Plant protein colloids in food emulsions

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Plant protein colloids in food emulsions

Plantaardige eiwit colloïden in emulsies voor voeding

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

Introduction

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Introduction

World's demand for protein is expected to double by 2050 (FAO), due to increasing population (9 billion by 2050), emerging economies and recognition of protein's role in a healthy diet. Currently, about half of the protein consumed by the Dutch population is derived from animal sources, i.e. meat and dairy products (Tijhuis et al., 2011). However, a shift from the use of animal protein to plant protein has been one of the most important food and nutrition trends over the past few years. This trend is expected to have positive effects on public health. Plant-based diets are linked with lower risks of heart disease, obesity, hypertension, type 2 diabetes and certain types of cancer. In addition, plant protein production offers a (more) sustainable solution and a lower environmental impact, since it requires less energy and water consumption and contributes less to atmospheric pollution than meat production. Plant protein sources include beans, lentils, peas, soy, nuts, rapeseeds, and cereals. Approximately 45% of the world protein consumed is plant- based and 55% is animal-based. Figure 1 shows the distribution of the world plant protein consumption in 2015/2016.

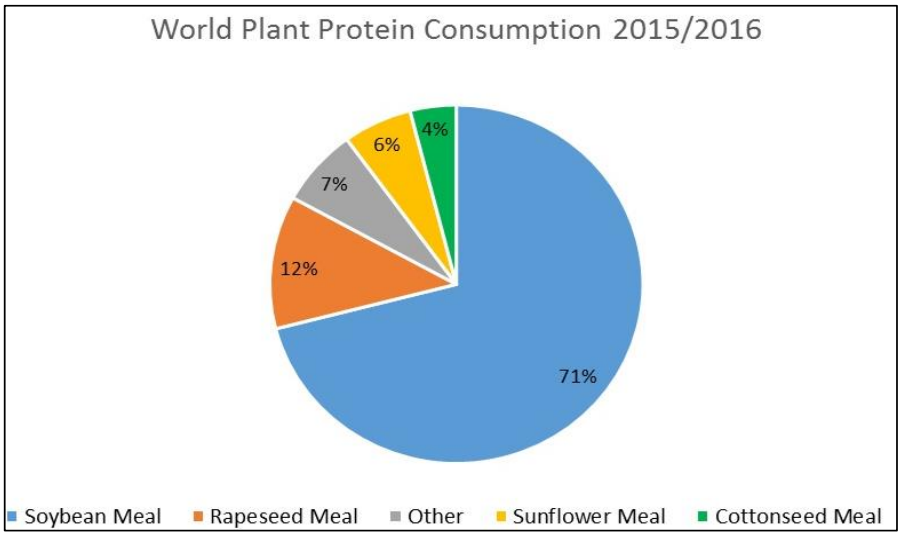

Figure 1. Distribution of the world plant protein consumption in 2015/2016 (USDA, 2016).

Some vegetable proteins, such as soy protein, are used in food applications. However, a significant number of plant proteins is underutilized in food industry due to their poor solubility. For example, the plant proteins zein and gluten are relatively hydrophobic, thus insoluble in water (they are soluble in aqueous ethanol). As a result, the only way they can be used in food preparation is if they are in a quasi-soluble form of sub-micron or nanoparticles. An efficient way to form aqueous particle suspensions from hydrophobic proteins is the anti-solvent precipitation method (Joye & McClements, 2013). In this thesis we focus on the stabilizing effect that insoluble protein particles have on oil-in-water and water-in-water emulsions.

Stabilization by particles has been known for a long time for oil-in-water emulsions (Pickering emulsions) (Aveyard, Binks, & Clint, 2003; Binks & Horozov, 2006; Chevalier & Bolzinger, 2013; Schmitt, Destribats, & Backov, 2014). It is presumed that particles need to have intermediate wettability to be the most effective emulsifiers. However, in this thesis (Chapter 2) it is shown that the proper wettability of particles is not necessarily sufficient to achieve emulsion stability. Earlier, this has been shown for foams (Deleurence, Parneix, & Monteux, 2014). We show that particle charge and interactions of particles with each other are sometimes more important in emulsion stabilization mechanism than adsorption of isolated particles.

Water-water interfaces can occur between phase-separating mixtures of incompatible water-soluble macromolecules, such as proteins and polysaccharides. Food products often contain such mixtures. The interfacial tension of water-water interfaces is orders of magnitude smaller than for oil-water interfaces, which makes their stabilization challenging. In recent years, the stabilization of water-in-water emulsions using particles has attracted much attention (Balakrishnan et al., 2012; Dewey et al., 2014; Firoozmand, Murray, & Dickinson, 2009; Hanazawa & Murray, 2013 & 2014; Murray & Phisarnchananan, 2014; Nguyen, Nicolai, & Benyahia, 2013; Nguyen et al., 2015; Poortinga, 2008; de Freitas, Nicolai, Chassenieux, & Benyahia, 2016; Firoozmand & Rousseau, 2014; Buzza, Fletcher, Georgiou, & Ghasdian, 2013). However, there are only a few studies where food-grade particles have been

utilized, because it is difficult to find water-insoluble food-grade particles suitable for water-in-water Pickering emulsions (Poortinga, 2008; Firoozmand, Murray, & Dickinson, 2009; Hanazawa & Murray, 2013 & 2014). In this thesis we show that interfacial aggregation of particles at the interface of emulsion droplets in these systems may complicate the simple picture of Pickering stabilization (Figure 2). Another parameter influencing the adsorption behavior of particles from insoluble protein is the possible adsorption of polymers on the particle surface. In the case of adsorption of hydrophilic polymers on the protein particles, the wetting characteristic of particles and their tendency to aggregate can be positively or negatively affected. Therefore, we conclude that the study of the particle-particle and particle-polymer interactions is crucial for understanding the stabilization mechanisms of water-water systems.

Instead of using particles, there is also another way of stabilization of water-in-water emulsions: the addition of either one or two oppositely charged polyelectrolytes that act through interfacial complexation (Ma et al., 2016; Hann, Niepa, Stebe, & Lee, 2016; Tromp, Tuinier, & Vis, 2016). Here, it is important to distinguish whether the added polyelectrolyte is strongly or weakly charged. Small quantities of weakly charged polyelectrolyte have been found to accumulate at the water-water interface, and impart some stability (Tromp, Tuinier, & Vis, 2016). On the other hand, strongly charged polyelectrolyte can induce thermodynamic incompatibility (demixing) in semi-dilute and highly compatible protein-anionic polysaccharide mixtures at pH>pI of protein (Antonov & Moldenaers, 2009), but it can induce compatibility (mixing) in cases of incompatible protein-uncharged polysaccharide mixtures (Antonov & Moldenaers, 2012).

Chapter 2 deals with the effectiveness of various food-grade sub-micron protein particles (zein, gluten, gelatin) of different hydrophobicity as stabilizers for oil-in-water emulsions. This chapter describes the important parameters that should be taken into account, besides particle wettability, in order to understand the Pickering stabilization. Chapter 3 focuses on the interactions between the above-mentioned food-grade protein particles with uncharged polysaccharide dextran. Particle-polymer interactions are also studied in Chapter 4, as a basis

for understanding the behavior of particles in aqueous polymer mixtures. This chapter explains the surface activity of zein particles at water-water interfaces between phase-separating mixtures of fish gelatin and dextran on the grounds of the interactions of particles with each polymer. Finally, Chapter 5 explores the possible stabilizing effect of strongly charged polyelectrolyte dextran sodium sulfate (DSS) on the phase behavior of gelatin-dextran mixtures.

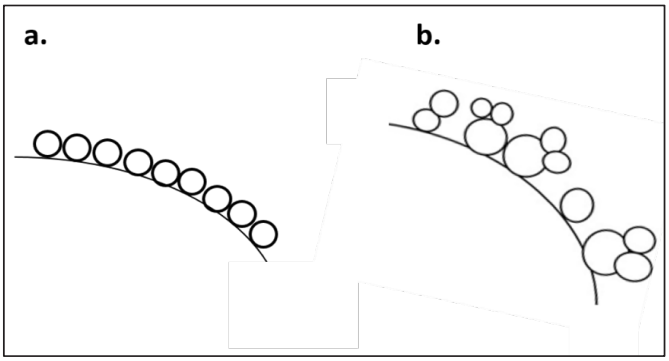

Figure 2. Classical picture of Pickering stabilization (**a**) and interfacial aggregation of particles at the interface of single droplets that complicates the simple ‘’Pickering picture’’ (**b**).

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Chapter 2

Protein particles at oil-water interfaces

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Abstract

Sub-micron food-grade protein particles (zein, gluten and gelatin) of different hydrophobicity are synthesized by the anti-solvent precipitation method and compared with respect to their effectiveness as stabilizers of oil-water emulsions. We show that the formation of a contact angle close to 90^{o} is not sufficient to predict emulsion stability. Instead, the charge of particles and the interactions of particles with each other are crucial parameters in emulsion stabilization mechanism. Gelatin particles are shown to be the most efficient stabilizers owing to their gelling properties.

Introduction

The use of solid food-grade particles for emulsion stabilization, so-called Pickering stabilization (Pickering, 1907; Ramsden, 1903) has several applications in food industry, such as encapsulation of active ingredients or improvement of the texture or structure of processed foods (Berton-Carabin, C. C., & Schroën, K., 2015). Particles adsorb at the oil-water interface and create a mechanical barrier that prevents the emulsion droplets from coalescence (Dickinson, 2012). The desorption energy of particles depends on their hydrophobicity, which is quantified by the three-phase contact angle θ made between particle surface and the liquid interface:

$$\Delta G = \pi r^2 \gamma_{ow} (1 \pm cos\theta)^2 \quad (1)$$

where ΔG is the free energy required to remove a particle from the interface, r the radius of the particle, θ the contact angle with the interface and γ_{ow} the interfacial tension. By convention, θ is measured from the water side, and particles mostly immersed in the aqueous phase are termed hydrophilic, whereas those exposed to the non-polar phase are hydrophobic. Small contact angles (<<90°) correspond to high wettability (by water) and are observed when the liquid spreads on the surface. Large contact angles (>>90°) correspond to low wettability and are observed in case of very hydrophobic surfaces, where the fluid tries to minimize its contact with the solid surface and forms a compact liquid droplet. When the contact angle is exactly 90°, we have the neutrally-wetting case and the highest emulsion stability (Yuan., & Lee, 2013).

The right wettability, i.e. not too different from 90°, of particles is not sufficient, though, to produce a stable emulsion. This has been already shown for foams (Deleurence, Parneix, & Monteux, 2014). The reason is that the ideal case of a monolayer of uniform spherical particles is rarely realized in practice due to the high polydispersity of most particle suspensions. Therefore, except for the right contact angle that the particles should form at the interface, other parameters are also crucial for emulsion stabilization mechanism. One is the charge of particles: positively charged particles tend to stabilize interfaces better than negatively charged particles. This happens presumably because

anionic particles are repelled by the electric charge of the bare liquid–liquid interface, which is negative. Another parameter is the interactions between particles. For example, the aggregation of particles may reduce their effectiveness in covering interfaces after adsorption. Alternatively, the formation of a three-dimensional network from particles adsorbed at different oil droplets may lead to a stronger gel-like emulsion, since oil droplets can be trapped in the network. This has been reported both in the case of gelatin and microgel particles (Li, Ming, Wang, & Ngai, 2009; Tan, Sun, Lin, Mu, & Ngai, 2014; Tan et al., 2017). Dickinson (2010) has summarized the possible arrangements of particles once they are adsorbed at the interface. Some of these structures involve aggregation of particles or flocculation of droplets (Figure 1a-c). An addition to this picture is the aggregation of particles with each other at the interface of single droplets (Figure 1d), as we showed in our previous work (Chatsisvili, Philipse, Loppinet, & Tromp, 2017). In this case, on the one hand, the elastic layer of aggregated particles that is formed does not allow the droplets to shrink or expand, but on the other hand, there are fewer particles available for adsorption.

In the present work, we report the study of three types of food-grade protein particles with different hydrophobicity as emulsion stabilizers: hydrophobic zein, more hydrophilic gluten and very hydrophilic mammalian gelatin particles. The goal is to explore if there is a connection of the right wettability and a favorable contact angle of particles at the oil-water interface with increased emulsion stability. In other words, we seek to demonstrate that a favorable contact angle close to 90^{o} is not sufficient to ensure emulsion stability. Zein is a class of prolamine protein found in maize (corn), it is insoluble in water and therefore considered hydrophobic. Gelatin is a denatured, biodegradable protein obtained by controlled hydrolysis of the triple-helix structure of collagen into single-strain molecules. This protein is soluble in water elevated temperatures and therefore considered hydrophilic. Gluten is a co-product of the wheat starch isolation industry and consists of the monomeric gliadin (soluble in 70% ethanol) and polymeric glutenin (insoluble residue) (Belitz, Grosch, & Schieberle, 2009), and therefore in terms of hydrophobicity it can be considered to be between zein and gelatin.

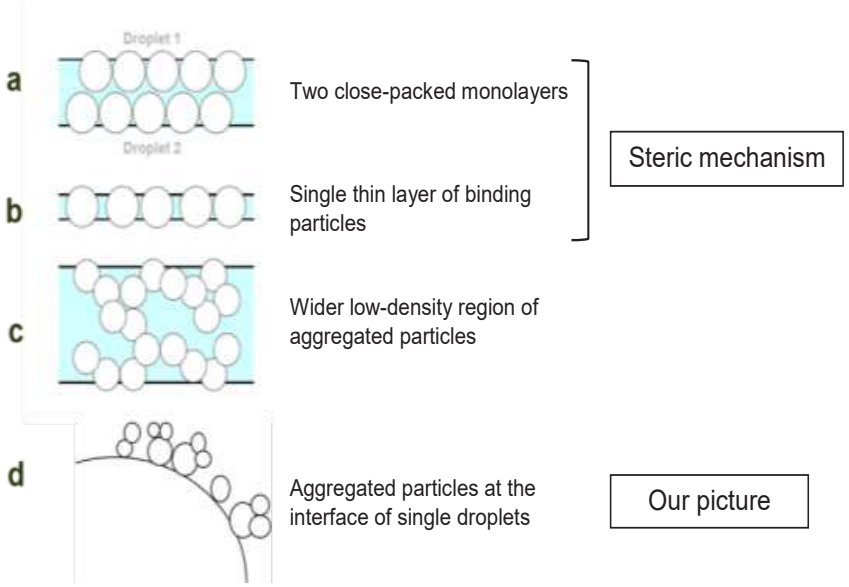


Figure 1. Schematic representation (**a, b, c**) of alternative stabilizing arrangements of particles in a thin film between closely approaching emulsion droplets (adapted and taken from Dickinson, 2010). Cartoon **d** depicts another arrangement of particles, shown in our previous work (Chatsisvili, Philipse, Loppinet, & Tromp, 2017).

Materials and Methods

Materials

As a source of particle material we used the proteins zein from corn (obtained from Flow Chemical Corporation, Ashburnham, MA, USA), gluten from wheat (obtained from Sigma-Aldrich), and pig skin gelatin (Bloom 250, from Darling Ingredients, Gent, Belgium). Medium chain triglyceride (MCT) oil was used without further purification. Phosphate buffer was used to control the pH. Absolute ethanol (99.9%) was obtained from Nedalco, Heilbronn, Germany. The dialysis tubing used for the removal of ethanol in particle dispersions (MWCO 12-14 kDa) was obtained from Medicell Membranes Ltd. Glutaraldehyde solution of 25% (w/w) was used as a crosslinker for gelatin particles. Water purified by a reverse-osmosis was used for all the experiments.

Synthesis of particles

Zein particles. 0.2 g zein was dissolved in an aqueous ethanol mixture (80 (w/v) % EtOH), prepared by mixing 80 g ethanol with 20 g water. The solution was stirred for 1-2 hours at 40^{o}C to achieve the dissolution of zein as much as possible, and centrifuged at 9,000 g for 30 min. Afterwards, 30 g of the supernatant was added dropwise to a beaker which contained 70 g water, under gentle stirring. A turbid dispersion with a milky appearance was formed. To remove ethanol and obtain an aqueous dispersion of zein particles, dialysis was performed against acidic water of pH 3.7 for 15-16 hours. The pH of the suspension of zein particles was 3.7. Increasing the pH gave rise to extensive aggregation, which was considered undesirable for the present investigation.

Gluten particles. 0.5 g gluten was dissolved in an aqueous ethanol mixture (70 (w/v) % EtOH), prepared by mixing 70 g ethanol with 30 g water. The solution was stirred for 1-2 hours until the ethanol-soluble part, i.e. gliadin, dissolved as much as possible, and then, centrifuged at 1,400 g for 10 min. Afterwards, 30 g of the supernatant was added dropwise to a beaker which contained 70 g water, under stirring. To remove ethanol and obtain an

aqueous dispersion of gliadin particles, dialysis was performed against RO water for 15-16 hours. The final aqueous dispersion had a pH of about 6.

Gelatin particles. 25% of gelatin solution in water was prepared by dissolving gelatin under stirring at 60°C. Afterwards, 5% of the above solution was added dropwise to a beaker which contained 95 g of an aqueous ethanol mixture (80 (w/v) % EtOH, under stirring. Glutaraldehyde was also added (0.06%, w/w) as a cross-linker in order to prevent the dissolution of particles in water. The solution was stirred for 1-2 hours. To remove ethanol and excess glutaraldehyde, dialysis was performed against water for 15-16 hours. The final aqueous dispersion had a pH of around 5.5.

Particle characterization

Particle size measurements. The size of particles in aqueous suspensions was determined by dynamic light scattering measurements carried out with the ALV-CGS4 Compact Goniometer System with 4 detectors. The pH of particle dispersions was 3, 5.7 or 8.1.

Electrophoretic mobility. The electrophoretic mobility of particles was measured with a Zetasizer Nano, employing cuvettes equipped with an electrode, and using the Smoluchowski equation for converting measured mobilities to zeta potentials. The surface potential properties of particles were investigated as a function of pH. At least two separate measurements were performed for each pH value.

Contact angle measurements. The oil-in-water three-phase contact angles were measured using a DataPhysics OCA15 setup. For this purpose, particle suspensions of pH 3, 7.1 and 8.1 were deposited on glass substrates and left in the fume hood to evaporate. After their evaporation, they were observed under the optical microscope to confirm the formation of particle films. Next, the particle-coated substrates were immersed in an oil phase. A needle of a syringe containing water phase was brought into contact with the substrate, and a water droplet (1–2 μL) was formed in the oil phase at the tip of

the needle. Subsequently, the needle was gently retracted, leaving behind a water droplet at the particle film (Figure 2). Contact angles were determined automatically with a Laplace–Young fit, from the contour of the droplet shapes recorded by a camera. Measurements were averaged over at least five droplets.

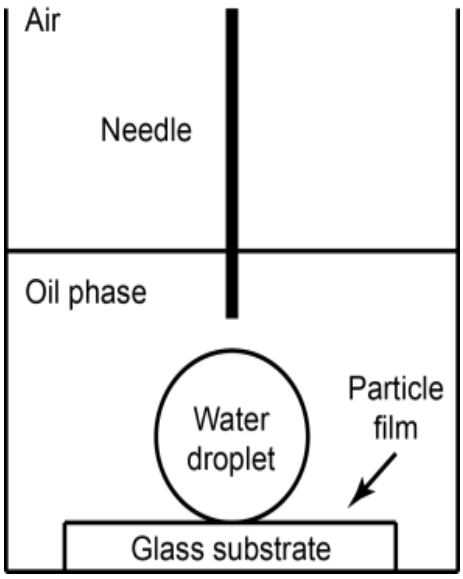


Figure 2. Schematic representation of the contact angle measuring setup**.**

Interfacial tension measurements. A certain volume of particle suspension (0.2% w/w) or water (the control) was put inside a flat cuvette (45x0.5 mm). MCT oil was slowly added on top of the water phase using a syringe. Care was taken to avoid disturbing the oil-water interface. After a few minutes, the profile of the oil-water interface in the cuvettes was photographed with a standard digital camera (Figure 3). Finally, the values of the interfacial tension were determined by fitting the curvature of the interface to the Young-Laplace equation which relates the capillary pressure difference to the shape of the interface. The calculations were done using Matlab.

Figure 3. Camera image of a cuvette containing the particle suspension in the bottom and the oil phase on the top.

Emulsion preparation and characterization

To prepare an oil-in-water emulsion, 5 g of particle suspension (0.2% concentration, w/w) and 5 g of MCT oil were brought into a glass vial. Mixing with an Ultra Turrax homogenizer followed immediately with a dispersing head operating at 800 rpm for 2 minutes. The time and speed of mixing was proven not to be crucial. pH adjustments were made in particle suspensions with HCl and NaOH solutions. The samples were stored in the refrigerator at 4^{o}C.

The layer formation due to instability of the emulsion was followed in time by measuring the position of the interface 1 hour and 2 weeks after sample preparation. The top (oil-rich) phase was visualized using the optical microscope Reichert-Jung (Leica Microsystems, Switzerland), as well as Confocal Scanning Light Microscope (CLSM).

Results and discussion

Synthesis and characterization of various particles

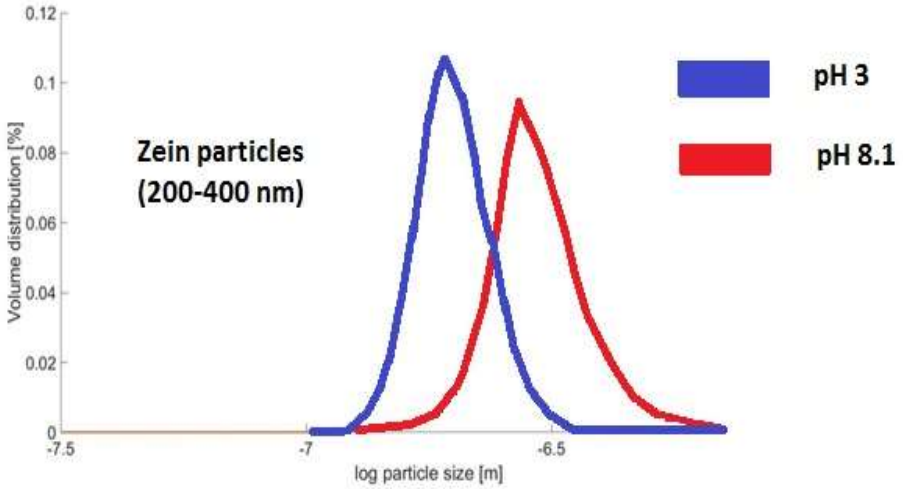
The anti-solvent precipitation method is a rapid, cost-effective and simple method to produce various kinds of sub-micron or nanoparticles (Langer et al., 2003; Patel, Hu, Tiwari, & Velikov, 2010; Subia & Kundu, 2013; Yoo, Choi, & Park, 2001). According to the method, a material is dissolved in an appropriate solvent, and then this solution is added to an anti-solvent. Nucleation occurs when the concentration of the material in the resulting mixture exceeds the critical supersaturation concentration. Above a critical nuclei size, the nuclei serve as seeds for the formation and growth of particles. The nucleation and growth rates of particles determine their final properties, and can be manipulated by careful selection of the solvent, anti-solvent, and process parameters/conditions (Joye & McClements, 2013; Kakran, Sahoo, Tan, & Li, 2012; Joye, Nelis, & McClements, 2015).

In this work, we used successfully the anti-solvent precipitation method for the synthesis of well-defined, non-aggregated particles from proteins with different hydrophobicity, i.e. zein, gluten and gelatin. We used aqueous ethanol as a solvent and water as an anti-solvent, except in the case of gelatin, where the water is the solvent and ethanol the anti-solvent. Because of gelatin's solubility, an extra step of crosslinking with glutaraldehyde was required in order to prevent the dissolution of gelatin particles in the final aqueous suspension. Glutaraldehyde is commonly used to harden protein particles by inducing the formation of covalent crosslinks between proteins (Migneault, Dartiguenave, Bertrand, & Waldron, 2004). Although its use as a hardening agent in food applications is not currently accepted, it can be used as a general preservative in food-contact adhesives and mineral slurries which are used during the fabrication of paper meant as food packaging material (FDA, 2013).

All particles prepared with anti-solvent precipitation method had sizes ranging from 100-500 nm dependent on pH (Figure 4). Therefore, they can be more appropriately called sub-micron particles instead of nano-particles. Zein particles had an average radius of 200-400 nm (except at pH 5.8-6.5, where they were extremely aggregated, so DLS

measurements were unfeasible), gluten particles between 70 and 300 nm and gelatin particles 200-250 nm. Still, a significant fraction of the particles was small enough to exhibit Brownian motion and resist sedimentation. For the latter, the charge is also an important factor that determines together with the hydrophobicity the overall stability of particle suspensions. These surface characteristics of particles (i.e. charge and hydrophobicity) are expected to be set during their production. Specifically, during the nucleation and particle growth processes, the protein molecules position themselves most likely in such a way that the majority of the non-polar residues are buried inside the particle, while the more polar ones are oriented towards the surrounding aqueous phase (Joye, Nelis, & McClements, 2015). In order to determine the surface potential of particles, electrophoretic mobilities were measured at different pHs. From these measurements, the zeta-potentials of particles and the isoelectric point of each protein were also determined (Figure 5).

a.



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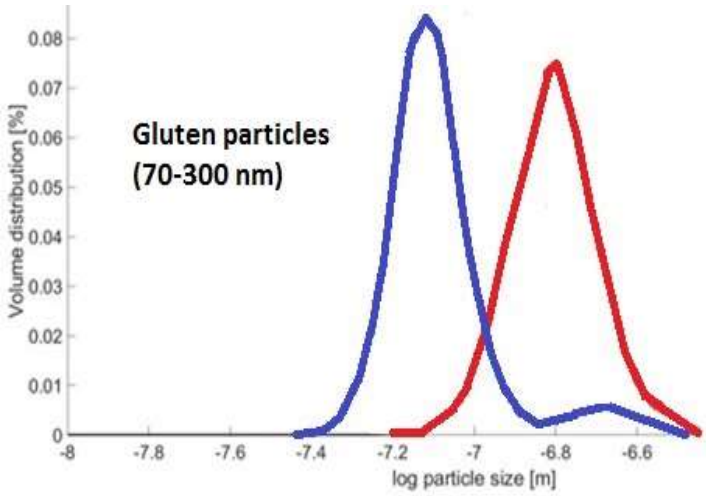

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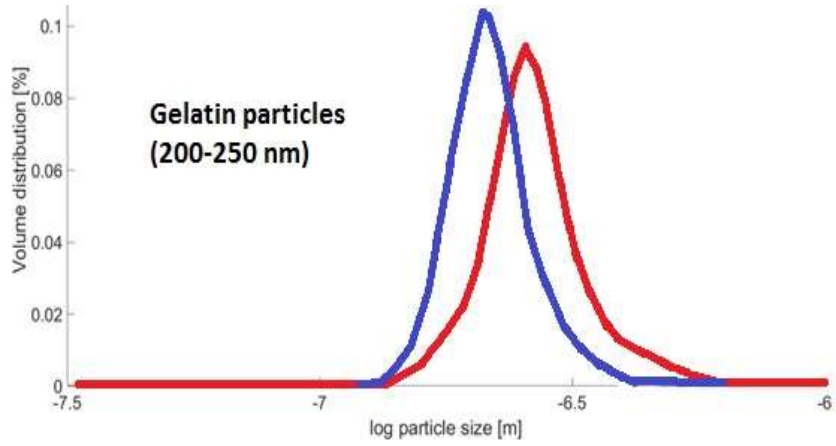

Figure 4. Hydrodynamic radius of zein (**a**), gluten (**b**) and gelatin (**c**) particles at pH 3 and pH 8.1 determined by dynamic light scattering measurements (scattering angle 17º).

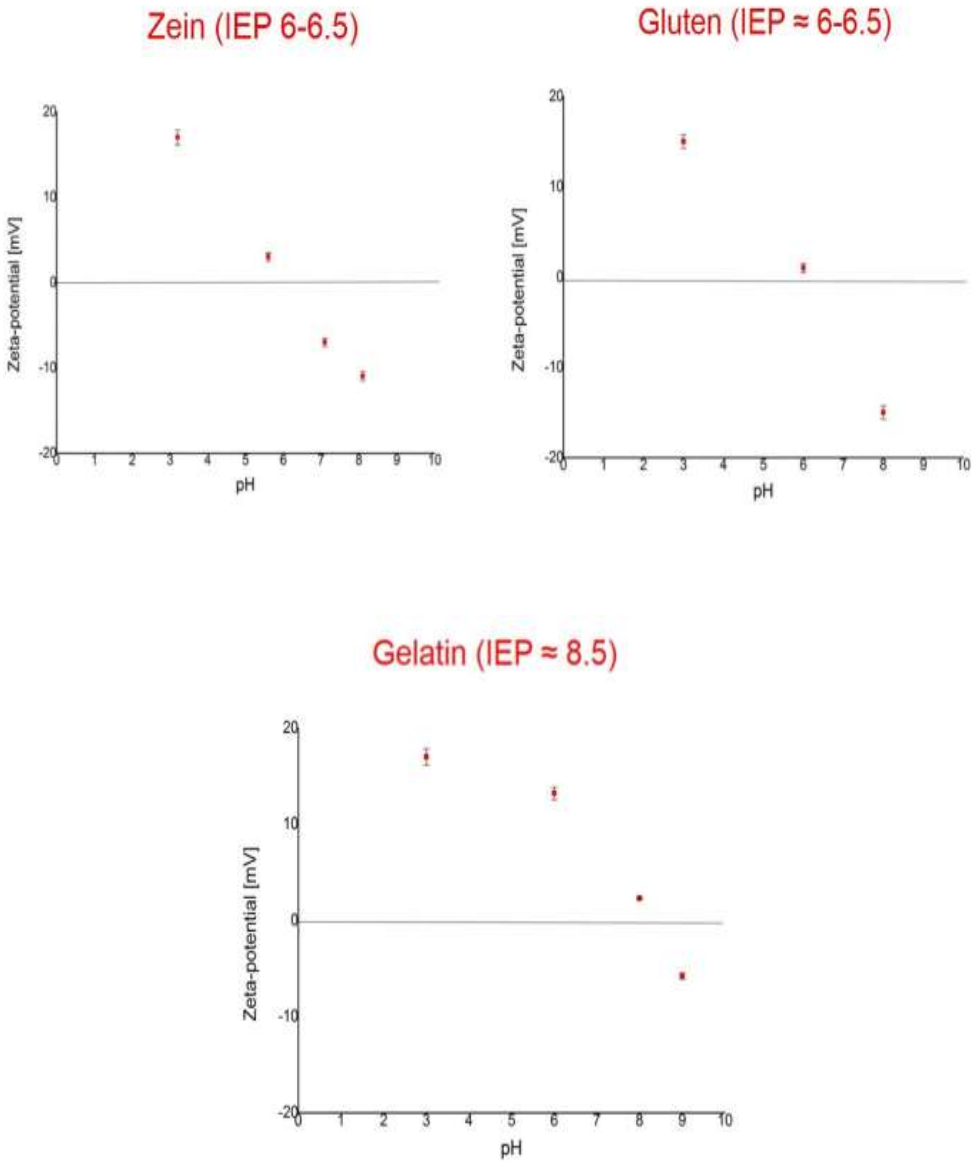

Figure 5. Zeta-potential values of zein, gluten and gelatin particles.

Wetting properties of particles and interfacial tension of oil-particle suspensions

The wettability of particles was determined by measuring the contact angles which were formed when a drop of water was spread on the particle film immersed in oil-continuous phase. Figure 6 shows the contact angles that zein, gluten and gelatin particles formed at the oil-water interface. For zein particles, at pH < pI and pH > pI we see a difference in wettability. Since in both cases we expect a low hydrophobicity on the basis of IEP, we could presumably say that charge (positive or negative) affects the exposure of hydrophobic and hydrophilic groups at the surface of zein particles. Negatively-charged zein particles appear to be more hydrophilic than positively-charged particles. Also, there are reports showing that for very hydrophobic particles, an increase in the pH raises their hydrophilicity (Binks & Rodrigues, 2007). For gluten particles, we also see that charge affects significantly their hydrophobicity: at pH 3 they seem to be quite hydrophilic (with small contact angle), whereas at pH 8 they become relatively more hydrophobic. On the contrary, the wettability of gelatin particles does not seem to be greatly affected by pH.

Figure 7 shows the interfacial tension calculations from which we conclude that zein particles were able to lower the interfacial tension the most, while oil-water interfaces with gelatin particles had the highest surface tension. Gluten particles showed better activity at pH 8; apparently at pH 3 they appear to be too hydrophilic to stabilize an oil-water interface.

Taking into account the contact angle measurements, the size of each type of particles and the measured interfacial tension values, we calculated the energy that is required to remove the particles from the interface using the equation (1) [$\Delta G = \pi r^2 \gamma_{ow}(1-\cos\theta)^2$] (Aveyard, Binks, & Clint, 2002) (Table 1). Gluten particles at pH 3 and gelatin particles at pH 8 seem to have the lowest adsorption energy at the oil-water interface. That should mean that they are the less efficient emulsion stabilizers.

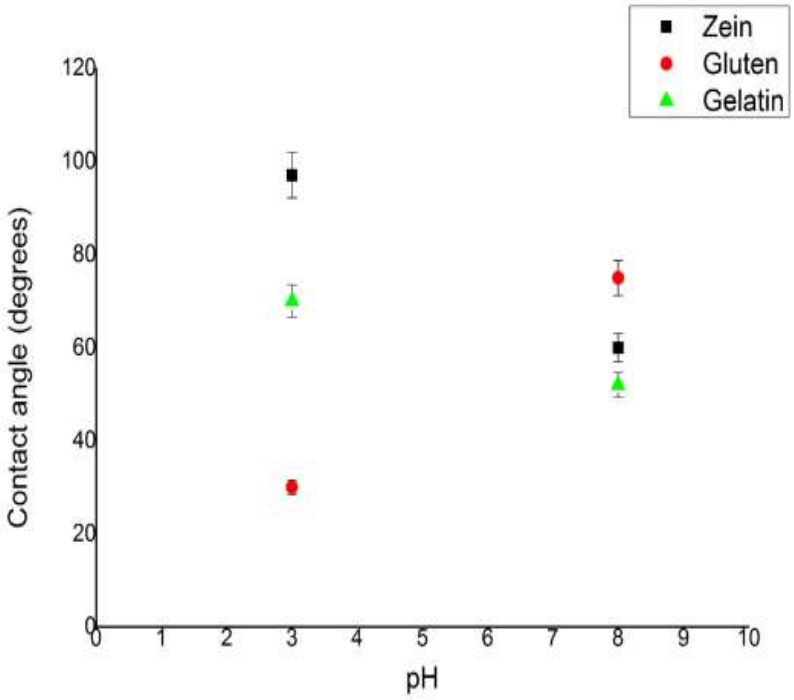

Figure 6. Three-phase contact angle of zein, gluten and gelatin particles, at different pH values, at the oil-water interface.

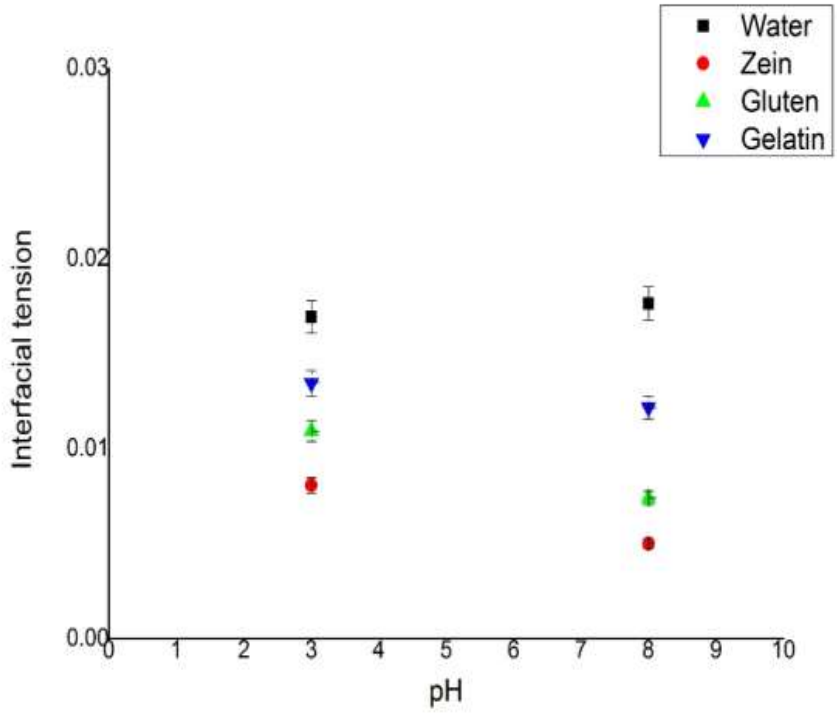

Figure 7. Measured interfacial tension values at the interface between oil and particles. The interfacial tension at the oil-water interface is lower than expected, possibly due to surface active minor components in oil.

Particles	Zein		Gluten		Gelatin	
pH	3	8	3	8	3	8
θ	97	60	30	75	70	52
ΔG (10^5 kT)	3	1.5	0.007	2.8	1.8	0.8

Table 1. Values of contact angle and adsorption energy of zein, gluten and gelatin particles.

Emulsion stabilization with different particles

As we mentioned above, we expect poor stability of gelatin particle-stabilized emulsions at pH 8 based on the grounds of particle wettability, compared to zein particle-stabilized emulsions. As for gluten particles, they must have good emulsion stabilizing ability at pH 8, and poor ability at pH 3.

We prepared emulsions containing zein, gluten or gelatin particles at pH 3 and pH 8. Figure 8 shows the macroscopic image of those emulsions after their phase separation into two layers. As we can see macroscopically, a stabilized oil-rich phase is formed in all emulsions. Quantitative determination of emulsion stability was achieved by measuring the volume of that oil-rich phase (Figure 9). We see that emulsions containing gelatin particles at pH 8 showed the highest stability, which is contradictory to the predictions made according to gelatin particles' wettability at that pH. At pH 8 which is close to IEP of gelatin, particles have a neutral charge and don't repel each other. However, in the case of zein or gluten particles, the preparation of stable emulsions at pH close to IEP was not possible due to the aggregated state of aqueous particle suspensions. In this case, the aggregation behavior of particles overrules, and the particles are unable to adsorb at the droplet interface. In general, zein particles and gluten particles show higher tendency to aggregate when compared to gelatin particles (data not shown). Far from the IEP, zein-stabilized emulsions showed a better stability compared to gluten-stabilized emulsion. At pH 3 gluten-stabilized emulsions showed higher stability compared to gelatin-stabilized emulsions, which is also unexpected according to their wettability.

Therefore, we conclude that the right contact angle that particles form at the oil-water interface is not enough to predict emulsion stability. Instead, we should also take into account the interactions of particles with each other due to charge and aggregation behavior.

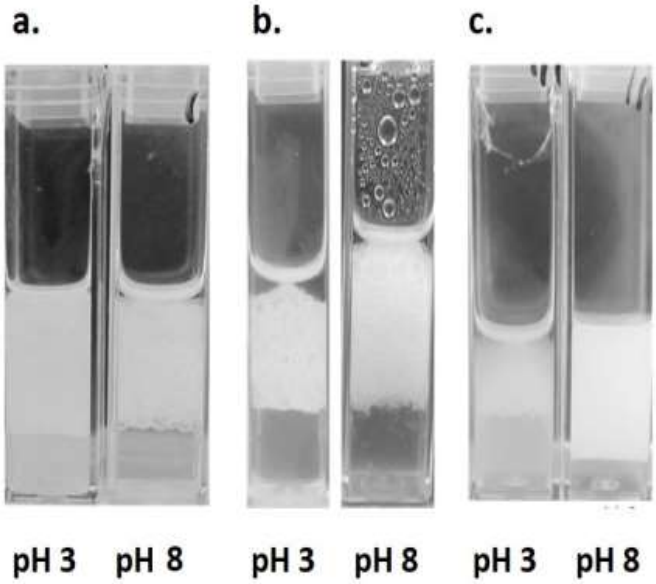

Figure 8: Macroscopic images after the layer formation in oil-water emulsions with zein (**a**), gluten (**b**) and gelatin (**c**) particles as potential stabilizers. The top layer is oil-rich.

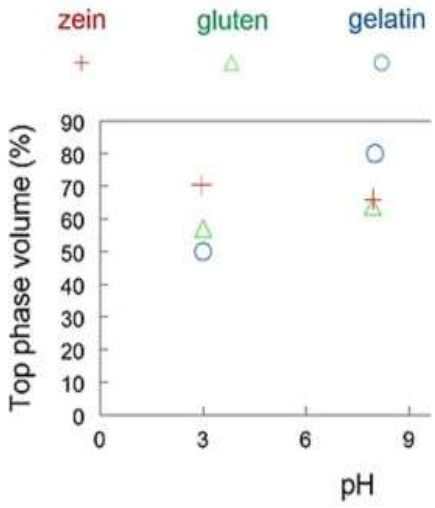

Figure 9: Volume (% of total volume) of the oil-rich layer after layer formation of the zein, gluten and gelatin particle-stabilized emulsions.

CLSM images of the top phase show layers of nearly touching particles inbetween droplets (Figure 10). These droplets are probably come closer to each other after the water is pulled out due to the gravity and the structure becomes more compressed.

In all emulsions with particles, the bottom phase water-rich phase does not appear to contain stabilized emulsion droplets, but only particles showing Brownian motion (data not shown).

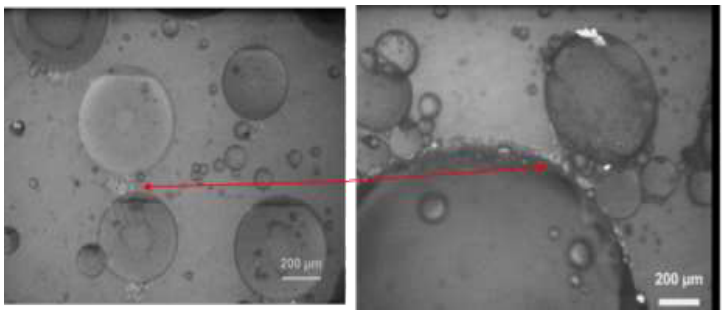

Figure 10. CLSM images of the particle-stabilized oil-rich phase. The droplets are oil droplets. The images appeared similar with all types of particles.

Special case of gelatin particles

Emulsions containing gelatin particles showed a very stable top phase, even weeks after their preparation (Figure 11). In contrast, the top phase of emulsions containing gluten (or zein) particles was shrinking even after 1 week.

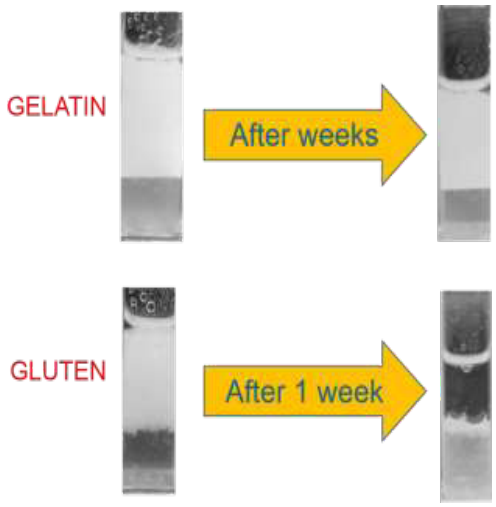

Figure 11. Macroscopic image of emulsions containing gelatin and gluten particles a few hours and after 1 or more weeks after their preparation.

This led us to examine the possibility of a different mechanism by which gelatin particles may stabilize an emulsion. Since gelatin gels at room temperature, a possible mechanism could be stabilization due to the gelling of the contact areas between the particles. In order to test this hypothesis, phase separation of emulsions containing gelatin particles was performed without heating and after heating in an oven at 45^{o}. As we can see in Figure 12, the macroscopic image of the top phase of the two emulsions is clearly different. Microscopically, the top phase of the emulsion with no heating contained stabilized droplets, whereas the top phase of the emulsion after heating contained only particles (many of which are not visible with the optical microscope due to their size in the submicron-range) and no droplets. This can be explained by the fact that at high temperature the ''contact gels'' melted. This experiment proved two things: first, gelatin particles are the most efficient stabilizers due to their gelling properties, and second, the interactions between particles (i.e. aggregation) is able to dominate the stabilization of an emulsion by particles. When gelatin particles did not have the additional interaction of merged contact zone (at high temperature), the emulsion was unstable.

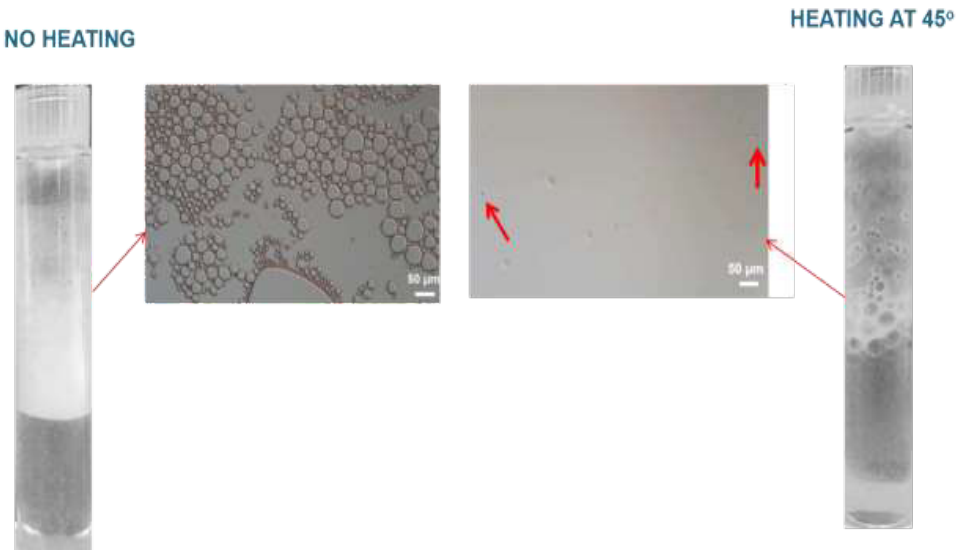

Figure 12. Emulsions containing gelatin particles after formation of water-rich and oil-rich layers without heating (left) or after heating at 45°C (right). The microscopy images are taken in the oil-rich top layer.

Conclusions

We examine the emulsion stabilizing ability of 3 types of food-grade sub-micron particles with different hydrophobicity (zein, gluten and gelatin). We conclude that only the wettability of Pickering particles is not sufficient to predict their effectiveness as emulsion stabilizers. Other parameters that should be taken into account is the particle charge and the aggregation behavior in the initial particle aqueous suspensions. We showed that gelatin particles are the most efficient stabilizers even though their adsorption energy at the oil-water interface is relatively low. Their stabilizing ability is tentatively attributed to their gelling properties.

Acknowledgements

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Chapter 3

Repeptization of protein particle aggregates by uncharged polysaccharide

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Abstract

Dynamic light scattering (DLS) is employed to investigate the interactions between food-grade protein particles from zein, gluten and gelatin, synthesized by the anti-solvent precipitation method, and the uncharged polysaccharide dextran. We demonstrate that hydrodynamic radii always decrease upon dextran addition, indicating that particle aggregates fall apart in an extent that increases at higher dextran concentration. This aggregate repeptization is considered to be caused by dextran's adsorption on the surface of particles. The differences in the importance of dextran's repeptizing effect for each type of particles at certain pH values is explained on the basis of particle aggregation behavior. The effect of the addition of neutral polysaccharides on the aggregation of sub-micron vegetable protein particles could be of practical importance when using these particles as Pickering stabilizers or in high-protein, low viscosity food formulations.

Introduction

Colloidal suspensions are produced by suspending particles of sizes between nanometers and micrometers in solvents. The stability of colloidal suspensions is governed by mutual interaction forces between the colloidal particles. The most relevant interaction forces are described with the classical theory from Derjaguin, Landau, Verwey, and Overbeek (DLVO) (Derjaguin, 1941; Russel, Saville, & Schowalter, 1989; Verwey, & Overbeek, 1948). According to the DLVO theory, the interaction forces can be approximated by the sum of the electrical double-layer repulsion and the van der Waals attraction. The balance between those two opposing forces explains why some colloidal systems aggregate while others do not. The stability of colloidal dispersions is highly sensitive to surface charge of particles (zeta-potential, pH) and salt concentration. In the case of aggregated particles, the aggregation can be reversed by e.g. a change of solvent pH, solvent dielectric constant or a polymer, as described in this thesis. The reversal of aggregation is, in particular in the case of protein particles, often called repeptization (Rasa, Philipse, & Meeldijk, 2004).

When mixing colloidal dispersions with polymer solutions, other interaction forces (i.e. steric repulsion, depletion etc) induced by polymer may also become important (Block & Helm, 2007; Christov, Danov, Zeng, Kralchevsky, & von Klitzing, 2010). In the study of interactions in colloid-polymer mixtures, it is important to distinguish between polymers that are adsorbed on the colloidal surfaces and those that are free in solutions, because the two situations lead to different effects (Ye et al., 1996), as illustrated in Figure 1. In a mixture of a colloid and a non-adsorbing polymer, attraction of colloidal particles takes place due to the depletion effect, in case of uncharged particles. In this case, polymer chains are expelled from the regions between colloidal particles, producing an unbalanced osmotic pressure difference that in turn results in an effective attraction between particles (Asakura & Oosawa, 1954). On the other hand, in a mixture of a colloid and an adsorbing polymer, either stabilization or flocculation of colloidal particles can occur, depending on a number of parameters such as the solvent quality, the amount of polymer adsorbed to the surface, and whether it is physically or chemically attached to the surface (de Gennes, 1980; Netz & Andelman, 2003). Adsorption stabilization, also termed steric stabilization, arises in

a good solvent and can be attributed to the osmotic (or entropic) interactions between the polymer segments on opposite surfaces (Dolan & Edwards, 1974). In this case, polymer adsorption makes the particles more hydrophilic, therefore their stability is not dependent anymore only on their charge. Adsorption flocculation occurs for two reasons: either due to the bridging of polymer chains adsorbed on both surfaces when there is not enough polymer to fully cover the surfaces, or due to bad solvent conditions for the adsorbed polymer layers (Israelachvili, 2011).

Although the adsorption of charged polymers on oppositely charged surfaces through electrostatic interactions has been widely studied (Joanny, Castelnovo, & Netz, 2000), the case of non-ionic polymer adsorption is less clear. Unlike polyelectrolytes whose chains are more or less stretched out or extended due to intramolecular charge repulsion, uncharged or non-ionic polymers tend to adopt a random coil conformation in a solution. For this reason, the latter are relatively poor flocculants of dilute particle dispersions (Theng, 1979). Another theory is that hydrogen bonding plays an important role in the mechanism of non-ionic polymer adsorption (Reid, Villalobos, & Cranston, 2017; Lindman, Karlstrom, & Stigsson, 2010; Medronho, Romano, Miguel, Stigsson, & Lindman, 2012). Yet, many studies show a varying adsorption behavior of chemically similar non-ionic polysaccharides, which suggests that the adsorption may not be driven by the common ability to hydrogen bond. Alternatively, the adsorption of an uncharged polymer on a hydrophobic surface may be entropically driven by the release of water from that surface (Reid, Villalobos, & Cranston, 2017). In other words, the non-ionic polymer adsorption onto a surface leads to the desorption of numerous solvent (water) molecules from that surface and replacement with a layer of hydrophilic polymer. The entropy that is so gained provides the driving force for adsorption, because the enthalpy change of the process is commonly very small and may even be positive. Adsorption also leads to a change in conformation of the polymer, which in solution tends to exist as a random coil, as we said, but at the solid/solution interface it uncoils and spreads out (Figure 2).

In the present work we investigate the interactions in protein particle suspensions in presence of low concentrations of dextran. Dextrans are a class of neutral (uncharged) polysaccharides composed of repeated monomeric glucose units mainly bonded by a-1,6 glycosidic bonds with some a-1,3 bonds. As a source of particle

material, we chose three food-grade proteins: gluten, zein and mammalian gelatin. Dynamic light scattering (DLS) was used to determine effective hydrodynamic radii of the colloidal particles, and how they are affected by the presence of non-charged dextran chains. Varying parameters were the polymer concentration and the pH. DLS has been widely used to determine the hydrodynamic radius of various particles: the measurement time is short, automated and the technique is extremely sensitive towards the presence of small aggregates, since the scattering intensity is proportional to the sixth power of the particles' radius. These features make DLS one of the most powerful techniques in monitoring the stability of colloidal suspensions (Lim, Yeap, Che, & Low, 2013).

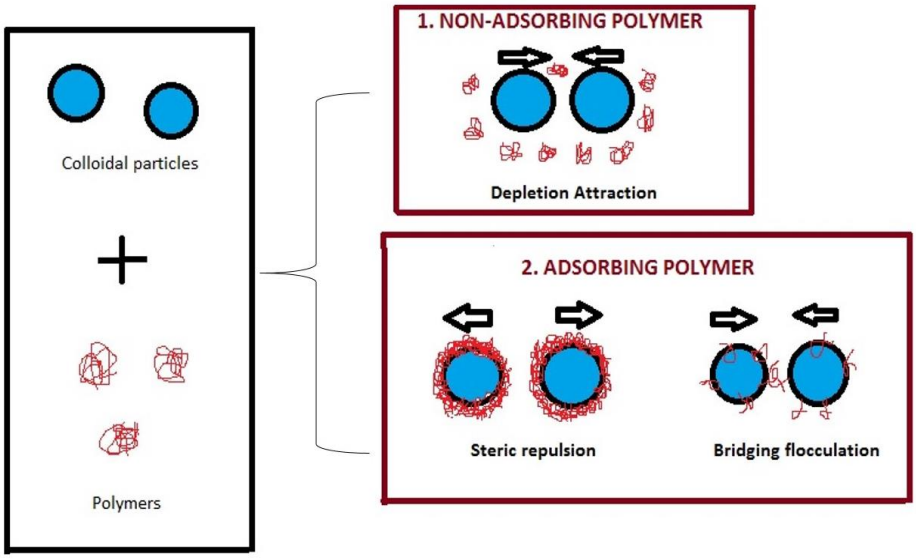


Figure 1. Polymer-induced interactions between colloids (scheme inspired by Gong et al., 2014).

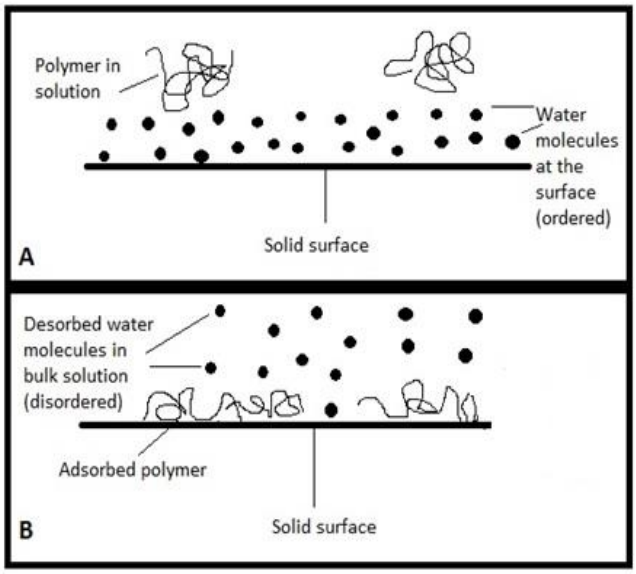


Figure 2. Schematic representation of the desorption of numerous water molecules from a (clay) surface during the adsorption of an uncharged linear polymer, leading to a net gain in entropy by the system. The change in polymer conformation from a random coil in solution to a more or less extended one at the clay/solution interface is also indicated (scheme inspired by Theng, 1979).

Materials and Methods

Materials

As a source of particle material we used three proteins, gluten from wheat (obtained from Sigma-Aldrich), zein from corn (obtained from Flow Chemical Corporation, Ashburnham, MA, USA), and pig skin gelatin (Bloom 250, from Darling Ingredients, Gent, Belgium). Dextran from Leuconostoc spp (450-650 kDa) was obtained from Sigma-Aldrich. Phosphate buffer was used to control the pH. Absolute ethanol (99.9%) was obtained from Nedalco, Heilbronn, Germany. The dialysis tubing used for the removal of ethanol in particle dispersions (MWCO 12-14 kDa) was obtained from Medicell Membranes Ltd. Glutaraldehyde solution of 25% (w/w) was used as a crosslinker for gelatin particles. Water purified by a RO system was used for all the experiments.

Methods

Preparation of mixtures of colloidal particles and dextran

In order to prepare colloid-polymer mixtures, first aqueous particle suspensions were synthesized using the anti-solvent precipitation method, as it is described in Chapter 2. Afterwards, stock solutions of dextran were prepared by dissolving dextran in RO water under magnetic stirring at room temperature. Finally, colloid-dextran mixtures were prepared by mixing appropriate amounts of particle suspensions, stock solutions of dextran and buffer. Two dextran concentrations were studied (0.1% and 0.01% w/w), whereas the particle concentration was always kept at 0.03%. The pH of samples was set at 3, 6.5 or 8.

Dynamic light scattering

Dynamic light scattering measurements were performed to determine the effect of addition of dextran on the stability of particles. The measurements were carried out

using the ALV-CGS4 Compact Goniometer System with 4 detectors at pH 3, 6.5 and 8 at 20°C for gluten, zein and gelatin particles (0.03% w/w) in buffer without polymer and in suspensions of dextran solutions of 0.1% and 0.01% w/w. The effective hydrodynamic radius R was calculated from the Stokes-Einstein equation:

$$D = \frac{kT}{6\pi\eta R} \quad (1)$$

where D is the diffusion coefficient, k the Boltzmann's constant, T the temperature, R is the effective hydrodynamic radius of particles, and η the viscosity of the particle suspension. The diffusion coefficient is calculated from:

$$D = \frac{\Gamma}{2q^2} \quad (2)$$

where Γ is the initial slope of the intensity correlation function and q the scattering vector. The scattering vector is calculated from:

$$q = \frac{4\pi n}{\lambda} sin\frac{\theta}{2} \quad (3)$$

where n is the refractive index of the solvent, λ the wavelength and θ the detection angle.

The results reported are averages of three measurements.

Results

In this work we used 3 food-grade proteins as particle material: zein, gluten and gelatin.

Zein is a relatively hydrophobic protein, due to the high proportion of hydrophobic amino acids, such as leucine, proline and alanine, and its relative deficiency of basic or acidic amino acid residues (Pomes, 1971). The low solubility of zein in water allows for the controlled synthesis of colloidal particles through the anti-solvent precipitation method (Chatsisvili, Philipse, Loppinet, & Tromp, 2017; de Folter, van Ruijven, & Velikov, 2012). The isoelectric point of zein is close to pH 6-6.5. At that pH, the net particle charge is zero, which leads to the aggregation of the particle dispersions. Far from the isoelectric point the particles become charged and repel each other. By lowering the pH to 3, zein particles become positively charged [ζ = +20 mV], whereas above the isoelectric point, the particles appear negatively charged (Chatsisvili, Philipse, Loppinet, & Tromp, 2017). Therefore, the overall stability and aggregation behavior of zein particle suspensions is determined by their surface characteristics, i.e. charge and hydrophobicity.

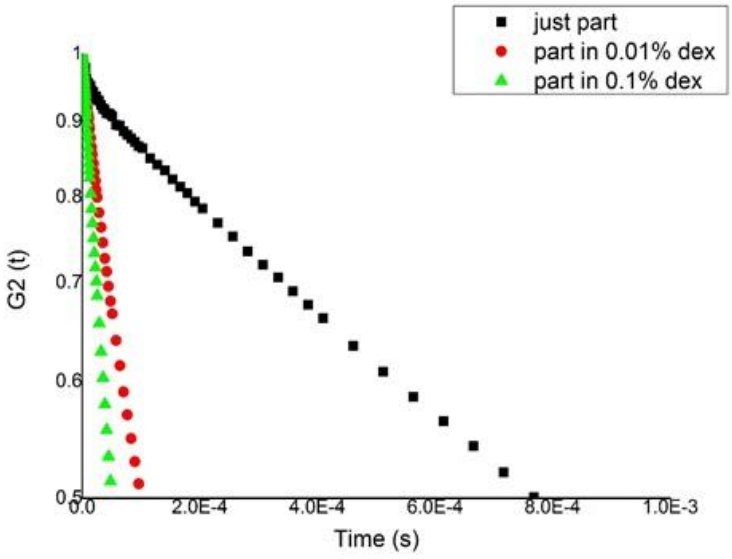
Gluten, a by-product from wheat starch isolation, has been used in previous studies to produce colloidal particles using the anti-solvent precipitation method (Joye, Nelis, & McClements, 2015). Based on solubility, two gluten protein types can be distinguished: monomeric gliadin (soluble in 70% ethanol) and polymeric glutenin (insoluble residue) (Belitz, Grosch, & Schieberle, 2009). The insolubility of gliadin in water makes these proteins interesting materials to produce (gliadin-based) particles. However, it was observed that gliadin particle dispersions have relatively poor stability compared to zein and gelatin particles in almost all pH values, and even more near their isoelectric point (pH≈6) (data not shown). This observation agrees with the findings of Joye, Nelis, & McClements (2015), who mention a slight improvement in gliadin particle stability only after chemical hardening with glutaraldehyde.

Gelatin is a hydrophilic protein obtained by controlled hydrolysis of collagen. Gelatin used in this work is type A and its isoelectric point lays in the pH around 8.5. Below the IEP gelatin carries a net positive charge, and above the IEP a net negative charge. At all pH values gelatin particle dispersions show a relatively high stability and

do not have a great tendency for aggregation. This may be explained by the hydrophilic character of gelatin, as well as the addition of glutaraldehyde to particle dispersions as an extra hardening step in order to prevent their dissolution in the final aqueous suspension.

Dynamic light scattering was used to investigate the interactions of particles with each other in presence and absence of dextran (0.01% and 0.1% w/w). Figure 3 shows a typical measurement for gluten and gelatin particles with and without dextran at pH 6.5. Same tendency was observed also at other pH values, so the results are not shown. As we can see, gluten particles are significantly affected by the addition of dextran. Specifically, a reduction in particle size is observed, which is more pronounced at high polymer concentration. On the other hand, the effect of dextran is almost invisible in case of gelatin particles, even at a high dextran concentration. The difference in dextran's effect for gelatin and gluten particles can be explained on the grounds of the aggregation behavior of particles: gluten (gliadin-based) particles are known to be relatively unstable, as we already mentioned, requiring additional strategies, such as coating with polysaccharide in order to remain stable (Joye, Nelis, & McClements, 2015). This makes the effect of dextran visible, since there is a large number of aggregates that break in presence of dextran. On the other hand, gelatin particles are proven to be relatively stable even at pH close to IEP, therefore there are almost no aggregates in their suspensions. As a result, the size of gelatin particles with and without dextran is almost the same.

a.



b.

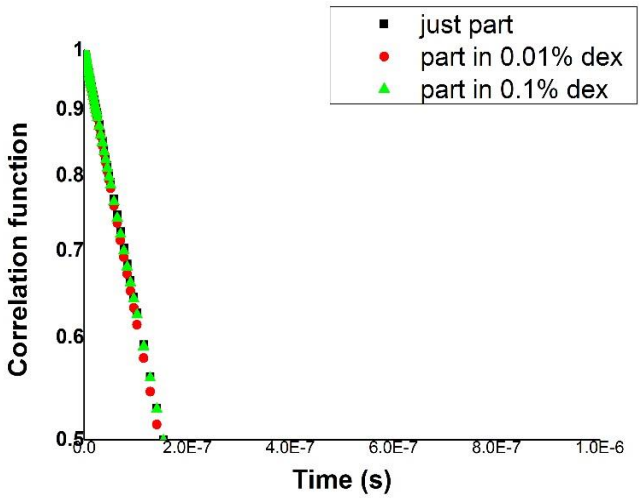
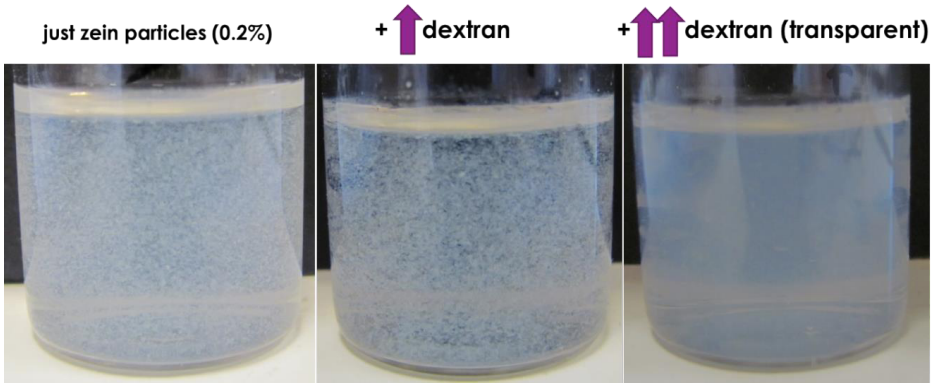


Figure 3. Typical graphs of time correlation functions for gluten (**a**) and gelatin (**b**) particles with and without the addition of dextran (pH 6.5). Similar effect of dextran was observed also at other pH values (data not shown).

In case of zein particles, the charge is an important factor for their aggregation behavior, as we already mentioned. At pH 6.5 (close to the IEP of zein), particles showed such an extensive aggregation that DLS measurements were not possible. Instead, the macroscopic images of particle suspensions at pH 6.5 gave a clear picture of particle stability in presence or absence of dextran (Figure 4a). As we see, without dextran, zein particle suspensions aggregate at pH 6.5, since the net charge at this pH is zero. However, it is obvious that in presence of 0.1% dextran, the aggregates break - repeptize to smaller- and the particle suspension becomes transparent. Lower dextran concentrations (0.01%) do not improve particle stability. At pH 3.5 and pH 8, below and above the IEP of zein, respectively, the effect of dextran on particles is also visible, but much less significant, due to the limited aggregation of particles at these pH values (Figure 4b).

a.



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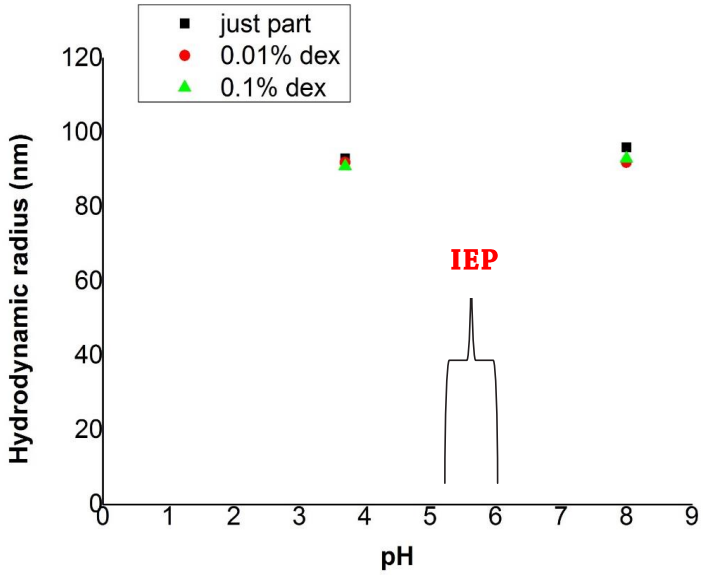

Figure 4. Effect of dextran on zein particle stability at pH close to IEP (**a**), and far from it (**b**).

To sum up, the addition of dextran has a greater effect in particle size in case of gluten particles at all pH values, much smaller effect in case of gelatin particles, and significant effect for zein particles at pH close to the IEP of zein. This tendency is shown in Figure 5, where we can see the estimated hydrodynamic radius for all particles with and without dextran at various pH values.

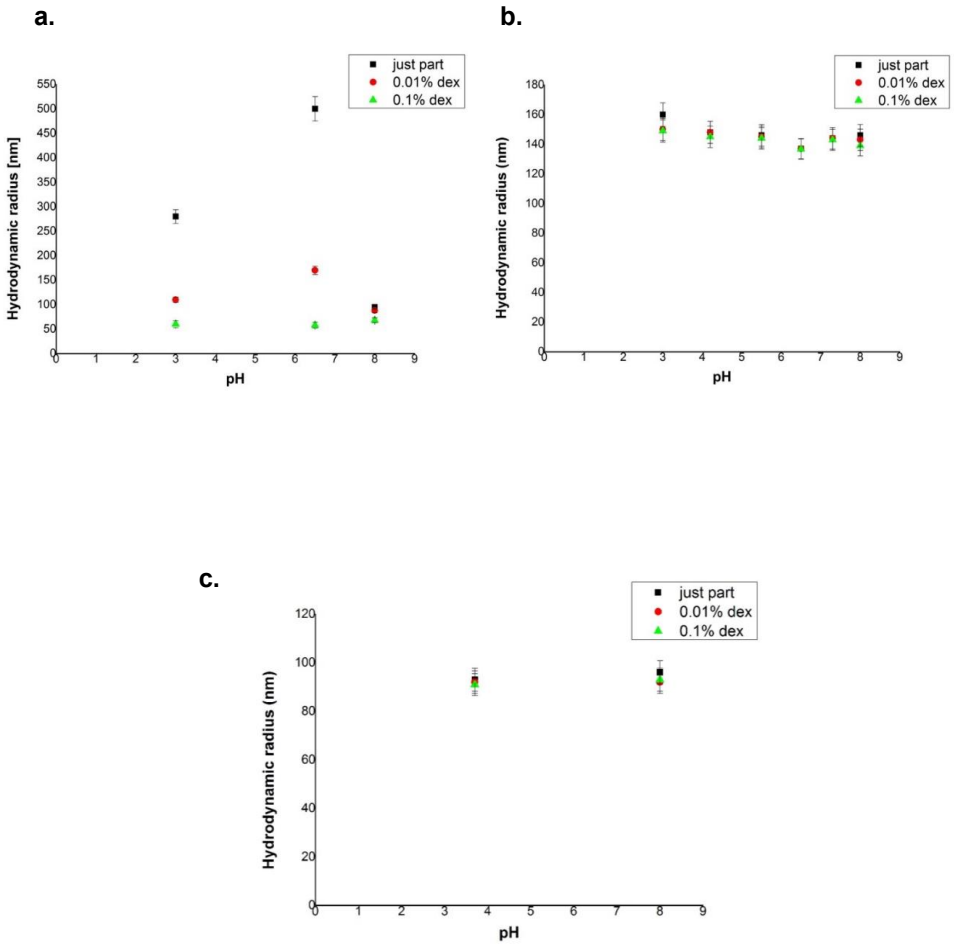

Figure 5. Calculated hydrodynamic radius for gluten (**a**), gelatin (**b**) and zein (**c**) particles without and with 0.01% or 0.1% (w/w) dextran at various pH values.

Discussion

We showed that the addition of dextran to protein particle suspensions causes repeptization of aggregates, which is reflected by decrease in effective particle size measured by DLS. In fact, the decrease in particle size is more pronounced at higher dextran concentration, and the disaggregation was most visible in cases of extensive particle aggregation.

Particle disaggregation induced by dextran is very likely the result of dextran adsorption on the surface of protein particles. The adsorbed dextran chains act as a steric barrier that prevents the aggregation of particle clusters, thus imparting a stabilizing effect on the particle suspension. The suppression of particle aggregation could be also the result of increase in their hydrophilicity induced by dextran. There have been reports regarding dextran's tendency to absorb on colloidal oxide particles (Li & Spencer, 1992; Baudin, Ricard, & Audebert, 1990). In fact, a striking specificity and selectivity in dextran adsorption has been observed with dextrans having a high affinity for clays (Glass, Ahmed, & Senecker, 1986) and very poor affinity for silica (Baudin, Ricard, & Audebert, 1990).

Furthermore, we showed that that the decrease in effective particle size due to dextran was observed for all pH values. The adsorption of polymers may, therefore, be not only a charge-controlled phenomenon. Apparently, the poor solubility or hydrophobicity of colloidal particles synthesized by the anti-solvent precipitation method induces the adsorption of uncharged polymers, such as dextran. In fact, we found in our previous work that colloidal particles interact in a similar way both with uncharged, i.e. dextran, or charged polymers, i.e. gelatin (Chatsisvili, Philipse, Loppinet, & Tromp, 2017). This is indicative of an energetically favorable interaction of particles with polymer phases (relatively to pure water). Especially for the case of adsorption of non-ionic polymers, there are suggestions that the adsorption is an entropically driven process. Still, the mechanisms of non-ionic polymer adsorption have not yet fully confirmed in general, since the diverse set of structures (e.g. branching) and hydrophilicities of polysaccharides can make it difficult to isolate interactions and driving forces.

Conclusions

We used dynamic light scattering (DLS) to investigate the effect of the addition of low concentrations of uncharged polysaccharide dextran to protein particles from gluten, zein or gelatin. We proved that aggregates of gluten particles repeptize upon the addition of dextran. In fact, a higher dextran concentration leads to more extensive dissolution of gluten aggregates. Furthermore, aggregates of zein particles repeptize at pH 6.5 (close to IEP of zein, where there is extensive aggregation) upon the addition of 0.1% dextran (the sample becomes transparent). However, gelatin particles are not significantly affected by dextran addition, because gelatin particles do not tend to aggregate, such that dextran has no effect. The tentative explanation of dextran's effect on particle aggregation is its adsorption on the surface of particles. The relatively hydrophobic particles become effectively hydrophilic and less prone to aggregation.

Acknowledgements

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Chapter 4

Colloidal zein particles at water-water interfaces

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Abstract

We synthesize colloidal zein particles using the anti-solvent precipitation method and study particle behavior at water-water interfaces. When added to phase-separating aqueous mixtures of fish gelatin and dextran, particles accumulate at the interface. In order to explain the mechanism of particle accumulation at the water-water interface, we investigate how zein particles interact with polymers (i.e. fish gelatin and dextran). We show that both polymers adsorb similarly on particle surface, which can explain why particles form contact angles close to 90°. Moreover, we show that particle accumulation is accompanied by aggregation. Those aggregates are able to arrest the late stage of the demixing process of the emulsion by the formation of a stable particle-rich layer at the water-water interface. This layer is referred as a 'foam-like layer' due to its morphology similar to that of a wet (non-drained) foam, and contains droplets of one phase, surrounded by particle-stabilized lamellae of the other phase.

Introduction

Phase separation of moderately concentrated aqueous solutions of thermodynamically incompatible polymers leads to a dispersed system called a water-in-water emulsion (Tolstoguzov, 1986). The situation is often encountered in food systems containing a mixture of protein *(e.g.*, gelatin) and polysaccharide (*e.g.*, dextran, maltodextrin), under conditions where the protein-polysaccharide interaction is net repulsive (Butler & Heppenstall-Butler, 2003; Firoozmand, Murray, & Dickinson, 2009; Loren, Langton, & Hermannson, 2002; Syrbe, Fernandes, Dannenberg, Bauer, & Klostermeyer, 1995; Tromp, Rennie, & Jones, 1995), usually due to a difference in affinity for the solvent. The interfacial tension between the aqueous phases is extremely low ($\gamma \approx 0.001\text{-}0.01$ mN m^{-1}) (Wolf, Scirocco, Frith, & Norton, 2000; Stokes & Frith, 2002), compared to the oil-water tension which is generally 10-30 mN m^{-1}. As a consequence of the low interfacial tension, the driving force for coalescence in water-in-water emulsions is low. Still, however, gravity-induced layer formation is observed within hours. A stabilizer is therefore needed. Kinetically stable water-in-water emulsions would be of great commercial interest, since they could lead to fat-free food or capsules of sensitive materials such as proteins.

Recently it was shown that water-in-water emulsions can be stabilized by the addition of particles. Particle-stabilized emulsions, so-called Pickering emulsions, have been studied extensively for oil-in-water emulsions. In water-in-water emulsions, though, the behavior of particles can be quite different, due to the much smaller interfacial tension of these systems, and the presence of interactions of particles both with the polymers in the mixture and with each other at the interface. These interactions play a crucial role in the understanding of stabilization of water-in-water emulsions and should be addressed in the study of these systems.

Repulsive interactions between particles and polymers were suggested by Firoozmand, Murray, & Dickinson (2009) as the driving force for the accumulation of particles at the water-water interface. However, this

explanation does not apply in every case, since particles and polymers do not always repel each other. In this study we show that polymers tend to adsorb on particle surface. Such an adsorption can affect the contact angle, and have consequences on the stability of the particle dispersion. Except for the particle – polymer interactions, one should also take into account the particle – particle interactions. Specifically, aggregation of particles may be induced as a result of increased particle concentration locally after their adsorption at the water-water interface. Due to this effect, aggregates of particles – instead of single particles - can interact in a stabilizing way with the water-water interface, forming an elastic layer around the droplets that cannot shrink or expand anymore (Murray & Phisarnchananan, 2014; Murray & Phisarnchananan, 2016). The formation of the viscoelastic particle layer at the liquid-liquid interface has the potential to arrest the process of layer formation (Firoozmand, Murray, & Dickinson, 2009), although it does not necessarily prevent droplets from coalescing. This has been proven lately to be one of the most important differences between oil-in-water and water-in-water emulsions: whereas for the former the presence of particles at the interface leads invariably to stabilization, this is not the case for water-in-water emulsions where coalescence of fully-covered droplets can still be observed. During coalescence, it has been reported that particles may even be driven off the droplet surface into the solution (Balakrishnan, Nicolai, Benyahia, & Durand, 2012; de Freitas, Nicolai, Chassenieux, & Benyahia, 2016).

This paper is an effort to study the interactions between particles and polymers, and particles with each other at the water-water interface, and to show how they can contribute or complicate the Pickering stabilization of water-in-water emulsions. In other words, first, we want to show the relevance of particle-polymer interactions, which will help us to better understand the driving force for particle accumulation at the interface. Second, we aim to show particle aggregation at the interface which does occur even at low particle concentrations (where is no crowding of particles at the interfaces of dispersed droplets) and complicates the simple picture of a monolayer of adsorbed particles. For this study, we chose zein, an extensively studied, food-grade and abundant protein that can be used as a particle emulsifier. The main advantage

of zein over other food-grade proteins is its solubility properties, as it is mentioned in Chapter 2: it is very hydrophobic (insoluble in water), but soluble in some organic solvents (*e.g*. ethanol), which allows the controlled fabrication of colloidal particles through the anti-solvent precipitation method without the use of any crosslinker. Furthermore, zein particles do not require any additional chemical surface modification or pretreatment to assure interfacial affinity, as opposed to other particle emulsifiers (*e.g*. chemically modified starch granules) (Yusoff & Murray, 2011).

Experimental details

Materials

As a model system, we used the polymers cold water fish gelatin and dextran. The system gelatin/dextran/water is a well-documented model system for phase separation studies (Scholten, Sprakel, Sagis, & van der Linden, 2006; Vis et al., 2015). Fish gelatin (type A or acid-extracted, gelling temperature 8–10 °C, approximately 100 kDa) was obtained from Norland Products Inc., via FIB Foods (Harderwijk, the Netherlands). Gelatin has an isoelectric point at approximately pH 8 (Karim & Bhat, 2009). Dextran (from Leuconostoc spp., 450-650 kDa) was obtained from Sigma-Aldrich. As a source of particle material, we used the protein zein from corn (obtained from Flow Chemical Corporation, Ashburnham, MA, USA). Absolute ethanol (99.9%) was obtained from Nedalco, Heilbronn, Germany. Phosphate and citrate buffer were used to control the pH. The dialysis tubing used for the removal of ethanol in particle dispersions (MWCO 12-14 kDa) was obtained from Medicell Membranes Ltd. Water purified by a RO system was used for all the experiments.

Methods

Synthesis of zein particles

Zein is a hydrophobic protein, poorly soluble in water except in the presence of polar organic solvents (*i.e.* ethanol). Therefore, it can be used as a stabilizer only in the form of particles. These are dispersible in water due to charge, but still have a hydrophobic surface. In order to synthesize zein particles, the classic anti-solvent precipitation method (Baars et al., 2015; de Folter, van Ruijven, & Velikov, 2012; Joye & McClements, 2013) is used. The preparation of zein particles by anti-solvent precipitation method is described extensively in Chapter 2.

Characterization of zein particles in aqueous suspension, in gelatin solution and in dextran solution

The size and shape of zein particles in aqueous suspensions were analyzed by taking cryogenic transmission electron microscopy (cryo-TEM) photographs using a Tecnai 200 (FEI Company, The Netherlands), operating at 200 kV. One drop of a particle dispersion was placed on a carbon grid, which was then freezed into liquid ethane at the temperature of liquid nitrogen. Larger size fractions of particle dispersions were observed with the optical microscope Reichert-Jung (Leica Microsystems, Switzerland). The size of zein particles was confirmed by dynamic light scattering measurements carried out with the ALV-CGS4 Compact Goniometer System with 4 detectors. Particle dispersions were diluted in buffer of pH 3.2, 7.1 and 8.1.

Dynamic light scattering measurements were performed to determine the effect of addition of polymer on the stability of particles. In other words, possible interactions between particles and polymers were investigated. The measurements were carried out using the ALV-CGS4 Compact Goniometer System with 4 detectors at pH 3.2, 7.1 and 8.1 at 20°C for zein particles (0.003% w/w) in buffer without polymer and in suspensions of dilute gelatin (0.01%, w/w) and dilute dextran (0.01%, w/w) solutions. Hydrodynamic radii were derived using the equations described in Chapter 3. Low polymer concentrations were chosen to avoid the depletion and subsequent attractive interactions between the particles. The results reported are averages of three measurements.

Preparation and characterization of emulsions containing zein particles

Water-in-water emulsions were prepared by dissolving fish gelatin and dextran at an initial ratio of 2/1 (total polymer concentration 12% w/w) in aqueous suspensions of particles of a concentration of 0.1-0.2% (w/w). Complete dissolution of polymers was achieved with magnetic stirring at 60ºC. No high shear treatment was necessary due to the ultra-low interfacial tension.

The pH of emulsions was 5.6. Sodium azide (0.05% w/w) was added in the final emulsions in order to prevent the microbial growth. All samples were stored in the refrigerator at 4^{o}C.

Emulsions with and without particles were assessed in terms of their macroscopic appearance and stability against coalescence, after they were left overnight at room temperature to allow the macroscopic separation into two phases. The process of the macroscopic separation or demixing of emulsions was recorded overnight using a USB digital microscope (Dino-Lite), which created time-lapse movies at a frame rate of one frame per 30 seconds. The interface of macroscopically separated emulsions was also studied in terms of its microscopic structure by using the horizontal Ultra View RS Confocal Imaging System; for this purpose, the emulsion was left in a cuvette until it was separated into two layers. The interface between the two phases was then observed in a horizontal direction. Rhodamine B was used as a fluorescent dye to stain the gelatin phase. Finally, optical microscopy was used to obtain images of the microscopic structure of fresh emulsions.

Results and discussion

The behavior of zein colloidal particles and their interactions at the water-water interface are studied using as a model system phase-separating aqueous mixtures of gelatin and dextran. The concentration of particles in our study was chosen to be low, 0.1-0.2% w/w, in order not to induce an increase of viscosity, even when all particles localize in the same phase. Besides, zein particles are prone to aggregation when concentrated, which did not allow their addition to emulsions in high concentrations.

Zein particles are synthesized by the anti-solvent precipitation method and dialyzed against acidic water of pH 3.7, in order to obtain aqueous dispersions of particles. Dialysis has proven to be a crucial step in this process, in order to obtain cleaned particles without any possible surface-active components which may alter the behavior of particles. Zein particles in aqueous dispersions (without buffer) appear to have a spherical shape and a number-averaged radius of 100 nm according to cryo-TEM images (Figure 1). However, a fraction of micron-sized particles is also present, resulting in a large polydispersity. Interestingly, these large-sized particles and their behavior at interfaces are directly observable using optical microscopy, as exemplified below in figures 3 and 4.

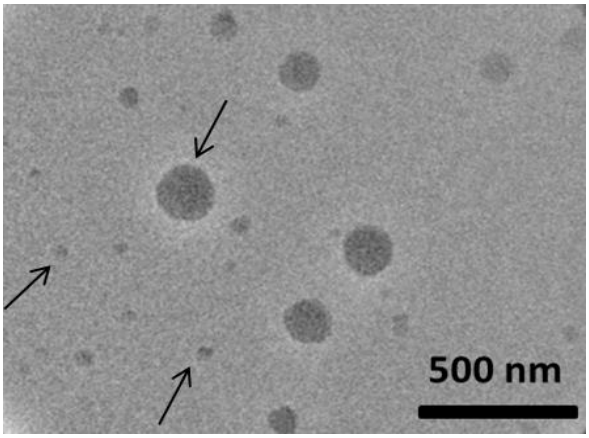
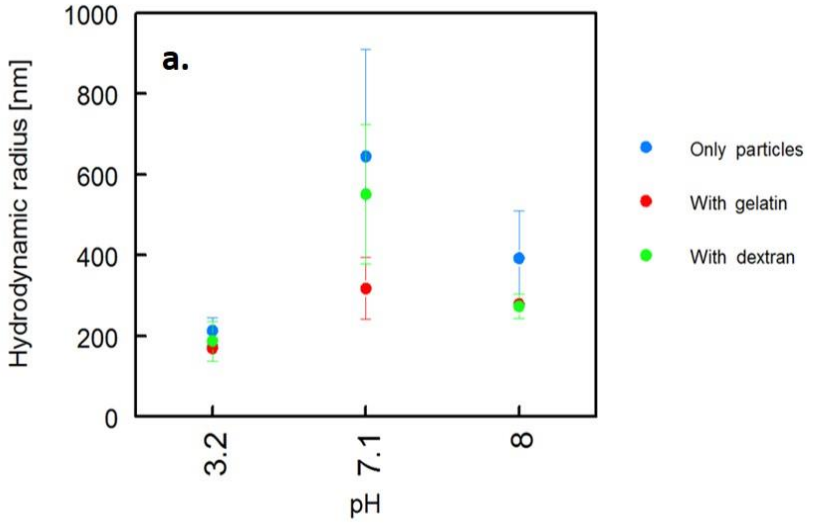


Figure 1. Typical cryogenic transmission electron micrograph of zein particles (arrows).

Interactions of particles with polymers. First, the interactions of particles with both gelatin and dextran were investigated by dynamic light scattering measurements. These interactions may be related to surface charge properties, which were determined by measuring the zeta-potential of particles, as we described in Chapter 2. As we have mentioned in Chapter 2, the isoelectric point of zein is in the range of pH 6-6.5. By lowering the pH to 3, the particles become positively charged [ζ = +20 mV], whereas above the isoelectric point, the particles appear negatively charged. Figure 2 shows the hydrodynamic radii derived from scattering data at scattering angle 90^{o} of particles in buffer of pH 3.2, 7.1 and 8 without polymer and in 0.01% (w/w) gelatin and dextran solutions. It turns out that at pH 7 the apparent particle size is larger than at higher or lower pH, most probably due to aggregation at neutral pH, close to the isoelectric point. However, in the presence of gelatin or dextran, the apparent particle size decreases (notice the faster decay in presence of polymer in Figure 2b, c and d). The scattering is dominated by the particles, which are much larger than the polymer molecules. This observed decrease in hydrodynamic radius might be a consequence of adsorption of polymer on the particles. That will lead to an increase of the hydrophilicity and a disaggregation of the particles in smaller ones. It is remarkable that gelatin and dextran have similar effects, in spite of the fact that dextran is an uncharged polymer that generally does not interact with proteins. Interactions between protein and dextran, though, were reported earlier, and tentatively ascribed to the formation of hydrogen bonds (Antonov & Wolf, 2005). The adsorption of polymers may, therefore, be not only a charge-controlled phenomenon. Apparently, the poor solubility or hydrophobicity of zein particles induces also the adsorption of uncharged polymers, such as dextran. The similar effects of dextran and gelatin on the hydrodynamic radius of zein particles in dilute polymer solutions are indicative of an energetically favorable interaction with both phases (relative to pure water) and this is in agreement with the fact that particles display an intermediate contact angle, as we will show below. These interactions between particles and polymers may also play a role in the dynamics of particle adsorption at the water-water interface.



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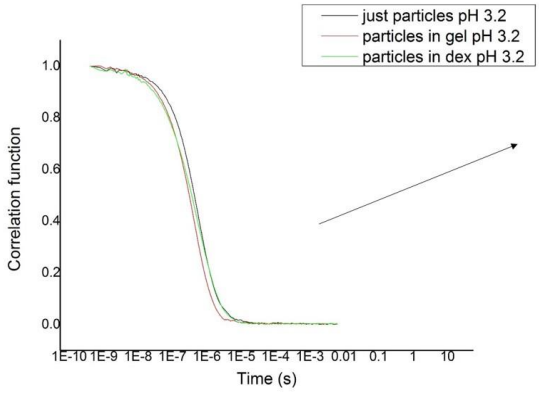

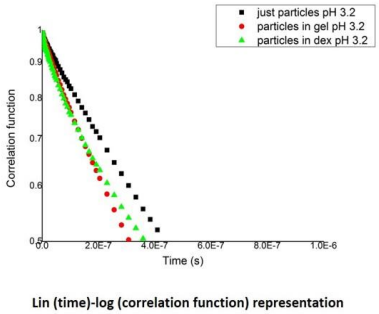

Lin (time)-log (correlation function) representation

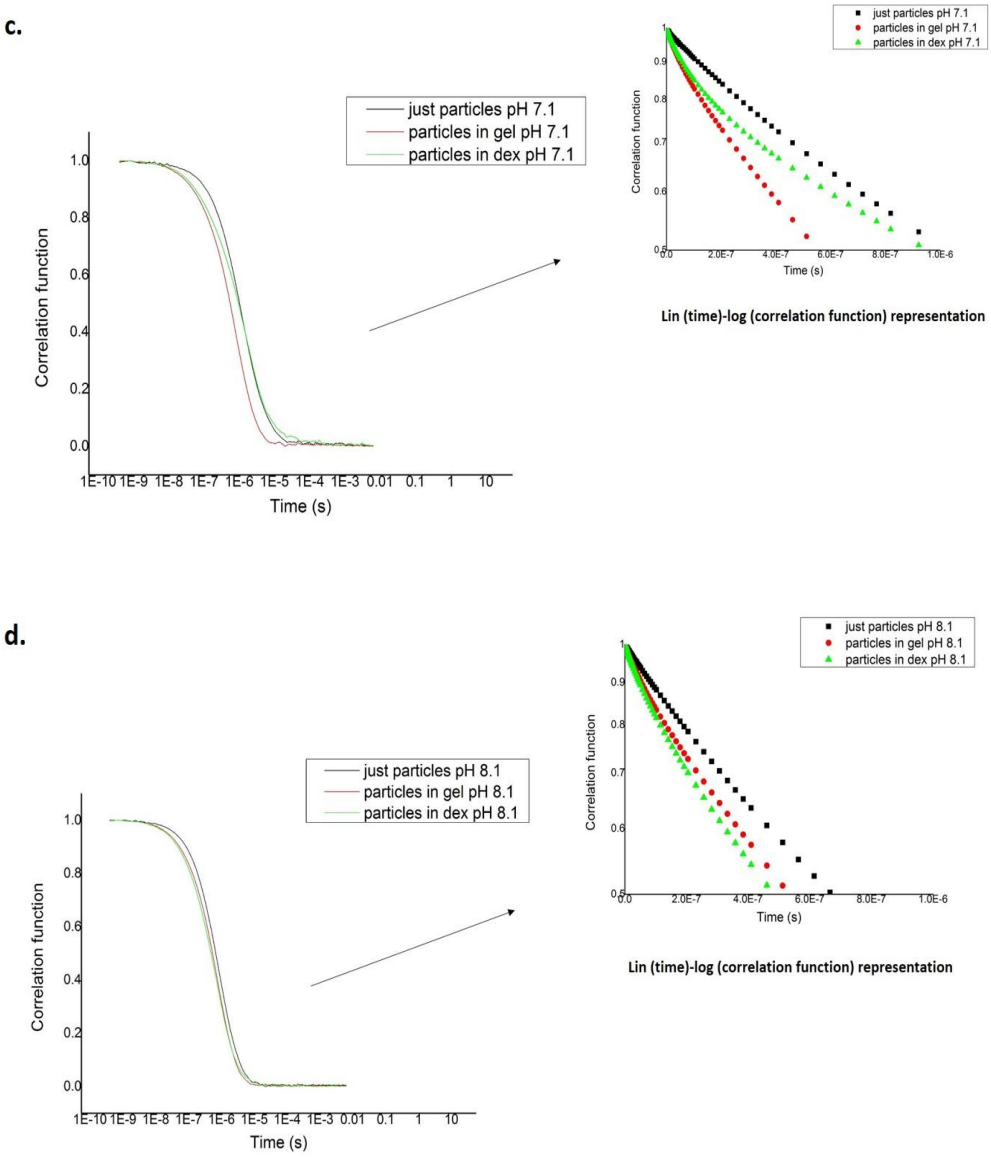

Figure 2. Hydrodynamic radius of particles in buffer and in gelatin/dextran solutions plotted as a function of pH (**a**), and time correlation function graphs at pH 3.2 (**b**), 7.1 (**c**) and 8.1 (**d**). We notice the faster decay in presence of polymer.

Particle accumulation and aggregation at the water-water interface. When added to phase-separating aqueous mixtures of fish gelatin and dextran, particles show a clear preference for the gelatin-rich phase. This can be understood from the hydrophobicity of zein and the relative hydrophobicity (in the gelatin-dextran pair) of gelatin. However, the particles are not fully wetted by the gelatin-rich phase. Figure 3 shows the contact angle of approximately 65° of an exceptionally large particle, which is clearly partly in one phase, and partly in the other. The presence of particles of sizes of the order of 1 μm or larger – the fraction visible by optical microscopy - at the interfaces is consistent with the adsorption energy of the order of 10^3 k_BT predicted by Young's law, provided that the contact angle is not close to 0° or 180°. Eventually, most of the particles end up accumulated at the interface between the gelatin-rich and dextran-rich phases. The accumulation is accompanied by a degree of particle aggregation (Firoozmand, Murray, & Dickinson, 2009), as is clearly seen in Figure 4. Apparently, the adsorption of particles at the interface led to an increased local particle concentration which followed by aggregation of particles with each other. So, particle aggregation is probably driven by depletion attraction (Tuinier, Dhont, & de Kruif, 2000). The increase of particle concentration and ionic strength can be expected to lead to aggregation of particles, as the electrostatic repulsion is weakened. Some evidence of aggregation is also provided by the pH dependence of the hydrodynamic radius.

To sum up, we form the hypothesis that particles first adsorb at the interface and form a contact angle, and then aggregate with each other. This aggregation occurs possibly due to the increased number of localized at the interface particles (depletion). This hypothesis is also supported by the fact that, when adding solutions of (already) aggregated particles to the emulsions, particles did not seem to adsorb at the droplet interface (data not shown). Aggregation of particles at interfaces is a known phenomenon (Sinn, Alishahi, & Hardt, 2015) and has been proposed as an alternative mechanism for the Pickering stabilization of oil-in-water emulsions in addition to the particle layer formation around the droplets (as discussed in Chapter 2). In this case, the steric particle-based barrier is not a simple monolayer or bilayer which is densely packed, but

a region of a network of particles adsorbed at the interface with the whole aggregated structure held together by attractive inter-particle forces (Gautier et. al., 2007; Tscholakova, Denkov, & Lips, 2008; Dickinson, 2010).

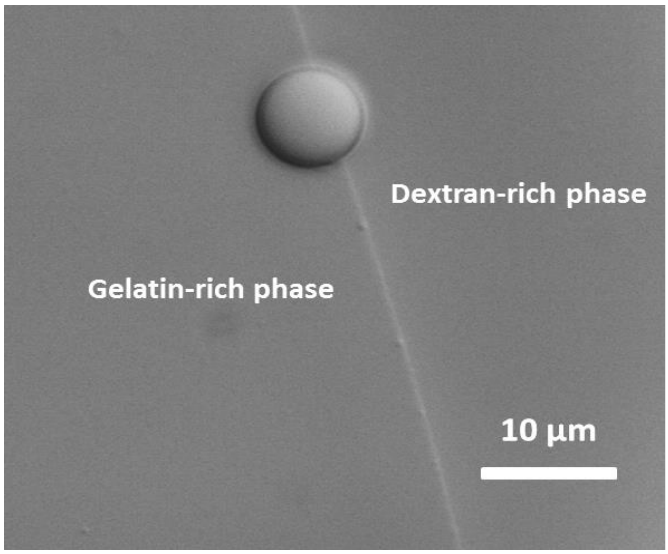

Figure 3. Particle residing mostly in the gelatin-rich phase with a contact angle of 65º (measured from the dextran-rich phase).

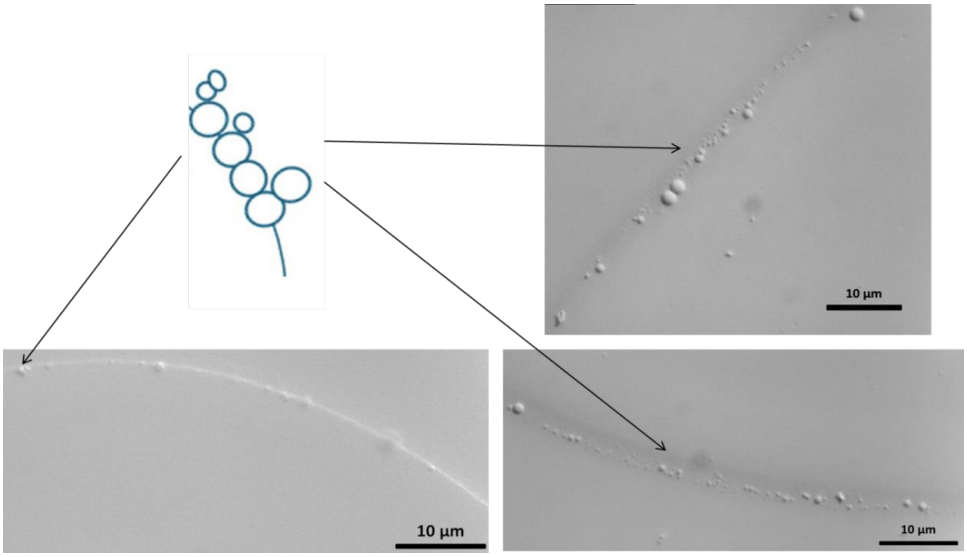

Figure 4. Optical microscopy images of a water-in-water emulsion containing zein particles, which show the particle accumulation at the curved water-water interface accompanied by aggregation of particles.

Interfacial particle-rich 'foam-like' layer. As it is mentioned above, zein particles accumulate and aggregate at the water-water interface. However, this microscopically observed particle accumulation at the interface did not lead to a stable emulsion. Nevertheless, particle accumulation did affect the coarsening kinetics and the final macroscopic morphology of emulsions at the late stage of the demixing process. Specifically, aggregates of particles were able to form and stabilize a macroscopically observable interfacial particle-rich zone in the late stage of the demixing process (Figure 5). Figure 5 shows stills of an overnight time-lapse movie of emulsions with and without particles where we can see the different stages of the macroscopic formation of the interfacial zone. This zone appears to have the macroscopic morphology of a wet (non-drained) foam, and therefore, from now on, it will be referred as a 'foam-like' layer. It should be mentioned that the foam-like layer is always formed in presence of particles regardless the pH.

In order to understand in greater detail the microstructure of the foam-like layer, horizontal confocal microscopy was used. Some results are shown in Figure 6. According to those images, we can conclude that the foam-like layer consists of large dextran-rich globules (appeared black) surrounded by gelatin-rich phase (binded to the dye rhodamine), with interfaces occupied by particles in an irregular, cluster-like arrangement. Some of these interfaces are part of lamellae of gelatin-rich phase which are in-between dextran globules. These lamellae appear to be stabilized by the particles, as concluded from an absence of development during several days. In other words, after the foam-like layer is formed, a meta-stable state of the emulsion is reached, in which the dispersed dextran droplets in the layer do not coarsen further. This can be explained by the fact that particles may provide some mechanical stability to the foam-like layer, and further coarsening is arrested due to the mechanical cohesion of the layer. The stability of the foam-like layer is outstanding even after centrifugation of the emulsion at 14,000 g for 2 hours (Figure 7).

From these results, we conclude that particle aggregation may have both negative and positive consequences for the ability of particles to stabilize water-in-water emulsions. On the one hand, particles involved in an aggregate are less available for adsorption at the interface and for lowering the interfacial tension than free particles. On the other hand, though, an interface lined by aggregates will obtain a degree of (visco) elasticity which may contribute to stabilization against coalescence. The latter is probably observed in the foam-like layer.

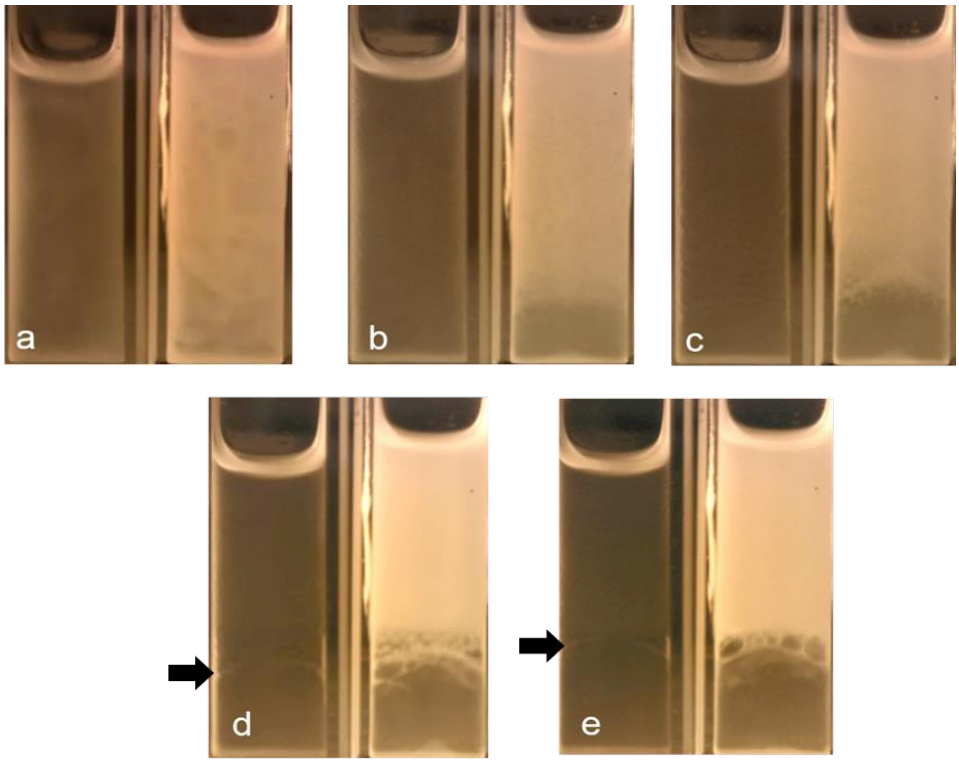

Figure 5. Movie stills from different stages of the interfacial particle-rich zone's formation during the macroscopic phase separation of emulsions without (left) and with particles (right). (a) 0 h, (b) 1h, (c) 1.5h, (d) 2h, (e) 3h. The pH of emulsions is 5.6. The movie was recorded overnight at one frame per 30 seconds. The width of the cuvettes was 1 cm. The black arrows indicate the position of the interface in emulsions without particles.

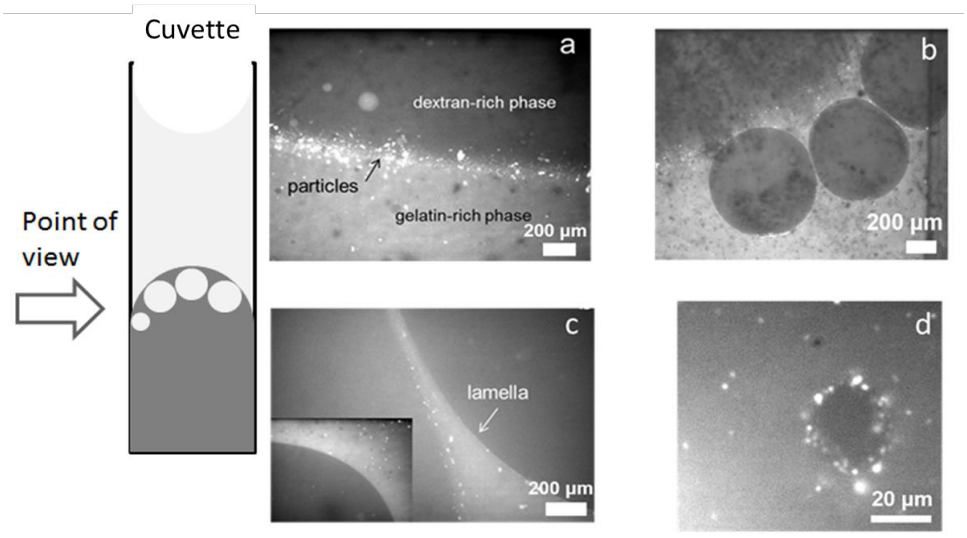

Figure 6. Horizontal microscopy images of the interface of a macroscopically phase-separated emulsion containing particles. We can see (a) particle accumulation at the interface between the gelatin-rich and the dextran-rich phase, (b) dextran droplets trapped in the interfacial particle-rich zone nearly touching each other without coalescing, (c) the interfacial particle-rich film/lamella which consists of a gelatin wetting layer containing particles, (d) magnification of a dextran droplet covered by particles.

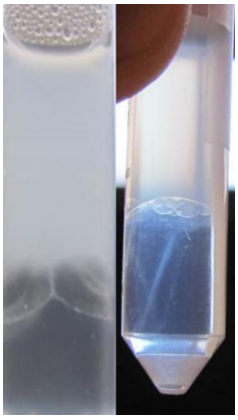

Figure 7. Foam-like layer of a macroscopically phase-separated emulsion (left) remaining stable even after centrifugation of the emulsion at 14,000 g for 2 hours (right).

Surface coverage and sedimentation as limiting factors for emulsion stability. When added to phase-separated mixtures of gelatin and dextran, zein particles do not lead to the macroscopic stabilization of the demixed solutions, as we already mentioned. In fact, after mechanical mixing, the demixing process stays almost unaffected by the presence of the colloidal particles. Nonetheless, we observed the expected stabilizing ability of particles at low particle concentration. Low particle concentration was chosen to avoid the increase in viscosity of emulsions, as well as the aggregation of particles in their initial aqueous dispersions.

Full emulsion stability against gravity-induced layer formation cannot be expected due to the low surface coverage of emulsion droplets that the particles can provide. Assuming that all the particles are adsorbed at the droplet interface, we can estimate the needed amount of particles to provide full coverage (assuming a close packed surface fraction). For droplets of size $R_{drop,}$ the full coverage is obtained when the mass concentration of particles c_{part} is equal to $4\varphi\rho R_{part}/R_{drop,}$ where φ is the volume fraction, ρ the density of droplets and R_{part} the particle radius. For droplets of radius of 25 µm, c_{part} is approximately 5 mg/mL, which is much higher than the particle concentrations we used (0.2%, w/w or 2 mg/mL). So, the chosen particle concentration is not sufficient to provide full coverage. At this particle concentration, the droplet size that will allow full particle coverage is 0.64 mm, which is reasonably close to the 0.7 mm diameter observed in experiments (Figure 6b) and confirms a link between particle adsorption at interfaces and the observed structures. In the foam-like layer, dispersed droplets do not coalesce further, so this is the maximum ‘equilibrium’ size they reach.

Even in the case of full coverage, though, the stabilization may not be reached due to the effect of gravity: fully covered droplets are made heavy by particle adsorption and they eventually sediment. This leads to poor stability of any emulsion, except if the mass density of colloidal particles closely matches that of the liquid. It is generally known that, when the effect of gravity dominates over thermal motion, sedimentation or creaming will occur. We can determine the droplet size R_{drop} fully covered by particles that still resists the gravitational

force and does not sediment, by equalizing the gravitational potential and the thermal energy:

$$R_{drop} = \left(\frac{3}{4\pi} \frac{k_B T}{g|\Delta h|\Delta\rho} \right)^{1/3} \quad (1)$$

where $\Delta\rho$ is the buoyant density of the particles and Δh is a distance of the same order of magnitude as of R_{drop}. R_{drop} is in the order of 10^{-7} m and is too small to be realistic.

The gravitational length $L_{grav,}$ which is defined as the length over which the gravitational energy of a droplet in a suspension equals the thermal energy, is given by: $L_{grav} = \frac{3}{4\pi} \frac{k_B T}{g|\Delta\rho|R^3}$ (Philipse & Koenderink, 2003). Here the equation refers to one droplet with radius R. However, when we have a droplet covered with particles, about $4(R_{drop}/R_{part})^2$ particles will fully cover the surface of the droplet. In that case we have to take into account the weight of the particles, which dominates the total weight of the droplet with particles. Therefore, neglecting the weight of the droplet, we end up to the following expression for the gravitational length:

$$L_{grav} = \frac{3}{16\pi} \frac{k_B T}{g|\Delta\rho|R_{drop}{}^2 R_{part}} \quad (2)$$

For the stability to be observed, L_{grav} should be much larger than the height of the container (typically of the order of 10^{-2} m), which is not the case. L_{grav} is in the order of 10 μm, so gradients due to gravity are sharp, indicative of strong sedimentation. If we consider the sedimentation length L_{grav} to be 10 times larger than the height of the container for the stability to be observed, then, from equation 2, the buoyant density $\Delta\rho$ of the particles should be in the order of 10^{-5} kg m^{-3}. The actual buoyant density is in the order of 100 kg m^{-3}.

Conclusions

Colloidal zein particles are synthesized by the anti-solvent precipitation method. When added to phase-separating aqueous mixtures of fish gelatin and dextran, particles show a contact angle of 65º. The non-zero contact angle is in accordance with the similar attractive interactions of particles with both polymers, as was investigated from dynamic light scattering measurements. Eventually, particles accumulate at the interface between the gelatin-rich and the dextran-rich phase, an accumulation that is accompanied by particle aggregation. Those aggregates are able to arrest the late stage of the demixing process of emulsions leading to the formation of an interfacial particle-rich zone, referred as a foam-like layer, due to its morphology similar to a wet foam. This layer remains stable even after centrifugation of the emulsion (14,000 g for 2 hours) and contains dispersed dextran droplets surrounded by particle-stabilized gelatin layer. The dextran droplets do not coarsen further leading the emulsion to an apparent equilibrium state. In the present conditions the particles cannot be expected to provide full stabilization of emulsions, as sedimentation is expected to be relevant. Sedimentation can be avoided if the mass density of the colloids closely matches that of the liquid or if the emulsion droplets are sufficiently small not to sediment. However, such a small droplet size is not realistic; therefore interactions between particles and droplets that lead to a network formation are required for stabilization against gravity.

Acknowledgements

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Chapter 5

Phase behavior of water-in-water emulsions containing small quantities of strongly charged polyelectrolyte

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(Manuscript in preparation)

Abstract

We investigated the effect of the strong polyelectrolyte dextran sodium sulfate (DSS) on the phase behavior of phase-separating aqueous mixtures of non-gelling (fish) gelatin and dextran by interfacial tension measurements, turbidity measurements, analytical centrifugation, IR spectroscopy, microscopy and dynamic light scattering. We found out that DSS concentrates in the gelatin-rich phase forming complexes with gelatin. Those complexes can be either soluble which appear transparent or insoluble which appear turbid. Gelatin/DSS complexation reduces the affinity of gelatin with water, therefore changes the water partitioning, and results in increased incompatibility between dextran-rich and complexes-rich phase. The enhanced incompatibility due to DSS is proven by increased interfacial tension and smaller gelatin-rich phase volume. Furthermore, the addition of DSS causes the formation of a turbid gelatin-rich phase, after phase equilibrium is reached. The turbid gelatin-rich phase is present even when the complexes are soluble and the gelatin/DSS mixtures are transparent; this proves the role of dextran in causing the turbidity of the gelatin-rich phase.

Introduction

The phase behavior of aqueous mixtures of thermodynamically incompatible polymers (also called water-in-water emulsions) has been studied extensively due to several technological applications of these systems, such as food and cosmetic microstructural design (Wolf, Scirocco, Frith, & Norton, 2000), protein separation and purification (Wang, Gao, & Dubin, 1996), and micro-encapsulation (Burgess & Ponsart, 1998; Kruif, Weinbreck, & de Vries, 2004). The applicability of water-in-water emulsions would be extended when the stability could be controlled and manipulated. This has been tried successfully with clay particles and protein particles (Nicolai & Murray, 2017; Vis et al., 2015). However, studies of the manipulation of thermodynamic compatibility by strong and weak polyelectrolytes are still relatively rare and the underlying mechanisms are not yet clear. It was recently found, that small quantities of weakly charged polyelectrolyte accumulate at the water-water interface, and impart some stability (Tromp, Tuinier, & Vis, 2016). It was also found that the strongly charged polyelectrolyte dextran sodium sulfate (DSS), on the one hand, can induce thermodynamic incompatibility (demixing) in semi-dilute and highly compatible sodium caseinate/sodium alginate mixtures (Antonov & Moldenaers, 2009), but, on the other hand, it can induce compatibility (mixing) in case of mixtures of gelatin type B/dextran and bovine serum albumin/dextran (Antonov & Moldenaers, 2012).

The aim of the present work is to study the influence of the addition of small quantities of the strong polyelectrolyte dextran sodium sulfate (DSS) on the phase behavior of the phase-separating mixtures of fish gelatin and dextran. Fish gelatin is a non-gelling gelatin with a pH-dependent charge, whereas dextran is an uncharged polysaccharide. The gelatin/dextran system was chosen mainly due to its good experimental accessibility, the avoidance of gelation and the extensive knowledge that is available (Edelman, 2003; Vis, Peters, Tromp, & Erne, 2014; Vis et al., 2015). DSS is a negatively charged polyelectrolyte and was chosen because it can be considered a charged form of dextran, so the addition of a polyelectrolyte to the gelatin/dextran system can be studied while staying from the chemical point of view as close as possible to the system without additive.

In our system (DSS/gelatin/dextran), two types of phase separation of polymers, as they have been described by Piculell et al. (Bergfeldt, Piculell, & Linse, 1996; Piculell & Lindman, 1992) are encountered: associative phase separation between gelatin and DSS, and segregative phase separation between gelatin (and DSS)-rich and dextran-rich phase.

An associative phase separation, also called complex coacervation, takes place when oppositely charged polymers form complexes (Basak et al., 2007; Izumrudov, 2012; Kayitmazer et al., 2013; Kruif, Weinbreck, & de Vries, 2004; McClements, 2014; Nakajima & Sato, 1972; Oskolkov & Potemkin, 2007; Overbeek & Voorn, 1957; Rubinstein & Papoian, 2012; Schmitt & Turgeon, 2011; Tiebackx, 1911; Veis & Aranyi, 1960; Veis 1961; Weinbreck, 2004). This results in one phase enriched in both biopolymers, while the other phase contains mostly solvent. The complexes that are formed can be either soluble or insoluble. Soluble complexes are formed when the overall charge is negative or positive, i.e. there is no full charge cancellation. Insoluble complexes are formed when the charges of the polyelectrolyte and the protein are equal and compensate each other, i.e. the complexes are neutral. The neutral complexes start to aggregate leading to the formation of coacervate droplets. The solubility of complexes of protein and (negatively) charged polysaccharides is determined by several factors, such as pH and the ratio of protein/polysaccharide (Li, Zhao, & Huang, 2014). The process of complex coacervation between oppositely charged polymers has been proposed by Veis and Aranyi (Veis & Aranyi, 1960; Veis, 1961). Figure 1 shows the process of the complex coacervation between a protein, such as gelatin, and a negatively charged polyelectrolyte below and above the iso-electric point (pI) of the protein. Below the pI (Figure 1a), when adding gelatin to a negatively charged polyelectrolyte (1), initially soluble complexes are formed due to the attraction of the oppositely charged molecules (2) combined with incomplete charge compensation. Adding more gelatin, charge compensation is eventually achieved and neutral complexes are formed (3). Aggregation of the neutral complexes leads to the formation of insoluble coacervate droplets (4). Further addition of gelatin causes re-arrangement of polyelectrolyte molecules, in order to incorporate the excess of positively charged gelatin chains (5). This inhibits the aggregation of the complexes due to their overall positive charge. Therefore, at this point no insoluble complexes are formed. Above

the pI, complexation is still possible (Dubin, Gao, & Mattison, 1994; Wen & Dubin, 1997) (2), even though both polymers are negatively charged. The reason is the non-homogeneous charge distribution along the protein backbone: gelatin still carries (randomly) positively charged patches despite its overall negative charge. Further addition of gelatin leads to larger soluble complexes (3). Insoluble complexes are not formed here, since charge compensation cannot be accomplished.

In a segregative phase separation, two polymers are separated into two different phases. This is typically the case for two non-charged polymers, or a non-charged polymer and a charged polymer (such as dextran and gelatin, respectively). Figure 2 shows a representative composition-composition phase diagram of an aqueous gelatin/dextran mixture at 20°C (Vis, Peters, Tromp, & Erne, 2014). The main source for segregative phase separation is the difference in water affinities between the polymers. Eventually, the polymer concentration in each phase will mainly be determined by the partitioning of water between the phases (Grinberg & Tolstoguzov, 1997).

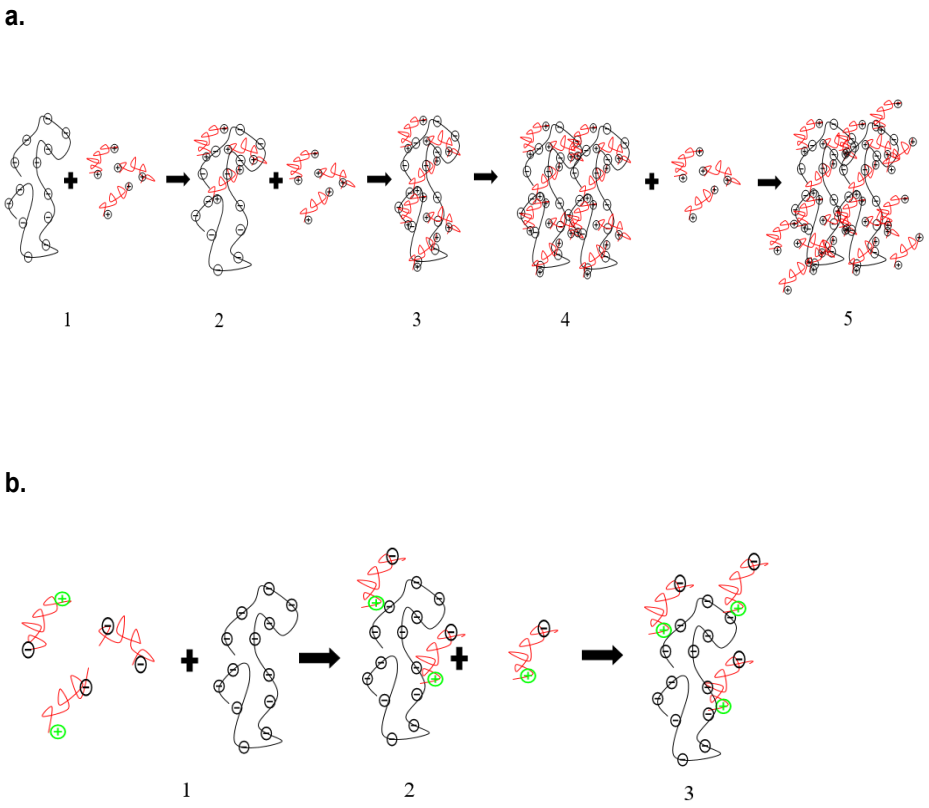


Figure 1. Schematic representation of the complex coacervation between gelatin and a negatively charged polyelectrolyte, at pH<pI of gelatin (**a**) and pH>pI (**b**).

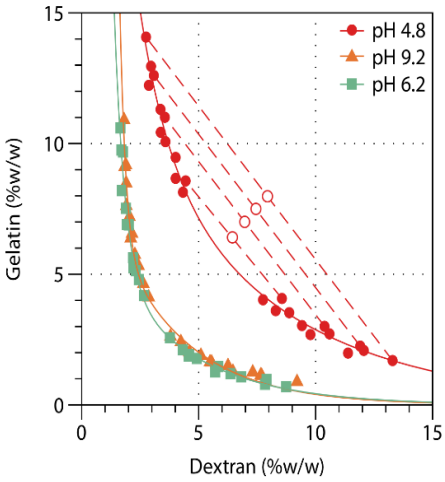


Figure 2. Phase diagram of aqueous gelatin/dextran mixtures at 20°C (Vis et al., 2014).

Experimental details

Materials

As a model system, the polymers cold water fish gelatin and dextran were chosen. Fish gelatin (type A or acid-extracted, gelling temperature 8–10 °C, approximately 100 kDa, iso-electric point at pH 7 to 8) was obtained from Norland Products Inc., kindly provided via FIB Foods (Harderwijk, the Netherlands). Dextran (from Leuconostoc spp., 450-650 kDa) was obtained from Sigma-Aldrich. As a polyelectrolyte, dextran sodium sulfate (DSS) was used (Sigma Aldrich, from Leuconostoc spp., 500 kDa). For the visualization of DSS in water-in-water emulsions, we used the FITC labelled DSS (40 kDa) purchased from Sigma-Aldrich. Water purified by a RO (reverse osmosis) system was used in all experiments. For the regulation of the pH, a dilute solution of NaOH or HCl was used.

Methods

Study of gelatin/DSS interactions

Dynamic light scattering measurements (DLS). The gelatin/DSS interactions were studied by dynamic light scattering (DLS) measurements, which give a diffusion coefficient (D). Hydrodynamic radii (R_h) of protein and polysaccharide molecules (as well as their complexes, if present) were derived using the equations described in Chapter 3.

First, the R_h of aqueous solutions of pure gelatin and DSS were determined, and then the R_h of mixtures of aqueous solutions of gelatin and DSS. The concentration of DSS in the mixtures was kept constant at 0.05% w/w, whereas the concentration of gelatin was varied from 0.05% (w/w) to 0.7% (w/w). The pH of the mixtures was set at 6, 8, 9.2 and 11.9. The experiments were carried out using an ALV Compact Goniometer System with four detector units (ALV/CGS-4) and two ALV-5000/E multiple tau digital correlators. A Coherent Verdi V2 diode-pumped laser was used operating with vertically linearly

polarized light with wavelength λ = 532 nm. The sample was placed in the cuvette housing, which was kept at a temperature of 20°C in a toluene bath. Using 4 detectors the scattering angle was varied from 0° till 148.75° in 4.25° steps. Each measurement was repeated three times at each angle. The detected intensity was processed by a digital ALV-5000 correlator. Finally, the viscosity of gelatin solutions was measured with an Ubbelohde viscometer at 20° C.

Turbidity measurements. In order to investigate the solubility of the complexes formed between gelatin and DSS, turbidity measurements were carried out on mixtures of gelatin and DSS solutions at pH 6 and 9 with a Cary 1E spectrophotometer (Varian, USA) at a wavelength of 600 nm. At this wavelength neither gelatin nor DSS absorb light. A significant increase in turbidity can be caused only by insoluble complexes between gelatin and DSS: in fact, insoluble complexes cause the solution to be significantly more turbid than soluble complexes (since the former phase-separate from water). This way, by varying the concentrations of gelatin and DSS, we were able to determine the transition point of soluble to insoluble complexes in the mixtures. The samples were put in a 1 cm path length cuvette and the turbidity was measured as a function of time at 20°C. Turbidity (τ) is defined as: $\tau = -\ln(I/I_0)$, with I_0 = incident light intensity and I = intensity of light passed through the sample volume. The turbidity measurements by spectrophotometer were combined with the visual assessment of samples' turbidity.

Preparation of water-in-water emulsions containing DSS

First, stock solutions of dextran, fish gelatin and DSS were prepared. Dextran was dissolved in RO water by magnetic stirring for approximately 2 hours at room temperature. Gelatin was dissolved in preheated water of 60 °C and subsequently stirred for approximately 1 hour at 60 °C. DSS was dissolved in RO water by magnetic stirring at room temperature. The stock solutions were stored in the refrigerator at 4 °C, after the addition of the preservative sodium azide (0.05% w/w).

Emulsions were prepared by mixing the desired amounts of stock solutions of dextran, gelatin and DSS. The weight ratio of gelatin and dextran was always 1:1 and the total polymer concentration 15% (w/w). The concentration of DSS was varied. Before use, the refrigerated, and therefore, gelled stock solutions of gelatin were heated again in a water bath of 60 °C for 30 minutes and subsequently allowed to cool to room temperature. The refrigerated stock solutions of dextran and DSS were allowed to warm up to room temperature. The mixtures were hand- shaken; no high shear treatment is necessary due to the ultra-low interfacial tension of these systems. The pH was adjusted with a diluted solution of NaOH or HCl.

Characterization of water-in-water emulsions containing DSS

Visual assessment. Phase-separating mixtures of gelatin (7.5% w/w) and dextran (7.5% w/w) containing low concentrations of DSS (0.01% w/w) were observed visually before and after phase equilibrium was reached.

Phase analysis. Phase analysis included the determination of phase composition and phase volumes in systems with and without DSS, after phase equilibrium was reached.

Fourier transform infrared spectroscopy (FTIR) was used to determine the composition of the co-existing phases in systems with and without DSS. The spectra were recorded using a Bruker Alpha-P FTIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) equipped with a diamond ATR window. Processing of the data was performed using Bruker OPUS software. The samples were centrifuged for 2 hours in order to achieve phase equilibrium. Afterwards, the two phases were collected separately and freeze-dried. Spectra were obtained from the dried solutes in the co-existing phases, in order to avoid the effect of the adsorption of gelatin on the ATR crystal. The compositions of the co-existing phases were determined by searching (using a Matlab routine) the weighted sum of pure component spectra which reproduced the measured data of the mixture.

Phase volumes were determined after the gravity-induced layer formation was reached, or after the centrifugation of emulsions at 800 g for 1 hour. For the second approach, the automated LUMiFuge® 110 system was used. This is an analytical centrifuge with an opto-electronic sensor system that measures NIR transmission profiles along the axis of the tube containing the phase separating sample (Lerche & Frömer, 2001; Luigies, Thies-Weesie, Erne, & Philipse, 2012).

Microstructure. The microscopic structure of systems with and without DSS was visualized using Confocal Scanning Light Microscope (CLSM). CLSM images were taken with a LEICA TCS SP Confocal Scanning Light microscope, in single photon mode, configured with an inverted microscope (model Leica, DM IRBE), and using an Ar/Kr laser. Fluorescent probes rhodamine and fluorescein-5-isothiocyanate labelled DSS were used. The excitation wavelength of FITC is 488 nm and the emission maximum is 518 nm.

Interfacial tension. Usually, the water-water interfacial tension is determined from spinning drop experiments or from the recovery of the droplet shape after a strain jump. However, the shear in dynamic methods can affect the equilibrium phase behavior (Antonov, van Puyvelde, & Moldenaers, 2004; Scholten, Visser, Sagis, & van der Linden, 2004). A good alternative is the analysis of the static, equilibrium shape of the water-water interface (Aarts, Wiel, & van der Lekkerkerker, 2003). Using this technique, the capillary length of the system can be obtained, only when it is much smaller than the lateral size of the cuvette.

For the determination of the interfacial tension between the co-existing phases, a phase-separating sample was left for 72 hours in a wide, flat (0.5x45 mm) glass cuvette. Gravity-induced layer formation resulted in a sharp interface, the profile of which was recorded by photography. The interfacial tension was derived from the capillary length obtained from the analysis of the static equilibrium profile of the interface. Specifically, the capillary length l_c was obtained from the profiles following the expression for the shape of the meniscus (Batchelor, 2002):

$$\frac{x(z)}{l_c} = \operatorname{acosh}\left(\frac{2l_c}{z}\right) - \operatorname{acosh}\left(\frac{2l_c}{h}\right) - \sqrt{4 - \frac{z^2}{l_c^2}} + \sqrt{4 - \frac{h^2}{l_c^2}} \qquad (1)$$

where x is the horizontal distance to the vertical wall, z is the elevation of the interface above its level at large x (where the profile is flat), h is the contact elevation (i.e., the elevation of the interface at x=0), and l_c is the capillary length (Figure 3).

The capillary length is related to the interfacial tension and the density difference by:

$$l_c = \sqrt{\frac{\gamma}{g\Delta\rho}} \qquad (2)$$

where γ represents the interfacial tension, $\Delta\rho$ the density difference between the two phases and g the gravitational acceleration. The density of each phase was measured using an Anton Paar DMA45 density meter.

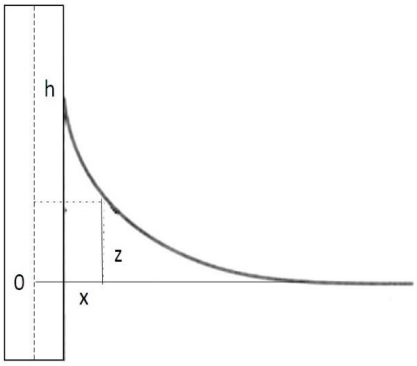


Figure 3. Schematic representation of the profile of a water-water interface.

Results

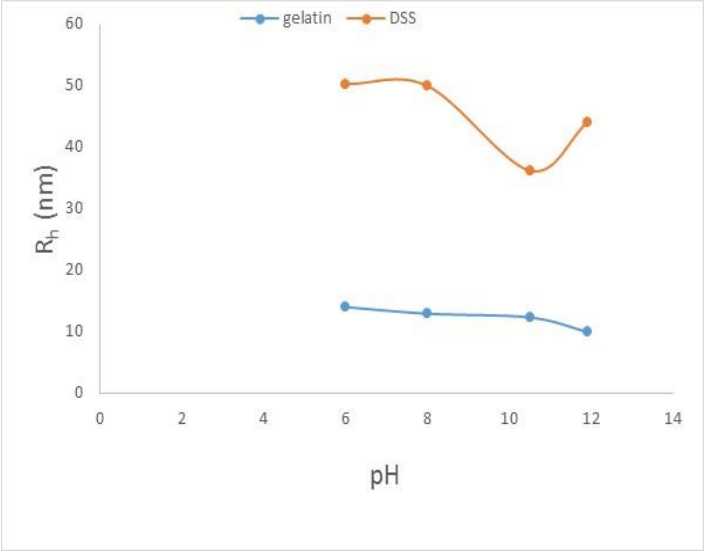
Intermolecular interactions between gelatin and DSS (complex coacervation)

To confirm the complexation between gelatin and DSS, DLS measurements were performed. Figure 4a shows the hydrodynamic radius R_h of aqueous solutions of pure gelatin and DSS at pH values below and above the pI of gelatin (approximately pH 8). The effect of viscosity of gelatin solutions on the estimated size was negligible. The R_h of DSS appeared to be about 5 times larger than that of gelatin, therefore DSS dominated the scattering intensity. There was no clear dependence on pH. In the mixtures of gelatin and DSS, the gelatin concentration was varied, whereas the concentration of DSS in those mixtures was kept constant at 0.05% (w/w). Figure 4b shows the R_h of gelatin/DSS mixtures with varied gelatin concentration at pH 6 (below the pI of gelatin). As we can see, by increasing the weight percentage of gelatin, the R_h became larger, which manifests complexation. Above the pI of gelatin, the R_h also increased with increasing the weight percentage of gelatin (data not shown). This suggests that complexation between proteins and polysaccharides is possible also when both polymers carry the same charge (Dubin, Gao, & Mattison, 1994; Grinberg & Tolstoguzov, 1997; Wen & Dubin, 1997).

After we confirmed with DLS that complexation between gelatin and DSS takes place at all polymer ratios tested both below and above the pI of gelatin, we investigated the solubility of the complexes that were formed. This was done at much higher concentration of gelatin, i.e. 1-10%, with the DSS concentration kept at the same concentration of 0.05%. When soluble complexes were formed, mixtures appeared to be transparent. When insoluble complexes were formed, mixtures appeared to be turbid. Turbidity was assessed both with UV-Vis spectrophotometry and visually. Figure 5 shows the turbidity measurements of gelatin/DSS mixtures, carried out with UV-Vis spectrophotometry at different gelatin concentrations at pH 6. It turned out that the turbidity had a sharp maximum at 1% (w/w) gelatin, indicating the formation of large, probably insoluble complexes. Figure 6 shows the macroscopic images of gelatin/DSS mixtures at pH 6 (a) and at pH 9 (b). At pH 6, soluble complexes were formed at lower gelatin concentrations,

and insoluble complexes were formed at around 1% gelatin concentration. This agrees with the UV-Vis spectrophotometry measurements. However, at pH 9, only soluble complexes were formed, therefore turbidity was much lower.

a.



b.

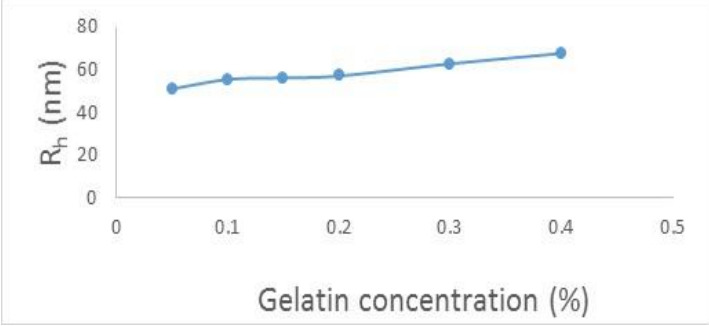


Figure 4. Hydrodynamic radii R_h of (**a**) pure gelatin (1.4%, w/w) and DSS (0.1%, w/w) molecules in aqueous solutions at various pH values, and (**b**) DSS/gelatin complexes in mixtures of their aqueous solutions, at pH 6. DSS concentration was kept 0.05% (w/w), whereas the gelatin concentration was varied.

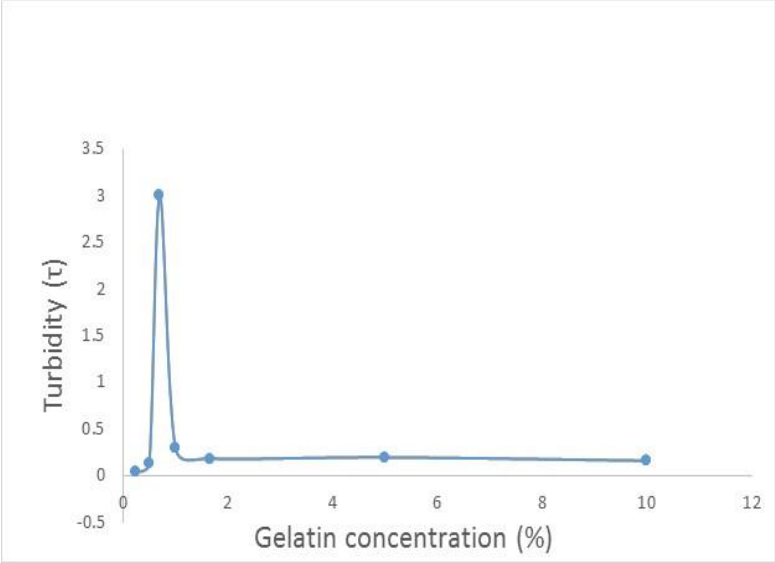
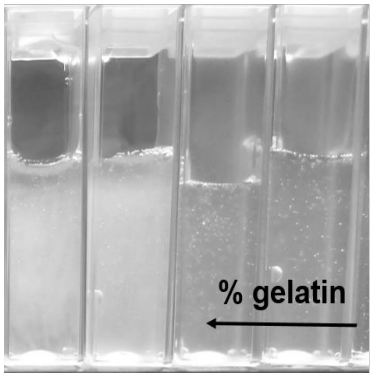


Figure 5. Turbidity values measured with UV-Vis spectrophotometer versus gelatin concentration (%) in gelatin/DSS mixtures at pH 6.

a.



b.

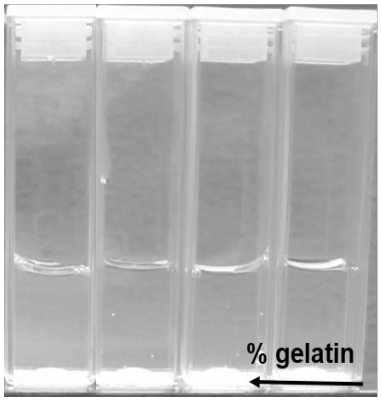


Figure 6. Macroscopic images of gelatin/DSS mixtures at pH 6 (**a**) and pH 9 (**b**). Gelatin concentrations (from right to left) are 0.5, 0.7, 1, and 1.66 % (w/w). DSS concentration is always 0.05% (w/w). At pH 6 soluble or insoluble complexes are formed, depending on the gelatin concentration. At pH 9 only soluble complexes are formed.

Effect of DSS

Visual assessment

Upon the addition of various concentrations of DSS (0.005-0.5%, w/w) to phase-separating mixtures of gelatin (7.5% w/w) and dextran (7.5% w/w), a turbid bottom phase was formed in all cases after phase equilibrium was reached (Figure 7). This turbid bottom phase was formed even at pH 9, where the system without DSS is not phase-separated. The turbid bottom phase may indicate either stabilized droplets of the top phase or the formation of some kind of structure induced by DSS.

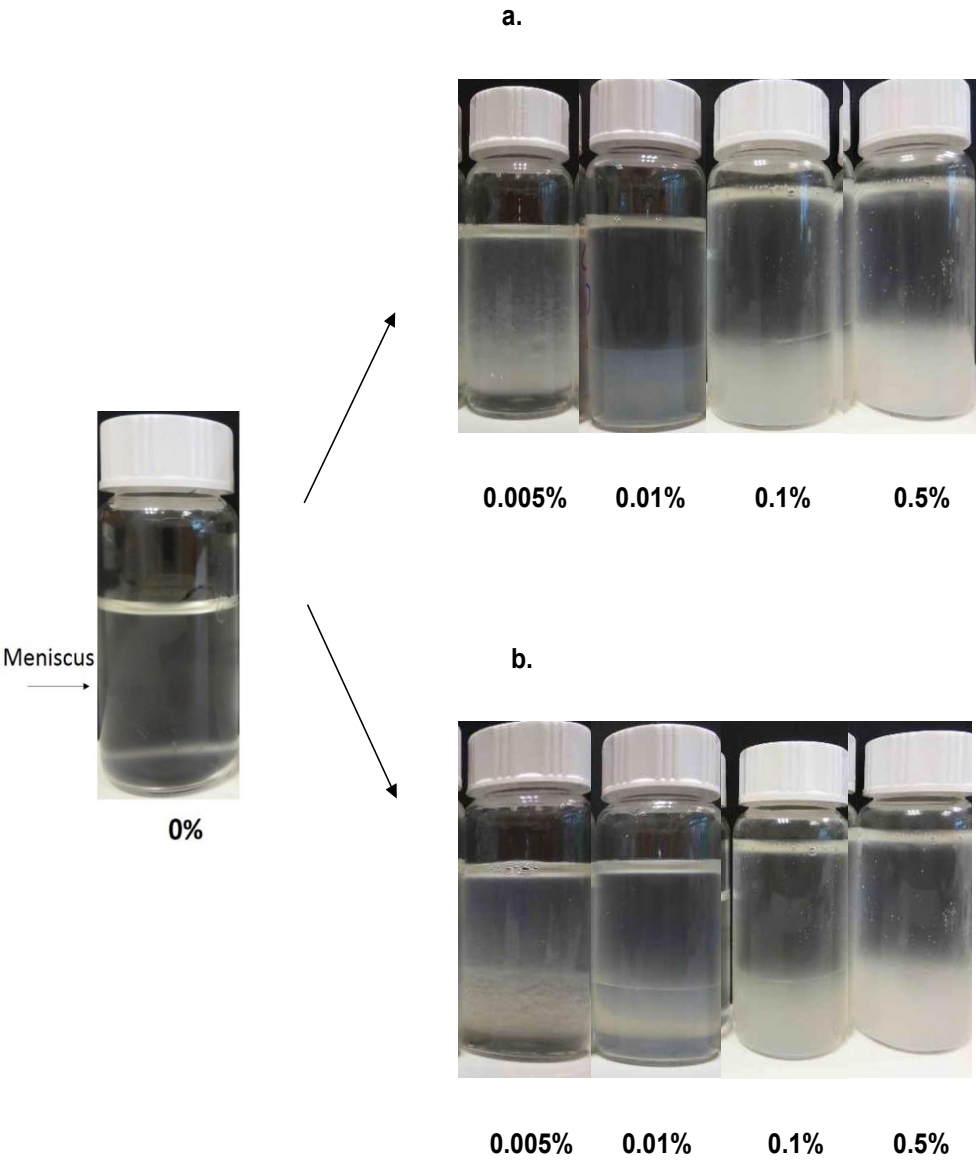


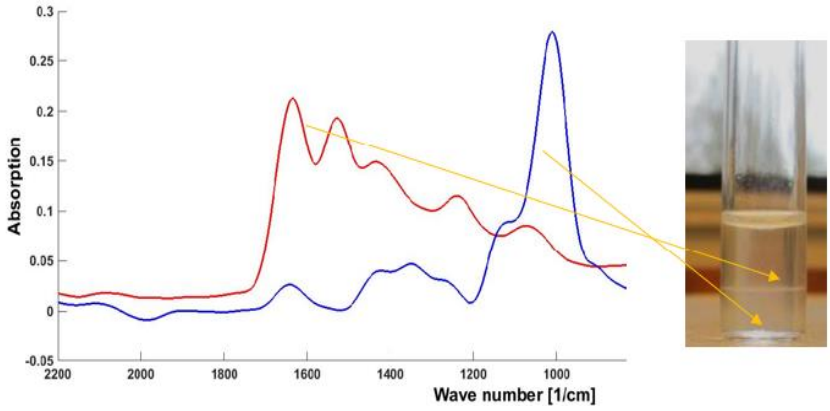
Figure 7. Effect of the addition of DSS in various concentrations on the macroscopic appearance of gelatin/dextran water-in-water emulsions, at pH 6.2 (**a**) and 9.2 (**b**). Note the formation of the turbid bottom phase.

Phase analysis

Phase composition. In order to investigate the composition and the origin of turbidity in the bottom phase of systems with DSS, FTIR measurements were performed on the solutes of both the top and bottom phase of those systems. Figure 8 shows the FTIR spectra of the solute in both phases in systems without (a) and with 0.1% (w/w) DSS (b) at pH 6. The signatures of gelatin, dextran and DSS are at 1630/1550 cm^{-1}, 1050 cm^{-1} and 1250 cm^{-1}, respectively. Table 1 summarizes the initial concentrations of gelatin, dextran and DSS in the emulsions and the measured concentrations of gelatin as part of all solutes in the top and bottom phases at the meta-stable equilibrium state (after centrifugation for 2 hours).

FTIR measurements showed that at pH 6 (below the pI) of gelatin, the top phase of the blank system consisted of gelatin and the bottom phase of dextran. Above the pI of gelatin, at pH 9, the opposite was the case. However, with the addition of DSS, the bottom phase was always turbid and turned out to always consist of gelatin and DSS (with hardly any dextran); the top phase, which showed no turbidity, contained almost exclusively dextran (and some DSS), regardless the pH value. This implies that there are neither dextran droplets stabilized in the (bottom) gelatin-rich phase nor gelatin droplets stabilized in the (top) dextran-rich phase. Therefore, the turbidity of the bottom phase appeared not to be due to droplet stabilization of the opposite phase, but to the formation of a structure directly induced by DSS. Because the turbidity in the gelatin-rich phase was accompanied by a density reversal of the phases, i.e. the top phase changed from gelatin-rich to dextran-rich, the DSS-induced structure must involve gelatin and an expulsion of water from the gelatin-rich phase.

a.



b.

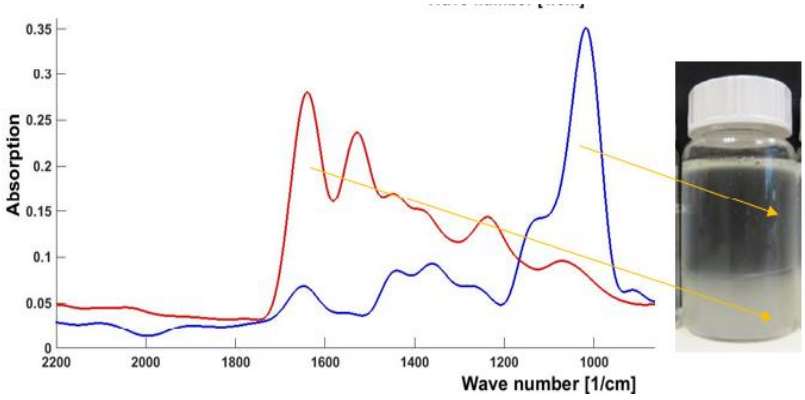


Figure 8. FTIR spectra of the blank system (**a**) and of the system with 0.1% (w/w) DSS (**b**) at pH 6. Arrows indicate the phase spectrum that belongs to each phase.

a.

Initial polymer concentration (% w/w)			Gelatin as part of all solutes (% w/w)	
Gelatin	Dextran	DSS	Bottom phase	Top phase
7.5	7.5	0	13.0	73.3
7.5	7.5	0.01	97.2	0
7.5	7.5	0.05	99.4	1.3
7.5	7.5	0.1	99.4	11.8

b.

Initial polymer concentration (% w/w)			Gelatin as part of all solutes (% w/w)	
Gelatin	Dextran	DSS	Bottom phase	Top phase
7.5	7.5	0	13.0	73.3
7.5	7.5	0.01	98.5	3.1
7.5	7.5	0.05	98.9	6.1
7.5	7.5	0.1	100	4.4

Table 1. Initial polymer concentrations and measured gelatin concentrations in the top and bottom phase after phase equilibrium was reached, at pH 6 (**a**) and pH 9 (**b**), according to FTIR measurements.

Phase volume. The phase volume of systems with and without DSS was measured under different phase separation conditions: under standard gravity after 72 hours, and after centrifugation at 800 g for 1 hour (using a LUMiFuge® 110 system).

Figure 9 shows the effect of DSS concentration on the volume of the bottom phase during the phase equilibrium of the systems. The smallest bottom phase volume was observed in the system with 0.01% DSS concentration.

Figure 10 shows the separation profiles in time, obtained by the LUMIFuge®, of emulsions without DSS, and with 0.005% and 0.07% DSS. The first significant observation here is the effect of DSS on the rate of demixing. At 800 g the blank system (without DSS) needed approximately 50 minutes to reach phase equilibrium (which is the point in the Lumifuge profile where the two phases are divided by the meniscus), whereas with the addition of DSS the required time was less (depending on the DSS concentration). This acceleration might have been caused by the formation of complexes between gelatin and DSS and the subsequent expulsion of water. This caused a larger density difference between the phases and a faster separation. Table 2 summarizes the time periods for emulsions containing various DSS concentrations to reach phase equilibrium. The second observation is the effect of DSS on phase volume after phase equilibrium was established (indicated by a sharp line representing the meniscus). As we mentioned earlier, the relative phase densities of the gelatin-rich and dextran-rich phases reversed upon the addition of DSS. This is consistent with the faster rate of phase separation: upon adding DSS, water is expelled from the gelatin-rich phase, causing it to become heavier and smaller. Figure 11 summarizes the bottom phase volumes of systems containing various DSS concentrations, obtained from the turbidity profiles of Lumifuge.

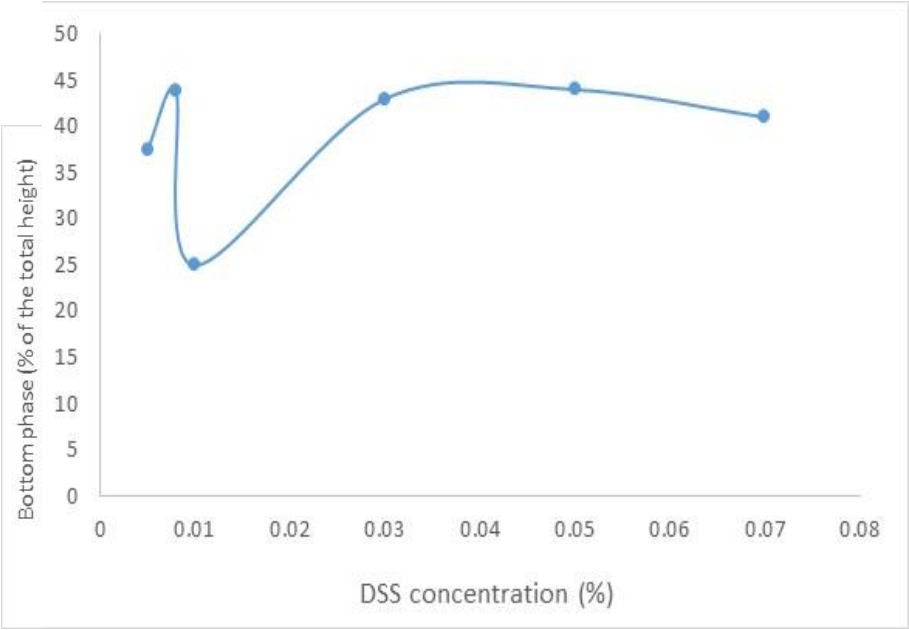


Figure 9. Bottom phase volumes after gravity-induced phase separation of systems containing various DSS concentrations.

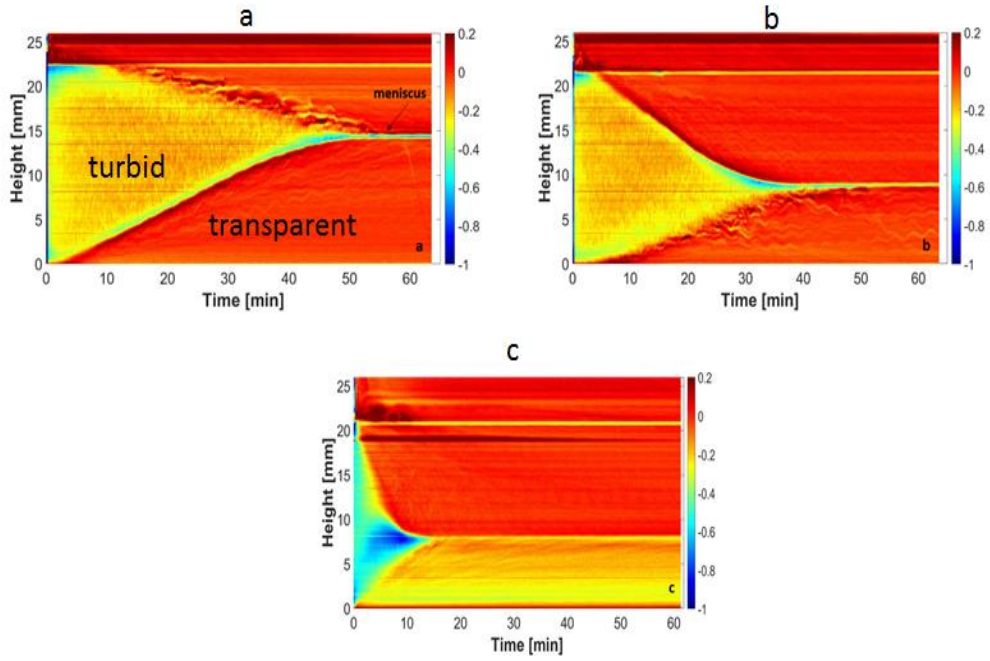


Figure 10. Turbidity-time profiles at 800 g of emulsions without DSS (a), and with 0.005% (b), and 0.07% (c) DSS.

DSS concentration in the system (%, w/w)	Time needed to reach phase equilibrium (min)
0 (blank)	53
0.005	48
0.008	38
0.01	32
0.05	14
0.07	14

Table 2. Required time for emulsions to reach phase equilibrium under centrifugal force at 800 g (data obtained from the turbidity profiles of emulsions).

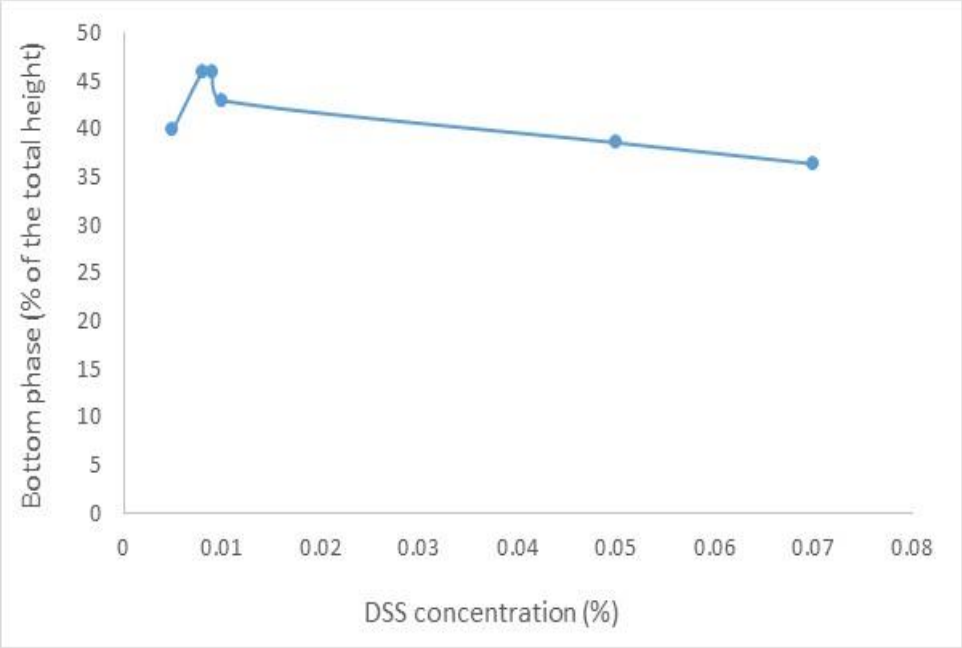


Figure 11. Bottom phase volumes after centrifugation at 800 g for 1 hour of systems containing various DSS concentrations (data obtained from Lumifuge profiles).

Microstructure

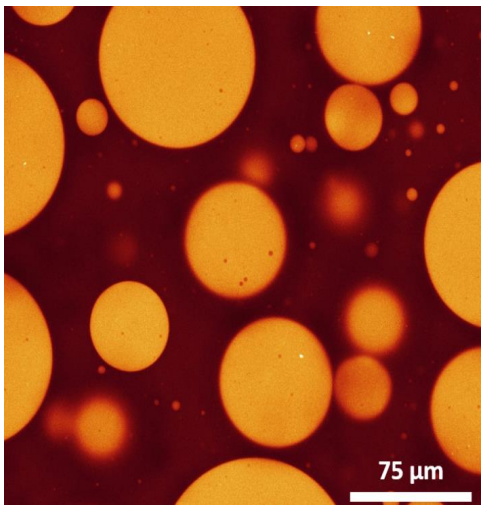
Figure 12 shows emulsions just after mixing, before layer formation. Figure 12a shows the microscope image of an emulsion without DSS (blank). Here the dispersed phase is formed by gelatin molecules (rhodamine-labeled) and the continuous phase by dextran molecules (dark). Figure 12b shows an emulsion with rhodamine and with 0.05% (w/w) FITC-labeled DSS. Here we observe three phases, instead of two: a bright phase, and two red phases which differ slightly in the degree of brightness. As we already know, gelatin and DSS form complexes, the solubility of which depends on the ratio DSS/gelatin. Therefore, the bright phase (1), rich in DSS must represent a phase consisting of complexes between gelatin and DSS. Phase (2) has a lighter color than the phase (3). Since rhodamine has no affinity with dextran, the phase (2) must consist of dilute gelatin

and some DSS causing the lighter color. The phase (3) must be dextran-enriched phase that has no affinity with either rhodamine or FITC labeled DSS.

Figure 13 shows CLSM images of the bottom phase (rich in gelatin and DSS) of a system with 0.05% (w/w) DSS, after it has reached its meta-stable equilibrium state (after 72 hours). Here we can observe two phases: insoluble complexes of gelatin and DSS that now form the continuous phase (1), and soluble complexes of gelatin and DSS which form the dispersed phase (2). Sporadically, some dark, spherical droplets can be found, representing probably 'trapped' dextran-rich droplets (3).

As mentioned before, adding DSS appears to generate a phase of gelatin/DSS complexes, which is heavier than the dextran-rich phase and also heavier than the original gelatin-rich phase. This can be explained by water migrating from this complex-rich phase to the dextran-rich phase. Figure 14 shows what happens when a droplet of dextran solution is brought into contact with a complex coacervate droplet of gelatin/DSS. The coacervate complex phase consisted of 7.5% (w/w) gelatin and 0.1% (w/w) DSS from which 0.05% was FITC-labeled. Dextran droplets were taken from a 25% (w/w) stock solution. Here the bright phase represents the complex coacervate phase of DSS and gelatin, and the black droplets are dextran-rich droplets formed by phase separation after interpenetration of the dextran and complex solutions. It is noticeable that denser complex coacervate regions are formed, as we can see in the magnified image. These regions are surrounded by dextran droplets.

a.



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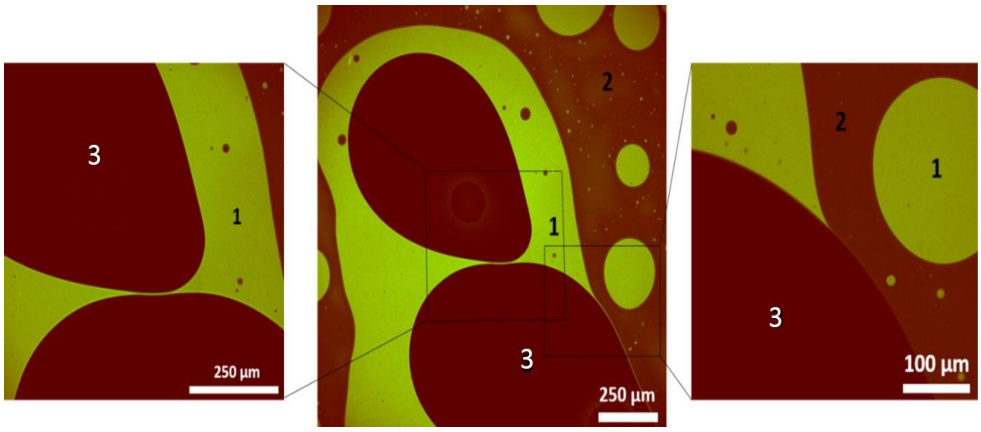

Figure 12. CLSM images of a water-in-water emulsion without DSS **(a)** and with 0.05% (w/w) DSS **(b)**. 1, 2, 3 are the different phases that are present.

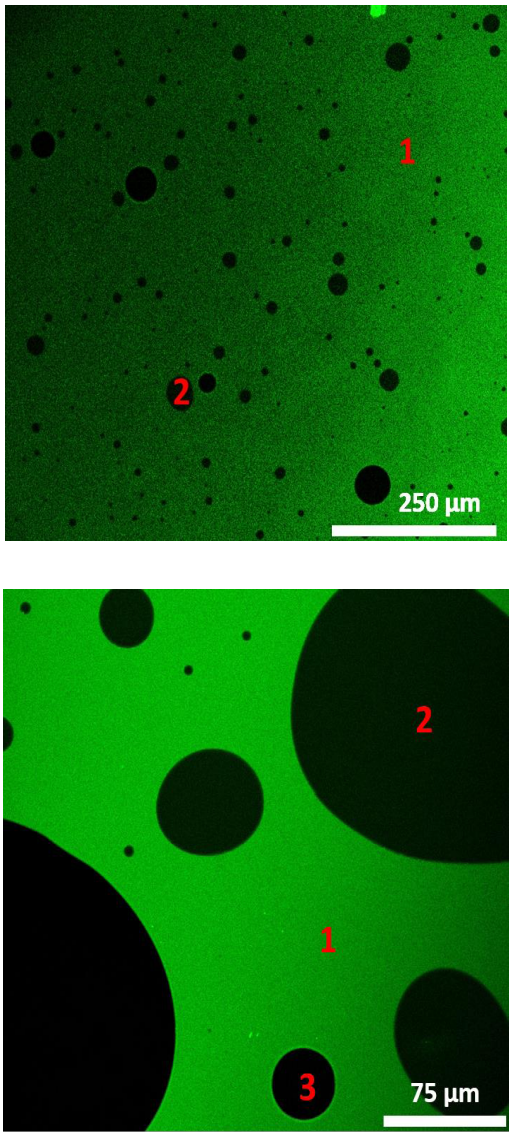

Figure 13. CLSM images of the bottom phase of a system with 0.05% (w/w) DSS, after phase equilibrium was reached. 1, 2, 3 are the different phases that are present.

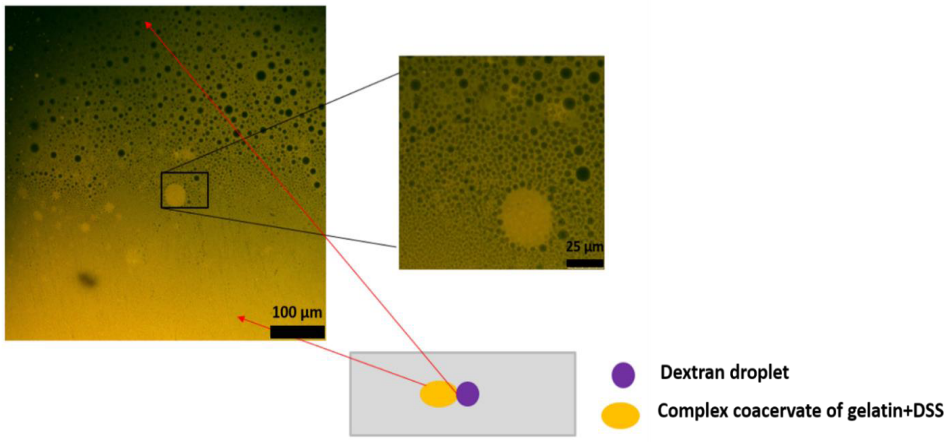

Figure 14. CLSM images showing the merging of a dextran droplet with a complex coacervate of DSS and gelatin. *Bottom*: scheme of the experimental setup of a microscope slide with the two droplets.

Interfacial tension

So far, we have seen that DSS did not lead to better compatibility between gelatin and dextran. Instead it increased polymer incompatibility and accelerated the demixing process of gelatin/dextran water-in-water emulsions. Furthermore, we saw that a new phase of gelatin and DSS was formed (due to complexation), which was actually less compatible with dextran than gelatin alone; the reason for that is the migration of water from the complexes to the more hydrophilic dextran phase.

To confirm the enhanced incompatibility due to DSS, interfacial tension measurements were performed on systems without and with various concentrations of DSS. Figure 15 shows the interface profiles of systems with various DSS concentrations (0.005%, 0.008%, 0.01%, 0.03%, 0.05% and 0.07%, w/w) at pH 6.2. After analyzing those profiles and measuring the density difference with the density meter, the values of

interfacial tension were calculated for each system (Table 3). Here we can observe an interesting tendency: by increasing the DSS concentration to 0.01% (w/w), the interfacial tension also increased (compared to the value of 4.72 μN/m in blank), and even reached a maximum of 24.25 μN/m. This proves that DSS indeed caused gelatin and dextran to become less compatible with each other. However, as the DSS concentration was further increased to 0.03% (w/w) till 0.07% (w/w), the interfacial tension started to decrease to 16.43 μN/m, which is still higher than in the blank system.

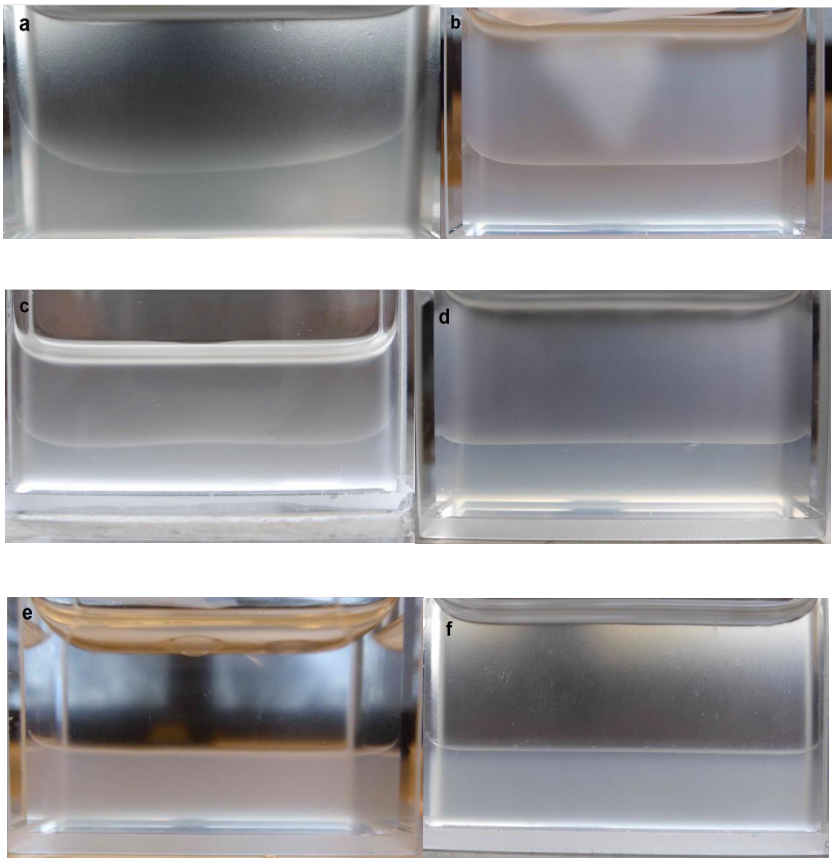

Figure 15. Effect of the DSS concentration on the static, equilibrium shape of the interface after the phase separation of the respective systems. (a) 0.005%, (b) 0.008%, (c) 0.01%, (d) 0.03%, (e) 0.05% and (f) 0.07% DSS. The pH was set at 6.2.

Concentration of added DSS (% w/w)	Capillary length (m)	Density difference $\Delta\rho$ (kg/m^3)	Interfacial tension (μN/m)
0 (blank)	-	-	4.72*
0.005	2.030×10^{-3}	0.15	6.06
0.008	1.411×10^{-3}	0.4	7.8
0.01	1.172×10^{-3}	1.8	24.25
0.03	0.822×10^{-3}	2.6	17.23
0.05	0.697×10^{-3}	3.6	17.13
0.07	0.597×10^{-3}	4.7	16.43

Table 3: Effect of DSS concentration on the interfacial tension of emulsions. *Value from the water/dextran (100 kDa)/gelatin (100 kDa) phase-separating mixture with a 1:1 polymer ratio and a total mass fraction of 10% (Edelman, 2003).

Discussion

The goal of this work was to study the effect of the addition of small quantities of the strongly charged polyelectrolyte dextran sodium sulfate (DSS) on the compatibility and phase behavior of phase-separating mixtures of fish gelatin and dextran.

We proved that DSS increases polymer incompatibility, since the interfacial tension in systems with DSS was always significantly higher than in the blank; the reason is the gelatin/DSS complexation, which reduces the solubility of gelatin and changes the affinity of polymers for the solvent - the main source of their phase separation. The change in polymer affinity for the solvent affects the partitioning of the solvent and consequently, the phase volume ratio of emulsions. Indeed, we saw that the bottom phase of systems with DSS had a smaller volume than that of the top phase, whereas the opposite applied for the blank system. The effect of DSS on water partitioning was also confirmed with CLSM visualization. Specifically, we observed a movement of water from the coacervate phase of gelatin/DSS to the dextran phase, which is probably caused by the reduced gelatin solubility due to its complexation with DSS. In other words, since the affinity of gelatin for water is reduced, water is pulled out of the gelatin-rich phase by the dextran phase (osmosis), forcing the gelatin-rich phase to split in a dense part containing insoluble, fully neutral complexes and a dilute part containing soluble charged complexes. Eventually, we have the formation of two co-existing regions/phases (soluble complexes within the continuous phase of insoluble complexes); these phases cause the turbidity of the bottom which was always observed in all systems with DSS.

However, dextran also played a role in causing the turbidity of the bottom phase in systems with DSS. In this context, we can consider the four-component system of dextran, gelatin, DSS and water from another point of view: the effect of dextran on the gelatin/DSS system. The role of dextran was confirmed when studying emulsions with pH values above the pI of gelatin. At these pH values, the complexes between gelatin and DSS are soluble and the gelatin/DSS mixtures (without dextran) appeared transparent. However, when combined with a dextran solution, the bottom phase of the ensuing phase-separating system was again turbid, as in the case of low pH and insoluble complexes. This means

that dextran is indeed involved in causing the turbidity of the gelatin-rich phase. Most likely, soluble complexes start to become at least partially insoluble, in presence of dextran. This can be explained on the basis of movement of water from the complexes to the dextran phase, which reduces eventually the water content in the complex coacervate phase. Consequently, the reduced water availability in the complex coacervate phase causes most likely re-positioning of the proteins bound in the interpolymeric complexes. This re-positioning makes it possible for neutral (insoluble) complexes with a low water affinity and charged (soluble) complexes with high water affinity to be formed. The two co-existing phases of insoluble and soluble complexes cause the macroscopic turbidity of the bottom phase in systems with pH>pI of gelatin.

Conclusions

In this work we studied the effect of the addition of the strong polyelectrolyte dextran sodium sulfate (DSS) on the compatibility and phase behavior of the phase-separating mixtures of fish gelatin and dextran. The addition of DSS (at concentrations 0.005-7%, w/w) increases the incompatibility between gelatin and dextran. The interfacial tension of the systems with DSS appears to be higher compared to the blank system, and the gelatin-rich phase volume is smaller. The latter is related to the change in the partitioning of water (due to DSS), which determines the phase behavior of these systems.

The increased incompatibility between the two polymers is explained on the grounds of the formation of intermolecular gelatin/DSS complexes via electrostatic interactions. The complexes that are formed can be either soluble or insoluble, depending on the available charges. These complexes cause the formation of a turbid gelatin-rich phase in systems containing DSS, after phase equilibrium is reached. The turbid gelatin-rich phase is present even at pH values above the pI of gelatin, where the complexes are soluble and the gelatin/DSS mixtures (without dextran) are transparent. This means that dextran is also involved in causing the turbidity of the gelatin-rich phase: due to DSS, dextran's affinity for water is relatively higher than that of gelatin, since gelatin forms complexes with DSS that reduce its solubility. Therefore, water is transferred from the coacervate phase of gelatin/DSS to the dextran phase. This leads to the formation of concentrated regions of insoluble complexes and less concentrated regions of soluble complexes. The presence of the two co-existing phases (soluble complexes within the continuous phase of insoluble complexes) causes the turbidity of the gelatin-rich phase.

Acknowledgements

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Chapter 6

Summary

Summary

The shift from the use of animal protein to plant protein has been one of the most important food and nutrition trends over the past few years, due to the positive effect of plant proteins on human health, sustainability and environment. However, a significant number of plant proteins is underutilized in food preparation because of their emulsifying properties. Utilization of plant proteins as emulsion stabilizers is limited by their poor solubility. The only way insoluble proteins can be used in food applications is if they are in the form of submicron or nanoparticles. Insoluble protein particles can be synthesized by using the anti-solvent precipitation method, and can increase stability of emulsions when incorporated during emulsification. This thesis reports on studies of the stabilizing effect of insoluble protein particles on two types of emulsions, the classical oil-in-water emulsions and the next generation water-in-water emulsions. Water-in-water emulsions have the potential application as oil-free emulsions.

In **Chapter 2** we show the synthesis of three types of food-grade sub-micron particles with different hydrophobicity (zein, gluten and mammalian gelatin) and study their stabilizing effect when incorporated in oil-in-water emulsions (50% w/w oil). Zein is a class of prolamine protein found in maize (corn) and it is insoluble in water, therefore it is considered hydrophobic. Gelatin is a denatured protein obtained by collagen hydrolysis. We use the type of gelatin derived from pork skin, which is soluble in water elevated temperatures, and therefore considered hydrophilic. Gluten is a co-product of the wheat starch isolation industry, and consists of gliadin (soluble in ethanol) and glutenin (insoluble). Therefore, in terms of hydrophobicity gluten can be considered to be between zein and gelatin. In this chapter, it is shown that the wettability of protein particles is not the only important factor that correlates with particle effectiveness as emulsion stabilizers. Instead, other parameters should be taken into account, such as the particle charge and the aggregation behavior of particles in the initial particle aqueous suspensions. It is also demonstrated that gelatin particles are the most efficient stabilizers, even though their adsorption energy at the oil-water interface is relatively low. Their stabilizing ability is attributed to their gelling properties (contact gelation).

When using the above colloidal particles as Pickering stabilizers (i.e. stabilizers that are particles, instead of molecules) or in food formulations, it is important to ensure their stability also in presence of biopolymers. So, it is important to understand the aggregation behavior of particles in colloid-polymer mixtures. When mixing colloidal dispersions with polymer solutions, interaction forces, such as depletion or steric repulsion, induced by polymer become relevant. In the study of colloid-polymer mixtures, it is important to distinguish between polymers that are adsorbed on the colloidal surfaces and those that are free in solutions, because the two situations lead to different effects. **Chapter 3** deals with the study of possible interactions of the three types of colloidal particles (zein, gluten and gelatin) with dextran in the particle-polymer mixtures. Dextran is a non-ionic branched polysaccharide. We show that the apparent hydrodynamic radii of the protein particles measured by dynamic light scattering measurements always decrease upon dextran addition, indicating that particle aggregates repeptize i.e. fall apart. In fact, we demonstrate that the particle repeptization increases at higher dextran concentration. This aggregate repeptization is attributed to dextran's adsorption on the surface of particles.

Particle-polymer interactions are also studied in **Chapter 4**, in which the behavior of colloidal zein particles at water-water interfaces is investigated. Water-water interfaces occur when two different polymers are present in aqueous solution forming aqueous two-phase systems which typically consist of 90% of water. These mixtures are explored by the food industry for the production of spreads, ice-creams and desserts, and are also known as water-in-water emulsions since their properties are comparable to normal oil-in-water emulsions. In this chapter we focus on a system consisting of the biopolymers dextran and gelatin in aqueous solution. In order to avoid gelation, a special non-gelling type of gelatin is used, produced from the collagen of cold-water fish. We show that, when added to phase-separating aqueous mixtures of fish gelatin and dextran, zein particles accumulate at the interface. Particle accumulation is explained on the grounds of similar interactions of particles with the two polymers. It is also demonstrated that particle accumulation is accompanied by aggregation of particles with each other at the interface of emulsion droplets. These aggregates are able to arrest the late stage of the demixing process of the emulsions by the formation of a stable particle-rich layer at the water-water interface.

Instead of using particles, the possible stabilization of water-in-water emulsions has been also studied with the use of polyelectrolytes. In **Chapter 5**, we focus on the phase behavior of water-in-water emulsions of fish gelatin and dextran after the incorporation of the strongly negatively charged polyelectrolyte dextran sodium sulfate (DSS). As it was expected, DSS accumulates in the gelatin-rich phase forming complexes with gelatin, as the gelatin is weakly positively charged. This complexation reduces the affinity of gelatin with water. As a result, water is expelled from the gelatin phase and we see increased incompatibility between dextran-rich and complexes-rich phase. The relative densities of the gelatin and dextran phases change sign, with the gelatin phase becoming the heavier, bottom phase. Furthermore, we show that the gelatin/dextran/DSS system contains three coexisting phases, one rich in insoluble gelatin/DSS complexes, one rich in soluble gelatin/DSS complexes and one rich in dextran.

Chapter 7

Samenvatting

Samenvatting

De verschuiving van dierlijk eiwit naar plantaardig eiwit is momenteel één van de belangrijkste voedingstrends, vanwege het mindere negatieve effect van plantaardige eiwitten op de volksgezondheid, duurzaamheid en milieu. De toepassing van plantaardige eiwitten als emulsiestabilisatoren wordt gehinderd door hun slechte oplosbaarheid. Echter, in de vorm van sub-micron deeltjes kunnen onoplosbare eiwitten wel gebruikt worden als stabilisator. Onoplosbare eiwitdeeltjes kunnen worden gesynthetiseerd door gebruik te maken van de anti-oplosmiddel precipitatie methode. Dit proefschrift rapporteert de resultaten van een studie naar het stabiliserende effect van onoplosbare eiwitdeeltjes in twee soorten emulsies, olie-in-water emulsies en water-in-water emulsies. Water-in-water emulsies hebben potentiële toepassingen als olievrije emulsies.

In **Hoofdstuk 2** beschrijven we de synthese van drie soorten sub-micron eiwitdeeltjes met verschillende hydrofobiciteit (zeïne, gluten en gelatine) en hun stabiliserende werking wanneer ze worden opgenomen in olie-in-water emulsies (50% w/w olie). Zeïne is een prolamine eiwit uit maïs en het is onoplosbaar in water. Daarom wordt het als hydrofoob beschouwd. Gelatine is een gedenatureerd eiwit verkregen uit hydrolyse van collageen. We gebruiken gelatine afkomstig van varkenshuid, oplosbaar in warm water. Daarom wordt het beschouwd als hydrofiel. Gluten is een co-product van de productie van tarwezetmeel, en bestaat uit gliadine (oplosbaar in ethanol) en glutenine oplosbaar in water). In termen van hydrofobiciteit ligt gluten tussen zeïne en gelatine. In dit hoofdstuk wordt aangetoond dat de bevochtiging (contacthoek tussen olie en water) van eiwitdeeltjes niet de enige factor is die de effectiviteit van eiwitdeeltjes als emulsiestabilisatoren bepaalt. Naast de contacthoek of hydrophobiciteit moeten andere parameters in aanmerking worden genomen, zoals de deeltjeslading en het aggregatiegedrag in de waterige suspensies. Ook wordt aangetoond dat gelatine deeltjes de meest efficiënte stabilisatoren zijn, hoewel hun adsorptie-energie aan het olie-water-interface relatief laag is. Hun stabiliserende vermogen wordt toegeschreven aan hun gelerende eigenschappen (contactgelering).

Bij het gebruik van de bovengenoemde colloïdale deeltjes als Pickeringstabilisatoren (dat wil zeggen stabilisatie met deeltjes in plaats van met moleculen) in voedingsformuleringen, is het belangrijk om hun stabiliteit ook in aanwezigheid van biopolymeren te waarborgen. Dus is het van belang om het aggregatiegedrag van deeltjes in colloïd-polymeermengsels te begrijpen. Bij het mengen van colloïdale dispersies met polymeeroplossingen worden interacties geïnduceerd door polymeer relevant, zoals depletie of sterische afstoting. Bij de studie van colloïd-polymeermengsels is het belangrijk om te onderscheid te maken tussen polymeren die geadsorbeerd zijn op de oppervlakken van de colloïddeeltjes en polymeren die vrij in oplossingen zijn, omdat beide situaties verschillende effecten hebben. **Hoofdstuk 3** behandelt de interacties van de drie soorten colloïdale deeltjes (zeïne, gluten en gelatine) met dextraan in de deeltjes-polymeer mengsels. Dextraan is een niet-geladen, vertakt polysaccharide. We laten zien dat de schijnbare hydrodynamische stralen van de deeltjes die worden gemeten met dynamische lichtverstrooiing altijd afnemen bij toevoeging van dextraan. Dit suggereert dat deeltjesaggregaten repeptiseren, dat wil zeggen uit elkaar vallen door de toevoeging van dextraan. Ook tonen we aan dat de repeptisatie toeneemt bij hogere dextranconcentratie. Deze totale repeptisatie wordt toegeschreven aan de adsorptie van dextran op het oppervlak van deeltjes.

Polymeer-eiwitcolloid interacties worden ook onderzocht in **Hoofdstuk 4**, waar het gedrag van colloïdale zeïne deeltjes in water-water grenslagen bestudeerd wordt. Water-water grenslagen worden gevormd wanneer een waterige oplossingen van twee polymeren ontmengen. Deze water-gebaseerde ontmengende systemen worden door de levensmiddelenindustrie onderzocht op hun toepasbaarheid in spreads, ijsjes en desserts en zijn ook bekend als water-in-water emulsies, aangezien hun structuureigenschappen vergelijkbaar zijn met die van normale olie-in-water emulsies. In dit hoofdstuk richten wij ons op een systeem dat bestaat uit de biopolymeren dextraan en gelatine in waterige oplossing. Om gelering te vermijden wordt een speciaal niet gelerende type gelatine gebruikt, geproduceerd uit het collageen van koudwatervissen. We laten zien dat zeïnedeeltjes adsorberen aan de grenslagen tussen gelatinerijke en dextraanrijke fasen. De adsorptie van deeltjes aan de grenslaag is het gevolg van ongeveer even grote interacties van de deeltjes met de twee polymeren. Er wordt ook aangetoond dat adsorptie

van deeltjes aan de grenslaag vergezeld gaat met aggregatie van deeltjes met elkaar. Deze aggregaten zijn in staat om de late fase van het ontmengingsproces van de emulsies te stoppen door de vorming van een stabiele deeltjesrijke laag aan de grenslaag.

Ook is onderzocht of in plaats van deeltjes, polyelectrolyten gebruikt kunnen worden voor de stabilisatie van water-in-water emulsies. In **Hoofdstuk 5** richten we ons op het fasegedrag van water-in-water emulsies van visgelatine en dextraan na de toevoeging van het hoog negatief geladen polyelektrolyt natrium dextraan sulfaat (DSS). Zoals verwacht, concentreert DSS zich in de gelatine-rijke fase waar het complexen vormt met gelatine. Deze complexatie vermindert de affiniteit van gelatine met water, waardoor water zich van de gelatine naar de dextraanfase verplaatst. De incompatibiliteit van de dextraanrijke en gelatinerijke fasen neemt toe. De relatieve dichtheden van de fasen verandert van teken, zodat de gelatinerijke fase de zwaardere fase wordt. Bovendien blijkt dat het gelatine/dextraan/DSS systeem drie fasen bevat: één rijk aan onoplosbare gelatine/DSS complexen, de tweede rijk aan oplosbare gelatine/DSS complexen en de derde rijk aan dextraan.

Chapter 8

List of publications

List of publications

- **Chatsisvili N.**, Philipse A, Loppinet B. & Tromp H. (2017). Colloidal zein particles at water-water interfaces. Food Hydrocolloids, 65, 17-23.

- Tromp H. & **Chatsisvili N.** (2016). Label Friendly Emulsifiers for Oil-Water Emulsions. The World of Food Ingredients, October/November issue, 50-52.

- Tromp H., de Jong S. & **Chatsisvili N.** (2015). Zein: A Future Insoluble Vegetable Protein? The World of Food Ingredients, October/November issue, 46-47.

- **Chatsisvili N.T.**, Amvrosiadis I. & Kiosseoglou V. (2012) Physicochemical properties of a dressing-type o/w emulsion as influenced by orange pulp fiber incorporation. LWT – Food Science and Technology 46: 335-340.

- **Chatsisvili N.T.**, Philipse A. & Tromp H. Protein particles at oil-water interfaces. Submitted to Food Hydrocolloids.

- **Chatsisvili N.T.**, Philipse A., & Tromp H. Repeptization of protein particle aggregates by uncharged polysaccharide. Submitted to Food Hydrocolloids.

- **Chatsisvili N.T.**, Kibbelaar H., Philipse A. & Tromp H. Phase behavior of water-in-water emulsions containing small quantities of strongly charged polyelectrolyte (manuscript in preparation).

Chapter 9

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Nina

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Chapter 10

Curriculum Vitae

Nino Chatsisvili was born on January 9th, 1986, in Georgia by Georgian father and Greek mother, and was raised in Greece. In 2003 she graduated from High School (19.3/20), and continued her education in the Faculty of Veterinary Medicine at the Aristotle University of Thessaloniki. In 2008 she graduated 9th among 119 graduates from Veterinary Medicine with the specialization in public health and food science. In that same year she started her Master study ''Food Chemistry and Technology'' in the Department of Chemistry at the Aristotle University of Thessaloniki. Her thesis was carried out in the laboratory of Prof. Dr. Vassilis Kiosseoglou, and was focused on the study of the physico-chemical properties of oil-in-water emulsions after the incorporation of orange pulp fiber. She published this work as a first author in LWT- Food Science and Technology Journal. After she obtained her MSc degree in 2011, she worked for one year as a Lecturer for Food Chemistry and Analytical Chemistry courses in a Private Institute for Higher Education in Thessaloniki (Greece). In February 2013 she obtained a highly-esteemed Marie Curie Fellowship for PhD studies in The Netherlands. She performed her PhD research at NIZO food research (Flavour and Texture Department) under the supervision of Dr. Hans Tromp, in collaboration with the Physical and Colloid Chemistry of Utrecht University under the supervision of Prof. Albert Philipse.