at pH 6.8 was higher after it was kept at 90 °C for 30 min and the viscosity increased significantly during the cooling step back to 25 °C (Fig. 4.9-A). Comparing the viscosity profiles of pH 5.5 particle dispersions during the heating and cooling step (Fig. 2.9-B), a small difference can be observed, which might be a result of additional denaturation of proteins. The viscosity of the dispersion of pH 5.5 particles after the cooling step is slightly lower than the initial viscosity. However, when the dispersion is kept at 25 °C for longer times, the viscosity increases back to the initial value (data not shown). The same is observed in the experiment shown in figure 4.7-B. These results indicate that the viscosity of the dispersion of protein particles prepared at pH 5.5 is stable when kept at 90 °C. This is not the case for the dispersion of pH 6.8 particles, showing clearly the difference in the heat stability of these two types of protein particles.

In these experiments, the volume fraction of protein particles prepared at pH 6.8 (Φ ~ 0.36) was higher than the volume fraction of particles prepared at pH 5.5 (Φ ~ 0.31). However when the volume fraction of particles prepared at pH 6.8 was

decreased to, e.g. Φ ~ 0.25, similarly an increase in the viscosity and shear thickening was observed, as discussed in Chapter 3. Therefore, a higher volume fraction of protein particles prepared at pH 6.8 in comparison to pH 5.5 is not the reason for the difference observed in the viscosity after heating.

Since no important aggregation was observed after heating, the increase in the size and viscosity of the particles prepared at pH 6.8 is most likely due to swelling of the particles, thereby increasing the volume fraction. Swelling of WPI-based microcapsules when dispersed in different aqueous media has been reported earlier [1]. In our case the rate of swelling was probably increased during heat treatment. It has been reported that the permeability of WPI gels increases significantly at gelling temperatures above 70 ^{o}C, as a result of altered protein aggregation [13]. Some other studies also have shown that whey protein aggregation and gel structure is sensitive to the temperature [25, 26]. Although heating experiments in our work were performed after the particles were already gelled at 80 ^{o}C, it is likely that during the second heating step particle swelling occurred due to structural rearrangements.

We did not observe a similar effect for the particles prepared at pH 5.5, although they have larger pores than protein particles formed at pH 6.8. We believe that the high density spherical domains in these particles prevent significant swelling. Also, the net charge of the protein is close to zero when the particles are prepared at pH 5.5, while the net charge of the protein is negative when the particles are prepared at pH 6.8. Due to electrostatic repulsion between the negatively charged groups, it is expected that the swelling capacity of the particles prepared at pH 6.8 will be significantly higher than the particles prepared at pH 5.5. Indeed lower equilibrium swelling ratios for bulk whey protein concentrate gels were reported both when the protein concentration of the gels was increased and when the pH of gelation was changed towards the isoelectric point of the protein [27].

Protein particles prepared at different NaCl concentrations also have shown similar changes as observed for the particles prepared at pH 6.8. In figure 4.10, size distributions of the particles prepared at different NaCl concentrations before and after heating are shown. For all the samples, the particle size increased after heating. The shift in the size distribution was slightly larger for the particles prepared at 200 or 400 mM NaCl concentrations.

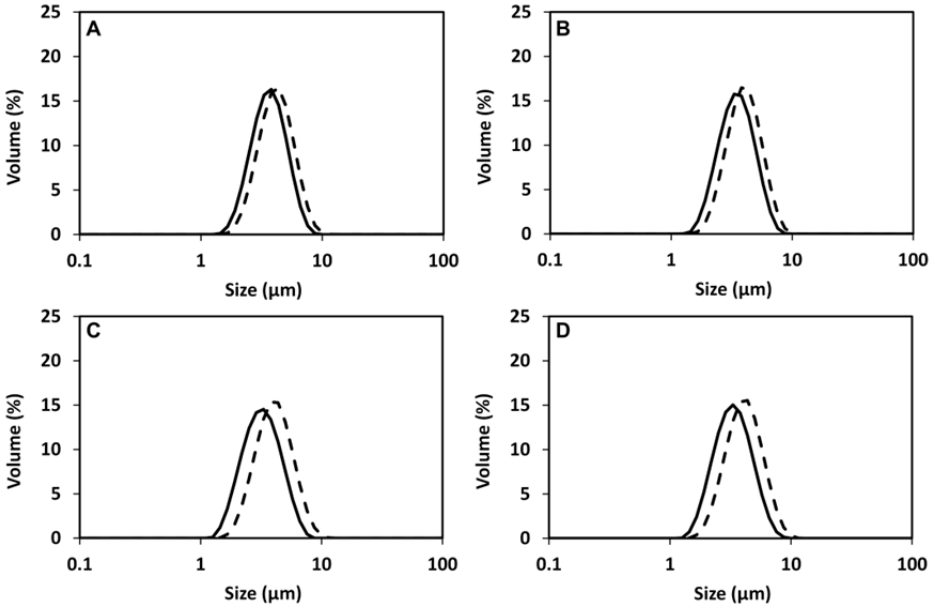

Figure 4.10 Influence of heat treatment (90 °C/30 min) on the size (diameter) distribution of protein particles prepared from a 25% (w/w) WPI solutions at no NaCl added (A), 50 mM NaCl (B), 200 mM NaCl (C) and 400 mM NaCl (D). Solid line: before heating, dashed line: after heating.

Shear viscosity of the dispersions containing particles prepared at different NaCl concentrations changed upon heat treatment; in all the samples viscosity increased and shear thickening was observed at different shear rates (Fig. 4.11). When particles are formed at 50 mM NaCl (Fig.4.11-B), the viscosity profile of the dispersion after heating was similar to the dispersion containing particles prepared at pH 6.8 with no added NaCl (Fig. 4.11-A). The increase in the viscosity of the dispersions containing whey protein particles prepared with either 200 mM NaCl (Fig. 4.11-C) or 400 mM NaCl (Fig. 4.11-D) was more significant than the viscosity increase observed for the dispersions containing protein particles prepared with 50 mM NaCl and the onset of shear thickening was shifted to lower shear rate values. Similarly no significant aggregation could be detected in these samples (optical microscopy, data not shown). This suggests that formation of particles at high NaCl concentrations might have resulted in greater particle swelling as reflected with the changes in viscosity profiles. Interestingly, this is opposite of what we observe when the particles were prepared at pH 5.5.

Effects of pH and ionic strength on the structure of WPI gels were extensively studied [13, 16, 17, 22, 25, 28-31]. Although some differences in the large deformation

properties of the gels, i.e. gel hardness, was observed [17, 18, 20, 28], it is generally accepted that adding salt has a similar influence on the structure of the gels as changing the pH towards the isoelectric point.

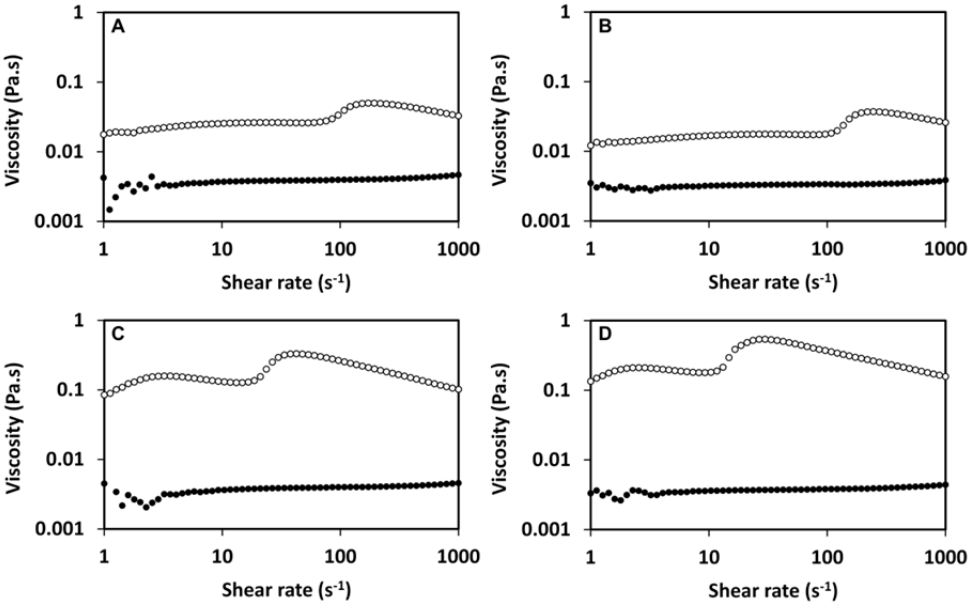

Figure 4.11 Influence of heat treatment (90 °C/30 min) on the shear viscosity of protein particle dispersions. Protein particles were prepared from a 25% (w/w) WPI solutions at no added NaCl (A), 50 mM NaCl (B), 200 mM NaCl (C) and 400 mM NaCl (D). Solid symbols: before heating, open symbols: after heating.

However our study shows clear differences in the properties of protein particles prepared either close to the isoelectric point of whey proteins (pH 5.5) or at high salt concentrations (200 and 400 mM). In the case of particle formation close to a neutral pH (~ 6.8) and at high salt concentrations, the formation of inter- and intramolecular covalent bonds through thiol/disulfide exchange reactions are expected to play a role in the aggregation process beside non-covalent interactions [15, 32], while the aggregation process is mainly driven through non-covalent interactions when particles are formed at pH 5.5 [15]. At this pH the net charge of the proteins is close to zero, but proteins still contain negative and positive charges and the electrostatic interaction between these oppositely charged groups may also be involved in the aggregation process [33]. Difference in the aggregation mechanism of the proteins (aggregation kinetics and amount of aggregated protein at gel

point) is reported to have a strong influence on the pore size distribution in the gel, which is reflected in the gel permeability [13, 31]. Here it was shown that the permeability of WPI gels increased significantly with increasing NaCl concentration and at pH's approaching the isoelectric point, corresponding to larger pore sizes. Based on those results, one might expect that particles formed at 200 or 400 mM NaCl might have a more permeable microstructure and it is likely that they also would have a higher swelling capability as we also do observe. This is not confirmed for protein particles prepared at pH 5.5, although their larger pore size was shown by SEM analysis. We expect that this is due to the presence of domains in the structure with a high protein density that prevents the swelling. We also note that, after dispersing in the same aqueous environment, protein particles prepared at different gelling conditions have resulted in a different final pH. Protein particles prepared at pH 6.8 and in the presence of NaCl increased the pH slightly above 7.0, whereas protein particles prepared at pH 5.5 decreased the pH slightly. This small variation in the pH may also influence the particle swelling during or after heating, because protein particles are expected to show a pH sensitive swelling due to their polyampholytic character.

Conclusions

This study has shown that changing gelation conditions during preparation of protein particles led to formation of different whey protein particles in terms of microstructure, internal protein density and physical properties.

Both the initial pH and NaCl concentrations had a strong influence on the microstructure of the whey protein particles and the changes observed in the microstructure of particles at different gelling conditions were comparable to the macroscopic WPI gels. Formation of protein particles either at pH 5.5 or in the presence of high NaCl concentrations (200 mM or 400 mM) resulted in formation of irregular and denser particles whereas spherical and less dense particles were formed at pH 6.8 or low NaCl concentrations (50 mM).

Protein particles prepared at pH 5.5 had a substantial higher protein content compared to the particles prepared at different conditions and these particles also showed an excellent heat stability because of their compact microstructure which does not swell during heat treatment. Even though increasing NaCl concentrations (200 or 400 mM) resulted in formation of particles with microstructures similar to those observed when the pH was 5.5, the viscosity of their dispersions increased

significantly after heat treatment. This is opposite of what is observed for the particles formed at pH 5.5 and implies some specific differences in the influence of pH and NaCl on the structure of whey protein microparticles.

Acknowledgements

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Chapter 5

The influence of pH and ionic strength on the swelling of protein particles

In this chapter the swelling and protein leakage of protein particles was investigated as a function of pH. Protein particle dispersions were stable over a wide pH range. Around pH 5.0, aggregation, presumably due to weak electrostatic repulsion, was observed. Protein leakage from the particles was found not to be higher than 8% (w/w) in most of the pH range, whereas it increased significantly at alkaline pH, most likely, as a result of disruption of the particle structure by OH- ions. The protein particles show a pH- and salt-responsive swelling, as shown by CLSM analysis. These findings are important, when the protein particles are used for development of high protein foods, such as, liquid medical drinks. In particular, because variation in pH can induce changes in the volume of protein particles and, thereby, considerably influencing the rheological properties of the concentrated particle dispersions. The results of this chapter show that whey protein particles can also be used in the applications, such as controlled delivery.

This chapter is submitted to *Soft Matter* as:

Sağlam, D.; Venema, P.; de Vries, R.; van der Linden, E., The influence of pH and ionic strength on the swelling of dense protein particles.

Introduction

Hydrogels are three-dimensional polymer networks, that can swell and retain large amounts of water in aqueous solutions, while maintaining their network structure [1, 2]. They are usually formed by cross-linking the polymer chains through covalent bonds, hydrogen bonding, Van der Waals interactions or physical entanglements [3, 4]. They may exhibit stimuli-induced swelling behavior (i.e. upon changes of pH, temperature, ionic strength) [1, 5]. This makes them interesting for applications, such as targeted delivery of bioactive compounds, separation and purification technologies, or tissue engineering [6-8].

The swelling behavior of hydrogels is explained by the contribution of the entropy of mixing of the polymers and solvent to the free energy, changing solvent quality upon dilution, and an elastic energy of the polymer network opposing the swelling (due to either energy consuming bond breakage and/or entropy decrease of the chains due to stretching) [4, 9, 10]. The type and the distribution of the functional groups on the polymer chain are important in this respect [1, 11]. For non-charged hydrogels, swelling or shrinkage of the hydrogel will depend mainly on the entropy of mixing and the opposing elastic energy. For the hydrogels containing ionisable moieties (polyelectrolyte gels), the interaction between ions and polymers will also contribute to the swelling of the hydrogels [4]. The swelling behavior of the latter depends mainly on the osmotic pressure inside the particles due to counter-ions [11] and can be highly pH sensitive depending on the particular groups on the polyelectrolyte chain. Polyelectrolyte gels that contain both positive and negative charges are called polyampholytic gels and they show different properties, due to presence of opposite charges within the gel structure [12]. For example, they may retain a robust structure in a wider pH range than polyelectrolyte gels [11]. Several studies have focused on preparation of polyampholytic hydrogels from synthetic or natural polymers. Formation of stable hydrogels from synthetic materials might require a chemical cross-linking step, which would prevent dissolution of the hydrogel in an aqueous environment [13, 14].

Proteins form polyampholytic gels due to the presence of acidic (carboxylic groups) and basic (amino groups) moieties in the polypeptide chain [9, 10, 13]. Formation of gels from proteins can be achieved by a simple heating step or by cold gelation [15-17] , which does not require a chemical cross-linking step. The physical properties of protein gels, such as mechanical properties, microstructure, water holding capacity, permeability or pH sensitivity, can be adjusted by small

modifications in the gelation parameters, which allows design of gels with various functionalities [18-21]. Moreover, proteins are completely biodegradable, explaining different types of proteins having been investigated for use as polyampholytic hydrogels [1, 22]. One of the early studies reported on pH-sensitive swelling of egg albumin [23]. Here, it was reported that albumin gels showed the least swelling when the pH of the environment was close to the isoelectric point of albumin (pH~ 4). When the pH was adjusted further away from the isoelectric point, the equilibrium swelling of the albumin gels increased. A few studies investigated swelling properties of heat-induced whey protein [9, 10, 13] and β-lactoglobulin gels [24]. These studies have shown that whey protein gels swell in aqueous media and this swelling was strongly influenced by the protein concentration of the gel, pH during gelation and pH of the swelling medium. These studies have suggested that whey proteins have good matrix properties to be used for the targeted delivery purposes.

The size of the delivery matrix is an important physical parameter for controlling the release properties, such as release rate. Decreasing the matrix size enables improved delivery properties: shorter and faster release of the compounds and a higher bioavailability can be some advantages of the decreased matrix size [22]. For food applications, in order to eliminate the negative effects on sensory quality, decreasing the size is essential as well [25]. Several studies investigated the formation of particles based on polymer networks, showing similar properties as hydrogels and these type of particles are usually defined as nano particles or microgel particles [11, 14].

Studies on the swelling behavior of whey protein particles are scarce [10, 26, 27]. Whey protein gel beads, containing internal oil droplets, were prepared by dropwise addition of an oil-in-water emulsion, in which a WPI solution formed the aqueous phase, into a $CaCl_2$ solution [26]. It was shown that the gel beads had the lowest swelling ratio close to the isoelectric point of the proteins and the swelling ratio was increased at neutral or acidic pH. These gel beads had an average diameter of ~ 2 mm, which might be too large for several applications. In another study, whey protein particles in the size of a few hundred nanometers were formed by heat-induced aggregation of whey proteins at pH 5.9 [27]. Small angle X-ray scattering (SAXS) analysis of the particle structure, as a function of the medium pH, suggested a dense structure close to the isoelectric point of the proteins and a more open structure at acidic pH, suggesting pH responsive swelling of the particles.

In Chapter 4, we have shown that whey protein particles with different morphology and internal protein density can be prepared by a simple pH adjustment. Particles have a rather uniform structure when prepared at pH 6.8, whereas they have an irregular structure consisting of highly dense protein domains and large pores distributed throughout the particles, when prepared at pH 5.5. Additional to their polyampholytic character, the altered morphology and internal protein density of the particles can be of use for improved encapsulation and controlled delivery purposes. Apart from the immediate relevance of swelling versus pH for delivery purposes, the swelling of the particles also influences the rheological properties and heat stability of protein particle dispersions significantly (Chapter 3 and 4). Therefore, in this chapter, we have studied the swelling of the whey protein particles as a function of pH and ionic strength. We have monitored the swelling of individual particles in different buffers by confocal laser scanning microscopy (CLSM). Additionally, the protein leakage from the particles, their zeta potential at different pH and pore size was studied.

Experimental

Preparation of whey protein particles

Protein particles were prepared according to the method described in Chapter 4. All the particle dispersions had a pH value close to 7.0 ±0.2 after preparation. Further adjustment of the pH was done by 6 M HCl or 2 M NaOH. For the CLSM analysis protein particles were prepared from a 25% (w/w) WPI solution that was covalently labeled with FITC (see labeling of proteins with FITC).

Optical microscopy

Optical microscopy analysis was performed as described in Chapter 4.

Labeling of proteins with FITC

1% (w/w) WPI solution was prepared in 0.1 M carbonate buffer (pH 9.1). FITC was dissolved in DMSO at 4 mg/ml. For each ml of protein solution, 50 µl of FITC solution was slowly added into the protein solution, while gently stirring. The sample was incubated in the dark for 6 h. After incubation, the protein solution was transferred into dialysis membranes (MWCO 12-14 kDa, Medicell International Ltd.) and the excess of FITC was removed by dialyzing this solution against Millipore water. Dialysis was performed in the dark at 4 ^{o}C for~ 60 h and water was refreshed every 12 h. The pH of the labeled protein solution was

adjusted to 6.8 by adding 6M HCl and this labeled protein solution was used for the preparation of protein particles.

Swelling of protein particles

Protein particles were dispersed in 10 mM phosphate buffer at pH 7 and fixed on a flow cell surface coated with Poly-L-Lysine (1 μ-Slide $I^{0.4}$ Luer coated, Ibidi, Munich, Germany) having a channel volume of 100 μl. Unattached protein particles were rinsed off by 10 mM phospate buffer at pH 7. The change in size of a few of the attached particles were monitored by CLSM (Zeiss 200M Axiovert, Thornwood, NY, USA) using an oil immersion objective (Plan-Apochromat, 100x/1.4 oil, Zeiss, Thornwood, NY, USA). The samples were analyzed at an excitation wavelength of 488 nm and an emission wavelength of 543 nm. The buffer was exchanged with the help of a syringe pump. The flow rate of the buffer in the flow cell was kept at 60 μl/min.

Determination of the pore size of the particles

FITC labeled dextran (molar masses of 4, 10, 70, 150, 250, 500 and 2000 kDa, SIGMA-ALDRICH, Steinheim, Germany) was dissolved in a phosphate buffer at pH 7 at 0.1% (w/v). Protein particles, at 0.6% (v/v), were added in dextran solutions of different masses and incubated in closed vials at 4-7 ^{o}C, while slightly stirring to avoid particle sedimentation. After 6 days of incubation, samples were examined by CLSM, to determine the FITC signal inside the particles and in the dispersing media. Samples were placed in a chamber made from a standard microscopy glass slide (Menzel-Glaser, 76x26mm, Thermo Scientific, Braunschweig, Germany) and a cover slip (Menzel-Glaser, 24x24mm, Thermo Scientific, Braunschweig, Germany), separated from each other by two strips of parafilm, giving a volume of~ 25 μl. Imaging of the samples was performed using the confocal microscope (Zeiss 200M Axiovert, Thornwood, NY, USA). The samples were analyzed at an excitation wavelength of 488 nm, an emission wavelength of 543 nm, by an oil immersion objective (Plan-Apochromat, 100x/1.4 oil, Zeiss, Thornwood, NY, USA).

Determination of particle size distribution and zeta-potential

The particle size distribution and zeta potential measurements of the protein particles were done according to the method described in Chapter 3.

Determination of protein concentration

Protein concentration of the dispersions was determined by DUMAS. A Flash EA 1112 N/protein analyzer (Thermo Scientific, Waltham, US) was used to determine total nitrogen content of the samples. Nitrogen values were multiplied by 6.38 to calculate the total protein content of the samples (% w/w).

Determination of protein leakage from the particles

Protein particles were dispersed in a 1% (w/w) WPI solution (total protein concentration ~ 5%) and incubated at least for 24 h at a certain pH. The incubation was performed at room temperature and the dispersions were mildly stirred to avoid sedimentation. The dispersions were centrifuged (25.000 RPM/1.5 h, Beckman ultracentrifuge L-60, USA) to separate protein particles from the dispersing media and supernatant was filtered (pore size ~ 0.22 μm) to warrant the absence of protein particles. The protein concentration of the supernatant was determined by a UV spectrophotometer (Cary 50 Bio, Varian). The absorbance of the samples at 280 nm wavelength was measured and the protein concentration was calculated from a calibration curve of WPI solutions at known concentration.

Results and discussion

Stability of protein particles as a function of pH

Protein particles were prepared either at pH 6.8 or pH 5.5. As shown in Chapter 4, this resulted in a significant change in morphology and internal protein concentration of the particles. In brief, protein particles prepared at pH 6.8 were spherical, whereas particles prepared at pH 5.5 had an irregular appearance (cauliflower-like) and a more compact structure. Figure 5.1 shows the protein particle dispersions as a function of pH. The upper row relates to the particles initially prepared at pH 6.8 and the lower row to the particles prepared at pH 5.5. The dispersion sedimented at pH 5.0, while at the other pH values, the dispersion was stable. A clear separation at pH 5.0 was visible within 1 h. Close to pH 5.0, the particles were weakly aggregated as indicated by their disassembly upon decreasing the pH, as observed by optical microscopy (data not shown). For the dispersions containing protein particles prepared at pH 6.8, some particle aggregates were still present close to pH 3.5. We can see from the pictures that at

alkali pH, e.g. pH 9.5, a more transparent appearance was observed than that of the samples at other pH values.

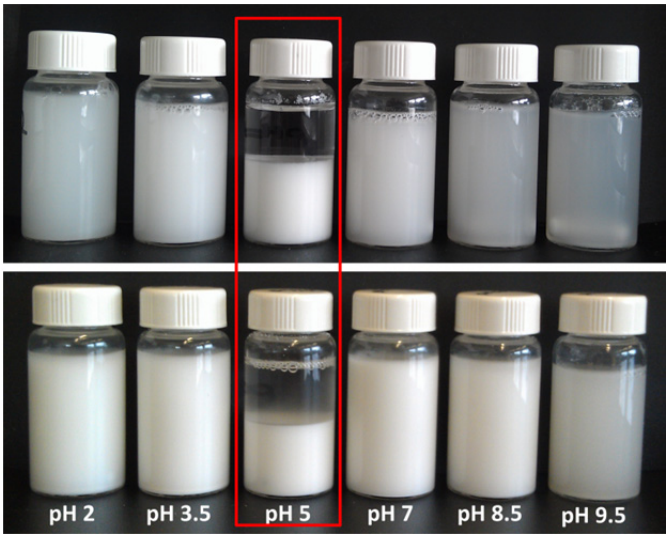

Figure 5.1 The protein particle dispersions as a function of pH. Protein particles were prepared from a 25% (w/w) WPI solution either at pH 6.8 (A) or pH 5.5 (B) and dispersed in a 1% (w/w) WPI. The total protein concentration of dispersions were ~ 5% (w/w) and the pH of the dispersions was adjusted by addition of either 6 M HCl or 2 M NaOH.

There can be several explanations for this increased transparency: firstly, the protein particles will swell at this pH due to increased amount of negative charges in the network leading to a less denser network inside the particles. This will lower refractive index of the particles. Secondly, due to the presence of NaOH, the bonds involved in the formation of the protein network, such as, disulfide bridges, may be disrupted and the protein particles may dissolve, as was earlier reported for heat-induced protein gels [24, 28, 29]. The refractive index difference between the particles and the continuous phase may decrease due to leakage of protein material into the continuous phase or due to swelling of the particles, thereby reducing the overall sample refractivity. Particles seemed larger at pH 9.5 (optical microscopy, data not shown) and the microstructure was not disrupted for the particles prepared at pH 6.8. Due to their irregular shape, it was difficult to evaluate the microstructural change for the particles prepared at pH 5.5, but it can be concluded that particles were not, or only to a small extend, dissolved at this pH.

In figure 5.2, the zeta-potential of the protein particles as a function of pH is shown. Protein particles, prepared at pH 5.5 and 6.8, both had a net surface charge close to zero once brought to pH 5.0 , which is close to the isoelectric point of whey proteins (pH ~ 5.1) [19]. The particles were negatively charged above this pH, whereas they were positively charged when the pH was lowered. These results are in agreement with the behavior of the dispersion (Fig. 5.1), where aggregation occurred around pH 5.0, with the electrostatic repulsion between the particles being minimal.

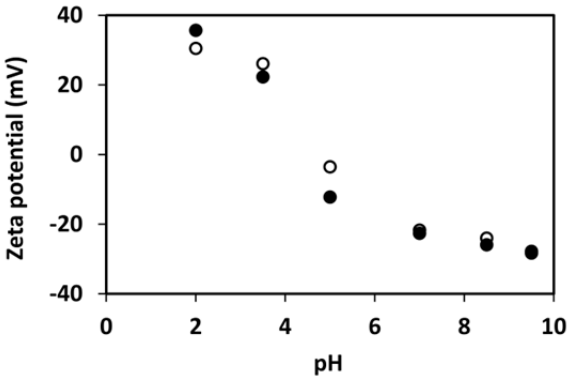

Figure 5.2 The zeta-potential of protein particles as a function of pH. The protein particles were prepared from a 25% (w/w) WPI solution either at pH 6.8 (solid symbols) or at pH 5.5 (open symbols) and dispersed in a 1% (w/w) WPI solution. The pH of the dispersions were adjusted either with 6 M HCl or 2 M NaOH. Zeta potentials were measured after the samples were equilibrated at least for 2 h.

Recently, a similar pH dependency was reported for whey protein particles in the size range of a few hundred nanometers, where the particles were prepared through heat-induced aggregation of whey proteins [27]. These authors have shown that their dispersions of whey protein particles were unstable in the pH range between 4.0 to 5.5 and that their surface charge was zero around pH 4.8.

During preparation of the particle dispersions, we have observed that protein particles can change the pH of the dispersions slightly. When dispersed in the same aqueous environment (either buffered or non-buffered solutions), protein particles prepared at pH 6.8 were observed to increase the pH. Oppositely, a slight decrease in the pH was observed when particles prepared at pH 5.5 were dispersed. In line with our observations, it was reported that whey protein

particles prepared through heat-induced aggregation of proteins in a pH range between 5.8 and 6.1, increased the pH of the medium, leading to increased surface charges and thereby stabilization against aggregation [30]. These authors suggested that, the mechanism of the pH increase during formation of protein particles might be caused by partial charge neutralization of proteins, leading to depletion of H^+ ions from the solution. In another recent study, the isoelectric point of the hydrogel particles formed through heat-induced gelation of whey proteins at different pH was determined [10]. The authors showed that the isoelectric point of the particles was lower (pI ~ 4.2) for the particles gelled at pH 7, compared to the isoelectric point of particles (pI ~ 5.0) gelled at pH 5.2. The decrease in the isoelectric point of particles, when gelled at neutral pH, was explained by the loss of protonable groups, due to formation of disulfide bridges. This led to an increase in net negative charge and, as a result, the pI of the particles has shifted towards the more acidic region. This could also explain the change in the pH that we have observed upon dispersing the protein particles in the same aqueous environment. If the protein particles formed at pH 6.8 have a higher net negative charge than the protein particles prepared at pH 5.5, the amount of H^+ ions diffusing into the protein particles and thereby decreasing the amount in the continuous phase might be more for the case of the pH 6.8 particles than for the case of the pH 5.5 particles, explaining a higher final pH.

To further investigate the stability of the particles, protein leakage from the particles was measured as a function of pH. After preparation, the dispersions were incubated at room temperature for 24 h, while being slightly stirred, to avoid sedimentation of the particles. Particles were removed by centrifugation (25.000 RPM/1.5 h) and protein leakage was determined by analyzing the protein concentration of the supernatants by UV spectrophotometer. The supernatants were filtered (pore size of 0.22 μm) to ensure no protein particles were present in the samples. The results are shown in figure 5.3. The protein leakage from the particles was around zero, close to pH 5.0, and it increased when the pH was changed. In the alkaline pH range, either at pH 8.5 or 9.5, the leakage was significantly higher in comparison to the leakage measured at neutral or in the acidic pH range. At pH 8.5 or 9.5, protein particles are highly charged, thereby expected to swell significantly, which may increase protein leakage from the particles. Besides, as mentioned above, it is reported that whey protein gels may dissolve in alkaline conditions, usually above pH 11.0-12.0 [24, 28, 29].

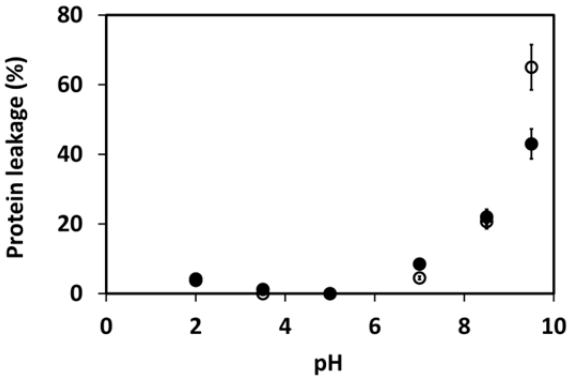

Figure 5.3 The protein leakage from the particles as a function of pH. Protein particles were prepared from a 25% (w/w) WPI solution either at pH 6.8 (solid symbols) or at pH 5.5 (open symbols) and dispersed in a 1% (w/w) WPI solution at a total protein concentration of 5% (w/w). Dispersions were incubated at room temperature for 24 h prior to analysis.

Although particles were not dissolved under these conditions, as found by optical microscopy analysis (data not shown), the increased protein leakage under alkaline conditions might be due to diffusion of OH^--ions into the protein particles leading to destabilization of the microstructure through cleavage of the covalent bonds. Apart from the large amount of leakage in the alkaline conditions (pH>7.0), the total protein leakage was never larger than 8% (w/w). The latter leakage is similar to what was reported by Schmitt et al.[27] for whey protein microgel particles prepared through heat-induced aggregation of proteins at pH 5.9. In another work on the swelling of whey protein bulk gels at a wide pH range (pH 1.5 to 10), a marginal dissolution (less than 2% total protein) of the gels was reported [10].

We also observe that the protein leakage at pH 9.5 is notably larger for the particles prepared at pH 5.5, compared to particles prepared at pH 6.8. This implies that the pH of the gelation influences the leakage of proteins from the particles. Protein particles that are formed at pH 5.5 are expected to have less intramolecular covalent linkages, such as disulfide bonds, in comparison to the particles prepared at pH 6.8 [31, 32]. This would make more proteins to be still available for being able to diffuse out of the particles once immersed in a solvent. Indeed, this has also been argued for alkaline dissolution of the whey protein bulk gels that, when the gels were formed at higher pH values, due to inclusion of more disulfide bonds a resistance against dissolution was observed [28].

Swelling of protein particles

Influence of pH

Swelling of the protein particles was measured either by light scattering or confocal laser scanning microscopy (CLSM). For the light scattering measurements, samples were equilibrated at room temperature at least for 2 h after the pH adjustment. To avoid the changes in the particle size due to pH fluctuations, the pH of the dilution solvent used during the measurement was kept the same as the pH of the dispersions. Figure 5.4 shows the particle size distribution of the protein particles prepared either at pH 6.8 (A) or pH 5.5 (B).

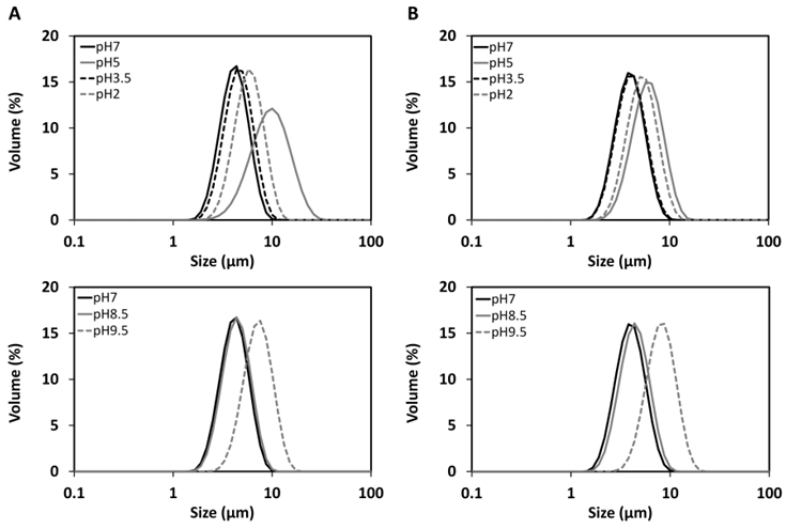

Figure 5.4 The particle size distribution as a function of pH. Protein particles were prepared from a 25% (w/w) WPI solution at pH 6.8 (A) and pH 5.5 (B) and dispersed in a 1% (w/w) WPI solution. The pH of the dispersions were adjusted by adding either 6 M HCl or 2 M NaOH and dispersions were equilibrated for 2 h prior to measurements.

For both type of particles, we observe similar changes in the size distribution: in the alkaline pH range, the size distribution shifted towards larger sizes, particularly, at pH 9.5. This increase in the size is due to particle swelling, as no particle aggregation was observed at this pH range (optical microscopy, data not shown). At pH 9.5, the average diameter of the protein particles prepared at pH 5.5 was larger (D [3,2]~ 7.10), than the particles prepared at pH 6.8 (D [3,2]~ 6.30), suggesting a larger swelling for the particles prepared at pH 5.5. In the acidic pH

range, an increase in the size was observed at pH 5.0, which is caused by the aggregation of the particles, as explained earlier. Only a small difference was observed in the size of the particles between pH 7.0 and pH 3.5 and the size of the particles became larger when pH was further lowered to pH 2.0. Both type of particles (prepared either at pH 6.8 or pH 5.5), showed a significant aggregation at pH 5.0 and, although not extensively, some clusters were still present, when the pH was lowered to pH 3.5 (optical microscopy, data not shown). This was observed particularly for the particles prepared at pH 6.8. Therefore, the results of light scattering measurement does not lead to a clear conclusion on particle swelling at acidic pH values.

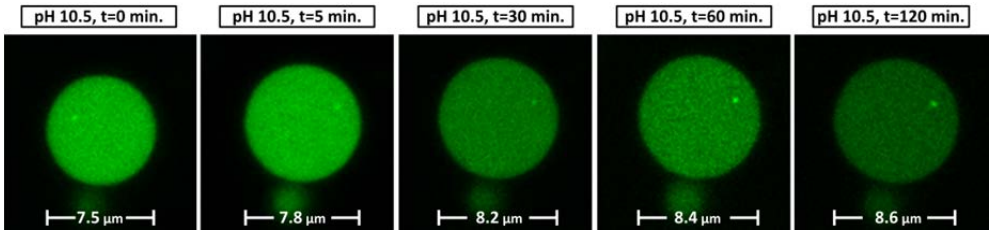

Figure 5.5 CLSM micrographs of protein particles during the incubation at pH 10.5. Protein particles were prepared from a FITC labeled WPI solution (25% w/w) at pH 6.8 and fixed on the microscope slide coated with poly-L-lysine. Swelling of the particles were monitored as a function of time after the buffer solution at pH 7 was exchanged by a buffer solution at pH 10.5.

We have further studied particle swelling by CLSM. Swelling of protein particles prepared at pH 5.5 could not be investigated by CLSM, because the particles did not attach to the microscope slide. This might be due to the difference in the surface character of the particles or due to their irregular shape. As a result, the data presented in this part includes only protein particles prepared at pH 6.8. CLSM micrographs showing the change in the diameter of protein particle, after the buffer at pH 7.0 was exchanged by a buffer solution at pH 10.5, are presented in figure 5.5. We have observed approximately ~ 1 µm increase in the size of a 7.5 µm particle after 2 h incubation at pH 10.5. After an incubation time of 5 min, the particles were already swollen. It should be noted, particles were scanned in the z-direction to determine the maximal circumference. From the radius of the particles, the volume was calculated. In figure 5.6, the relative volume change of the particles, being the ratio between the volume of the particles at a given pH and the initial volume at pH 7.0, is plotted against time.

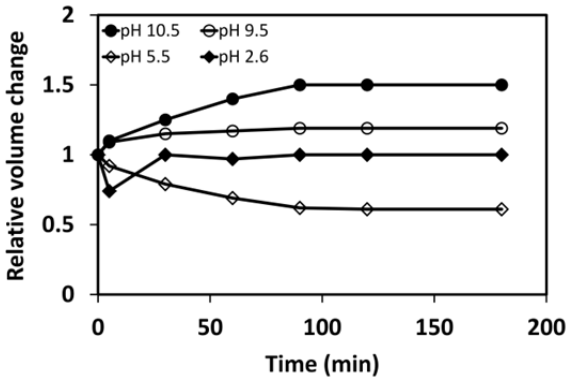

Figure 5.6 Relative volume change of protein particles as a function of time at different pH. Protein particles were prepared from a 25% (w/w) WPI solution at pH 6.8. The change in the volume of the particles at different dispersing media was calculated from the diameter change monitored by CLSM.

The relative particle volume increased at pH 10.5 and 9.5, the final relative volume being significantly larger at pH 10.5, showing that particles swell in the alkaline pH range, which is in line with the light scattering measurements. When the buffer was changed from pH 7.0 to pH 5.5, the volume of the particles decreased, showing that particles have shrunk.

As it is already shown for bulk gels of whey proteins [9, 10, 13], and confirmed by the zeta-potential measurements in this work, protein particles have a polyampholytic character: they have acidic and basic residues, and therefore they show pH-sensitive swelling behavior. When the pH of the medium was 9.5 or 10.5, due to ionization of the negatively charged groups, protein particles started swelling. Due to the larger number of negatively charged groups at pH 10.5, the equilibrium volume ratio was larger for pH 10.5 than pH 9.5. When pH of the buffer was changed from pH 7.0 to pH 5.5, the net charge of the protein particles approached zero, causing the particles to shrink, resulting in a decreased volume change. After the buffer exchange to pH 2.6, we first observe a decrease in the relative volume change (after ~ 5 min) followed by an increase and a final value close to 1. This might be due to the shrinking of the particles during the pH decrease from 7.0 to 2.6, which includes neutralization of the charges around isoelectric point of the particles. On the time scale of the measurement, we have not observed any significant difference in the final volume of the particles, when the pH was changed from 7.0 to 2.6, showing that protein particles should have similar charge densities at these pH values, leading to a similar swelling ratio. It should be noted

that small changes in the size of the particles, such as, 100-150 nm could not be accurately measured due to resolution limit of the CLSM images. On the time scale of the measurement, we see that most of the water uptake occurred within the first 90 min and that after this period no significant change in the volume of the particles was observed. Our results show that protein particles have considerably higher swelling ratios in alkaline pH than in acidic pH.

In general, our observations are comparable with the swelling behavior reported for the whey protein hydrogel tablets [9, 10, 13, 33] and for whey protein beads prepared through Ca^{+2}-induced cold gelation [26]. In these studies, the minimum swelling was observed close to isoelectric point of whey proteins, whereas the relative swelling increased significantly at alkaline conditions. In the acidic pH range, swelling of the gels were not significantly different than the swelling observed at neutral pH (~ pH 7.0) [9, 13]. Recently, the change in the internal structure of whey protein particles (average diameter of a few hundred nanometers) prepared through heat-induced aggregation was studied by small angle X-ray scattering (SAXS) [27]. It was shown that the diameter of protein particles decreased, when the pH was close to 5.0, confirming that particles have shrunk, when the net charge of the network is close to zero. The authors have reported that the size of the particles increased at acidic pH and reached a maximum at pH 2, whereas the increase at higher pH values was less significant. Only when the pH was above 8, further increase in the particle size and as well as disruption of the particles due to alkaline hydrolysis was reported. In the current study, we have not observed a large increase in the particle size at acidic pH. Besides, during the swelling experiments in the buffer solutions at high pH values (~ 9.5), particles were not significantly disrupted as observed by CLSM, which is also confirmed by an observed reversibility of the swelling, as will be discussed below. Differences in the internal structure, such as protein concentration or the amount of covalent bonds included in the network, might have caused the reported differences for particle swelling.

To measure the reversibility of particle swelling, particles were brought to pH 5.5 and pH 9.5, consecutively. After each buffer exchange, the particles were hold at the new pH long enough to reach equilibrium. The result of this swelling experiment is presented in figure 5.7. As can be seen, the particles were able to shrink or swell reversibly. This means that after being exposed to large pH differences or alkaline conditions, they have not lost their pH-responsive character, as well as they maintained their network structure.

A small increase in the swelling capacity can be observed after the first swelling. As shown in the previous part, at pH values above 7.0, there was a significant increase in the protein leakage from the particles. Thus protein leakage from the particles might have occurred during the swelling at alkaline pH. This would result in lower internal protein concentrations, altering swelling capacity of particles.

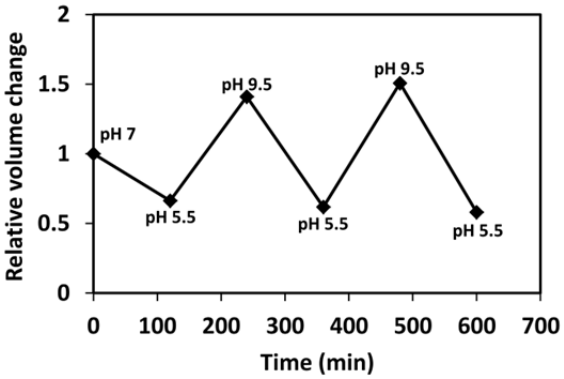

Figure 5.7 Reversibility in the swelling and shrinking of protein particles. Protein particles were prepared from a 25% (w/w) WPI solution at pH 6.8. The change in the volume of the particles at different dispersing media was calculated from the diameter change as measured by CLSM.

Influence of ionic strength

The ionic strength is also expected to influence the swelling of the particles. Therefore, we have studied the effect of NaCl concentration on particle swelling at different pH values. In figure 5.8, the relative change in the particle volume is plotted against NaCl concentration.

At all pH values, an increasing NaCl concentration decreased the relative volume increase, indicating shrinking of the particles. We observe a larger effect of the ionic strength on shrinking at either pH 9.5 or pH 2.6, compared to pH 5.5. Protein particles already have significantly shrunk at pH 5.5 (see fig 5.6). As can be seen in figure 5.8, at pH 5.5, there is a small decrease in the volume ratio at 50 mM and 100 mM NaCl, whereas increasing NaCl concentration further to 500 mM did not result in a further decrease in the particle volume. At both pH 9.5 and 2.6, the protein particles have a high charge density, resulting in water uptake due to strong electrostatic repulsion. At increasing ionic strength, the electrostatic repulsion

becomes weaker due to screening of the charges and particles shrank. It should be noted that influence of NaCl on particle swelling is less pronounced for the measured salt concentrations, compared to the swelling of the particles due to pH changes (see fig. 5.6). The latter effect is more than twice as strong compared to the former effect.

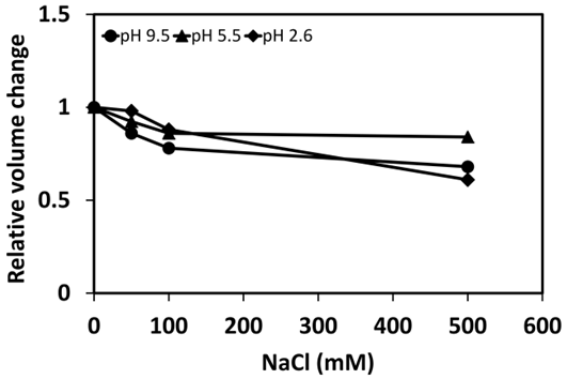

Figure 5.8 Influence of NaCl concentration on particle swelling. Protein particles were prepared from a 25% (w/w) WPI solution at pH 6.8 and dispersed in a 10 mM phosphate buffer at pH 7.0. The change in the volume of the particles was measured in different dispersing media by CLSM.

Pore size of protein particles

The average pore size of the protein particles was estimated based on the diffusion of dextran molecules, with molar masses between 4 to 2000 kDa, into the particles [34]. After the protein particles were equilibrated in FITC-labeled dextran solutions for 6 days, the samples were analyzed by CLSM to determine the partition coefficient, which is the ratio of the fluorescence intensity inside the protein particles to the fluorescence intensity in the continuous phase. In figure 5.9, CLSM micrographs of the protein particles after 6 days equilibrium in dextran with an average molar mass of 10 kDa are shown. For protein particles prepared at pH 6.8 (Fig. 5.9-A), a rather homogenous intensity distribution throughout the particles was observed, whereas for protein particles prepared at pH 5.5, the intensity distribution was quite inhomogeneous (Fig. 5.9-B), as can be seen by the local darker areas, as well as the domains having a high fluorescence signal inside the particles. The intensity profiles (Fig. 5.9-B, lower row) also confirms inhomogeneous distribution of the dextran molecules for the protein particles prepared at pH 5.5. As we have shown by scanning electron microscopy (SEM)

analysis, protein particles prepared at pH 5.5 have a cauliflower like structure, consisting of highly dense domains and large pores distributed throughout the particles (Fig. 4.2-B, Chapter 4). Presumably, these highly dense domains, which are depicted as darker areas in the CLSM images, are not very accessible for dextran molecules due to a very small pore size, whereas they can easily diffuse into the particle structure through larger pores. Similar behavior was observed for the other dextran molecular masses, therefore the pore size of the protein particles prepared at pH 5.5 could not be accurately estimated.

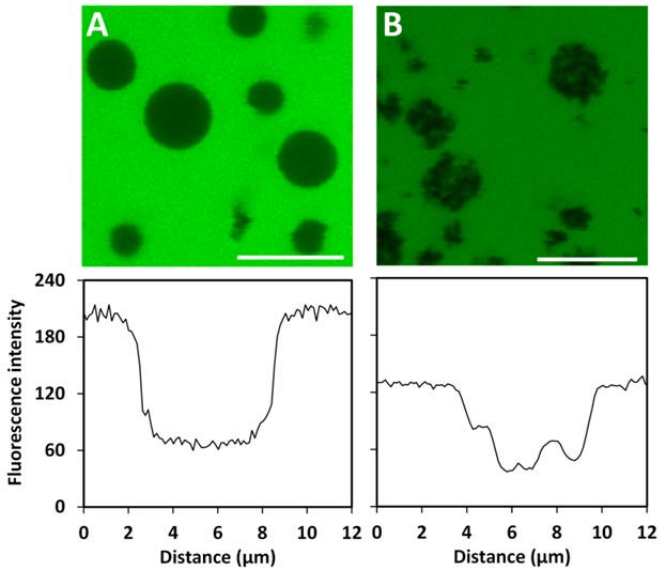

Figure 5.9 CLSM micrographs of protein particles prepared at pH 6.8 (A) and pH 5.5 (B) in a solution of FITC-labeled dextran molecules with a molecular mass of 10 kDa. Micrographs (upper row) and the intensity profiles (arbitrary units, lower row) show that the fluorescence signal inside the particles prepared at pH 5.5 was inhomogeneous. Scale bar: 10 μm.

In figure 5.10, the partition coefficient as a function of dextran molecular masses is shown for protein particles prepared at pH 6.8. We have observed that mainly dextran molecules with the molecular masses between 4 to 250 kDa (Stoke's radii ~ between 2 to 10 nm), diffused into the particles. Above 250 kDa, the intensity inside the particles was relatively low and not a large difference was observed between the partition coefficient for dextran molecules from 500-2000 kDa. This suggest that most of the pore sizes of the protein particles are in the range between

4 to 20 nm in diameter. The fact that even for 4 kDa dextran, we did not observe very large partition coefficients implies that the protein particles must also contain significant amount of pores that are smaller than 4 nm.

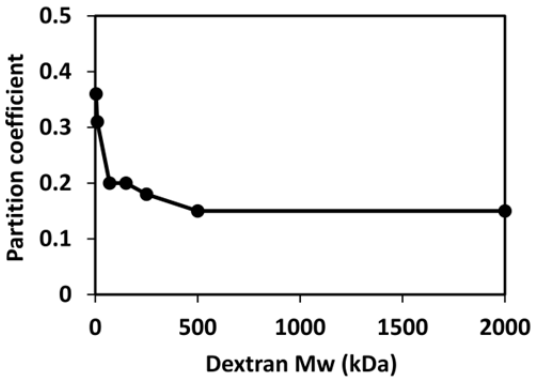

Figure 5.10 Partition coefficient as deduced from the ratio between fluorescence signal inside the protein particles and in the continuous phase.

Conclusions

Whey protein particles with tunable physical properties, such as morphology, size and mechanical properties, are interesting for targeted delivery purposes, particularly for the food applications due to their biodegradability. In this chapter, we have investigated the stability of whey protein particles in a wide pH range. Dispersions of whey protein particles are mainly stabilized through electrostatic repulsion. As a result, at the pH values close to isoelectric point of the particles, the particles tend to aggregate, leading to sedimentation. However, the aggregation was weak and reversible: when the pH was adjusted away from the isoelectric point, the aggregated particles redispersed again. Whey protein particles showed a pH- and salt-sensitive swelling and in the pH range studied, they maintained their network structure, which allowed them to swell and shrink reversibly. The protein leakage from the particles was also studied. The leakage was close to zero around pH 5.0, whereas it was between 2-8% (w/w) at the other pH values studied. Only increasing the pH above 8.5 resulted in a significant increase in the leakage, due to large amount of swelling and partial disintegration of the particles under the alkaline conditions. These results indicate that whey protein particles prepared in the current work have a potential to be used for targeted delivery purposes due to

their pH- and salt-responsive character and their stable network structure over a wide pH range. The pH sensitive swelling of the particles may result in significant changes in the volume of the particles. As presented in Chapter 3, increased volume fraction of particles significantly influenced the rheological properties of the particle dispersions, especially in the concentrated systems. The findings of the current chapter are important when the protein particles are used for development of high protein foods, such as liquid medical drinks.

Acknowledgements

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Chapter 6

Comparing heat stability of high protein content dispersions containing whey protein particles

The aim of this chapter was to replace whey proteins with whey protein particles to increase the total protein concentration and heat stability. Dispersions of whey protein particles formed at pH 5.5 showed an exceptional heat stability (at pH ~ 7.0); the viscosity of the dispersions containing a total protein concentration around 18% (w/w) did not change after heating at 90 ^{o}C for 30 min, while a WPI solution already gelled under the same heating conditions at protein concentrations around 11% (w/w). Additionally, no gelation was observed in the dispersions prepared by pH 5.5 particles, when the total protein concentration was increased above 20% (w/w). However, due to the increased particle concentration, shear thickening was observed in these samples. Whey protein particles prepared at pH 6.8 showed a rather weak stability against heat treatment, mainly as a result of swelling. Protein particles were not resistant to gastric digestion and complete degradation of the particles was observed after a short incubation time under pancreatic conditions.

This chapter is submitted to Food Hydrocolloids as:

Sağlam, D.; Venema, P.; de Vries, R.; van der Linden, E., Comparing heat stability of high protein content dispersions containing differently designed whey protein particles.

Introduction

Protein give rise to a stronger feeling of satiety in comparison to carbohydrates and fats [1-4]. Also, high protein diets result in increased energy expenditure following the consumption than low protein diets [5, 6]. Therefore high protein foods are considered to be a potential candidate for body-weight control and treatment of obesity. In addition, liquid high protein formulations play an important role in clinical nutrition. Protein energy malnutrition is a common problem among hospitalized patients [7-10] and continuation of a balanced oral diet is not usually possible in some of the patients suffering from for example cancer or AIDS, due to development of anorexia, eating difficulty and reduced intestinal function [8, 11]. Those patients are at the risk of losing weight and developing impaired function in the organs and muscles, therefore there is a need for a dietary supplement such as a small volume of liquid with high content of nutrients, in particular proteins and minerals [8, 9, 11]. High-protein, high-energy liquid supplements were suggested to be beneficial: for example routine consumption of a high-protein energy sip feed supplement, containing approximately 6.25% (w/v) protein, resulted in an improved energy intake and reduced weight loss in elderly patients [9]. Similar findings were also reported in another study focused on a patient group infected with HIV [11].

Therefore, in view of the above issues, the development of novel food products with substantially higher protein content is of importance. When developing high protein foods maintaining a desired product structure is often difficult due to unwanted interactions between the ingredients, which may occur especially during heating and storage. Dairy proteins such as caseins and whey proteins are usually used in formulations of liquid products with high protein levels. In the concentrated systems caseins may aggregate during thermal sterilization [12]. Whey proteins have a high nutritional value and they are a good source of leucine, which is an important amino acid in the muscle protein synthesis [13]. Similar to most proteins, whey proteins are also not stable at high protein concentrations: viscosity increase and gelation after heat treatment may result in the formation of undrinkable products.

Design of protein particles with controlled size, protein content, and surface properties can be useful to reduce or even eliminate such effects of increased protein content on food structure. Improved heat stability was shown for micro-aggregates formed through heat treatment and high-pressure shearing of whey

proteins [14]. The authors reported that the heat coagulation time at 140 °C was significantly higher for whey protein aggregates in comparison to native whey proteins. In another recent study it was also reported that using whey protein nano-particles can enhance heat stability of liquid protein formulations [15]. Whey protein nano-particles were prepared (average diameter smaller than 100 nm) through addition of a whey protein isolate solution in a w/o micro-emulsion containing reverse micelles of surfactant and subsequent heating at 90 °C for 20 min. The dispersion of those whey protein nano-particles at 5% (w/v) protein content was reported to be transparent and liquid-like after heat treatment.

The aim of the current study was to increase the total protein content that can be included in a liquid formulation without showing gelation after heat treatment. For this purpose native whey proteins were replaced by dense whey protein particles and the effect on the viscosity of dispersions before and after heat treatment was analyzed at neutral (~ pH 7.0) and acidic pH (~ pH 3.6). In Chapter 4, we have shown that whey protein particles prepared at different gelling conditions have different viscosity profiles after heat treatment. Therefore we have compared the dispersions of two different types of particles with a reference protein solution consisting only of native WPI. In view of their potential application in foods, we also have checked the in-vitro digestibility of the protein particles under gastric and pancreatic conditions.

Experimental

Preparation of whey protein particles

Protein particles were prepared according to the method described in Chapter 4.

Heating experiments

Approximately 20 ml from each sample was transferred into a glass tube and closed tightly. For the heat stability measurements at acidic pH, samples were slowly acidified with glucono-δ-lactone (GDL) which resulted in a pH value of 3.6 (±0.05). Samples were heated at 90 °C for 30 min in a temperature-controlled heating plate (RT15, IKA Werke, Germany). Samples were stirred mildly by a magnetic stirrer during heat treatment to avoid particle sedimentation and to facilitate the heat transfer. The experiments were performed in duplicate.

Determination of particle size distribution

The particle size distribution of the protein particles was determined according to the method described in Chapter 2. The average diameters reported are volume-averaged.

Determination of hydrodynamic volume fraction

Hydrodynamic volume fraction (Φ) of the protein particles was determined according to the method explained in Chapter 2.

Determination of protein content

Protein contents were determined by DUMAS. A Flash EA 1112 N/protein analyzer (Thermo Scientific, Waltham, US) was used to determine the total nitrogen content of the samples. Nitrogen values were multiplied by 6.38 to calculate the total protein content of the samples (% w/w).

Confocal laser scanning microscopy (CLSM)

Protein particles were diluted in Millipore water (100x) and stained with a few drops of 0.2% (w/w) Rhodamine B (Sigma-Aldrich, Steinheim, Germany) in water prior to microscopy analysis. Samples were placed in a chamber made from a standard microscopy glass slide (Menzel-Glaser, 76x26mm, Thermo Scientific, Braunschweig, Germany) and a cover slip (Menzel-Glaser, 24x24mm, Thermo Scientific, Braunschweig, Germany), separated from each other by two strips of parafilm, giving a volume of ~ 25 μl. Imaging of the samples was done using the confocal microscope (Zeiss 200M Axiovert, Thornwood, NY, USA) using an oil immersion objective (Plan-Apochromat, 100x/1.4 oil, Zeiss, Thornwood, NY, USA). The samples were analyzed at an excitation wavelength of 550 nm and an emission wavelength of 585 nm and at constant acquisition conditions.

Rheological measurements

Rheological measurements were performed with a Paar Physica MCR 300 Rheometer (Anton Paar, Graz, Austria). The viscosity of the samples were measured using a Couette geometry (CC17/T200/SS, cup diameter: 18.08 mm, bob diameter: 16.66 mm). For this measurement 5 ml of each sample was placed in the cup and the surface of the sample was covered with paraffin oil to avoid evaporation of water. The shear viscosity of samples was measured over the shear rate range 1-1000 s^{-1}. Dynamic rheological measurements at small deformation were performed with a parallel plate geometry with a diameter of 50 mm (PP50). A

time sweep test was performed for 30 min at a strain of 0.1% and a frequency of 1 Hz. This measurement was directly followed by a strain sweep test with a strain ranging from 0.01% to 10% at a frequency of 1 Hz. All measurements were performed in duplicate at 25 °C.

Digestibility of the particles

Protein digestion was simulated by utilizing a static model (NIZO static simulation of physiological digestion as part of the NIZO SIMPHYD platform). 5% (w/w) protein solutions were mixed with artificial saliva (main component α-amylase) before simulated gastric acidification towards pH 1.5 – 2.0 was initiated (typically pH 1.5 – 2.0 is reached within ~ 15 min). At pH 1.5 – 2.0 simulated gastric secretions (main components pepsin and lipase) were added and further incubated for 1.5 h. To simulate duodenal conditions, the pH was neutralized towards 6.0 and pancreatic and bile acids were added. At each step samples were taken to study the protein breakdown. RP-HPLC (Reversed-phase high-performance liquid chromatography) was applied to characterize the digests. Digestion was expressed as the reduction of the initial area of peaks representing the two major whey protein fractions, α-lactalbumin and β-lactoglobulin, in the RP-HPLC chromatographs.

Results and discussion

Heat stability of protein particle dispersions

Heat stability at neutral pH

Whey protein particles (prepared either at pH 5.5 or pH 6.8) were dispersed in a 1% (w/w) WPI solution (pH ~ 7.0) at increasing particle concentrations. Due to the buffering effect of the particles, the final pH of the dispersions could not be easily adjusted to the same pH value. As a result, for the dispersions containing protein particles prepared at pH 5.5 the final pH was ~ 6.9, while for the dispersion containing protein particles prepared at pH 6.8 the final pH was slightly above 7.0.

The heat stability of the dispersions was assessed after a heat treatment at 90 °C for 30 min. The microstructure of the particles before and after heat treatment was analyzed with CLSM after the samples were stained with Rhodamine B (Fig. 6.1). Protein was homogeneously distributed inside the particles when particles were prepared either at pH 5.5 or 6.8 (Fig. 6.1, depicted as green areas in the micrographs). Some darker areas present inside the particles prepared at pH 5.5

are due to larger and inhomogeneously distributed pores present in these particles (Fig. 6.1, first row). As can be seen, there is no aggregation or significant change in the microstructure of the particles prepared at either pH 5.5 (Fig.6.1, first row) or pH 6.8 (Fig. 6.1, second row) after heat treatment.

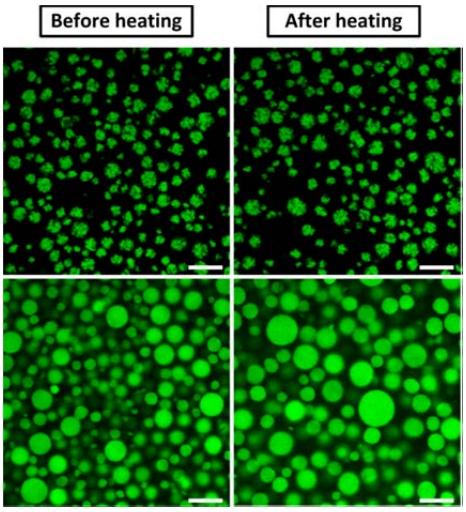

Figure 6.1 Effect of heat treatment on the microstructure of protein particles analyzed by CLSM. Particles were prepared from a 25% (w/w) WPI solution at pH 5.5 (first row) and at pH 6.8 (second row) and dispersed in a 1% (w/w) WPI solution. The dispersions were heat treated at 90 °C for 30 min. Samples were diluted 100x and stained with Rhodamine B prior to analysis. Scale bar: 10 μm.

In figure 6.2, viscosities of the dispersions containing whey protein particles (prepared at pH 5.5) and native whey proteins are compared at increasing total protein concentration, at a shear rate of 20 s^{-1}. Due to increased particle volumes, the viscosities of the dispersions increased with increasing protein concentration before heat treatment (Fig. 6.2-A). For all protein particle concentrations, there was no significant difference between the viscosities of the dispersions before and after heat treatment. A total protein concentration close to 20% (w/w) could be obtained without a significant increase in the viscosity after heat treatment. The viscosity of the reference sample, a whey protein isolate (WPI) solution at the same pH, increased significantly after heat treatment at all protein concentrations. Above a total protein concentration of 10% (w/w), the samples were gelled after heat treatment (Fig. 6.2-B). Although the increase in the viscosity before heat treatment was more pronounced with increasing protein concentration, the protein particles

improved the heat stability of the dispersions significantly compared to the reference system. In images 6.2-C and 6.2-D, the inverted glass vials containing the whey protein particle dispersion and WPI solutions before and after heat treatment at 12.1% (w/w) total protein concentration are shown.

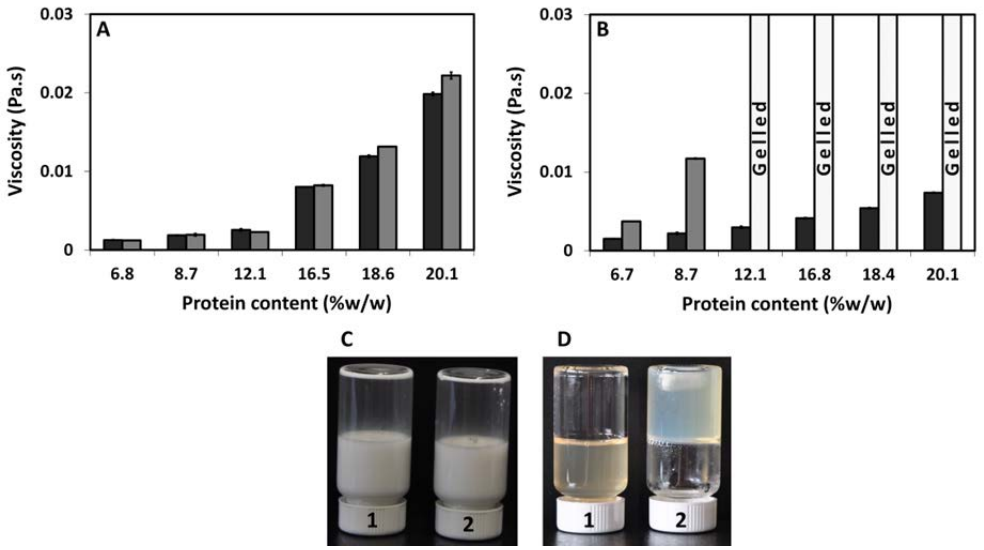

Figure 6.2 Change in the viscosities of whey protein particle dispersions (A) and WPI solutions (B) before (black) and after (gray) heat treatment at 90 °C for 30 min. Whey protein particles were prepared from a 25% (w/w) WPI solution at pH 5.5 and dispersed in a 1% (w/w) WPI solution. Viscosities of the dispersions at 20 s^{-1} are shown. Images (C) of an inverted glass vial showing non-gelled whey protein particle dispersions before (1) and after (2) heat treatment. Images (D) of an inverted glass vial of WPI solution before (1) and after (2) heat treatment at a total protein concentration of 12.1% (w/w). The heated sample (D-2) is gelled.

The particle dispersion had an opaque, milk-like appearance (Fig. 6.2-C1) and no visual change after heat treatment was observed (Fig. 6.2-C2). At the same protein concentration, the reference system containing just WPI gelled during heat treatment (Fig. 6.2-D2). This gelled sample had also a more turbid appearance due to formation of protein aggregates during heat treatment. In a recent work, whey protein nanoparticles (with an average diameter smaller than 100 nm), formed through emulsification and thermal treatment at 90 °C for 20 min [16] or enzymatic cross-linking [15], were shown to improve the heat stability in comparison to native whey proteins. Although a less turbid appearance after heat treatment could be obtained due to the small size of the particles, the protein concentrations, where no

gelation was observed, were low (5% w/v) compared to the protein concentrations obtained in the current work (~ 20% w/w). In another recent study whey protein particles were prepared by drying and milling a 40% (w/w) WPI gel [17]. The authors studied the heat stability of the dispersions of these whey protein particles at total protein concentrations of 15, 20 and 30% (w/w). At all protein concentrations the viscosity was reported to increase noticeably and non-flowing structures were obtained after heat treatment, which was explained by the high swelling capacity of the particles.

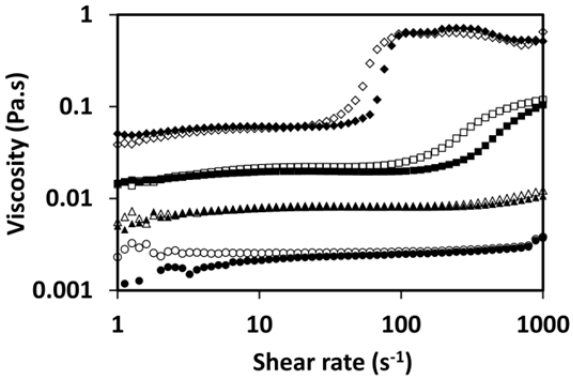

Figure 6.3 Viscosities of whey protein particle dispersed in a 1% WPI solution at increasing particle volumes before (solid symbols) and after (open symbols) heat treatment at 90 °C for 30 min. Whey protein particles were prepared from a 25% (w/w) WPI solution at pH 5.5 and dispersed in a 1% (w/w) WPI solution. Particle volume fraction and total protein contents of the dispersions were; Φ ~ 0.30, $C_{p,t}$ =12.1% (w/w) (●), Φ ~ 0.45, $C_{p,t}$ =16.5% (w/w) (▲), Φ ~ 0.55, $C_{p,t}$ =20.1% (w/w) (■), Φ ~ 0.59, $C_{p,t}$ =21.1% (w/w) (♦).

In figure 6.3, some of the flow curves for whey protein particle (prepared at pH 5.5) dispersions at increasing protein concentrations are presented. Although increasing protein concentration did not result in a significant change in the flow curves after heat treatment, a strong shear-thickening was observed when the total protein concentration ($C_{p,t}$) was increased above 20% (w/w). At this protein concentration the system is highly packed with a volume fraction of particles (Φ) being ~ 0.59. As discussed in Chapter 3 and Chapter 4, we have observed shear-thickening in the whey protein particle dispersions (for protein particles formed at pH 6.8) after heat treatment due to increased particle volume. Here, we also see a similar behavior both in the non-heated and heated dispersions in the high concentration regime, which further confirms the significant influence of particle

volume fraction on the onset and magnitude of shear-thickening. There was a small shift in the onset of shear-thickening after the heat treatment, which might be due to structural arrangements in the particles during heat treatment. The first heating step (80 °C/20 min) applied during particle formation (to gel the proteins inside the particles) might be not enough for the complete denaturation of whey proteins [16]. Therefore, in the second heating step (90 °C/30 min), further denaturation of the proteins might have resulted in more hydrophobic sites to be exposed at the interface, thereby changing the surface properties of the particles. It is reported that the shear-thickening transition is sensitive to the changes in the surface properties of the particles such as surface asperities, roughness and inhomogeneity [18, 19]. A possible change in the surface properties of the particles upon the second heating step may have influenced the onset of shear-thickening. Another explanation for this observation might be swelling of the particles after heat treatment. Protein particles prepared at pH 5.5 are highly dense and they have a very compact structure as shown in Chapter 4. Therefore, they do not show a significant swelling at this pH (~ 6.9) after heat treatment, which was confirmed by particle size distribution (data not shown) and microstructure analysis (Fig. 6.1-A). However, the sample in which a pronounced shear-thickening was observed (Fig. 6.3) has ~ 21.1% (w/w) total protein concentration ($C_{p,t}$). This would correspond to a particle volume fraction of ~ 0.59. A small amount of swelling (even too small to be detected by light scattering) can still significantly influence the viscosity profile at this high particle concentrations, including the onset of shear-thickening.

In order to investigate the dependence of the heat stability on the type of particles, we discuss the properties of the dispersions of protein particles prepared from a 25% (w/w) WPI solution at pH 6.8. These particles had a spherical shape and had similar particle size distribution as the particles prepared at pH 5.5. However, their internal protein concentration (~ 18.5% w/v) was significantly lower than the particles prepared at pH 5.5 (~ 39% w/v). This required the use of a higher particle concentration, in order to reach similar protein concentrations as in the systems discussed above. After dispersing the particles in a 1% WPI (w/w) solution, the final pH of the dispersion increased slightly above pH 7.0. Similar to the dispersion of particles prepared at pH 5.5, the viscosity of these dispersions increased steeply with increasing particle concentration before heat treatment (Fig. 6.4-A).

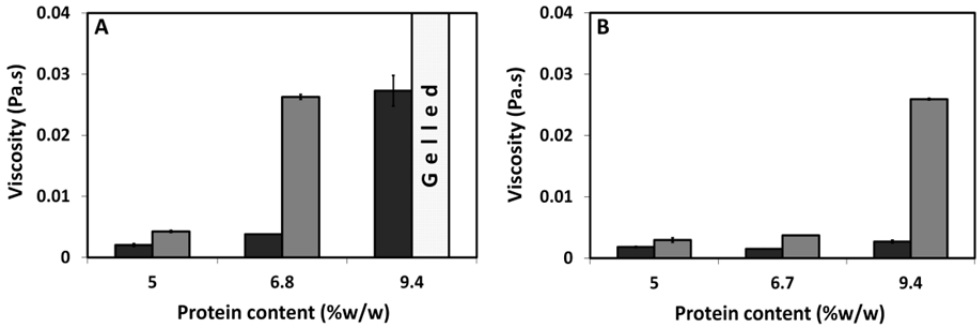

Figure 6.4 Change in the viscosities of whey protein particle dispersions (A) and whey protein isolate solutions (B) before (black) and after heat treatment (gray) at 90 °C for 30 min. Whey protein particles were prepared from a 25% (w/w) WPI solution at pH 6.8 and dispersed in a 1% (w/w) WPI solution. Viscosities of the dispersions at 20 s^{-1} are shown. Approximate volume fraction of the particles were $\Phi \sim 0.26$ for $C_{p,t}$= 5% (w/w), $\Phi \sim 0.36$ for $C_{p,t}$ = 6.8% (w/w) and $\Phi \sim 0.55$ for $C_{p,t}$ = 9.4% (w/w).

Due to the higher volume fraction of the particles, the increase in the viscosity was more pronounced at the same protein concentrations, when compared to the dispersions containing pH 5.5 particles (Fig. 6.2-A). After heat treatment viscosity increased significantly at all protein concentrations and formation of a weak gel at a total protein concentration of ~ 9.4% (w/w) was observed. The rheological data in figure 6.5 shows the storage (G′) and loss (G″) moduli of this gel as a function of strain. The gel had a scoopable structure (Fig. 6.5, insert) and a relatively small strain value (~ 0.14%) was sufficient to fracture the gel structure.

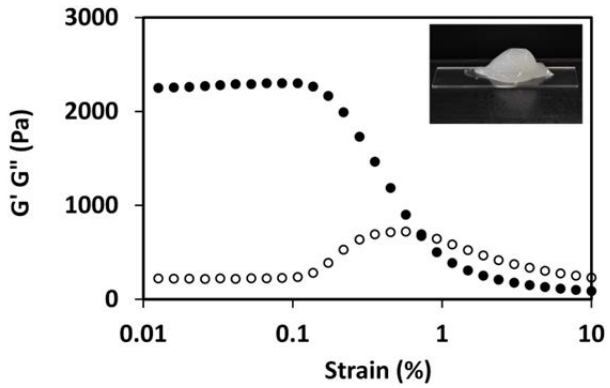

Figure 6.5 Storage (G′, solid symbols) and loss (G″, open symbols) modulus of whey protein particle dispersions at total protein content of 9.4% (w/w) after heat treatment at 90 °C for 30 min. Protein particles were prepared from a 25% (w/w) WPI solution at pH 6.8 and dispersed in 1% (w/w) WPI at a volume fraction of ~ 0.55. Insert: Appearance of the gelled sample.

Although a significant viscosity increase was observed for all protein concentrations, the reference system containing only native whey proteins showed a better heat stability (Fig. 6.4-B) in comparison to the dispersions containing protein particles prepared at pH 6.8 (Fig. 6.4-A).

The whey protein particles act as polyelectrolyte gels that may swell or shrink when dispersed in a different continuous phase, as we have also shown in Chapter 5. The internal structure and protein density of the particles were reported to influence the equilibrium swelling ratio of the whey protein gels [20-22]. The particles prepared at pH 5.5 have a much higher internal protein concentration than the particles prepared at pH 6.8, which might have decreased their swelling ratio [21]. As a result they are more heat stable compared to the particles prepared at pH 6.8. In addition, when dispersed in the same aqueous phase, compared to the pH 5.5 particles, particles prepared at pH 6.8 changes the pH of the dispersions more towards alkaline pH, something which could also influence the swelling of the particles. These observations may explain, at least partially, the difference in the heat stability of protein particles prepared at different pH values.

Heat stability at acidic pH

Dispersions of protein particles at a total protein concentration of 6.8% (w/w) were slowly acidified by addition of 1.5% (w/w) of glucono-δ-lactone (GDL), which resulted in a final pH value of 3.6 (±0.05).

Figure 6.6 shows the microstructure of the dispersions (containing protein particles prepared at pH 5.5 or pH 6.8) at different pH values during acidification. Close to pH 5.0, some aggregation was observed in the dispersions containing protein particles prepared at pH 6.8 and when the pH was further dropped to pH ~ 4.5, both types of particles showed clear aggregation. When the pH was further decreased to 3.6, aggregates of the particles were completely disassembled when they were prepared at pH 5.5 (Fig. 6.6, first row). This was also further confirmed by particle size distribution analysis, where no significant change in the particle size distribution at pH 7.0 or at pH 3.6 (Fig. 6.7-A) was found. These observations suggest that the pH-induced aggregation of protein particles prepared at pH 5.5 is reversible. Although only some moderate aggregation was observed in the CSLM micrographs (Fig. 6.6, second row) when the pH of the dispersion reached 3.6, the shift towards larger particle sizes in the particle size distribution (Fig. 6.7-B)

suggests the presence of some aggregates in the dispersion containing protein particles prepared at pH 6.8.

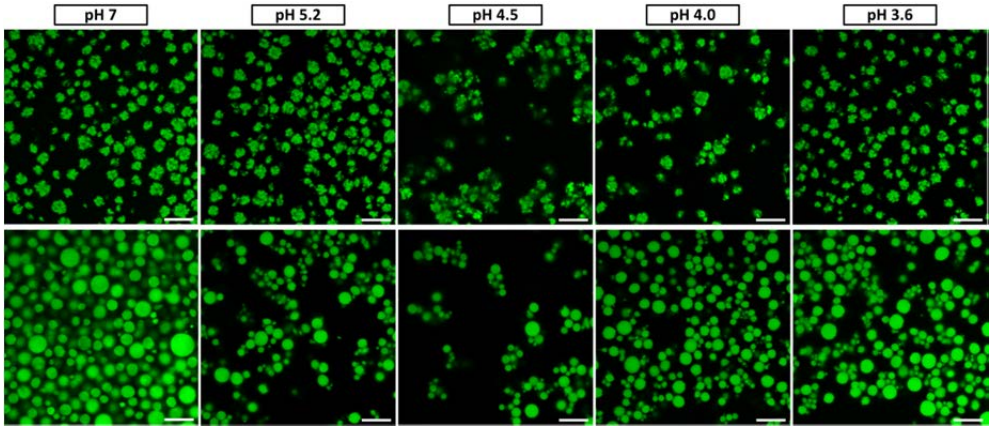

Figure 6.6 Effect of acidification by GDL on the microstructure of whey protein particles visualized by CSLM. Protein particles were prepared from a 25% (w/w) WPI solution either at pH 5.5 (first row, Φ ~ 0.15) or at pH 6.8 (second row, Φ ~ 0.35) and dispersed in a 1% (w/w) WPI at a total protein concentration of ~ 6.8% (w/w). Samples were diluted 100x and stained with Rhodamine B.

It should be noted that the volume fraction of the two types of protein particles were not the same for these measurements. Significantly higher volume fraction of the protein particles prepared at pH 6.8, compared to the particles prepared at pH 5.5 might be one of the factors influencing particle-particle interactions.

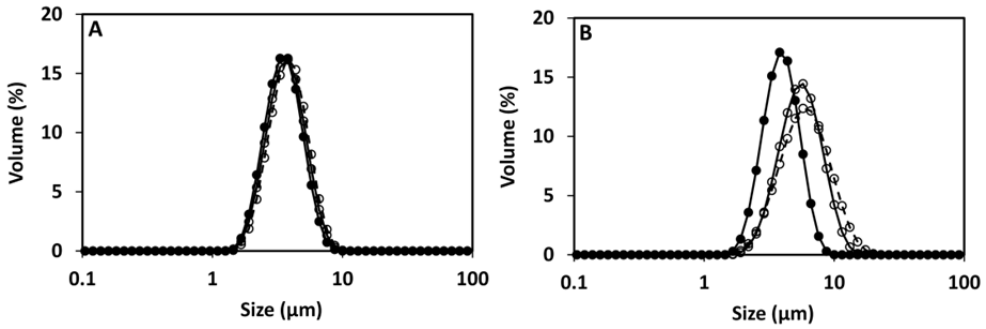

Figure 6.7 Effect of pH on the size distribution of protein particles. Protein particles were prepared from a 25% (w/w) WPI solution at pH 5.5 (A, Φ ~ 0.15) or at pH 6.8 (B, Φ ~ 0.35) and dispersed in a 1% (w/w) WPI solution at a total protein concentration of ~ 6.8% (w/w). Solid symbols: pH 7.0, open symbols with solid lines: pH 3.6 before heat treatment, open symbols with dashed lines: pH 3.6 after heat treatment.

Heat treatment of these dispersions after acidification to pH 3.6 further resulted in a small shift in the particle size distribution for the particles prepared at pH 6.8 (Fig. 6.7-B), whereas no significant change in the size distribution of the particles was observed for the particles prepared at pH 5.5 (Fig. 6.7-A).

The flow curves of these dispersions before and after heat treatment is presented in figure 6.8. Dispersions of protein particles prepared at pH 5.5 had a Newtonian viscosity profile and heat treatment did not significantly influence the viscosity (Fig. 6.8-A). Although microscopy and particle size analysis revealed particle aggregation, we do not observe higher viscosity values (when compared to the same sample at neutral pH) or shear-thinning in the dispersions of particles prepared at pH 6.8 (Fig. 6.8-B). The aggregates might be weak and already disrupted at low shear rates. After heat treatment a very small increase in the viscosity can be observed (Fig. 6.8-B).

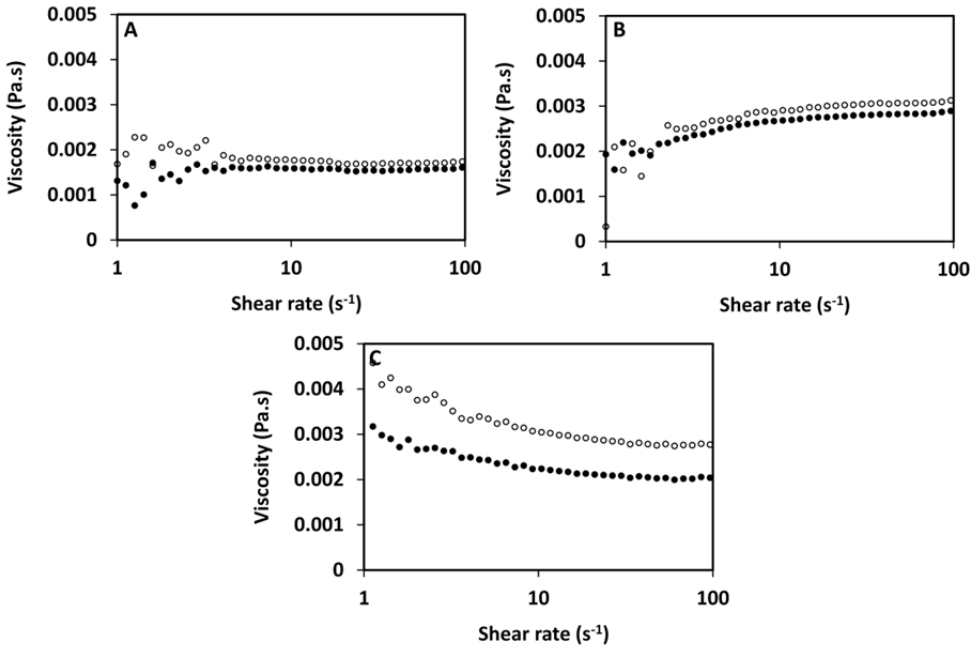

Figure 6.8 Viscosities of whey protein particle dispersions and whey protein isolate solution at pH 3.6 before (solid symbols) and after (open symbols) heat treatment at 90 °C for 30 min. Protein particles were prepared from a 25% (w/w) WPI solution either at pH 5.5 (A, Φ ~ 0.15) or at pH 6.8 (B, Φ ~ 0.35) and dispersed in a 1% (w/w) WPI solution. C: Whey protein isolate solution. All samples were prepared at a total protein concentration of ~ 6.8% (w/w).

In figure 6.8-C flow curves of the reference system containing native whey proteins at a protein concentration of 6.8% (w/w) are shown. We see an increase in the viscosity after heat treatment, most likely due to the formation of heat-induced protein aggregates, something also supported by the shear-thinning behavior. Similarly, a shear-thinning behavior in the same sample before heat treatment also suggest existence of protein aggregates prior to heat treatment.

The viscosity increase (after heat treatment) at pH 3.6 for the system consisting of pH 6.8 particles (Fig. 6.8-B) is significantly less than the viscosity increase observed for the same sample at pH ~ 7.0 (Fig. 6.4-A). This hints in the direction that the swelling of the protein particles after heat treatment might depend on the acidity of the dispersing phase. Indeed it is reported that a whey protein gel (prepared in the bulk by heat gelation) has a higher equilibrium swelling ratio in the high pH range than in the low pH range [21]. This is attributed to the sensitivity of the charge density of the gel network to pH [21, 22]. We do not observe a similar effect for the particles prepared at pH 5.5. This might be due to differences in the gel morphology between the particles.

When protein particles were prepared at pH 6.8, disulfide bonds are involved in their gelation [23, 24] and their equilibrium swelling ratio was influenced significantly when pH is lowered towards acidic pH. Due to involvement of disulfide bonds during gelation, loss of protonable groups, such as alkaline amino acids occurs, which is reported to increase the net negative charge of whey protein gels at alkaline pH [22]. This is not expected to occur for the protein gels prepared at pH 5.5, where the aggregation of proteins during gelation is mainly driven by non-covalent interactions [23].

Digestibility of whey protein particles

In vitro digestibility of the protein particles were assessed with a 3-step digestion assay: first, samples were incubated in saliva conditions that contained α-amylase as main component, then samples were exposed to gastric digestion at pH 1.5-2 and finally hydrolysis at pancreatic conditions was assessed close to neutral pH. Figure 6.9 shows the change in the relative areas, which correspond to the main whey proteins (β-lactoglobulin and α-lactalbumin), during the digestion assay. Digestion of both the reference sample (native whey protein isolate solution) and protein particles started at gastric conditions (Fig. 6.9-B).

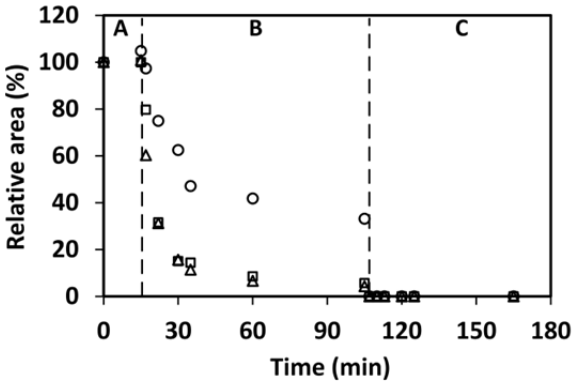

Figure 6.9 Relative area of the major whey proteins (beta-lactoglobulin and alfa-lactalbumin) as a function of digestion time. Digestion was performed in A: saliva incubation (0-15 min.), B: gastric incubation (15-100 min.) C: pancreatic incubation (100-170 min.). Samples had a total protein content of 5% (w/w): Reference (○), whey protein particles prepared at pH 5.5 (□),whey protein particles prepared at pH 6.8 (Δ).

In the gastric conditions protein particles were digested more quickly than the reference sample, which shows that they were more accessible to digestive enzymes in comparison to the native whey proteins. It is known that in their native state, major whey proteins, particularly β-lactoglobulins, are resistant to the gastric digestion (digestion by pepsin) [25-27], because hydrophobic amino acids which are more susceptible to digestion are buried inside the protein structure [27, 28]. Whey protein particles in this work were prepared through heat-induced gelation of whey proteins (20 min at 80 °C) which results in conformational changes in the tertiary and secondary structure of the proteins. As a result of these changes, functional groups such as side chain amide groups or amino acids are exposed to the surface of the particles, which might have increased the susceptibility of the protein particles to gastric digestion. Our observation is in accordance with the study of Reddy et al. [27], reporting significant increase in the digestibility of β-lactoglobulin after denaturation of proteins through a heat treatment at either 80 °C or 90 °C. In the pancreatic digestion, protein particles and native proteins were completely degraded already at the very early stage of the incubation (Fig. 6.9-C). These results show that protein particle formation did not negatively altered in vitro digestibility of the whey proteins.

Conclusions

This study has shown that replacement of native whey proteins with dense protein particles in highly concentrated systems improved or even eliminated changes, such as the increase of viscosity or gelation. Total protein concentration where no gelation after heat treatment was observed could be doubled (in comparison to native whey proteins) by the use of protein particles that are prepared close to the isoelectric point of whey proteins (at pH 5.5). Swelling of the particles may occur during heat treatment, thereby influencing the final properties of the dispersions, particularly the viscosity. Since the protein particles are polyelectrolyte gels, the swelling is expected to be pH and salt sensitive. The conditions at which the particles were prepared influences their internal structure and thereby also their swelling properties. Control over particle swelling by controlling acidity and salt concentration of the dispersing environment is expected to help to increase the heat stability of protein particle dispersions.

Acknowledgements

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Chapter 7

Whey protein particles modulate mechanical properties of gels at high protein concentrations

In this chapter, we have studied the influence of whey protein particles on the mechanical properties of whey protein isolate (WPI) gels at high protein concentrations (16-22% w/w). Incorporation of dense whey protein particles in the gel matrix, while keeping the total protein concentration constant, led to a considerably lower storage modulus (G'). By adding protein particles, the total protein concentration of the WPI gels could be increased between 25 to 55% (w/w), without increasing the G' of the gel. The large deformation properties of the WPI gels were also influenced in the presence of dense protein particles. The gels containing protein particles fractured at lower strain values than pure WPI gels, at the same protein concentration. These findings lead us to conclude that protein particles can be used to modulate mechanical properties of WPI gels and are promising candidates for the development of high protein foods with improved textural properties.

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Introduction

The ability of whey proteins to form gels under specific conditions is an important functional property of whey proteins, thereby giving several food products desirable textural and sensory characteristics [1-4]. The physicochemical properties of whey protein gels, e.g. rheological, optical and water holding properties, are mainly determined by the molecular interactions and microstructure of the gels and those can be tailored by adjusting the protein concentration, pH and NaCl concentration of the solution [2, 3, 5-8]. Heat-induced gelation of whey proteins may occur at high enough protein concentrations, at temperatures above the denaturation temperature of whey proteins. Heating whey protein solutions at lower protein concentrations leads to formation of protein aggregates. Through addition of salt or decreasing the pH towards acidic pH values, these pre-heated protein solutions may gel, which is known as cold gelation of proteins [9-11].

Additional to their functional properties, whey proteins are also used in food products, due to their high nutritional value and balanced amino acid composition [12, 13]. Several health-related benefits of high protein foods are reported. Due to the strong satiating effect of proteins, compared to carbohydrates and fats [14-18], increasing the protein concentration of processed foods gained a significant interest. It is believed that elevated protein concentrations in a diet may help to lose weight and maintain a healthy body weight. However, increased protein concentrations are often of inferior sensory properties. These negative effects of increased protein concentrations were also reported for protein gels. For example, Kangli et al.[19] studied the effect of protein concentration on the texture of soy protein gels formed through heat treatment, and found a very strong protein concentration dependence of the 'hardness' and 'toughness'. Gels formed at higher protein concentrations were described as firm, tough and could not be fractured. Similar results for soy and whey protein gels have been reported in other studies as well [11, 20-22].

One way to reduce or eliminate negative textural properties, reported for gels at high protein concentration, can be the incorporation of dense protein particles into the protein gel matrix. Mixed gels, containing protein as the continuous phase (or matrix) and oil (or other types of particles) as the dispersed phase (or filler), showing improved physical properties, have been studied extensively [23-27]. The mechanical properties of the mixed gels are determined by several factors, such as the mechanical properties of the matrix and particles, the volume fraction and size

distribution of the particles and the interaction between the matrix and particles [23-26, 28, 29]. Incorporation of protein-stabilized oil droplets into a heat-induced protein gel is reported to increase the rigidity of the gel, as a result of interaction between the proteins in the matrix and the proteins on the surface of the particles [23, 25, 28]. In this case, the particles are classified as 'active fillers'. Oppositely, if the oil droplets were covered by a non-ionic surfactant, lower gel rigidities were reported by increasing the volume fraction of particles [30]. This was attributed to the weak interaction between the matrix and the filler particles and in this case the particles are classified as 'inactive fillers'.

Mixed protein gels, containing protein aggregates or particles, were also shown to improve textural and mechanical properties [31-36]. It was reported that in the presence of whey protein aggregates, softer whey protein gels were formed, in comparison to the whey protein gels containing no aggregates or less amount of aggregates [31, 34]. Recently, whey protein particles (average diameter ~ 70 μm) formed under high shear treatment, referred as 'microparticulated whey proteins', were incorporated in WPI gels and the effects on the textural properties were studied at relatively high protein concentrations (15-20% w/w) [35]. The mixed protein gels were reported to be weaker than WPI gels without protein particles at the same total protein concentration. Increasing the volume fraction of the whey protein particles led to a further decrease in the gel strength. However, due to the large swelling capacity and thereby low internal protein density of those particles, the increase in total protein concentration of the gels was limited.

In Chapter 4, we have shown that dense protein particles, having approximately 39% (w/v) internal protein concentration can be formed by heat-induced gelation of whey proteins at pH 5.5. These particles are rather compact, heat stable and do not significantly swell upon heat treatment. These properties of the particles may be an advantage to modulate the rheological properties of protein gels at high protein concentrations. Thus, this chapter investigated the influence of dense whey protein particles on the mechanical properties of whey protein isolate gels (WPI). Mixed systems at different particle volume fractions and whey protein concentrations were prepared. These systems were heat-treated and based on their gelling behavior a state diagram was prepared. The visco-elastic properties of the gels, with and without added protein particles, but at the same total protein concentration were determined. To study the influence of particle type, protein particles formed at pH 6.8 were also included in this study. These particles have a

significantly lower internal protein concentration (~ 18.5% w/v) than the particles prepared at pH 5.5 (~ 39% w/v).

Experimental

Preparation of whey protein particles

Protein particles were prepared according to the method described in Chapter 4.

Optical microscopy

Optical microscopy of the samples taken from different steps during the preparation of protein particles were done according to the method described in Chapter 4.

Preparation of mixed systems

Protein particles were initially dispersed, at a known volume fraction, in a 1% (w/w) WPI solution. Dispersing was done by 5 min mixing with a high speed mixer (9500 RPM) and then homogenization at 150 bar (6 passes). These dispersions were mixed with either 12, 16, 20 or 24% (w/w) WPI solutions, with a weight ratio 1:1 and stirred for at least 30 min prior to analysis.

Volume fraction of particles

Volume fraction of the particles in the mixed systems were determined according to the method described in Chapter 2

Determination of particle size distribution

The particle size distribution of the protein particles was measured according to the method described in Chapter 2.

Determination of protein content

The protein concentration of the samples was determined either by UV spectrophotometer (Cary 50 Bio, Varian) or by DUMAS. For UV measurements, the absorbance of the samples at 280 nm was measured and the protein concentration was calculated from a calibration curve of WPI solutions at known concentrations at the same wavelength. For DUMAS, a FLASH EA 1112 N/protein analyzer (Thermo Scientific, Waltham, US) was used to determine total nitrogen content of the samples. Nitrogen values were multiplied by 6.38 to calculate the total protein content of the sample (% w/w).

Heating experiments

Approximately 20 ml from each sample was transferred into a glass tube and closed tightly. Samples were heated at 90 °C for 30 min in a temperature-controlled heating plate (RT15, IKA Werke, Germany). Samples were stirred mildly by a magnetic stirrer during heat treatment to avoid particle sedimentation and to facilitate the heat transfer. The experiments were performed in duplicate.

State diagram

The state diagram for the mixed systems, containing native whey proteins in the continuous phase and dispersed protein particles, was determined, based on the gelation behavior of the samples after a heat treatment.

Rheological measurements

Rheological measurements were performed using a Paar Physica MCR 300 Rheometer (Anton Paar, Graz, Austria) with a Couette geometry (CC17/T200/SS, cup diameter: 18.08 mm, bob diameter: 16.66 mm). For the measurement, approximately 5 ml of each sample was placed in the cup and the surface of the sample was covered with paraffin oil to avoid evaporation of water. The viscosity of the samples was measured over the shear rate range 1-1000 s^{-1} at 25 °C. Time sweep oscillatory measurements were performed at a constant frequency of 1 Hz and a strain amplitude of 1%. The temperature was gradually increased from 25 °C to 80 °C at a heating rate of 2.75 °C/min, kept constant at 80 °C for 30 min, and decreased from 80 °C to 25 °C at a cooling rate of 1.83 °C/min. After this heating profile, the temperature was kept constant at 25 °C for 30 min. Subsequently, a strain sweep was performed by increasing the strain from 1% to 1000% in 20 min, at a constant frequency of 1 Hz. All measurements were performed in duplicate.

Scanning electron microscopy (SEM)

Mixed gels of whey protein and protein particles were prepared by heating (80 ± 0.5 °C for 30 min) in the cylindrical glass tubes that were coated with Sigmacote (Sigma-Aldrich, St. Louis, USA). The sample preparation and analysis by SEM was done following the procedure described in Chapter 4 for WPI gels (see materials and methods). An additional fixation, with 1% (w/w) osmium tetroxide (OsO4), was applied over night, after the gels were already treated with gluteraldehyde.

Results and discussion

State diagram

In figure 7.1, the state diagram of aqueous dispersions of protein particles (prepared at pH 5.5) and native whey proteins is shown. The samples were defined as gel, based on the rheological measurements, when the storage modulus (G') was larger than the loss modulus (G''). It is observed that for the same total protein concentration, e.g. for 16% (w/w), an increase in the amount of native whey protein shifts the system towards gelation, whereas increase in the amount of particles shifts the system towards the liquid region.

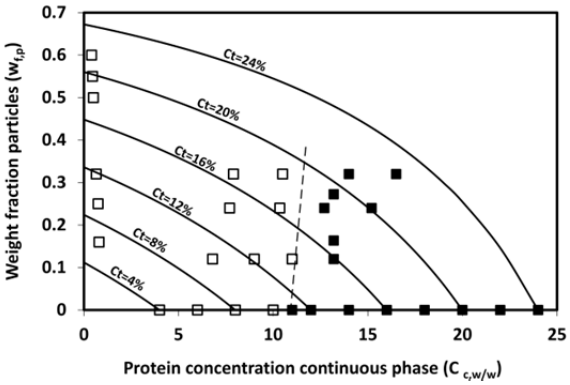

Figure 7.1 The state diagram of the mixed systems containing whey protein particles as dispersed phase and native whey proteins as continuous phase. The samples were heated at 90 °C for 30 min. The gelled samples are indicated by the solid symbols, while the non-gelled samples are indicated by the open symbols. The solid lines indicate systems that have an equal total protein concentration (Ct). The dotted line separates the regions of the state diagram that are gelling and non-gelling. The protein particles were prepared from a 25% WPI solution at pH 5.5 and the final pH of the mixed systems was 6.8.

From figure 7.1, it follows that even at a very high weight fraction (~ 0.65) of only protein particles, the system does not form a gel. Therefore, the formation of a particle gel, that is a gel network formed through aggregation of particles [29], is not expected in these mixed systems. This indicates that the proteins in the continuous phase are responsible for the gel formation in these mixed systems. In order to obtain a gelled system, the concentration of the protein in the continuous phase should be above the critical gelling concentration, which is around 11% (w/w) protein for whey proteins at neutral pH. A dotted line is drawn between the liquid and gel region in the state diagram. If particles do not have an influence on the gel

formation, the line between the liquid and gel regions would be a vertical line. Whereas, if the particles interact with each other, or with the continuous phase, thereby contributing to the gel network, the dotted line would not be vertical. It is observed that the dotted line is rather vertical (Fig. 7.1), which suggest that incorporation of particles do not influence the gel formation. This point will be later addressed in more detail.

Heat stability of mixed systems

The viscosity changes in the mixed systems that did not form a gel after heat treatment, was analyzed with a shear sweep test in the shear rate range 1-1000 s^{-1}. They typically showed Newtonian behavior and the viscosities of different systems at the same volume fraction of particles were compared at 20 s^{-1} (Fig. 7.2). For the same volume fraction of particles, the increase in the total protein concentration (as shown on the horizontal axis in Fig. 7.1) was obtained by increasing the amount of WPI in the continuous phase.

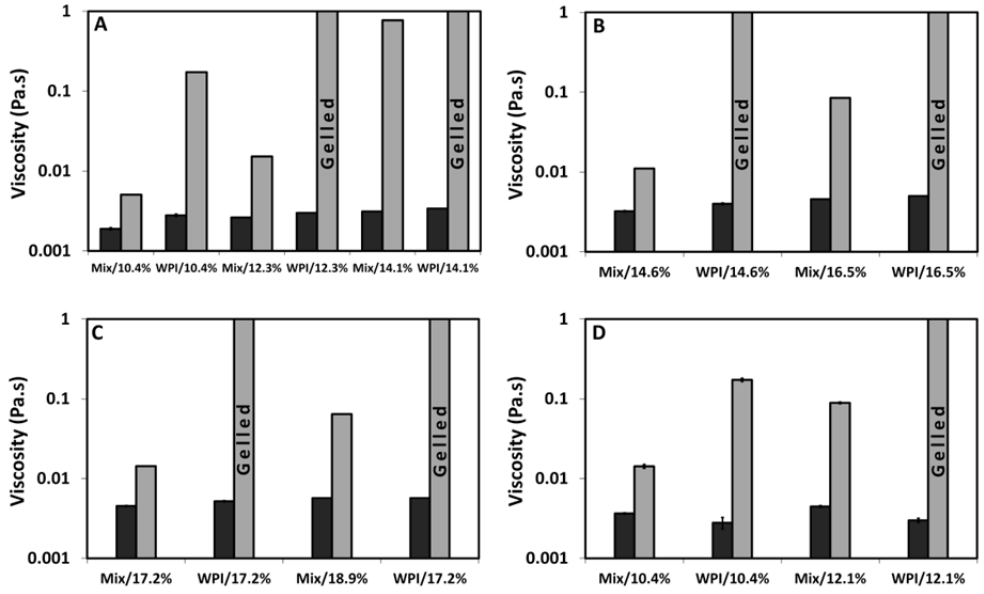

Figure 7.2 The viscosities of the mixed systems (Mix), containing whey protein particles (prepared at pH 5.5 (A,B,C) and pH 6.8 (D) compared to pure systems (WPI), containing only native whey proteins and no added particles, at the same total protein concentration. The vertical bars represent the viscosity at 20 s^{-1}. The black bars represent the viscosity before heating, while the gray bars represent the viscosity after heating. Whey protein particles are dispersed at volume fractions (Φ) of 0.11 (A), 0.22 (B), 0.30 (C), 0.24 (D).

For all particle volume fractions, an increase in the viscosity was observed after heat treatment, and this increase became more pronounced at increasing WPI concentration in the continuous phase. As shown in Chapter 6, the liquid systems containing protein particles prepared at pH 5.5 (Φ between 0.30 to 0.60) did not show any viscosity change upon heat treatment under similar conditions. Thus, the observed viscosity increase must be due to denaturation and aggregation of whey proteins present in the continuous phase. Obviously, due to the presence of native whey proteins in the continuous phase, mixed liquid systems of whey proteins and whey protein particles prepared at pH 5.5 were less heat stable compared to the dispersions containing mainly protein particles. The system containing only whey proteins, but no added particles either had a larger viscosity increase in comparison to mixed systems (at the same total protein concentration) or they formed a gel after heat treatment (Fig. 7.2).

We have observed an opposite effect for the mixed systems containing whey protein particles prepared at pH 6.8. As shown in figure 7.2-D, mixed systems containing particles at a volume fraction of ~ 0.24, did not form a gel at a total protein concentrations ~ 12% (w/w), whereas pure WPI systems gelled at this protein concentration, after heating. In the previous chapter, swelling of the particles during heat treatment was shown to cause a large increase in the viscosity of the dispersions and the dispersions gelled at a total protein concentration of ~ 9.4% (w/w). The swelling of the particles after heating was confirmed by a shift found in the particle size distribution towards larger sizes (Chapter 6).

In figure 7.3, we compare the particle size distribution of protein particles prepared at pH 6.8, dispersed either in 1 or 8% (w/w) WPI solution. We can only see a small increase in particle size after heating, in comparison to the previous study, indicating that particle swelling might have been suppressed in the mixed systems. This observation is also in agreement with the results presented in Chapter 3, showing less pronounced increase of particle size after heat treatment when larger amount of Na-caseinate was present in the continuous phase. There can be a few reasons explaining the reduced swelling: firstly, the particle might swell less after heat treatment, as a result of increased osmotic pressure in the continuous phase due to a higher concentration of protein. Secondly, a higher concentration of protein influences the pH and ionic strength, which might also contribute to the suppressed particle swelling. Thirdly, as reported in the previous chapters, protein particles changed the pH of the environment to a small extend, which may

influence the swelling ratio. This was not observed in the mixed systems containing a larger amount of whey proteins in the continuous phase, presumably due to the buffering effect of the protein.

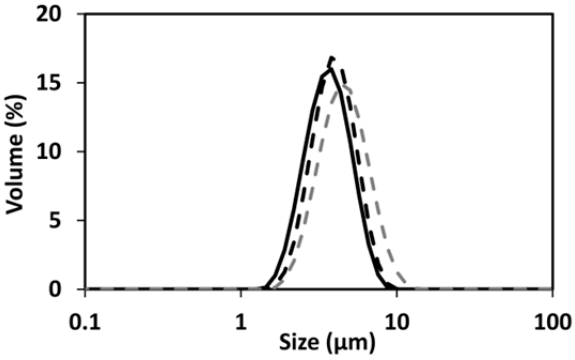

Figure 7.3 Particle size distribution of protein particles before (continuous line) and after heating (dashed lines) at 90 °C for 30 min. Protein particles were prepared from a 25% WPI solution at pH 6.8 and dispersed either in a 1% (w/w) WPI (dashed line in gray) or 8% (w/w) WPI (dashed line in black) solution.

Therefore, it is likely that contribution of particle swelling to the viscosity increase observed in the mixed systems after heat treatment is rather small. In this case, the viscosity increase after heating is most likely due to aggregation of whey proteins present in the continuous phase.

Visco-elastic properties of mixed systems

Small deformation properties of the mixed systems, containing protein particles (prepared either at pH 5.5 or pH 6.8) and whey protein isolate as the continuous phase, that formed a gel upon heat treatment were investigated at this part. In figure 7.4, the temporal evolution of the storage modulus (G') and tan δ (G''/G') of the mixed systems (with particles prepared at pH 5.5) and pure whey protein gels (no particles added) at the same total protein concentration are shown. For both systems, G' increases rapidly with increasing temperature and levels off to reach a stable plateau value upon cooling to 25 °C. When increasing the temperature to 80 °C, the whey proteins denature and their hydrophobic groups, which are initially buried inside of the proteins, become exposed. Due to interaction between these hydrophobic groups and formation of intermolecular disulfide bonds a protein network is formed [7]. The G′ continues to increase during the cooling step. We

notice that, for the whey protein gels containing protein particles, the increase in the G' upon cooling is more significant at all particle volumes, compared to the pure WPI gels (Fig. 7.4-A, -B and -C).

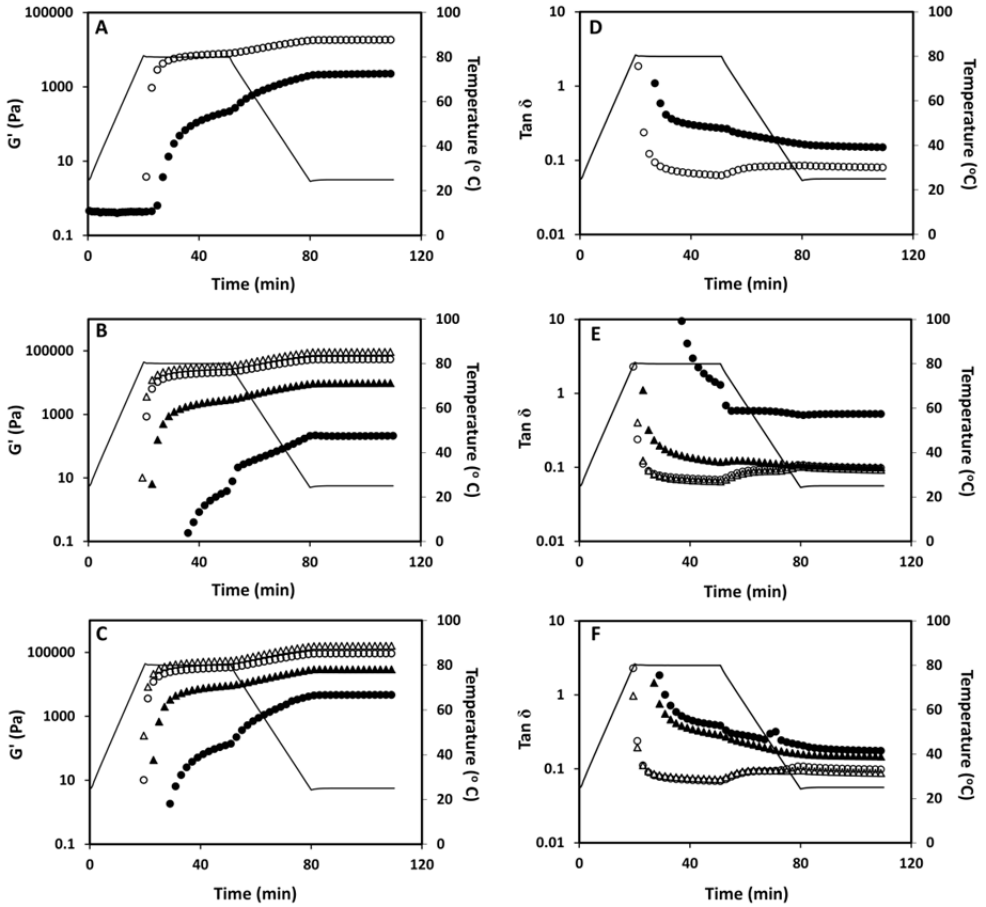

Figure 7.4 The temporal evolution of the storage modulus G′ and tan δ for WPI gels (open symbols) and WPI gels filled with whey protein particles prepared at pH 5.5 (solid symbols) at volume fractions (Φ) of ~ 0.11 (A and D), 0.22 (B and E) and 0.30 (C and F). The total protein concentration of the samples in A (and D) was 16% w/w, in B (and E) 18.3% w/w (circles), 21% w/w (triangles), in C (and F) 20% w/w (circles), 22% w/w (triangles). The heating profile is shown by the solid line. All measurements were performed at a frequency (f) of 1 Hz and a constant strain (γ) of 1%.

In figure 7.4 (D,E,F), the evolution of tan δ as a function of time is also shown. For the pure WPI gels, containing no protein particles (open symbols), we observe a

strong decrease in tan δ, when the temperature reached 80 °C, followed by a slow decrease when the temperature was kept at 80 °C. During cooling, a small increase in tan δ is observed and it reached a stable plateau value at 25 °C, at a value, that is significantly lower than 1, showing the elastic character of the gels. For the WPI gels containing protein particles, tan δ slowly decreases after the gelation point and reaches a stable plateau value at 25 °C. All the WPI gels containing protein particles at varying volume fractions have smaller tan δ values compared to the pure WPI gels at the same total protein concentration, showing that WPI gels are more elastic in the absence of protein particles.

For all volume fractions of the particles studied, the final G' values of the mixed systems were significantly lower compared to the G' values of the pure WPI gels at the same total protein concentration. G' became approximately one order of magnitude smaller with addition of protein particles at a volume fraction of 0.11 (Fig. 7.4-A). When the volume fraction of the particles was further increased to either 0.22 or 0.30, the decrease in the storage modulus became even more pronounced (Fig. 7.4-B and 7.4-C). When the whey protein gels are compared at equal particle volume fraction, an increase in the G' with increasing protein concentration can be observed. This is due to a higher concentration of whey proteins in the continuous phase.

In table 7.1, we present the protein concentration of pure WPI gels, that would have a G' value equal to that of a mixed gel. For instance, a mixed gel containing protein particles at a volume fraction of ~ 0.11 and having a total protein concentration of about 16% (w/w), as shown in the first row in the table, has a G' of 2250 Pa (Fig. 7.4-A). A pure WPI gel having a similar G' value (2250 Pa), would have a total protein concentration of ~ 12.8% (w/w). This means, addition of protein particles, for this specific mixed system, results in a 25% relative protein gain at the same G' value. As we can see from the other estimated protein gains for the same G' values, a pure WPI gel has significantly lower protein concentration in the absence of protein particles and addition of protein particles led to a protein gain between 25-55% (w/w) relative to the pure WPI gels at the same G'. When the particle volume fraction was increased and the protein concentration in the continuous phase was decreased, the relative protein gain for the same G' value became considerably higher. This implies that the ratio between whey protein particles to pure whey proteins is an important parameter determining visco-elastic behavior of these mixed gels.

Table 7.1 Increase in the total protein concentration of whey protein gels at the same G' upon addition of protein particles

Particle volume fraction (Φ)	Total protein concentration mixed gels (% w/w)	Total protein concentration WPI gels at the same G'* (% w/w)	Relative protein gain at the same G'
0.11[b]	16.0	12.8	25%
0.22[a]	18.3	11.8	55%
0.22[b]	20	14.7	35%
0.30[a]	20	13.7	45%
0.30[b]	22	17.0	30%

[a] Contribution of continuous phase to the total protein concentration is ~ 10 % (w/w)

[b] Contribution of continuous phase to the total protein concentration is ~ 12% (w/w)

* Total protein concentration of WPI gels that would give the similar G' value as in the mixed gels

Mixed systems were prepared at different particle to WPI ratios. For instance, the mixed system shown in the first row contains a total protein concentration of 16% (w/w), to which protein particles contribute about 4% (at volume fraction ~ 0.11) and WPI (continuous phase) contributes 12% protein.

Similar dependency of large deformation properties for mixed gels of whey protein aggregates and whey proteins was also reported [34]. In a recent work, also weakening of the WPI gels was observed by addition of whey protein particles, that were formed under high shear application [35]. The authors reported a maximum protein gain of only 15% (w/w). This is much lower than the protein gain obtained in our work. One reason might be that the internal protein concentration of the protein particles used in this study is much higher than the particles used in the reported study.

In gelled systems filled with protein particles prepared at pH 6.8, similar results were obtained as found for the gelled systems filled with particles prepared at pH 5.5. For example, the visco-elastic properties of WPI gels containing pH 6.8 protein particles at a volume fraction of 0.30 is shown in figure 7.5. The G' of mixed gels were lower, compared to the G' of WPI gels without protein particles, at the same total protein concentrations. Likewise, this effect became smaller, when the whey protein concentration in the continuous phase was increased (Fig. 7.5, solid triangles). It should be noted that, due to the lower internal protein concentration (18.5% w/v) of the protein particles prepared at pH 6.8, the relative protein gain (which was between 8 to 16%) at the same G' was not very high in this case.

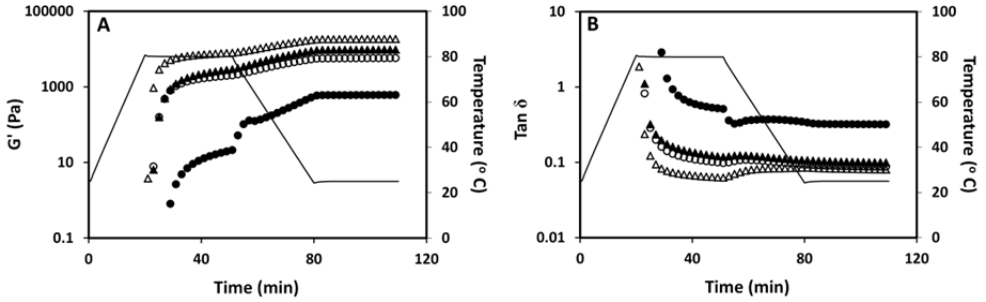

Figure 7.5 The temporal evolution of the storage modulus G′ (A) and tan δ (B) for WPI gels (open symbols) and WPI gels filled with whey protein particles prepared at pH 6.8 (solid symbols) at volume fraction (Φ) of ~ 0.30. The total protein concentration of the samples was 14% w/w (circles), 16% w/w (triangles). The heating profile is shown by the solid line. All measurements were performed at a frequency of 1 Hz and a constant strain (γ) of 1%.

Large deformation properties of the WPI gels containing protein particles were determined by a strain amplitude sweep from 1 to 1000%. Pure WPI gels (without particles), having a protein concentration equal to the mixed gels, stayed in the linear visco-elastic region until strain values over 100%.

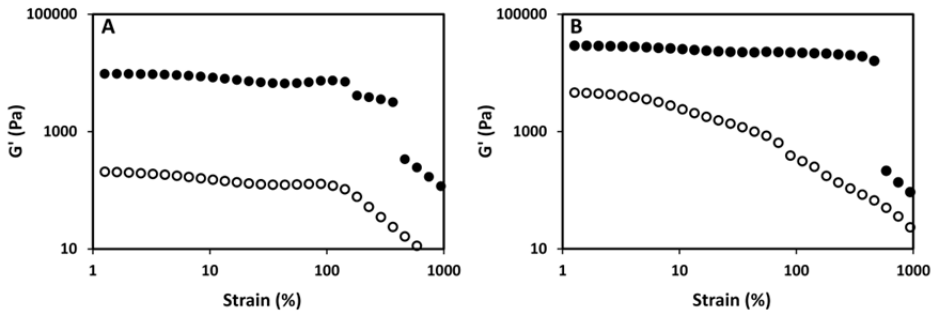

Figure 7.6 Large deformation properties of WPI gels in the presence of whey protein particles prepared at pH 5.5 (A) and pH 6.8 (B). Volume fraction (Φ) of protein particles were ~ 0.22 (A) and 0.24 (B). Protein concentration of the mixed gels for A was 18.3% w/w (open symbols) and 21% w/w (solid symbols) and for B was 13.5% (open symbols) and 15.5% w/w (solid symbols). The protein contribution of the continuous phase to the total protein in the mixed gels was either 10% (open symbols) or 12% (solid symbols), as explained in table 1.

The fracture point of WPI gels at this protein concentrations could not be measured accurately, because the maximum torque of the rheometer was reached during the

measurements, before the gels were fractured. Therefore, we present the data on WPI gels containing protein particles, prepared either at pH 5.5 (Fig. 7.6-A) or pH 6.8 (Fig. 7.6-B). We can see that for both mixed systems, the gels were in the linear visco-elastic regime, until a strain value of ~ 10-15 %, and then a small increase in the storage modulus was observed, before the gels were fractured at ~ 150 %. We note that, lower fracture strain values were observed, when the volume fraction of the particles was increased at similar continuous phase concentrations (data not shown).

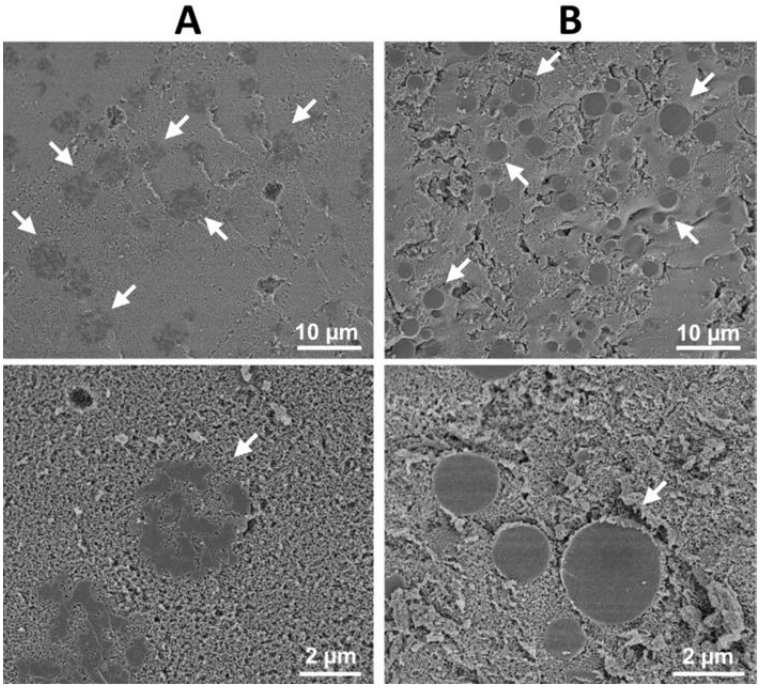

Figure 7.7 Microstructure of the mixed gels of WPI and whey protein particles as observed by SEM. Protein particles were prepared from a 25% WPI solution at either pH 5.5 (A) or pH 6.8 (B). Protein concentration of the continuous phase is 13% (w/w) and the volume fraction of particles (Φ) is 0.15.

It was shown that whey protein particles, formed under high shear application, showed aggregation during heat treatment [32, 33]. This aggregation was reported to influence the final rheological properties of the protein gels, formed in the presence of protein particles. To secure that the observed changes in the visco-elastic properties of the protein gels in the current study was not influenced by any type of particle aggregation, we have analyzed the mixed gels by scanning electron

microscopy (SEM). Gel samples were freeze-fractured and critical point dried prior to SEM analysis. The darker and denser domains in the SEM micrographs, as pointed out by the arrows, are the protein particles prepared at pH 5.5 (Fig. 7.7-A) and prepared at pH 6.8 (Fig. 7.7-B), embedded in the whey protein gel matrix. No aggregation of the particles could be observed in the gel samples. It is also clear from the micrographs that protein particles, either prepared at pH 5.5 or at pH 6.8, have a higher density than the gel matrix, in which the total protein concentration is about 13% (w/w).

Interaction between particles and continuous phase

When particles strengthen the gel matrix, where they are dispersed in, they are classified as 'active' fillers [26, 28-30]. Oppositely, if the particles weaken the gel matrix, they are classified as 'inactive' fillers. In this case, the particles only have a weak or even no interaction with the surrounding matrix. As a result, the particles behave as 'holes' in the gel matrix, thereby lowering the elastic modulus (G') of the matrix. In the first part of this chapter, we have shown that at equal protein concentration, significantly lower G' values were obtained in WPI gels containing dense whey protein particles, in comparison to the pure WPI gels containing no added particles. In order to investigate whether the particles act as active or inactive fillers, the mechanical properties of a gel matrix with and without particles were compared. Care was taken that the protein concentration in the continuous phases of both systems, were as close to each other as possible. Taking into account the excluded volume effects upon particle incorporation, mixed gels at increasing particle volumes, that would contain the same protein concentration in the continuous phase, were prepared. It should be noted, that the volume fraction of the particles was determined using the Einstein viscosity expression after measuring the kinematic viscosities of sufficiently diluted particle dispersions. Therefore, there might be approximately 5% variation from the actual particle volume. Additionally, as shown in Chapter 5, molecules smaller than the pore size of the protein particles might diffuse inside the particles and reversely protein leakage from the particles, depending on the pH, might also occur. Due to these limitations, calculated protein concentration in the continuous phase might differ from the actual protein concentration. Therefore, additional to the calculated values, the protein concentration in the supernatant was measured by a UV-spectrophotometer after the protein particles were sedimented by centrifugation.

As can be seen in table 7.2, the calculated and measured protein concentrations for the continuous phase of the mixed systems are satisfactorily comparable.

Table 7.2 Protein concentration of the continuous phase for mixed gels at different particle volume fractions

Particle volume fraction (Φ)	Protein concentration continuous phase (% w/w) [a]	Protein concentration continuous phase (% w/w) [b]
0.11	13.22	ND
0.15	13.21	13.24
0.25	13.20	13.10

[a] Calculated protein concentration in the continuous phase

[b] Measured protein concentration in the continuous phase

ND: not determined

In figure 7.8, the G' of the mixed systems, presented in table 7.2, are compared to the G' of a WPI gel at 13.2% (w/w) total protein concentration, representing the continuous phase.

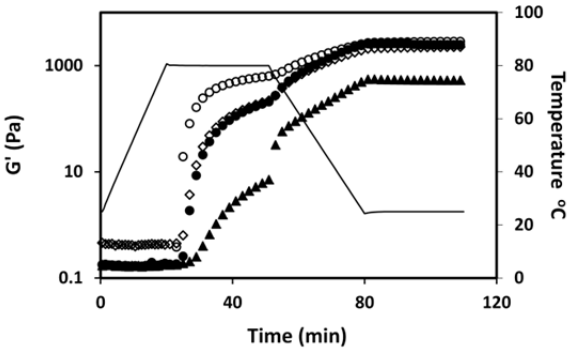

Figure 7.8 Temporal evolution of storage modulus (G') of WPI gels containing no protein particles (○) or protein particles at volume fractions (Φ) of 0.11 (◊), 0.15 (●) and 0.25 (▲). Continuous line shows the evolution of temperature (°C). Measurements were performed at a frequency (f) of 1 Hz and a constant strain (γ) of 1%.

For lower particle volume fractions (Φ), either at 0.11 or 0.15, there was a small decrease in the final G', whereas when the particle volume fraction was increased to 0.25, we have observed a pronounced decrease in the final G', suggesting that protein particles do not strongly interact with the proteins in the gel matrix. These

results are in agreement with the results presented in the previous sections. As we have shown, gelation of the mixed systems after heating was observed only when the protein concentration of the continuous phase was higher than 9-10% (w/w) protein. This value is close to gelling threshold of whey proteins at pH 6.8. If protein particles were strongly interacting with the proteins in the continuous phase, gelation of the mixed systems at much lower continuous protein concentrations than 9% (w/w) would be expected. As covered in a greater detail in a recent review paper on mixed gels containing oil droplets, several studies have shown that addition of active fillers, for example protein-stabilized oil droplets, to a WPI solution at relatively lower protein concentrations resulted in gelation and significantly higher storage modulus values [29].

The influence of oil droplets on the small and large deformation properties of a protein gel matrix has been studied extensively [24, 26, 28, 30, 37-39]. It was shown, that when oil droplets were stabilized with proteins, there was a strong interaction between the proteins on the oil surface and proteins in the continuous phase which resulted in an increased storage modulus of the gels [26, 30, 39]. Influence of whey protein particles on the mechanical properties of protein gels (β-lactoglobulin or WPI) were also reported in a few studies [32, 33, 35]. These studies have concluded that protein particles were active fillers, because they had increased the rigidity of the gel matrix. Good binding properties between matrix and protein particles was also shown by SEM analysis of the WPI gels containing whey protein particles prepared under high shear [35]. These observations are to be expected, because the proteins on the surface of the particles may interact with the proteins in the gel matrix. Our results suggest, that protein particles in the current work, behave as inactive fillers.

The surface properties of the particles is an important factor, which influences how the particles interact with the matrix. In the current study, protein particles were prepared through a 2-step emulsification method, which included usage of an oil-soluble emulsifier (PGPR), as presented in Chapter 2. It is expected that after removal of the excess of the oil by centrifugation, PGPR is still present on the surface of the particles. Indeed, characterization of the particles by CLSM (Chapter 2 and 3) and SEM (Chapter 4) have suggested presence of oil on the surface of the particles. Presence of PGPR on the particle surface, in addition to the proteins, might influence the interaction between the protein particles and proteins in the

gel matrix. For the protein gels, containing oil droplets, it was shown that the surface properties of the droplets have a significant effect on the interaction between the particles and the gel matrix [30, 38, 40, 41]. Oil droplets containing a mixture of protein and oil-soluble surfactants on their surface, such as lecithin, showed a rather weak interaction with the proteins in the gel matrix, resulting in a small decrease in the gel rigidity [41].

The above results still needs to be interpreted with caution. We have observed that the G' of the WPI gels is very sensitive to the small changes in the protein concentration, due to a strong power law relation between G' and protein concentration [42]. For example, the decrease in the protein concentration from 13% (w/w) to 12% (w/w) resulted in G' values of 2300 Pa and 100 Pa, respectively. Therefore, the decrease observed in the G' after addition of protein particles at a volume fraction of 0.25, as shown in figure 7.8, may be within the experimental error. Therefore, it is not possible to draw the conclusion that the particles are completely inactive fillers with certainty. However, it would be reasonable to conclude that even when there is an interaction between the protein particles and the protein matrix, this interaction cannot be very strong.

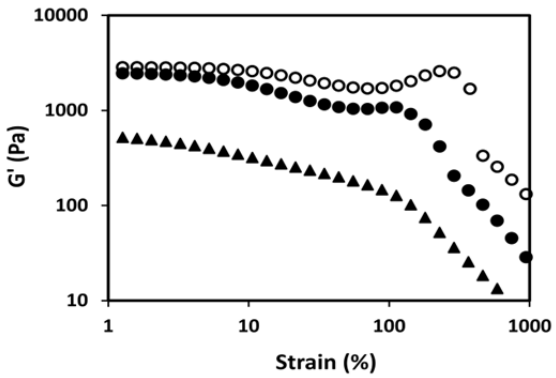

Figure 7.9 Large deformation properties of WPI gels containing no protein particles (○) at 13.2% (w/w) total protein or containing protein particles at volume fractions (Φ) of 0.15 (●) and 0.25 (▲).

Figure 7.9 compares the fracture of the mixed gels at increasing particle volume fraction with the fracture of the gel matrix. For the gel matrix, we see a linear-viscoelastic behavior until a strain value of ~ 50-100% and then an increase in the storage modulus (strain hardening) before the gels fractured at ~ 300% strain. The

gels in the presence of protein particles fractured at lower strain values (~ 100%). The strain hardening was also less pronounced at a particle volume fraction (Φ) of 0.15 and was not observed when the volume fraction of the particles was further increased to 0.25. A similar observation was reported for the WPI gels containing protein-stabilized oil droplets [30]. Before fracture occurs, due to resistance of the network against disentanglement, strain hardening occurs in protein gels. WPI gels consist of strands that can extend with increasing strain rates and structural rearrangements may take place before the fracture of the gel [43, 44], which is probably not very pronounced in a WPI gel containing protein particles, due to less connectivity in the protein network.

Conclusions

Development of negative sensory properties, such as tough and unfracturable texture formation, was reported for the protein gels at high protein concentrations. In the current work, it was shown that dense protein particles may be used to improve the mechanical properties of whey protein gels at high protein concentrations. WPI gels had significantly lower storage moduli (G') in the presence of dense protein particles, when compared to the gels containing no added protein particles at the same total protein concentration. Additionally, WPI gels containing protein particles were fractured at much lower strain values, than WPI gels without particles. Our results suggest that there is not a strong interaction between protein particles and the proteins in the gel matrix, which might be due to the surfactant layer present on the surface of the particles. Due to a strong power law relation between the G' and the protein concentration of the WPI gels, small variations in the calculated protein concentration resulted in large variations in the G' values. Therefore, it is not possible to draw the conclusion that the particles are completely inactive fillers with certainty. However, we can conclude that if there is an interaction between the protein particles and the protein matrix, this interaction cannot be very strong. The results presented in this chapter suggest that dense protein particles can be useful for the development of high protein foods having desired textural properties.

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Chapter 8

General Discussion

Introduction

Preparation of high protein foods appealing to consumers is a challenging task, due to negative effects of increased protein concentrations on product characteristics, such as heat stability and sensory properties. Several approaches, including addition of other components and modification of proteins by heat or enzyme, have been investigated to solve some of these problems [1-9]. Although promising results were presented by some of the suggested methods, protein concentrations reported were rather low and the effects at higher protein concentrations still remain uncertain, particularly concerning the heat stability. One way to increase the protein concentration of foods, without influencing the other properties of the product, is the use of pre-fabricated protein structure elements. An example of such a structure element can be dense protein particles with controlled size, internal protein concentration and surface properties. When the particles are small enough, their effect on the food texture will be independent of their internal protein concentration and also protein source. Thus the effects on food texture can be controlled largely through the surface and mechanical properties of the particles and their interactions with the food matrix.

In this thesis, dense whey protein particles formed through heat gelation of whey proteins were investigated as a possible tool for development of high protein foods. In figure 8.1, the content of the thesis is summarized in a graphical presentation. In the first part of the thesis, we have presented a rather simple method to prepare protein particles with high internal protein concentration. In the second part, physical properties of the protein particles and their dispersions were studied. The last part of the thesis focused on applications, in particular heat stability and textural aspects of the systems, that contain protein particles at high protein concentrations.

In this last chapter, we put the findings of the previous chapters in perspective, by evaluating the successes and limitations of the dense protein particles, as a possible approach for development of high protein foods. Firstly, preparation of protein particles and their physical properties will be discussed. Besides the method described in this thesis, there are several other ways to prepare protein particles with varying properties and functionality. We will review these other methods, and discuss a range of possible applications of such particles, apart from their use in high protein food systems. Additionally, we will present some data on the sensory properties of high protein systems, that contain protein particles and show

that mixed systems of native proteins and protein particles allow formulation of high protein foods with good sensory properties. In the final part, we will comment on possible future research and the relevance of the findings in this thesis regarding applications.

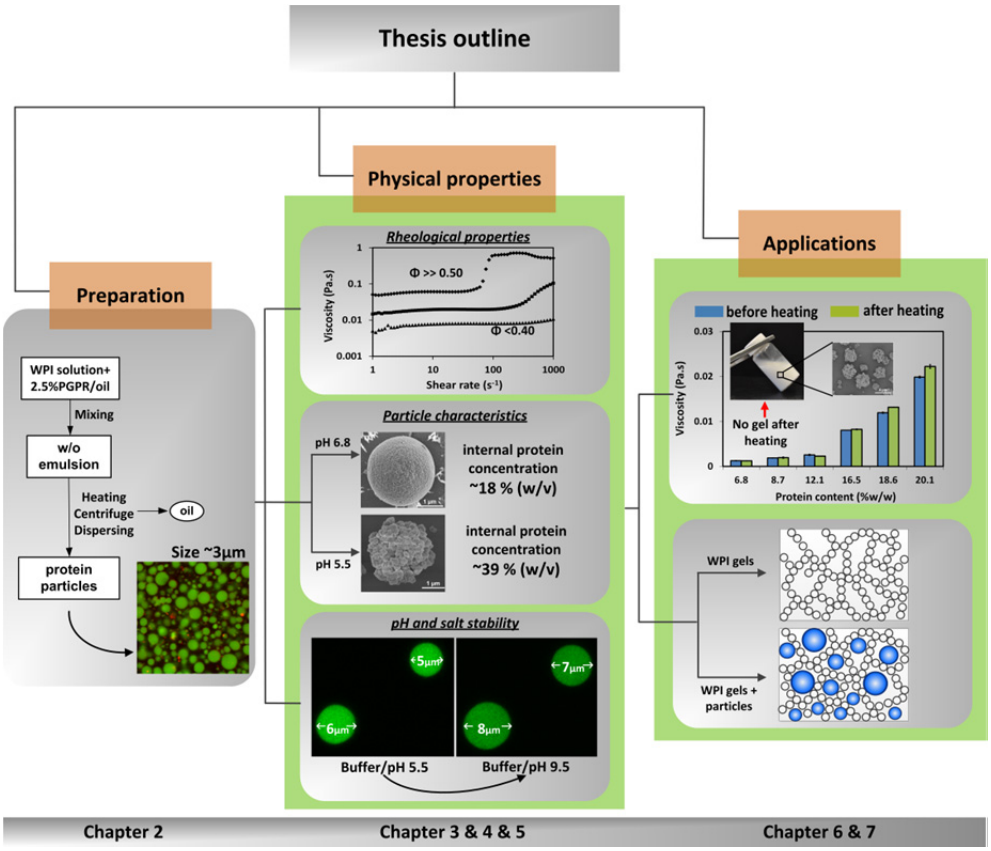

Figure 8.1 Graphical presentation of the thesis content.

Protein particles: preparation, physical properties and applications

Preparation of protein particles

Preparation of protein particles, or particles containing proteins, have been studied for several purposes. Most studies focused on double emulsification [10, 11], encapsulation and controlled release [12-21] properties of protein particles, while some other studies investigated protein particles as possible structuring materials [22-25].

Protein particles with varying microstructure, size and final functional properties can be produced by different methods. In this part, we will first review the methods to prepare protein particles and then discuss to what extent these methods are appropriate for the formation of dense protein particles.

Proteins denature when heated above their denaturation temperature and protein aggregates are formed through hydrophobic and covalent interactions [26, 27]. Depending on the protein concentration, heating conditions, pH and the type and amount of salt added, aggregates varying in microstructure, size, shape and functionality can be obtained [27]. It was shown that this heat-induced aggregation of proteins can be used to form protein particles. For example, aggregation of whey proteins close to pH 6 upon heat treatment at 95 °C for 15 min at a protein concentration between 0.5 to 10% (w/w) resulted in formation of whey protein particles [28]. Similarly, formation of spherical particles from whey proteins and β-lactoglobulin (β-lg) after heat treatment (85 °C/15 min) of 1% (w/w) protein solutions was also shown by other studies [29-31]. β-lg particles formed in these studies were reported to have a rather low polydispersity and the average diameter of the particles was between 160 and 220 nm depending on the initial pH before heat treatment [30, 31]. In another study, soluble aggregates of whey proteins were prepared through heat treatment at 85 °C for 15 min [32]. Acidification of these soluble aggregates (to pH 6, pH 5.5 and pH 5) during continuous stirring resulted in formation of whey protein particles in the size range from 100 to 300 nm.

Protein particles can also be formed through segregative phase separation in a protein-polysaccharide mixed system. When two incompatible biopolymers are mixed, they undergo phase separation, which leads to formation of regions that are rich in one of the biopolymers. The phase separation can be influenced (or freezed) through gelation of one of the phases, before a completely separated two-phase system is formed [33]. By gelation of one or both of the biopolymers and varying the type and the ratio of the two phases, the final gel microstructures can be manipulated [34]. Formation of spherical gelatin particles (average diameter between 10-20 μm) was reported upon rapid gelling in gelatin/dextran [35-37] and gelatin/locust bean gum (LBG) [38] mixed systems. Donato et al. [39] showed that β-lg particles, having an average diameter of 5 μm, were formed by heat-induced gelation of β-lg/low methoxyl (LM) pectin mixed systems. Addition of calcium was reported to decrease the size of the particles in this study. Similarly, spherical whey protein particles were formed through phase separation and cold gelation of

whey protein/polysaccharide mixtures [40]. In this study, first, soluble whey protein aggregates were formed by heat treatment of a 9% (w/w) whey protein isolate (WPI) solution (68.5 °C/2.5 h). Then protein/polysaccharide mixed systems, containing 3% (w/w) of this pre-heated WPI solution, were slowly acidified close to isoelectric point of proteins. This resulted in increased protein-protein interactions and protein-rich spherical domains were formed. The size of those domains varied from 2 μm to 50 μm depending on the type of polysaccharide used. More recently, monodisperse particles of gelatin-maltodextrin mixtures in the size range from 115 to 160 μm were prepared by combining microfluidic emulsification and phase separation technology [21].

These studies show that aggregation of proteins by heating or phase separation, combined with the gelation of proteins, allow formation of protein particles in the size range of a few hundred nanometers to micrometers. However, both of these methods are not suitable to prepare protein particles with high internal protein concentrations. For example, if the initial protein concentration is high (> 11% w/w), a bulk protein gel will be formed during heat induced aggregation, instead of individual protein particles [27, 41]. Similarly, increasing protein concentrations in the protein/polysaccharide mixed systems, will change the kinetics of phase separation and also the viscosity of the system. This may result in either a very fast gelation, before the particles are formed, or the formation of a bi-continuous phase. As a result, the formation of dense protein particles, by using above discussed methods, is rather limited. Additionally, both of these methods are highly sensitive to the environmental parameters, such as, pH and ionic strength. For example, if the pH of the protein solution was above 6.1, protein strands in the size of 15-20 nm were formed, instead of spherical protein particles after heat treatment to form protein particles [41].

Preparation of protein particles under shear, referred as 'microparticulation', is another extensively studied technique. Heat treatment of whey proteins, at high concentrations under shear application will prevent the formation of a bulk whey protein gel and lead to formation of individual protein particles. As described in the preparation of Simplesse®, whey protein particles can be formed through simultaneous application of heat (80-120 °C) and high shear conditions (7500-10.000 s^{-1}) in a scraped surface heat exchanger [42]. Extrusion cooking at an acidic pH (pH range 3.5-3.9) was also shown to form whey protein particles having an average diameter ~ 11.5 μm [43]. Alternatively heat treatment and high pressure

shearing (microfluidisation) can also be used [44, 45]. Depending on the processing conditions applied and the composition of the initial protein source, whey protein particles having different particle sizes, internal structure, denaturation degree and solubility can be prepared by these methods [46]. Higher internal protein concentrations can be obtained with this method, when compared to heat-induced aggregation or phase separation. However, it may be difficult to control the final particle morphology and surface properties due to high shear requirements.

Another way to obtain protein particles with better controlled internal and surface properties can be a combination of emulsification and gelation of proteins. For this purpose, particles having an internal oil phase (oil droplets), were prepared through two step emulsification and proteins in the secondary phase were gelled to solidify the particles. Microspheres with an average diameter between 15 to 75 μm were produced through w/o emulsification and glutaraldehyde crosslinking of whey proteins [47]. In another study, drop wise addition of a pre-denatured WPI solution, containing emulsified oil droplets, into a calcium chloride solution resulted in formation of whey protein gel beads with an average diameter of 2 mm [12]. The resulting protein beads were spherical and the internal structure consisted of multiple oil droplets entrapped in a whey protein gel matrix. The concentration of protein inside the beads was lower than 5.6% (w/w). In another study, oil-in-water-in-oil (o/w/o) emulsification combined with heat-induced gelation of whey proteins was used for the formation of microcapsules (diameter 10-100 μm), containing fat as core material and gelled whey proteins as a wall solid [11]. Similarly, another study has shown formation of double emulsion particles (water-in-oil-in-water) in the size of 10 μm, having gelled WPI particles as the internal aqueous phase [10]. As the droplets contained a significant amount of oil, the average protein content of the composite droplets was much lower than 15% (w/w).

More recently, whey protein particles were prepared through treating a w/o micro-emulsion, containing WPI in the aqueous phase, either by a heating step or transglutaminase cross-linking [48, 49] to gel the whey proteins. This method resulted in formation of protein particles with an average size smaller than 100 nm. The size of the particles formed in this study was highly sensitive to the initial concentration of whey proteins: upon increase of the initial whey protein concentration from 2% (w/w) to 5% (w/w), the size of the particles increased from approximately 100 nm to 2.5 μm. Due to a narrow stability window of micro-emulsions, it is very unlikely that protein particles with a high internal protein

content, e.g. 20% (w/w), can be obtained by this method, which is the primary aim of this thesis.

Not much attention was given to formation of dense protein particles in the methods reviewed above. In Chapter 2, by using a combination of emulsification and heat-induced gelation of proteins, we have shown that protein particles with controlled size and internal protein content could be prepared. In the first step, whey protein particles were formed by addition of a 25% (w/w) WPI solution into the oil phase containing an oil-soluble emulsifier during mixing. Subsequently, the oil phase was removed by centrifugation. The viscosity difference between the two phases in this initial step was small, and the rather mild mixing conditions (6500 RPM) resulted in protein particles having an average diameter of 3 to 4 μm. By adjusting the mixing conditions, the average diameter of the particles could be decreased to 1 μm (Chapter 2). The gelation of the particles in the following step allowed to keep the individual particles and avoid particle coalescence in the further steps. Apart from particle swelling occurring in the dispersing media (Chapter 3, 4 and 5), the size of the particles was well controlled with the method we have presented in Chapter 2. Microstructural analysis of the particles showed that particles formed at neutral pH had a spherical shape with a homogeneous internal protein distribution. The method we have presented in Chapter 2 is robust in the sense that it allows the use of various protein sources (animal-derived or plant-derived), a large range of concentrations inside the particle, and numerous stabilizers, that are able to vary the particle surface properties and thereby the interaction of the particle with the surrounding matrix.

Physical properties of protein particles

In the second part of the thesis, the focus was on the physical properties of the protein particles and their dispersions. Incorporation of particles in an aqueous or gelled system may have significant influence on the textural and rheological properties, as well as on the stability. Therefore, we have studied particle properties and behavior of the concentrated dispersions.

Rheological properties of particle dispersions at high volume fractions ($\Phi \sim 0.35$) and the influence of heat treatment on these properties were investigated in Chapter 3. Particle dispersions had a Newtonian viscosity profile at this volume fraction, which showed that they did not aggregate, when dispersed in a continuous phase containing a stabilizer, like Na-caseinate or WPI. After a heat

treatment at 90 °C for 30 min, a shear-thickening behavior in the high shear regime was observed, which is typically reported for colloidal particle dispersions [50, 51]. Although no aggregates were formed after heat treatment, there was an increase in particle size, suggesting that the protein particles might have swollen upon heating. It has been reported that the onset and magnitude of shear-thickening is strongly influenced by the volume fraction of the particles [50, 52]. Indeed, an increase in volume fraction resulted in a shift of the onset of shear-thickening towards lower shear rates. This swelling effect on shear thickening was further confirmed in Chapter 6, where shear-thickening behavior was observed in non-heated dispersions of protein particles (prepared at pH 5.5) at higher volume fractions (Φ ~ 0.60 to 0.65).

For the dispersions of protein particles prepared by other methods, diverse rheological properties were reported. For example, Mleko and Foegeding [53] observed shear-thickening, shear-thinning and thixotropy in the whey protein particle dispersions prepared through a two-step heating of WPI solutions at different pH and protein concentration range. In another study, shear-thinning was reported for the dispersions of whey protein particles (prepared under high shear application) in water [54]. The authors concluded, by analysis of the particle dispersions through stress relaxation and modulus measurements, that these dispersions contained structures of aggregated particles. These structures were disrupted at increasing shear rate, which resulted in a decrease in the viscosity. We have not observed shear-thinning before heat treatment, showing that in our case, the protein particles were stabilized, probably through electrostatic repulsion. It must be noted that in the current study, particle dispersions were prepared in the presence of a stabilizer in the continuous phase, such as WPI, Na-caseinate or gum arabic. This might also influence the interaction between the particles.

We have shown in Chapter 3, that the rheological behavior of the protein particles is highly dependent on the properties of the continuous phase, mainly because of the different degree of particle swelling observed after heat treatment. The decrease observed in the swelling degree upon increasing stabilizer concentration in the continuous phase may be a result of an increase in osmotic pressure difference, caused by the larger number of protein molecules. Additionally, slight differences in the pH and salt concentration might also contribute to a decreased swelling.

We found that swelling of the particles after heat treatment was suppressed, and the Newtonian viscosity profile was maintained, when the particles were washed with and dispersed in an aqueous phase containing 1% (w/w) gum arabic. This noticeable improvement in the heat stability of the protein particle dispersions, when compared to the particle dispersions containing other stabilizers (Na-caseinate and WPI) may be a result of altered surface character of the protein particles. The findings of Chapter 3 can be useful to tune the rheological properties of the particle dispersions, as well to control the influence of heat treatment on particle swelling.

It was shown by several studies that WPI and gum arabic may interact with each other to form either soluble or insoluble complexes, depending on pH, ionic strength and the ratio between these two biopolymers [55-57]. The interaction is mainly of electrostatic origin, therefore the phenomenon occurs in a pH range, where WPI and gum arabic have opposite charges, in our case below the isoelectric point of WPI (pI ~ 5.1). However, it was also reported that at low salt concentrations, soluble WPI and gum arabic complexes may form above this pH, due to interaction between positively charged groups on the protein surface and negatively charged gum arabic [56]. Recently, it was shown that very stable o/w emulsions were obtained between pH 5.0 and pH 7.0, when a mixture of WPI and gum arabic was used as emulsifier [58]. The authors concluded, that in this pH range, there was a weak interaction between WPI and gum arabic, due to positively charged domains of the proteins. Additionally, it was also shown that complexation of gum arabic with β-lactoglobulin was influenced by the presence of β-lactoglobulin aggregates, suggesting a possible adsorption of the gum arabic onto the surface of the aggregates [59]. These studies suggest, that in a system containing protein particles and gum arabic, it is likely that gum arabic adsorbs onto the particles.

In figure 8.2, protein particle dispersions prepared either in 1% (w/w) gum arabic or 1% (w/w) WPI are shown as a function of pH. It should be noted that protein particles were formed in water-in-oil emulsions containing an oil soluble emulsifier (PGPR). Gum arabic or WPI are the stabilizers that are used in the aqueous phase the particles are dispersed in, after the removal of the oil. Aggregation of protein particles and sedimentation was observed close to pH 3.5, when particles are dispersed in gum arabic. Similar behavior was observed close to pH 5.0, when the particles are dispersed in WPI. Zeta-potential measurements have shown that the

net surface charge of the protein particles was close to zero at these pH values (data not shown), implying a weak electrostatic repulsion between the particles, causing aggregation

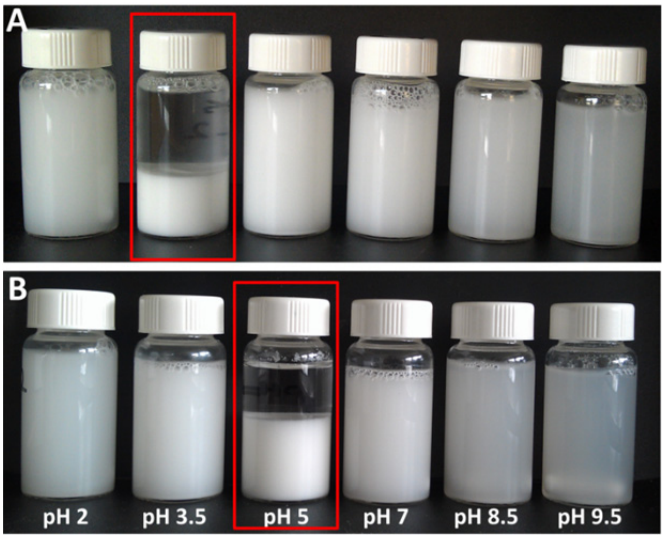

Figure 8.2 Protein particle dispersions as a function of pH. Protein particles were prepared from a 25% (w/w) WPI solution at pH 6.8 and dispersed either in 1% (w/w) gum arabic (A) or 1% (w/w) WPI (B) solution.

Similar to our findings, it was reported that the zeta potential was close to zero for a WPI/gum arabic mixed system with a ratio 3:1 at a pH around 3.8 [58]. This, together with the fact that aggregation of protein particles was observed at pH 3.5 when dispersed in gum arabic, indicates that gum arabic molecules are likely to be adsorbed onto the surface of the whey protein particles at pH 3.5. This variation in the pH stability of protein particles with different type of stabilizers can be used for the development of stable protein drinks at different pH values.

A significant increase in the protein concentration of a system, by adding protein particles, can be possible only if the internal protein content of the particles is high enough. With the method we have presented in Chapter 2, it is possible to increase the protein concentration of initial protein solutions. In this thesis, a 25% (w/w) WPI solution was used to prepare the particles. We have also investigated the formation of particles from protein solutions at 30%, 35% and 40% (w/w). When the whey protein concentration was increased to 30% and 35% (w/w), spherical

particles are formed (Fig. 8.3). A further increase in the protein concentration, to 40% (w/w), resulted in the formation of particles with internal inhomogeneities. The internal structures were only observed after the w/o emulsion (that was formed in the first emulsification step) was heated, in order to gel the protein inside the emulsion droplets. An increase in the size of the protein particles or a bimodal size distribution was also observed with increasing initial WPI concentrations in the emulsion droplets. It was shown that the viscosity of the WPI solutions increases steeply, when WPI concentrations are above 30% (w/w) [60]. When a WPI solution of 40% (w/w) was used for the preparation of protein particles, the viscosity ratio between the dispersed (μ^d) and continuous phase (μ^c) became significantly higher (~ 7) in comparison to the ratio (~ 0.3) when WPI at 25% (w/w) concentration was used. This ratio has a large influence on the viscous stress, that is needed for droplet break-up. If $\mu^d > 4\mu^c$, droplet break up becomes more difficult [61]. Therefore, when using a WPI solution of 40% (w/w) in the emulsions, to obtain protein particles at similar sizes as reported for 25% (w/w) WPI, higher shear rates or increased amount of emulsifier in the oil phase is necessary.

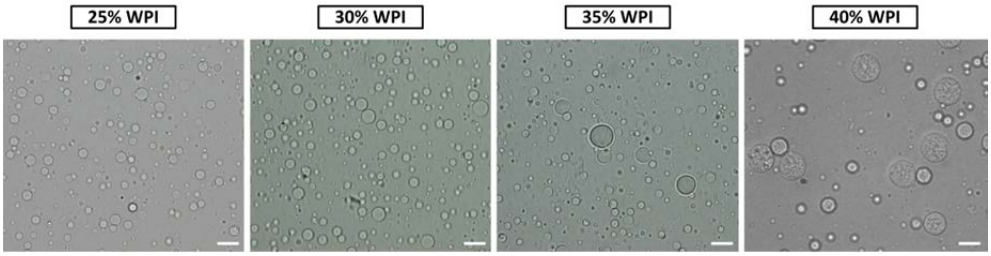

Figure 8.3 Optical microscopy images of protein particles prepared from WPI solutions at increasing initial protein concentration. Samples were diluted 100x prior to analysis and an oil immersion objective (100x) was used for imaging. Scale bar: 10 μm.

Another complication, regarding the increased internal protein concentration, is particle swelling. As reported in the literature for whey protein gels [62-64] and as also shown in this thesis (Chapter 5), protein particles prepared through gelation of whey proteins do swell, when dispersed in aqueous media. This swelling results in a lower internal protein concentration. Due to this swelling, an increase of the initial protein concentration does not always result in a similar gain in protein concentration inside the protein particles.

Considering the above mentioned complications, it was important to investigate other ways to increase the internal protein content of the particles. In Chapter 4, it was shown that adjusting the pH of the initial WPI solution close to the isoelectric point of whey proteins (pH 5.5), resulted in the formation of much denser protein particles, in comparison to the particles prepared at pH 6.8. The internal protein concentration of the particles (~ 39% w/v) prepared at pH 5.5 was approximately twice the internal protein concentration of the particles (~ 18.5% w/v) prepared at pH 6.8. We have observed that particles prepared at pH 5.5 shrank during the heating step (required to gel the whey proteins inside the emulsion droplets), resulting in a highly dense internal structure. Similarly, addition of NaCl (100 mM or higher) to the WPI solution, also resulted in denser particles, as shown in Chapter 4. Whey protein particles of an average diameter of ~ 100-300 nm and an internal protein concentration of 0.2-0.4 g/ml were reported to be formed through heat-induced aggregation in the pH range between 5.8 to 6.2 [41]. Somewhat denser particles were reported to form through addition of Ca^{2+}. Similarly, formation of more compact protein particles after addition of calcium ions were reported in another study [32].

Besides the increased internal protein concentration, the particle morphology is also an important parameter for the physical properties. This can be tuned by adjusting the initial pH or salt concentration. This can be of importance in the applications, like encapsulation or controlled release. For example, due to denser domains, as shown by scanning electron microscopy (SEM) analysis in Chapter 4, protein particles formed at pH 5.5 may show different release properties compared to protein particles formed at pH 6.8, since the latter exhibits a more open internal microstructure. Another way of tuning the morphology of protein particles was recently studied [21]. In this study, protein particles were formed through microfluidic emulsification of biopolymer mixtures containing WPI. The microstructure of these particles, i.e. the domain sizes and the type of microstructure, could be tailored by controlling the biopolymer type, mixture composition and cooling rate (determining the gelling rate of the biopolymer mixture).

The results of Chapter 3 and 4 have suggested that protein particles might swell or shrink, depending on the changes in the environmental factors, such as temperature, pH and ionic strength. In Chapter 5, we have taken an additional step to understand the stability of the particles at different pH and salt concentrations,

by analyzing the particle swelling and the protein leakage from the particles. There was almost no leakage of protein, when pH was close to the isoelectric point (pH ~ 5.0), whereas at the other pH values, a leakage between 2-8 % (w/w) was found. When the pH was above 8.5, the particles maintained their microstructure, but a significant increase in the protein leakage was observed. This may be due to disruption of the covalent bonds in the protein network, taking place at alkali conditions [65, 66].

Swelling of single protein particles was visualized by CLSM, using a fluid cell containing protein particles fixed on a poly-L-lysine coated surface. This method allows a controlled solvent exchange. As shown in Chapter 5 and 6, protein particles aggregate around pH 5 and, some clusters were still present when the pH was adjusted to pH 3.5. Therefore, particle size analysis at this pH value cannot be used to study particle swelling. By directly monitoring the swelling of a single particle, fixed on a surface, we exclude the influence of the particle-particle interactions. We have shown in Chapter 5 that protein particles have pH- and salt-responsive swelling properties. When application of protein particles in high protein foods is considered, this information can be useful to estimate and control the rheological properties and heat stability in a particle containing system. Additionally, these results imply the potential of protein particles to be used in the controlled delivery of compounds larger than the pore size of the particles.

Applications

In the last part of the thesis, attention was given to applications. Because the primary aim of this work was to investigate the potential of dense protein particles for the development of high protein foods, liquid and gel model systems at high protein concentrations were studied. Due to their interesting characteristics, like the variation in swelling, surface activity, gelling or digestibility, protein particles possess a wide range of functionalities. In this part, we will first review the possible applications of protein particles and then discuss, whether protein particles are suitable for the development of high protein foods.

Protein particles from various protein sources can be used for encapsulation and controlled release purposes. Protein particles prepared through cold gelation of whey proteins were shown to give a good protection against enzymatic attack during the stomach digestion and oxidation of fat-soluble biomaterials [12]. Similarly, whey protein micro-beads loaded with probiotic bacteria were shown to have good

targeted delivery properties [17]. Those micro-beads were reported to be resistant to acidic conditions and peptic digestion during 3h of in vitro stomach incubation (pH 1.8, 37 ^{o}C), whereas they disintegrated rapidly with concomitant release of probiotic bacteria under intestinal incubation conditions. In another recent study, the probiotic yeast *Saccharomyces boulardii*, was encapsulated in whey protein-alginate micro-particles (ratio of whey proteins to alginate 62:38) [19]. Those micro-particles were resistant against gastric digestion, which increased the survival rate of yeast cells from 10% to 40%, and a subsequent controlled release of yeast cells was observed at simulated intestinal conditions. Alginate-whey protein isolate particles prepared by emulsification and subsequent internal gelation were also shown to delay the release of riboflavin in the gastric conditions [15]. The same authors have recently presented good controlled release properties of particles prepared through cold gelation of soy/zein protein blends [14].

Some studies have shown that protein particles may be helpful to improve the heat stability of whey proteins. O' Kennedy et al. [67] studied the heat stability of the whey protein particles (average size of a few hundred nanometers) formed through addition of calcium phosphate to a pre-heated protein solution. The authors showed that this dispersion of whey protein particles was stable against heating at neutral pH. In another study, whey protein particles formed under high shear were shown to decrease the thermal aggregation temperature of WPI solutions, which was explained by the presence of more reactive groups leading to an earlier protein aggregation [68]. Recently, Dissanayake and Vasiljevic studied the functionality of whey protein particles formed by microfluidization [45, 69]. They have shown that the functionality of these whey protein particles could be tuned by changing the processing conditions. For example, whey protein particles formed through both heat and high pressure shearing enhanced the thermal stability of whey proteins, whereas the particles formed through only high-pressure shearing showed improved foaming properties, but lower heat-stability [45]. Preparation of the same particles at higher acidity (pH ~ 3) also improved the functional properties of the particles: a better stability was reported for the emulsions that are stabilized by these particles [69]. It has been shown that the composition of whey protein concentrate, usually used for the preparation of protein particles under high shear application, may also influence the functional properties of protein particles [70]. In a recent work, replacement of lactose with inulin was shown to form protein particles with improved functional properties, such as increased stability

against sedimentation [71]. In another recent work, whey protein nanoparticles were shown to improve heat stability: the dispersion of whey protein nano-particles was transparent and liquid-like after thermal treatment, while a WPI solution at the same protein concentration gelled after heat treatment [48, 49]. The emphasis of this work was on the formation of small particles to be used in clear beverages and the protein concentrations studied was rather low (5% w/v).

For the development of medical drinks and liquid high protein foods, it is important to increase the protein concentrations, while keeping the product heat-stable. Usually, when the protein concentration is increased above 5% (w/w) total protein, heat treatment results in significant changes in the product characteristics. The viscosity increase, due to heat treatment, is one of the most important problems observed in drinks enriched with proteins [72, 73]. This increase in viscosity can result in complications during processing. Besides this, formation of a thick texture is usually not appreciated by the consumers and is also not desirable for the medical drinks, that have to be consumed by patients, having for example swallowing problems [73]. Therefore, in Chapter 6, we have studied heat stability of whey protein particle dispersions at increasing protein concentrations. Viscosity changes upon heat treatment was the main focus in this study. It was shown that a heat-stable model drink, with a total protein concentration of 20% (w/w), could be obtained after replacement of native whey proteins by whey protein particles prepared at pH 5.5. No significant increase in the viscosity of this particle dispersion was observed after heat treatment, whereas a reference system consisting of native whey proteins was already gelled after heat treatment at a protein concentration of approximately 11% (w/w). To our knowledge, a similar heat stability at this high total protein concentration, by using any other type of protein particles or other ingredients, has not been found previously. These findings suggest that dense protein particles can be used for the development of heat stable liquid protein formulations. We have presented in Chapter 6, that protein particles are easily degraded under stimulated gastric and pancreatic conditions, which is another important requirement for the application of protein particles for the development of high protein foods.

One practical consideration is the rheological properties of the particle dispersions at high particle volumes. Protein particles prepared at pH 5.5 have an internal protein concentration of ~ 39% (w/v). In order to obtain a total protein concentration above 20% (w/w), high particle volume fractions (Φ ~ 0.55) are

necessary. Although the particles are quite heat stable, a strong-shear thickening behavior was observed in the dispersions at volume fractions above $\Phi \sim 0.55$ (Chapter 6). The particles prepared at pH 6.8 have a lower internal protein concentration (~ 18.5% w/v) compared to the particles prepared at pH 5.5, which would require even higher particle volume fractions to obtain a significant increase in the protein content. Mixed systems, containing protein particles as dispersed phase and native whey proteins in the continuous phase, can be helpful to remedy this. For example, a mixed system containing 6% (w/w) native whey proteins and 30% of particles (prepared at pH 5.5), would give a total protein concentration of approximately 17% (w/w). Because particles are heat stable, only a small increase in the viscosity after heating may be expected due to aggregation of the native whey proteins present in the continuous phase. Shear-thickening is not expected to occur, because the particle volume fraction is sufficiently low. This was confirmed with the data presented in Chapter 7, as will be discussed later in this chapter.

Another consideration for applications is the sensory properties of the dispersions containing the protein particles. To assess the sensory attributes, we have prepared model protein beverages by mixing native whey proteins and whey protein particles at different ratios, in a 8% (w/w) sucrose solution. The total protein concentration was adjusted to 12% (w/w) in all samples and a qualitative descriptive analysis (QDA) was conducted by 9 trained panelists. It should be noted that, due to scaling–up considerations, the average diameter of the protein particles used in this sensory study was slightly larger in comparison to the average sizes reported earlier in this thesis.

The most dominant mouthfeel attributes of model beverages were defined as 'thickness' and 'powdery' in this sensory evaluation. In Figure 8.4-A, the change in these attributes were compared with the reference sample containing only 12% (w/w) WPI. The mixed systems containing whey protein particles (WPP), were prepared at pH 6.8, either at WPI:WPP protein ratio of 8:4 or 4:8. The perceived thickness of both mixed systems was rated significantly lower than the thickness of the reference system at the same protein concentration. The 'powdery' attribute of the reference system and mixed systems were not significantly different. In Figure 8.4-B, a similar comparison for protein particles prepared at pH 5.5 is shown. In accordance with the results shown in Chapter 6 and 7, a decrease in the thickness of the product was perceived, when particles prepared at pH 6.8 were replaced by the particles prepared at pH 5.5, for both mixed systems. The effect was more

pronounced at increased particle concentration (when WPI:WPP ratio was 4:8). Oppositely to what is observed for the particles formed at pH 6.8, the protein particles formed at pH 5.5 increased the mouthfeel attribute 'powdery' significantly (Fig. 8.4-B). When the particle concentration was decreased and WPI concentration was increased the 'powdery' perception decreased (8:4), whereas the samples were perceived thicker, compared to the samples containing a higher amount of protein particles (4:8).

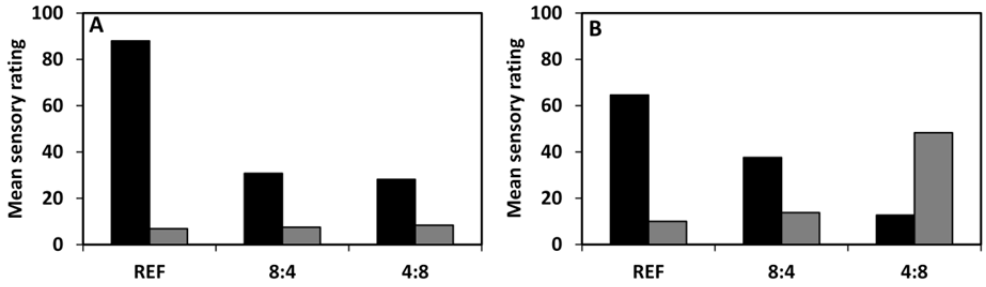

Figure 8.4 The mouthfeel attributes (expressed as arbitrary QDA-scores) thickness (bars in black) and powdery (bars in grey) for the protein particles prepared at pH 6.8 (A) and pH 5.5 (B). The total protein concentration of the samples were 12% (w/w), reference sample (REF) contained only WPI and the samples 8:4 and 4:8 contained WPI and protein particles with a protein weight ratio to 8:4 and 4:8, respectively.

These results show that mixed systems containing native whey proteins and protein particles can be used to optimize the sensory properties in the beverages, while allowing for an increase in the total protein concentration.

Considering the effects of the particles on the sensory and rheological properties, we have further investigated liquid and gelled mixed systems in Chapter 7. Mixed systems were prepared at 4 different native whey protein concentrations and 3 different particle volume fractions. The samples, which flow upon inverting the vial after heat treatment, were defined as liquid, whereas samples that did not flow and having a G' value larger than G" were defined as a gel. As expected, the viscosity of the liquid samples increased after heating, mainly due to aggregation of whey proteins in the continuous phase. In Chapter 6, we have shown that dispersions containing protein particles prepared at pH 6.8 were less heat-stable, in comparison to the dispersions of protein particles prepared at pH 5.5. Formation of a weak gel after the heat treatment at a protein concentration of about 9.4% (w/w)

was observed for protein particles prepared at pH 6.8. In Chapter 7, we have observed that mixed systems of protein particles prepared at pH 6.8 showed a better heat stability compared to the dispersions studied in Chapter 6: no gelation was observed in these dispersions at total protein concentrations between 12 and 14% (w/w). Viscosity increased after heat treatment, again as a results of aggregation of whey proteins that are present in the continuous phase.

Several studies have investigated the use of protein particles for improving the textural properties of food products. Protein particles prepared under high shear applications were mainly used as fat replacers and their texture and creaminess improving properties were reported for several foods, such as ice cream, cheese and yoghurt [22, 24, 70]. Whey protein particles, prepared in a phase separating system, were studied for their functionality to improve the properties of gluten-free dough and bread [23, 25]. The authors observed improved properties, both in the dough and bread, upon addition of protein particles, when compared to gluten-free systems. Whey protein particles, prepared through heat-induced aggregation, have also shown to have interesting other textural properties. For example, a creamy appearance in coffee was reported upon addition of whey protein particles [28].

Negative sensory properties is an important problem in high protein foods [74, 75] or in the products enriched with proteins [76]. Formation of a 'tough' and 'unfracturable' texture was reported at increasing protein concentrations for protein gels [77, 78]. Whey protein particles can be used to modify mechanical properties of protein gels at high protein concentrations. Some early studies reported formation of softer whey protein gels in the presence of aggregated whey proteins [79, 80]. In a recent study, it was shown that whey protein aggregates (prepared by heating a 9% (w/w) WPI solution) formed weaker gels in comparison to native whey proteins at equal protein concentration [81]. The same authors have also investigated texture modifying properties of whey protein particles, prepared under high shear [82]. Here, WPI gels mixed with whey protein particles (average diameter ~ 70 μm) were investigated at total protein concentrations between 15-20% (w/w). The authors reported that WPI gels containing protein particles were weaker, compared to WPI gels without protein particles, at the same total protein concentration. Increasing the amount of whey protein particles further decreased the gel stiffness. This decrease was not large, mainly caused by the interaction between the protein particles and the proteins in the continuous phase. The particles used here showed a significant swelling upon dispersing them in the WPI

solutions, which was also reported to be one of the important reasons for not having substantially higher protein concentrations.

Dense protein particles that do not show a significant swelling, might be useful to obtain a higher increase in the total protein concentration, while modulating the textural properties. The data presented in Chapter 6 and in the first part of Chapter 7 suggested that protein particles prepared at pH 5.5 may be suitable for this purpose, because no gelation was observed in the dispersions of these particles, even at considerably high particle volume fractions. In the second part of the Chapter 7, we have investigated the influence of protein particles on the mechanical properties of the WPI gels at high protein concentrations (16-22% w/w). The results have shown, that dense protein particles are promising ingredients to modulate the mechanical and textural properties of the gelled systems at high protein concentrations. In the presence of whey protein particles, WPI gels had much lower storage moduli (G') and fractured at lower strain values compared to the WPI gels without protein particles, at the same total protein concentration. The gain in the protein concentration, without significantly influencing the G', was relatively high (25 to 55% increase in the total protein concentration) and resulted from the combination of the high internal protein concentration of the particles and the weak interaction between the protein particles and the surrounding matrix.

Our results suggest that the interaction between the protein particles and the proteins in the continuous phase must be weak (Chapter 7). Other studies on the protein gels containing protein particles, prepared under shear, have shown an opposite effect and concluded that protein particles are 'active' fillers [54, 82, 83]. This is probably due to the fact that the proteins at the surface of the particles interact with the proteins in the continuous phase. In our work, the protein particles were prepared through a two-step emulsification method, using PGPR as the primary emulsifier. When the PGPR remains present on the protein particle surface, the interaction with the protein in the matrix can be substantially reduced. This conjecture is in accordance with a study on the presence of a surfactant on a particle surface, weakening the interaction between particles and the gel matrix [84].

Concluding remarks and outlook

This work initiated the preparation of highly dense protein particles with controlled size, morphology and internal protein concentration for the development of high protein foods. We have shown that dense protein particles

can be successfully used to obtain considerably high protein concentrations in both liquid and gelled systems. In the liquid model system, containing protein particles, a good heat stability was found, as shown by the absence of a viscosity increase after heating. For the gelled system, it was shown that weaker protein gels at high protein concentrations can be obtained by incorporation of dense protein particles.

We have studied the formation of the particles, as well as the physical properties and functionality in concentrated particle dispersions. The physical properties of the protein particles have been shown to considerably influence the final properties of the particle dispersions, such as rheological behavior, heat stability or textural properties. More research on determining the mechanisms responsible for the observed physical changes in the particle properties would be beneficial for the possible future applications of the protein particles.

An essential research line would be the investigation of the surface properties of the protein particles. In Chapter 2 and 3, we have shown that an oil layer or other adsorbed molecules (WPI, gum arabic or Na-caseinate used as stabilizers in this study) might be present on the surface of the particles. The interaction between the protein particles is an important parameter for the stability of the particle dispersions and will be influenced by the changes in the surface properties. Additionally, as discussed in Chapter 7, the surface properties of the particles is an important factor, determining the final rheological properties of a gelled matrix containing protein particles. We have investigated the surface of the protein particles by different microscopy techniques, such as CLSM, SEM and Cars-CLSM (data not presented in the thesis). The results suggested the presence of an oil layer or oil patches on the particle surface, however, due to labeling artifacts, experimental limitations of the methods used, and/or the porous structure of the particles allowing diffusion of small molecules, a complete elucidation could not be reached. Although pH stability experiments, as shown in Chapter 5 and in the current chapter suggested that stabilizers might be adsorbed onto the particle surface, we have not explicitly studied this. For example, a further characterization of the particle surface by CLSM analysis, using covalently labeled gum arabic, could clarify whether gum arabic adsorbs at the surface of the particles and/or forms a complex with the whey proteins present on the particle surface. Small angle x-ray scattering (SAXS) or small angle neutron scattering (SANS) techniques could also provide information on the surface properties of the particles on a nanometer length scale.

Swelling of the protein particles, when dispersed in aqueous media, is an important parameter controlling the rheological properties and heat stability of the particle dispersions. By particle size and CLSM analysis, we have shown that the particles may swell or shrink, depending on the changes of pH or ionic strength (Chapter 5). It was also observed that the size of the protein particles was increased after heat treatment at different temperatures, suggesting particle swelling (Chapter 3). In Chapter 4, we have shown that when dispersions of protein particles, prepared at pH 6.8, were heated inside the rheometer, a viscosity increase was observed both during heating and subsequent cooling step. This implies, that swelling of the particles did not reach an equilibrium yet, during the heat treatment and that swelling continued afterwards. Swelling after heating may be due to structural re-arrangements inside the particle network. Because our primary interest was on the functionality of protein particles, the mechanisms behind the temperature-induced changes in the protein particles was not studied. By further investigating this, a better control over the physical properties of the protein particles could be achieved. Temperature sensitive swelling of the protein particles is an interesting property for some applications, such as controlled delivery. It would be interesting to monitor the kinetics of particle swelling, as a function of temperature in different buffers. The use of controlled heating combined with an microscopy flow chamber, can be useful to observe, in situ, the swelling of the particles.

Considering applications, an important topic is to control the sedimentation of the particles. Due to their high internal density and relatively large size (the average size in the range of few micrometers), a rather fast sedimentation was observed for the particles prepared at pH 5.5 (data not shown). By increasing mixing conditions in the initial mixing step up to 20.000/25.000 RPM, the size of the particles would be reduced to a large extent (average diameter of a few hundred nanometers). However, a bimodal size distribution, consisting of a main particle population at an average size of 100 nm was observed (data not shown). This might be a result of particle coalescence due to high shear forces. We have made some effort to separate smaller particles from the larger ones. Centrifugal separation decreased the number of larger particles, but a complete fractionation was not possible. By filtering the samples, smaller particles could be fractionated from the larger particles. However, due to fouling of the membrane, this method is only suitable for small sample volumes and highly diluted samples. A possible way to separate

large particles from the smaller ones can be using shear-induced fractionation techniques, such as high flux membrane fractionation [85]. Reduction of the particle size and tuning the surface properties of the particles can broaden the application window of the protein particles. For example, small enough protein particles prepared at pH 5.5, having highly dense structure, can be interesting candidates to be used in the Pickering stabilization of emulsions.

Another interesting research line is preparation of protein particles from other protein sources. As shown in Chapter 2, the method for preparing the particles is flexible relative to the protein source and can be extended in the direction of sustainable protein sources, like plant-storage proteins.

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Summary

Food products that contain high levels of protein can help to control food intake and to maintain a healthy body weight due to their strong satiating properties. High protein foods are also beneficial in the nutrition of elderly people and they are commonly used in medical nutrition. Preparation of food products with desired product properties is difficult at high protein concentrations. This is mainly due to protein aggregation, occurring during processing and storage. This aggregation becomes more prevalent at higher protein concentrations and leads to undesired sensory properties and decreased product stability. A possible route in controlling this undesired aggregation is using pre-fabricated protein structures, such as protein particles with controlled internal and surface properties. The aim of this thesis was to design dense protein particles and to study their functionality in the systems at high protein concentrations (**Chapter 1**).

In the first part of the thesis, we have described a method to prepare dense protein particles with controlled size and surface properties (**Chapter 2**). Protein particles were formed through a simple water-in-oil emulsification step, where a 25% (w/w) whey protein isolate (WPI) solution was the aqueous phase and where sunflower oil, containing the emulsifier Polyglycerol Polyricinoleate (PGPR), was the oil phase. After emulsification, whey proteins inside the emulsion droplets were gelled by a heating step and the oil was removed by centrifugation. The average diameter of the protein particles was in the order of a few micrometers. Analysis of particle microstructure by Confocal laser scanning microscopy (CLSM) and Scanning electron microscopy (SEM) suggest that the protein is homogeneously distributed throughout the particles. Due to labeling artifacts and presence of hydrophobic sites on the protein particles, a complete elucidation of the nature of the particle surface could not be reached. The CLSM analysis suggested that there may be oil associated with the particles, either surrounding the particles and/or distributed throughout the particles. However, it was found that the amount of oil does not exceed 1.8% (w/w).

In the second part of the thesis, physical properties of the protein particles and their dispersions were investigated (**Chapter 3, 4 and 5**). The heat stability and rheological properties of concentrated whey protein particle dispersions in different dispersing media were addressed in **Chapter 3**. We have shown that protein particles swell during heat treatment, which considerably influences the rheological properties, especially at high particle volumes. Dispersions of protein particles have a Newtonian viscosity profile before heat treatment, implying that

there is no aggregation and that the particles are, presumably, stabilized by electrostatic repulsion. After heat treatment, due to increased particle volume upon swelling, a shear-thickening behavior was observed. We have also shown in **Chapter 3**, that the concentration and type of the stabilizer, present in the continuous phase, influences the changes in the dispersions after heat treatment. For example, increased concentration of Na-caseinate in the continuous phase suppressed the viscosity increase observed after heat treatment. The shear-thickening was also less pronounced, and was only observed at higher shear rates, which implies a suppressed particle swelling. We suggest, that the change in the osmotic pressure, due to presence of higher amount of protein, is one of the main factors responsible for the difference in particle swelling. It should be noted that, in the samples containing a lower amount of Na-caseinate, shear-thinning, after heat treatment, was observed in the low shear regime, which might be due to particle aggregation. Therefore, it is likely that the amount of stabilizer used in the continuous phase, also influences the interaction between the particles. Another interesting finding of this chapter is that, in the presence of 1% (w/w) gum arabic in the continuous phase, no change in the viscosity of dispersions was observed after heat treatment. These findings can be useful to modulate the rheological properties as well as the heat stability of dispersions containing protein particles.

While we have looked at the dispersing media in **Chapter 3**, we have made progress in tuning the particle morphology and density in **Chapter 4**. Particles were prepared either at different pH (pH 6.8 or pH 5.5) or at different ionic strength (NaCl at 50, 200 or 400 mM). Particles formed at pH 6.8 were spherical in shape, whereas those formed at pH 5.5 were irregular and had a cauliflower-like appearance. Particles formed at pH 5.5 had substantially higher internal protein concentration (~ 39% w/v), than the particles formed at pH 6.8 (~ 18.5% w/v). Similarly, particle morphology and internal protein density were also affected by initial NaCl concentration. Particles formed at 50 mM NaCl (pH 6.8) were spherical, whereas particles formed at either 200 mM NaCl (pH 6.7) or 400 mM NaCl (pH 6.6) were irregular, and protein density of the particles increased with increasing initial NaCl concentration. The rheological properties and heat stability of the particle dispersions were shown to be strongly influenced by the type of particle. We conclude, that the type of interactions, included during particle formation (gelation of the proteins inside the particles), might have resulted in

these differences in the particle morphology, as well as the final physical properties in their dispersions.

We have further investigated the stability of particles by studying protein leakage and swelling of the particles at different pH (**Chapter 5**). Protein particle dispersions show limited swelling and protein leakage in a wide pH range, while around pH 5.0, particles aggregated due to reduced electrostatic repulsion. Protein leakage from the particles was found not to be higher than 8% (w/w) in most of the pH range. It increased significantly at alkaline pH, most likely, as a result of disruption of the particle structure by OH^- ions. The protein particles show a pH and salt-responsive swelling, as shown by CLSM analysis. These findings are important, when the protein particles are used for development of high protein foods, such as liquid medical drinks. Especially, because the pH-induced change in the volume of protein particles may influence the rheological properties of the concentrated dispersions considerably. Additionally, these results imply the potential of protein particles to be used in the controlled delivery of compounds larger than the pore size of the particles.

Functionality of protein particles was addressed in **Chapter 6 and 7**, both for liquid and gelled systems at high protein concentrations. Due to aggregation and/or gelation during thermal treatment, the amount of whey proteins that can be used in the formulation of high protein foods, e.g. protein drinks, is limited. We have shown, that protein particles prepared at pH 5.5 can considerably increase the protein concentration of model drinks (**Chapter 6**). After heat treatment at 90 ^{o}C for 30 min, no change in the viscosity of the protein particle dispersions (particles prepared at pH 5.5) was observed at a total protein concentration of about 18% (w/w), whereas, a WPI solution already gelled under the same heating conditions at protein concentrations around 11% (w/w). Additionally, no gelation was observed in the dispersions prepared by pH 5.5 particles, when the total protein concentration was increased above 20% (w/w). The results presented in **Chapter 6** have shown, that the use of dense whey protein particles is a useful strategy to counter aggregation and/or gelation problems in high protein foods. In **Chapter 7**, we have investigated gelled systems containing dense protein particles at high protein concentrations (16-22% w/w). Incorporation of dense whey protein particles in a whey protein gel, while keeping the total protein concentration constant, led to a noticeably lower storage modulus (G'). A total protein increase between 25 to 55% (w/w) could be obtained in the presence of whey protein

particles, without significantly changing the G' of the gels. The gels were also fractured at lower strain values in the presence of protein particles compared to the WPI gels, without added particles, at the same protein concentration. Results of **Chapter 7** suggest that protein particles can be used to modulate mechanical properties of WPI gels and that they are promising candidates for the formation of high protein foods with improved textural properties.

Finally, the results of this thesis and the contribution of our findings to the general knowledge on how to control the interaction between proteins at high protein concentrations was critically reviewed in **Chapter 8**. A discussion on alternative methods to prepare protein particles, and their suitability for preparation of dense protein particles, was also included in this chapter. We presented information on the sensory properties of a model beverage containing whey protein particles, showing that mixtures of protein particles and native whey proteins are serious candidates to formulate high protein drinks with the desired heat stability and final sensory properties.

We have made a significant progress in understanding the properties and functionality of protein particles at high protein concentrations. The work presented in this thesis has shown that dense protein particles possess a promising potential for the development of food products at high protein concentration.

Samenvatting

Levensmiddelen met een hoog gehalte aan eiwitten (proteinen) hebben potentie om voedselinname te controleren en om een gezond lichaamsgewicht te behouden door hun verzadigende werking. Deze levensmiddelen rijk aan eiwitten hebben ook een positieve werking in het dieet bij ouderen en ze worden veelvuldig gebruikt als medische en klinische voeding. De productie van levensmiddelen met bepaalde gewenste eigenschappen is vaak lastig bij zulke hoge eiwitconcentraties. Dit wordt vaak toegeschreven aan aggregatie van eiwitten wat plaatsvind tijdens het productieproces en tijdens opslag. Een mogelijke route om ongewenste aggregatie te voorkomen is het gebruik van voorgevormde eiwitstructuren zoals eiwitdeeltjes waarbij zowel de interne als oppervlakte eigenschappen gecontroleerd kunnen worden. Het doel van het onderzoek beschreven in dit proefschrift was om geconcentreerde eiwitdeeltjes te produceren en om hun functionaliteit te onderzoeken in systemen met een hoog eiwitgehalte (**Hoofdstuk 1**).

In het eerste deel van dit proefschrift hebben we een methode beschreven om deze geconcentreerde eiwitdeeltjes te maken met een bepaalde grootte en oppervlakte eigenschappen (**Hoofdstuk 2**). De gemiddelde diameter van de deeltjes was een aantal micrometers en het eiwit was homogeen verdeeld in de deeltjes. CLSM analyse suggereerde dat olie geassocieerd kan zijn met de deeltjes, zowel aan het oppervlak als binnenin de deeltjes. Echter, de totale hoeveelheid olie zal niet meer zijn dan 1.8% (op gewichtsbasis).

In het tweede deel van dit proefschrift hebben we een overzicht gegeven van de fysische eigenschappen van deze eiwitdeeltjes en hun dispersies. (**Hoofdstuk 3, 4 en 5**). The hittestabiliteit en reologische eigenschappen van geconcentreerde eiwit deeltjes dispersies in verschillende media is besproken in **Hoofdstuk 3**. We hebben aangetoond dat de eiwitdeeltjes zwellen tijdens het verhittingsproces, wat een grote invloed heeft op de reologische eigenschappen en zeker bij hoge concentraties van deeltjes. Dispersies van eiwitdeeltjes hebben een Newtoniaans viscositeitsprofiel voor de hitte behandeling, wat duidt op de afwezigheid van aggregatie en dat de deeltjes waarschijnlijk worden gestabiliseerd door middel van elektrostatische repulsie. Na de hitte behandeling vertonen de dispersies een dilatant gedrag als gevolg van een verhoging van de volume fractie door zwelling van de deeltjes. Een ander interessant fenomeen beschreven in dit hoofdstuk is dat in de aanwezigheid van arabisch gom (1%) in de continue fase, geen verandering in de viscositeit was waargenomen na eenzelfde hitte behandeling. Deze

bevindingen kunnen worden gebruikt om zowel het reologisch gedrag als de hittestabiliteit te reguleren van dispersies die deze eiwitdeeltjes bevatten.

Terwijl we de invloed van het oplosmedia onderzocht hebben in **Hoofdstuk 3**, hebben we progressie gemaakt in het controleren van de morfologie en de dichtheid van de eiwitdeeltjes in **Hoofdstuk 4**. Deeltjes zijn gemaakt bij verschillende pH (pH 6.8 of pH 5.5) of bij verschillende ion sterkte (50, 200 of 400 mM NaCl). Deeltjes die bij pH 6.8 zijn gevormd zijn bolvormig, terwijl de deeltjes die bij pH 5.5 zijn gevormd een onregelmatig uiterlijk hebben vergelijkbaar met een bloemkool. De deeltjes die bij pH 5.5 gevormd zijn hebben een hoger interne eiwitconcentratie (~39%) dan de deeltjes die bij pH 6.8 worden gevormd (~18.5%). Vergelijkbaar, zijn de morfologie en dichtheid ook afhankelijk van de NaCl concentratie. De deeltjes die bij 50 mM NaCl (pH 6.8) zijn gevormd zijn bolvormig, terwijl deeltjes gevormd bij 200 of 400 mM NaCl (pH 6.7) een onregelmatig uiterlijk hebben en hun dichtheid wordt verhoogd bij een toenemende zoutconcentratie. De reologische eigenschappen en de hittestabiliteit van de deeltjes dispersies zijn erg afhankelijk van het type deeltje. We hebben de stabiliteit van de deeltjes onderzocht door de eiwitdiffusie en het zwelgedrag van de deeltjes te bestuderen bij verschillende pH (**Hoofdstuk 5**). De dispersies laten een geringe zwelling zien in een groot pH bereik, terwijl bij een pH van 5.0, de deeltjes aggregeren als gevolg van een gereduceerde elektrostatische repulsie. De diffusie van eiwit uit de deeltjes was niet hoger dan 8% (op gewichtsbasis) bij de meeste pHs. Bij een basische pH was deze diffuse het hoogst, waarschijnlijk als gevolg van het afbreken van de deeltjesstructuur door OH^- ionen. De eiwitdeeltjes hebben een pH- en zout-gevoelige zwelgedrag zoals aangetoond door CLSM analyse. Deze bevindingen zijn belangrijk wanneer deze deeltjes worden gebruikt voor het ontwikkelen van levensmiddelen met een hoog eiwitgehalte, zoals vloeibare medische drankjes. Vooral omdat de veranderingen in het volume door de pH een groot effect kan veroorzaken in het reologische gedrag van deze geconcentreerde dispersies. Deze resultaten laten ook de potentie zien van deze deeltjes om ze te gebruiken voor een gecontroleerde afgifte van bepaalde componenten die groter zijn dan de poriegrootte van de deeltjes.

De functionaliteit van de eiwitdeeltjes is besproken in **Hoofdstuk 6** en **7**, voor zowel vloeibare en gegeleerde systemen met een hoge eiwitconcentratie. We hebben aangetoond dat eiwitdeeltjes gevormd bij pH 5.5 de eiwitconcentratie aanzienlijk kan verhogen in vloeibare drankjes (**Hoofdstuk 6**).

Een hittebehandeling van 90 °C gedurende 30 minuten resulteerde niet in een verandering van de viscositeit voor dispersies met eiwitdeeltjes (gevormd bij pH 5.5) met een totale eiwitconcentratie van 18%, terwijl een eiwitoplossing (wei-eiwit) al een gel vormde bij een concentratie van 11% en dezelfde condities. Gelvorming trad niet op in deze dispersies met pH 5.5 deeltjes wanneer de concentraties waren verhoogd tot meer dan 20%. De resultaten in **Hoofdstuk 6** laten zien dat het gebruik van deze geconcentreerde deeltjes een goede strategie zou zijn om aggregatie en geleringsproblemen te voorkomen in levensmiddelen met een hoog eiwitgehalte. In **Hoofdstuk 7** hebben we gegeleerde systemen onderzocht die geconcentreerde eiwitdeeltjes bevatten bij hoge concentraties (16-22%). Het incorporeren van geconcentreerde eiwitdeeltjes (wei-eiwit) in een wei-eiwit gel met eenzelfde totale eiwitconcentratie resulteerde in een lagere modulus (G'). Een totale concentratie tussen de 25 en 55% (op gewichtsbasis) was behaald in de aanwezigheid van eiwitdeeltjes, zonder de G' significant te veranderen. De gelen vertoonden breukgedrag bij lagere deformaties in de aanwezigheid van deze deeltjes in vergelijking met gelen gemaakt van wei-eiwit zonder deeltjes bij eenzelfde totale eiwitconcentratie. De resultaten uit **Hoofdstuk 7** suggereren dat eiwitdeeltjes kunnen worden gebruikt om de mechanische eigenschappen van eiwitgelen te reguleren en dat de deeltjes een veelbelovende kandidaat zijn voor de ontwikkeling van levensmiddelen met een hoog eiwitgehalte en een verbeterde textuur.

Ten slotte zijn de resultaten in dit proefschrift en de contributie van onze bevindingen aan de algemene kennis om interacties tussen eiwitten te controleren bij een hoog eiwitgehalte kritisch bediscussieerd in **Hoofdstuk 8**. Dit hoofdstuk bevat een discussie over alternatieve methoden om eiwitdeeltjes te produceren en de bruikbaarheid van deze methoden om hoog geconcentreerde eiwitdeeltjes te maken. We hebben vooruitgang geboekt in het begrijpen van de eigenschappen en functionaliteit van eiwitdeeltjes bij hoge eiwitconcentraties. De resultaten in dit proefschrift laten zien dat geconcentreerde eiwitdeeltjes een veelbelovende kandidaat zijn in de ontwikkeling van levensmiddelen met een hoog gehalte aan eiwitten.

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Dilek

List of publications

Sağlam, D.; Venema, P.; de Vries, R.; Sagis, L. M. C.; van der Linden, E., Preparation of high protein micro-particles using two-step emulsification. *Food Hydrocolloids* **2011,** 25, (5), 1139-1148

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Sağlam, D.; Venema, P.; de Vries, R.; van der Linden, E., The influence of pH and ionic strenght on the swelling of dense protein particles.*Submitted to Soft Matter.*

Sağlam, D.; Venema, P.; de Vries, R.; van der Linden, E., Comparing heat stability of high protein content dispersions containing differently designed whey protein particles. *Submitted to Food Hydrocolloids*.

Sağlam, D.; van den Berg, Merel.; Venema, P.; de Vries, R.; van der Linden, E., Whey protein particles modulate mechanical properties of gels at high protein concentrations. *To be submitted*.

R. ter Haar, H.A. Schols, L.A.M. van dern Broek, D. Sağlam, A.E. Frissen, C.G. Boeriu, H.G. Gruppen, Molecular sieves provoke multiple substitutions in the enzymatic synthesis of fructose oligosaccharide-lauryl esters. *Journal of Molecular Catalysis B: Enzymatic 62/2 (2010) 183-189.*

Curriculum vitae

Dilek Sağlam was born on 29th of July 1981 in Trabzon, Turkey. She has studied in Istanbul Technical University (Istanbul, Turkey), where she has received her BSc diploma in Food Engineering. After her graduation in June 2004, she has worked as a marketing and product development specialist for TAMEK, in Istanbul.

In September 2006 she started her master studies in Food Technology at Wageningen University, specializing in Product Functionality. In May 2008, she was appointed as a Ph.D. fellow of Top Institute Food & Nutrition (TI F&N) to perform her research at the Physics and Physical Chemistry of Food group at Wageningen University. The outcomes of her Ph.D. research is covered in the present dissertation.

Overview of completed training activities

Discipline specific activities

Courses

Han sur Lesse, Physical Chemistry School, 2009, Han sur Lesse, BE

12th European School on Rheology, 2009, Leuven, BE

Food Hydrocolloids, 2009, Wageningen, NL

Conferences and Workshops

5th International Symposium on Food Rheology and Structure, 2009, Zurich, CH

3rd International Symposium on Delivery of Functionality in Complex Food Systems, 2009, Wageningen, NL

11th International Congress on Engineering and Food, 2011, Athens, GR

13th Student Colloid Conference, 2011, Falkenberg, SE

6th International Symposium on Food Rheology and Structure, 2012, Zurich, CH

Food Colloids, 2012, Kopenhagen, DK

6th European Workshop on Food Engineering and Technology, 2012, Singen, DE

General Courses

Project and Time Management, 2008, Wageningen, NL

Basic Statistics, 2008, Wageningen, NL

PhD Introduction Week, 2008, Eindhoven, NL

Techniques for Writing and Presenting a Scientific Paper, 2010, Wageningen, NL

Interdiciplinary Research, 2011, Wageningen, NL

Lecturing, 2012, Wageningen, NL

Optional courses and activities

Organized and Participated in the PhD Trip to Japan, 2010

Work meetings project ' High Protein Foods', TI Food and Nutrition, 2008-2012, Wageningen, NL

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