Tomas Bata University in Zlín
Faculty of Technology

Doctoral thesis

Stabilization of dispersion systems by polymeric emulsifiers

Stabilizace disperzních systémů polymerními emulgátory

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CONTENT

ABSTRACT

Biopolymers and biopolymer-based particles are a natural alternative to replace potentially toxic synthetic surfactants stabilizing the oil-water interface in emulsions. These more friendly species allow for the preparation of biocompatible, surfactant-free emulsions for pharmaceutical and cosmetic uses. However, the application of bio-based emulsifiers may, in some cases, be insufficient to prepare stable emulsions. Under these conditions, the ability of some natural emulsifiers to form and stabilize emulsions in synergy with other biopolymers or particles can be advantageously utilized. Therefore, the thesis is at first focused on stabilization of emulsions by single emulsifying protein, sodium caseinate, and in the next step on the investigation of the interactions between the biopolymer and particles at the phase interface. More specifically, the emulsions stabilized by mixtures of sodium caseinate and cellulose nanocrystals are investigated to understand processes of adsorption, complexation or layer-by-layer formation taking place at the oil-water interfaces. The knowledge gathered in the thesis enables to control the emulsion properties *via* variations in the composition of their stabilizing layer. The practical application of such stabilized emulsions is further verified through the preparation of emulsion-based oleogels, which can serve for delivery of bioactive lipophilic substances.

Keywords: biopolymer, stabilization, protein, polysaccharide, nanocellulose, interaction, emulsion.

ABSTRAKT

Biopolymery a biopolymerní částice jsou jednou z alternativ pro nahrazení potenciálně toxických syntetických surfaktantů stabilizujících rozhraní olej-voda v emulzích. Tyto mírnější druhy stabilizátorů umožňují připravit biokompatibilní emulze, vhodné pro farmaceutické a kosmetické využití, bez přítomnosti syntetických surfaktantů. Použití těchto stabilizátorů však může být v některých případech nedostačující, protože nejsou schopny vytvořit stabilní emulze. V takových případech pak může být výhodná schopnost některých přírodních emulgátorů vytvořit a stabilizovat emulze v synergii s jinými biopolymery anebo částicemi. Tato práce je proto nejprve zaměřena na problematiku stabilizace emulzí pouze proteinem, kaseinátem sodným, a v dalším kroku na studium interakcí mezi biopolymerem a částicemi na fázovém rozhraní. Konkrétně jsou studovány emulze stabilizované směsí kaseinátu sodného a nanokrystalické celulózy, se snahou porozumět stabilizaci emulzí prostřednictvím adsorpce, komplexace nebo vrstvení kaseinátu a nanocelulózy na rozhraní olej-voda. Znalosti získané v práci tak umožní kontrolovat vlastnosti emulzí pomocí změn ve složení stabilizující vrstvy na povrchu kapek. Praktické použití studovaných emulzních systémů je zaměřeno na přípravu oleogelů, které mohou sloužit jako nosiče bioaktivních lipofilních látek.

Klíčová slova: biopolymer, stabilizace, protein, polysacharid, nanocelulóza, interakce, emulze.

1. INTRODUCTION TO DISPERSION SYSTEMS

Dispersion systems are two-phase systems with one of the phases (dispersed phase) dispersed in the second, continuous phase. The continuous phase can be gas, liquid or solid in which particles, droplets or bubbles (with at least one dimension in the range 1–1000 nm) are dispersed. The basic types of colloidal dispersions can be classified to aerosols (liquid/particles in gas), foams (gas in liquid), suspensions (solid particles in liquid) and emulsions (liquid in liquid) (Fig. 1.1). However, in practice, the systems may be more complex, and dispersions with all three types of dispersed phases occur (Cosgrove 2005).

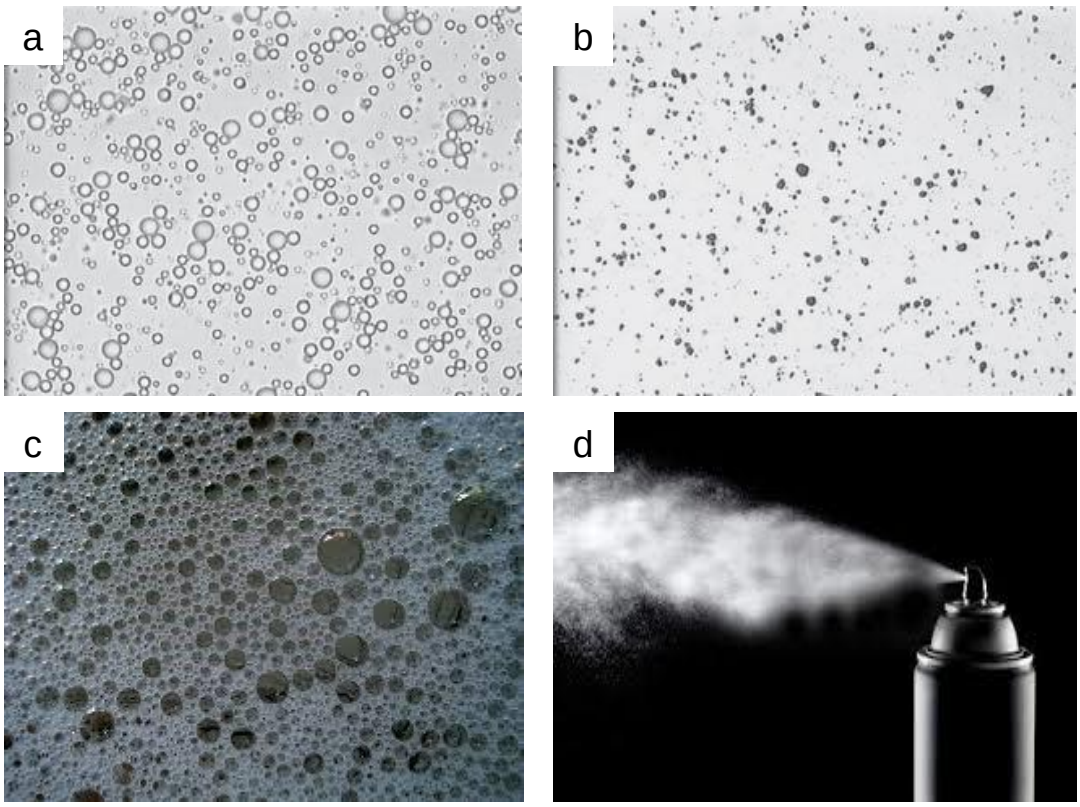


Fig. 1.1 Main types of dispersion systems: a) emulsion (spherical, regular droplets of the dispersed phase in the second phase); b) suspension (solid asymmetric particles in liquid); c) foam (gas bubbles) and d) aerosol.

Recently, these systems are widely applied in various disciplines, products and industrial processes (Schramm 2006), and the scientific interest is paid to their formation, properties and stability. The thesis, therefore, focuses on the topics related to dispersion systems, more specifically to emulsions, mechanisms of their stabilization (steric and electrostatic), and emulsions stabilized with biopolymers and particles. In addition, the investigation of interactions of biopolymers and particles at the oil-water interface brings a new approach in the understanding of emulsion stabilization, as the synergy between stabilizing agents leads to their adsorption, complexation and/or layer-by-layer deposition at the oil-water interface. Moreover, the composition of the surface layer formed around the

droplets can control the emulsion properties, as it is discussed below in more detail.

2. EMULSION SYSTEMS

Emulsions are, besides others, used in many industrial fields as delivery vehicles for aqueous- or oil-based actives. They can be, for instance, applied in the pharmaceuticals where they serve as drug delivery systems for parental, oral, and topical routes. As it was already mentioned above, emulsions are colloids consisting of mixtures of at least one liquid dispersed in another in the form of fine droplets. Moreover, the liquids used in emulsions should be immiscible (Schramm 2006; Bouyer, et al. 2012; McClements, D. J., et al. 2007; McClements, David Julian 2015).

The main role of emulsions is to encapsulate hydrophilic or lipophilic active molecules inside the dispersed phase, which ensures their protection against environmental stress and degradation and allows for their controlled delivery. Emulsions may also mask an unpleasant smell or taste, reduce drug toxicity, enhance its penetration through the skin or behave as detoxifying systems to entrap toxic molecules in the dispersed phase (Schramm 2006; Bouyer, et al. 2012; McClements, D. J., et al. 2007; McClements, David Julian 2015).

Two basic types of emulsion are readily distinguished depending upon which type of liquid forms the continuous phase. The first type is oil-in-water (o/w) emulsion, where oil droplets are dispersed in water; the second type is water-in-oil (w/o) emulsion with water droplets dispersed in oil. More complex emulsion types include double emulsions, which can be oil-in-water-in-oil or water-in-oil-in-water types (Schramm 2006; Bouyer, et al. 2012; McClements, D. J., et al. 2007; McClements, David Julian 2015).

Commonly, most of the emulsions are not thermodynamically stable due to the positive free energy associated with the contact of oil and water phases. The positive free energy originates from the interaction of oil and water molecules, and can mainly be attributed to the fact that water molecules are capable of forming strong hydrogen bonds with neighbouring water molecules, but not with molecules of oil. This thermodynamic instability is the main challenge when working with emulsions, as they are prone to destabilization and phase separation. The increase of thermodynamic stability can be accomplished by the addition of emulsifiers[1] (emulsifying/stabilizing agents, surfactants) with surface activity and/or thickening properties, which allow for emulsion formation and stabilization. Emulsifiers are surface-active molecules that rapidly adsorb onto the oil-water interface during the emulsification, creating thus a protective barrier that prevents droplets from coming close together and aggregating. Emulsions are

[1] *For purpose of this thesis, the terms as surfactant, emulsifier and stabilizer are used in followed meaning: emulsifier – general term, emulsifying and stabilizing agents; surfactant – small amphiphilic molecule; stabilizer – for Pickering emulsions.*

commonly stabilized by synthetic surfactants, nevertheless, their use can increase toxicity and induce various adverse effects, such as skin irritations in case of topically applied emulsions. Substituting synthetic surfactants with more friendly natural molecules (biopolymers) and solid particles have attracted a lot of attention, as they enable to prepare biocompatible, surfactant-free emulsions (Schramm 2006; Bouyer, et al. 2012; Dickinson, Eric 2017; McClements, David Julian 2009). The application of natural polymers, such as proteins and/or polysaccharides as emulsifiers and stabilizers, has been a successful approach for the formulation of emulsions in the food industry. This knowledge has, however, led to a better understanding of a new generation of emulsions for pharmaceutical and cosmetic applications (Bouyer, et al. 2012).

2.1 Emulsion stabilization

The main role of the emulsifier is to adsorb at the surface of the freshly formed droplets and thus prevent them from coalescing with their neighbours and form larger droplets again. Emulsifiers commonly comprise both hydrophobic and hydrophilic components that become integrated at the oil-water interface to lower interfacial tension. For a fixed rate of energy dissipation during emulsification, the final droplet size distribution is determined by the time taken for the interface to be covered with an emulsifier, as compared with the average time interval between droplet collisions. When the emulsifier adsorbs too slowly or is present at too low concentration, most of the individual droplets formed during the intense energy dissipation of emulsification are not retained in the final emulsion. This may be due to breakage of the thin film between colliding droplets (coalescence) or sharing of the adsorbed layer between two droplets (bridging flocculation) (Dickinson, Eric 2009; Lam, R. S. H. and Nickerson 2013).

One of the most important factors affecting the overall stability and physicochemical properties of emulsions is the nature of the forces acting between the droplets. Interactions between droplets depend on the sign, magnitude and range of the interactions, such as steric, electrostatic, hydrophobic, van der Waals, etc. To stabilize emulsions, emulsifying and stabilizing agents are commonly used. Emulsifiers may be in the form of low molecular weight synthetic surfactants, e.g. sucrose esters, polyglycerol esters or natural molecules, or be comprised of solid particles or larger macromolecules, such as proteins and polysaccharides (McClements, David Julian and Gumus 2016).

Stabilization of emulsions can be accomplished using the following ways. The first mechanism lies in an action of classical amphiphilic surfactants (Fig 2.1a), such as sodium dodecyl sulphate. These molecules decrease interfacial tension and increase emulsion stability but can be irritating and potentially toxic towards the environment. The second way comprises the use of polymers, such as proteins (Fig. 2.1b) and polysaccharides (Fig. 2.1c). In addition to their ability to lower interfacial tension, polymers induce steric or electrostatic interactions, changes in the interface viscosity or elasticity, or changes in the bulk viscosity of the system.

Finally, the third possible way of stabilization is related to action of small non-surfactant colloidal solids (Fig. 2.1d) that adsorb at interface and form a physical barrier between droplets, thereby delaying or preventing coalescence (Bouyer, et al. 2012; McClements, David Julian 2009; Lam, R. S. H. and Nickerson 2013).

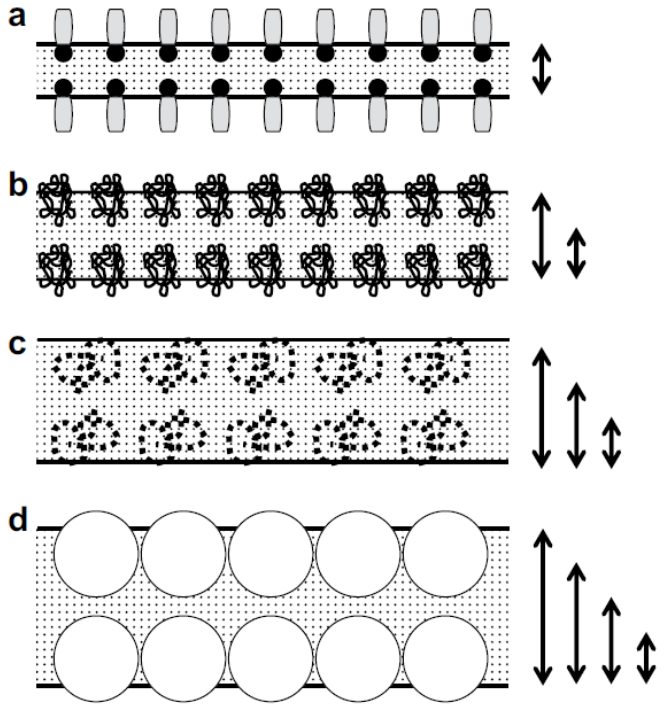


Fig. 2.1 Possible ways of stabilization of emulsions by a) surfactants, b) proteins, c) polysaccharides and d) particles (Dickinson, Eric 2009).

An efficient emulsifier is one that (i) rapidly reduces the interfacial tension at the freshly formed oil-water interface, (ii) binds strongly to the interface once adsorbed, and (iii) protects the newly formed droplets against flocculation or coalescence. This protection against immediate recoalescence occurs at first *via* dynamic surface tension effects and later *via* repulsive colloidal interactions (electrostatic and steric stabilization mechanism) (Dickinson, Eric 1992). This thesis is mainly focused on stabilization through steric/electrostatic interactions (biopolymer-stabilized emulsions) and stabilization through a physical barrier formed by solid particles (Pickering emulsion). These approaches will be, therefore, discussed in more details.

2.1.1 Electrostatic and steric stabilization

To introduce electrostatic and steric stabilization, it is important to mention polymers, which can act as emulsifiers with a broad range of applications, especially, in emulsion polymerizations, stabilization of crude oil (Kang, et al. 2011), or in medicine (de Vries, et al. 2010).

Besides synthetic polymers, also biopolymers, such as proteins and polysaccharides can serve as natural emulsifiers/stabilizers. Many proteins can act as emulsifiers thanks to their ability to adsorb at the oil-water interface. On the other hand, most of the polysaccharides are more thickening agents than

emulsifiers. They increase the viscosity of the continuous phase of emulsions by forming an extended network improving thus the emulsion stability. Contrary to proteins, only a few polysaccharide derivatives exhibit surface activity and are able to adsorb at the oil-water interface due to their surface properties (Bouyer, et al. 2012; Friberg, et al. 2003).

Biopolymers prevent flocculation and coalescence of emulsion droplets by combined mechanisms; non-adsorbing thickening polysaccharides reduce movement of droplets by increasing the viscosity of the continuous phase, whereas adsorbing biopolymers act on inter-droplet interactions. Flocculation and coalescence occurring in the biopolymer-stabilized emulsions can be avoided by inducing repulsive electrostatic interactions or steric hindrances between droplets (Bouyer, et al. 2012).

In general, stabilization of emulsions by a biopolymer depends on various parameters, such as polymer nature and concentration, as well as pH, and ionic strength of emulsion continuous phase. (Bouyer, et al. 2012; Friberg, et al. 2003). According to these parameters, two types of stabilization, electrostatic and steric, can be distinguished.

Electrostatic stabilization

In the case of biopolymer molecules, mainly charged proteins, stabilization by electrostatic repulsion occurs (Fig. 2.2a). The charges of molecules lead to electrostatic repulsion between the neighbouring protein molecules inside the adsorption layers, as well as between the layers adsorbed on two neighbouring emulsion droplets. This electrostatic repulsion keeps the adsorbed protein molecules at a certain distance from each other and hinders the formation of both non-covalent and covalent bonds between the adsorbed layers. Therefore, the stability of such emulsions is governed mainly by long-range electrostatic and van der Waals forces (Lam, R. S. H. and Nickerson 2013). The electrostatic stabilization is based on the mutual repulsive forces that are produced when electrically charged surfaces approach each other. In an electrostatically stabilized emulsion, a charged layer is formed at the oil-water interface. If the repulsive forces are strong enough, the droplets are repelled before they can come to contact and coalesce; thus the emulsion is stabilized. In general, electrostatic stabilization is significant only for o/w emulsions as the electric double-layer thickness is bigger in water than in oil (McClements, David Julian 2009; McClements, David Julian and Gumus 2016).

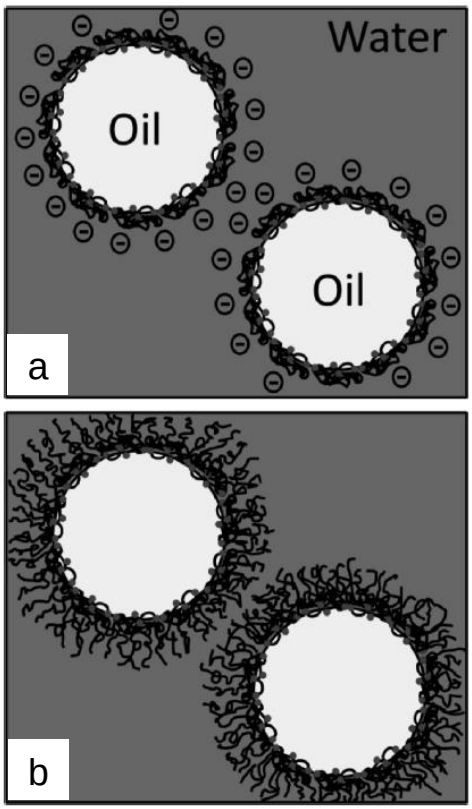


Fig. 2.2 Mechanism emulsion stabilization by a) electrostatic repulsion and b) steric stabilization.

Steric stabilization

Steric stabilization occurs mainly at low protein concentration, or when emulsions are stabilized with non-charged polysaccharides. Under these conditions, the electrostatic repulsion is negligible, due to the small net charge (Lam, R. S. H. and Nickerson 2013). The emulsion stability is controlled by steric repulsion between adsorption layers present on the surfaces of two neighbouring droplets, which touch each other. The magnitude and range of the steric repulsion (Fig 2.2b) between droplets are determined by the thickness and packing of the emulsifier molecules at their surfaces. Typically, the denser the packing and thicker interface, the stronger and longer range is the steric repulsion. Emulsifiers differ considerably in their molecular organization at oil-water interfaces, which influences their ability to generate steric repulsion between droplets. For example, polysaccharides that form thick interfacial layers are highly efficient in inhibiting droplet aggregation through steric interaction. On the other hand, the efficacy of globular proteins in preventing droplet aggregation through steric repulsion is low as they form only a thin interfacial layer. It is because the range of the van der Waals attraction exceeds the range of the steric repulsion. The thickness of the interfacial layer that implies steric interactions can be increased by choosing natural emulsifiers with large, extended structures or by using electrostatic deposition to form multi-layered interfaces (McClements, David Julian and Gumus 2016).

Both electrostatic and steric forces can prevent aggregation or coalescence, and thus stabilize emulsions. The electrostatic repulsive interactions acting between droplets dispersed in water are influenced by the surface charge density, as well as by the properties of the solution, such as ionic strength and solvent type. Typically, the higher the surface charge density and lower the ionic strength, the stronger is the electrostatic interaction (McClements, David Julian and Gumus 2016).

2.1.2 Stabilization with solid particles

Contrary to surfactant-based emulsions, Pickering emulsions are stabilized by steric barrier composed of solid particles, which are adsorbed at the oil-water interface. The particles can function in similar ways as surfactants; however, differences between surfactant and Pickering stabilizers are obvious. For instance, individual particles do not assemble to form aggregates in the same way as surfactants form micelles. Particles are able to irreversibly attach to the oil-water interface, which leads to more effective stabilization in comparison with the adsorption of surfactants. Moreover, particles are not capable of solubilizing powder materials, when present in a liquid (Dickinson, Eric 2017; Binks, Bernard P. 2002; Yang, et al. 2017; Lam, S., et al. 2014).

Pickering emulsions retain the basic properties of conventional emulsions stabilized by surfactants and can, therefore, substitute these emulsions in a broad range of applications. The stabilization by solid particles brings about specific properties to Pickering emulsions. Assuming the density of stabilizing particles at the oil-water interface is high enough to form an effective mechanical barrier, long-term protection against droplet-droplet coalescence is achieved. The high resistance to coalescence is a major benefit of the stabilization by solid particles. Their accumulation at the oil-water interface gives rise to a densely packed layer, which prevents droplet flocculation and coalescence (Dickinson, Eric 2017; Yang, et al. 2017; Lam, S., et al. 2014; Chevalier and Bolzinger 2013; Dickinson, Eric 2010). Though discovered a century ago (Pickering 1907), Pickering emulsions have recently drawn significant research interests as templates in many fields due to the following advantages: (i) solid particles reduce the possibility of coalescence, bringing about higher stability to emulsions; (ii) many solid particles can endow (as prepared materials) useful characteristics such as conductivity, responsiveness, porosity, etc.; (iii) many types of solid particles have low toxicity, thus leading to higher safety for *in vivo* usage (Dickinson, Eric 2017; Yang, et al. 2017; Lam, S., et al. 2014) and the “surfactant-free” character makes them attractive for utilization in cosmetic and pharmacy, where surfactants often show adverse effects (Chevalier and Bolzinger 2013).

Solid stabilizing particles are necessarily smaller than emulsion droplets and stabilize emulsions by their adsorption at the droplet surfaces. Nevertheless, in comparison with surfactants, the mechanism of adsorption is completely different. In Pickering stabilization, the particles do not exhibit amphiphilic character but

the stabilization takes place through partial wetting of the surface of the solid particles by water and oil, which is the origin of the strong anchoring of particles at the oil-water interface (Chevalier and Bolzinger 2013). Particles at the interface form elastic monolayer of high rigidity. When two droplets approach close together, the physical barrier associated with the re-arrangement of this particle bilayer prevents coalescence. The ideal particles capable of stabilizing emulsions are monodisperse hard spheres arranged in monolayer (Fig. 2.3a); however, this situation only rarely occurs in practice. The most limiting factor here is the actual concentration of dispersed particles that are available for adsorption at the freshly formed interface during emulsification. At low surface coverage, droplet-droplet coalescence leads immediately into the formation of bigger spherical droplets. However, if the particle density on the surface is close to full coverage, a pair of droplets may join together and form a stable non-spherical droplet (Fig. 2.3b). Colliding pairs of droplets may also become stabilized through the sharing of a single bridging layer of adsorbed particles (Dickinson, Eric 2017; Yang, et al. 2017; Lam, S., et al. 2014).

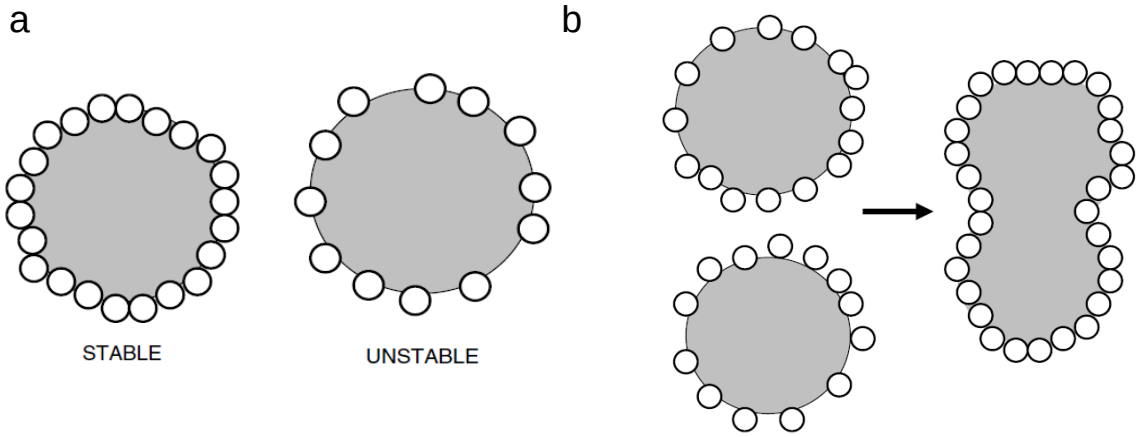


Fig. 2.3 Schematic representation of the stabilization of emulsion droplets by solid particles: a) adsorbed monolayer at the droplet surface; b) stable non-spherical droplet (Dickinson, Eric 2013).

The efficacy of the physical barrier in inhibiting coalescence is enhanced by the stability of the thin film of continuous phase between the particle-coated droplets. The strength of the steric barrier depends on how difficult it is to remove particles from the interface. The steric barrier is more effective when the particle layer lies predominantly on the outer side of the dispersed droplets, and the particles are preferentially wetted by the continuous phase (particles that are preferentially wetted by water form o/w emulsions, whereas particles that are preferentially wetted by oil will form w/o emulsions). Location of particles at the interface (Fig. 2.4) depends on the contact angle Θ (Dickinson, Eric 2010). Particles with Θ smaller than 90° will form an o/w emulsion, while particles with a contact angle higher than 90° usually form w/o emulsions (Tavernier, et al. 2016; Aveyard, et al. 2003). To remove particles from the interface, high energy,

which depends on the particle size and the contact angle, is required (Chevalier and Bolzinger 2013; Aveyard, et al. 2003).

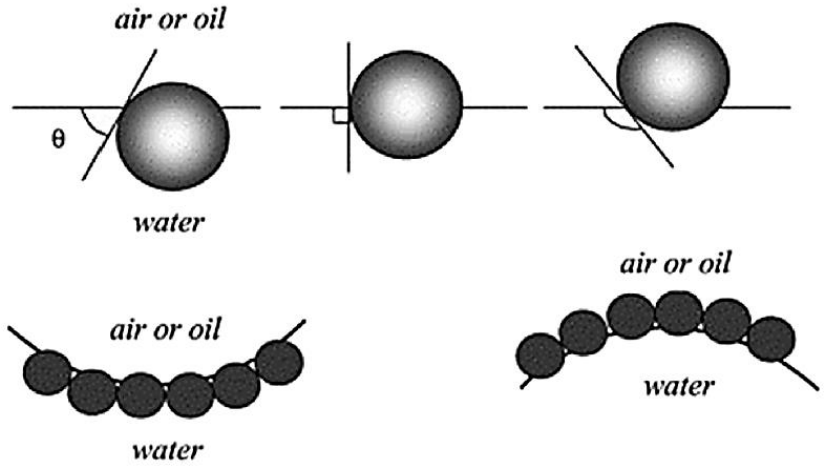


Fig. 2.4 Bending behaviour of the oil-water interface for a contact angle less than 90° (left) and greater than 90° (right). The contact angle equal to 90°corresponds to the point of phase inversion (Aveyard, et al. 2003).

3. BIOPOLYMERS AS EMULSIFIERS

Besides synthetic polymers, also biopolymers, such as proteins and polysaccharides can serve as natural emulsifiers/stabilizers with ability to improve texture and stability of emulsions. The interfacial activity of many biopolymers results from their composition, as they can contain both hydrophilic and lipophilic regions distributed along their backbones. Similarly, many proteins can act as emulsifiers thanks to their ability to adsorb at the oil-water interface. These amphiphilic proteins comprise both polar and non-polar groups, which are oriented towards the water and oil phases, respectively. On the other hand, polysaccharides work mostly as thickening agents with the ability to increase the viscosity of the continuous phase by forming an extended network, thus improving the emulsion stability. Contrary to proteins, only a few polysaccharide derivatives exhibit surface activity and are able to adsorb at the oil-water interface (Bouyer, et al. 2012; Friberg, et al. 2003).

When proteins are used as emulsifying agents, the complex interfacial structures are formed thanks to many conformational states accessible to such molecules. Adsorption of protein molecules to different hydrophobic materials causes differences in their conformation after being adsorbed. It was proved that proteins adsorbed to hydrocarbon-water or triacylglycerol-water interfaces exhibited different conformations (Friberg, et al. 2003).

The major driving force for adsorption of proteins to an oil-water interface is the hydrophobic effect. The conformation of the biopolymer at an interface and physicochemical and structural properties of the interfacial layer depends on the molecular structure and interactions of biopolymer molecules. Biopolymers with the conformation of flexible random coils adapt in emulsions an arrangement with the predominantly non-polar segments protruding into the oil phase, the predominantly polar segments protruding into the aqueous phase, and the neutral regions lying flat at the interface (Fig. 3.1). On the other way, globular biopolymers adsorb onto an interface, the predominantly non-polar regions of the molecule face the oil phase, while the predominantly polar regions are oriented towards the aqueous phase, and the macromolecules tend to retain their particulate shape at an interface. The organization of macromolecules at the interface can change in time, as they usually undergo rearrangement. Random coils are relatively flexible and can, therefore, rearrange their structures fairly rapidly, whereas globular biopolymers are more rigid and they reorganize slowly (McClements, David Julian 2009; Rodríguez Patino and Pilosof 2011). As the doctoral work is focused on the preparation and stabilization of emulsions by biopolymer-based emulsifying and stabilizing agents, especially by sodium caseinate and its combination with nanocrystalline cellulose, these types of emulsifiers and their mechanisms of stabilization are discussed in more details below.

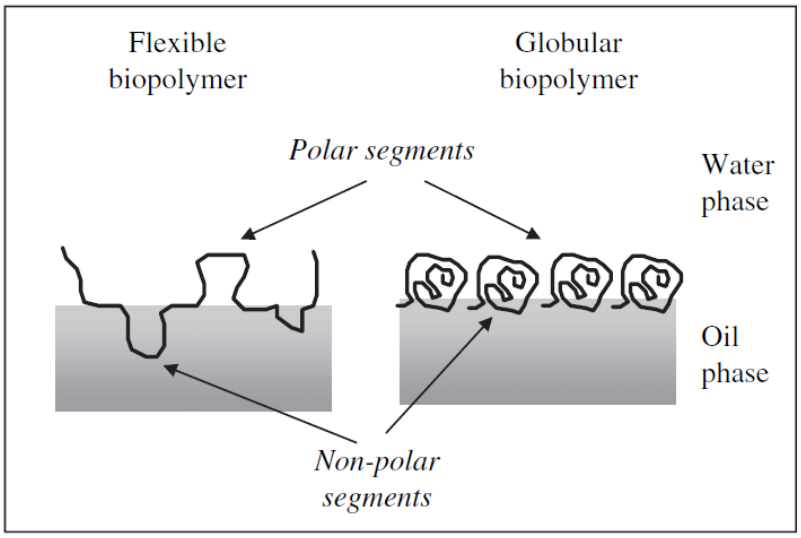


Fig. 3.1 Schematic representation of adsorption of biopolymers containing polar and non-polar segments at the interface (McClements, David Julian 2009).

3.1 Proteins in the stabilization of emulsions

Commonly used proteins suitable for the emulsion formation include, for example, whey protein, casein, ovalbumin, soy and bovine serum proteins (Lam, R. S. H. and Nickerson 2013). The interfacial layers formed by the proteins are usually relatively thin and electrically charged; therefore the major mechanism of stabilization is electrostatic repulsion. Thanks to their amphiphilic structure, proteins are able to adsorb at the oil-water interface, stabilize emulsion droplets by reducing the interfacial tension and slow down droplet coalescence by forming protective layers. Unlike low-molecular-weight emulsifiers that diffuse rapidly to the interface, proteins tend to be bulkier and diffuse at a much slower rate. Once at the interface, some level of partial denaturation is often needed to expose buried hydrophobic amino acids to the surface. Proteins then re-align themselves to a position with their surface hydrophobic amino acids inside the oil and hydrophilic acids being positioned in the aqueous phase (McClements, David Julian 2009; Lam, R. S. H. and Nickerson 2013). Moreover, at the interface strong viscoelastic protein films, which resist mechanical stresses, can be developed providing to droplets electrostatic and steric stabilization (Tcholakova, et al. 2006). Synthetic surfactants are generally more effective than proteins in lowering the interfacial tension; nevertheless, proteins lead to emulsions with better thermodynamic and kinetic stability. The ability of proteins to generate repulsive interactions (steric and electrostatic) between oil droplets and to form an interfacial membrane resistant to rupture also influences the long-term stability of these systems (Bouyer, et al. 2012; Lam, R. S. H. and Nickerson 2013; Rodríguez Patino and Pilosof 2011; McClements, David Julian 2004).

The physicochemical properties of proteins play an important role in their emulsifying abilities. Surface activities of various proteins are not equal, even though they all are amphiphilic, and a majority of them contains similar

percentages of polar and non-polar amino acid residues. The considerable differences in the surface activities of proteins must, therefore, be related to their physical, chemical, and conformational properties, which include size, shape, amino acid composition and sequence, charge, and the charge distribution. In their primary structure, proteins contain hydrophilic and hydrophobic residues randomly distributed over their structure. Surface hydrophobicity influences the ability of the protein to adsorb to the oil side of the interface. At interface, the protein adsorption proceeds in three main steps: i) diffusion of macromolecule from the bulk to the interface, ii) adsorption and iii) reorganization of the adsorbed protein (Bouyer, et al. 2012; Lam, R. S. H. and Nickerson 2013; Rodríguez Patino and Pilosof 2011; McClements, David Julian 2004).

The concentration of proteins occupying the oil-water interface tends to be in equilibrium with that in the continuous phase; therefore, changes of polymer amount in the system may destabilize/stabilize this equilibrium and influence the concentration of proteins at the interface. The experiments confirmed that concentration changes caused changes in the interfacial tension at the oil-water interface (Tcholakova, et al. 2006; Bos and van Vliet 2001). In contrast, the surface charge of the protein influences its solubility within the aqueous phase, where high solubility is desired for having higher diffusion rates to the interface. Once the viscoelastic film is formed, droplets can adopt a negative or positive charge depending on whether the emulsion pH is above or below the isoelectric point (*pI*) of the protein used. High electrostatic repulsion between oil droplets leads to better emulsion stability, whereas at pH close to the protein *pI* flocculation/aggregation of droplets dominates leading to their coalescence and instability of emulsion. Depending on the protein size, structure and conformation, “loops” or “tails” of protein segments may protrude from the interface covered mainly by hydrophilic amino acids and assure steric stabilization, physically restricting droplets from coming together. The presence of protein within the continuous phase also increases emulsion viscosity by reducing mobility and diffusion of oil droplets within the emulsion (Bouyer, et al. 2012; Lam, R. S. H. and Nickerson 2013; Rodríguez Patino and Pilosof 2011; McClements, David Julian 2004).

Protein-based emulsions have been found to hold better stability in comparison with surfactant-based emulsions, which was ascribed to differences in the interfacial organisation of the protein or surfactant. Proteins namely form an elastic interfacial layer, due to the bonds formed between neighbouring molecules. The superior emulsifying properties of proteins are attributed to their slower changes in conformation at the interface leading to a lower-density network between protein molecules, in comparison to surfactant-based emulsions. In contrast, faster conformational changes take place at the interface of surfactant-based emulsions, resulting from their small molecules providing a well-packed and concentrated stabilizing layer (Lam, R. S. H. and Nickerson 2013).

Typically, 1–3 wt% protein is used to stabilize emulsions. The proteins applicable in emulsions are characterised in more details in the following sections (Bouyer, et al. 2012).

3.1.1 Whey proteins

Whey proteins (WP) are globular proteins composed of a mixture of *α*-lactalbumin, *β*-lactoglobulin, and other minor proteins. Beta-lactoglobulin possesses net negative charge at pH 7 and is able to stabilize emulsion because it induces relatively strong electrostatic repulsion between the droplets. The *pI* for this protein is 5.2. Beside its stabilizing effect by electrostatic interactions, *β*-lactoglobulin also decreases the oil-water interfacial tension. In emulsions, *β*-lactoglobulin shows preferential interfacial adsorption over α-lactalbumin. At low concentration, when the first molecules reach the interface in optimal conditions, they are able to fully adsorb at any locus and occupy a maximum interface area. However, at higher concentration, abundant lateral protein-protein interactions at the interface induce a more compact conformation of the proteins present (Bouyer et al. 2012, Schröder et al. 2017). The heat treatment of whey proteins conducted for stabilizing of emulsions was investigated by Dybowska (2011). The study revealed that under heat treatment, which induces protein denaturation and aggregation, it may be possible to generate small stable structures (nanoparticles and/or aggregates). These structures are then able to improve the stability of the emulsions by increasing the viscosity of the continuous phase (Dybowska 2011). This is in correlation with the findings of a study using WP aggregates for the stabilization of emulsions. The aggregates not only stabilized emulsions, but they were also able to decrease their creaming (Sobhaninia et al. 2017). The effect of WP on the formation and stability of rice bran o/w emulsions was compared with the impact of other biopolymer types (gum arabic and modified starch). WP produced small droplets at low protein concentrations; nevertheless, the emulsions had poor stability to environmental stress (pH, ionic strength, temperature). This was attributed to changes in electrostatic interactions between droplets induced by changes in these variables (Charoen et al. 2011, Ravindran et al. 2018). On the other hand, gum arabic produced large droplets and high concentrations of this emulsifier were needed for droplet protection. Similarly to WP, the modified starch also formed emulsion with small droplets at low concentrations. Nevertheless, the two latter emulsions were more resistant towards the effect of pH, ionic strength and temperature, which can be due to the steric stabilization mechanism involved in their formation (Charoen et al. 2011).

3.1.2 Caseins

Caseins are the main protein components of mammalian milk. They mainly consist of a partially aggregated mixture of four individual flexible caseins (α_{S1}, α_{S2}, β and κ). Two of the caseins (α_{S1} and β) are efficient emulsifying agents capable of decreasing the interfacial tension during emulsification and protecting newly formed droplets against flocculation and coalescence by a combination of electrostatic and steric repulsion. In emulsion formulations, soluble sodium caseinate (CAS) is commonly used. CAS-stabilized emulsions destabilize by a flocculation mechanism in the presence of calcium salts. Calcium ions strongly bind to negatively charged phosphoserine groups of casein, which reduces the net negative charge (Bouyer, et al. 2012).

In o/w emulsions, CAS has been often used as a surface stabilizing and encapsulating agent (Drusch, et al. 2012; Dickinson, Eric 1999). The barrier formed by CAS at the oil-water interface is essential for protecting the encapsulated bioactive agents against oxidation and provides also an effective shield against flocculation and coalescence due to the mentioned combination of electrostatic and steric repulsion. For example, CAS used for encapsulation provided good protection of fish oil against oxidation through a physical barrier effect (Day, et al. 2007; Dickinson, Eric and Davies 1999; Livney 2010; Nielsen and Jacobsen 2009).

The emulsifying capacity of CAS depends on its concentration, the pH of the aqueous phase as well as on the type of oil being encapsulated. It was reported that high CAS concentration decreases the droplet size of emulsions leading to better emulsions stability, which was for example observed with oil phase composed of medium-chain triacylglycerols (Amine, et al. 2014). This finding is in correlation with studies conducted on emulsions of vegetable oils (soybean, sunflower and olive oils) stabilized with low CAS concentration, which showed low stability and poor resistance against creaming. To slow down the creaming process and improve the stability of emulsions, reduction of droplet size and modification of continuous phase and/or interface are the commonly used processes. The studies showed that the increase of CAS content available in continuous phase notably contributed to the formation of more stable emulsions prepared in studies of Perrechil, Cunha (2010) and Hebishy et al. (2017); in addition, the viscosity of continuous phase was increased by locust bean gum addition, which appeared to be a fundamental factor for emulsion stability (Perrechil and Cunha 2010; Hebishy, et al. 2017). The other way how to improve stability of CAS emulsion was reported in the work of Klaypradit, Huang (2008) and consists of the disaccharide trehalose addition (Klaypradit and Huang 2008). Here, the stability of CAS emulsions was strongly affected not only by the oil-to-protein ratio but also by the processing condition and composition of the aqueous phase. The interaction protein-sugar (CAS-trehalose) played an important role in the regulation of creaming and flocculation processes. The molecular interactions of disaccharides and proteins at the oil-water interface also explained lower

flocculation in emulsions, which can be controlled by suppression of protein aggregation caused by the sugar addition (Huck-Iriart, Cristian, et al. 2016). CAS was also successfully employed for encapsulation of essential oils. Stable emulsions with clove essential oil (droplet diameter ~172 nm) were obtained with 5 wt% CAS in the presence of pectin (0.1 wt%) (Sharma, et al. 2017). However, emulsions with the same formulation and processed in various ways may have very different behaviours. The properties and stability of emulsions are also affected by mutual interactions of emulsion components. These interactions (in this case between protein and other components of aqueous phase) can change the structure of the protein at the interface and thus modify emulsion structure and its behaviour (Huck-Iriart, Cristian, et al. 2011).

3.2 Polysaccharides in emulsion stabilization

Polysaccharides are natural polymers consisting of one or more types of monosaccharides linked together by glycosidic bonds. They are known for water-holding and thickening properties originating from their hydrophilic character and high molecular weight. Some of the polysaccharides covalently attach peptides or lipids, which often influence their ability to act as emulsifiers. Polysaccharides are usually not good emulsifiers because they mainly comprise hydrophilic monosaccharides and are, therefore, not particularly surface-active and do not adsorb at oil-water interfaces. These non-adsorbing polysaccharides enhance the emulsion stability by gelling or modifying the viscosity of the aqueous continuous phase, which slows down droplet movement. On the other side, polysaccharides such as gum arabic, modified starch or cellulose derivatives contain non-polar and polar groups and are, therefore, amphiphilic molecules that can adsorb at interfaces. They display surface/interfacial activity and are classified as adsorbing polysaccharides. They can stabilize emulsions by two mechanisms 1) by adsorption at the surfaces of oil droplets and 2) by preventing droplet flocculation and coalescence through electrostatic and/or steric repulsive forces. The surface activity of these polymers results mostly from the presence of a protein fraction in their structure or the combination of hydrophobic and hydrophilic groups along the polysaccharide backbone (cellulose derivatives) (Bouyer, et al. 2012; McClements, David Julian and Gumus 2016). Gum arabic, beet pectin and corn fibre gum working with different stabilization mechanisms were studied and compared with respect to their emulsifying properties in o/w emulsions. It was found out that emulsions can be produced by using all three polysaccharides, with the mean particle diameter decreasing with biopolymer concentration and homogenization pressure applied during their formation. Nevertheless, gum arabic and beet pectin were more efficient than corn fibre gum because of their higher surface activity and stronger adsorption at the oil-water interface (Bai, et al. 2017).

3.2.1 Cellulose

Besides starch and chitosan, cellulose is a biopolymer frequently used in emulsion systems. The cellulose is the most abundant natural polysaccharide and it is the major structural component of plants. Chemically, it is a linear homopolymer of β(1-4)-D-glucopyranose units linked together by glycosidic bonds. Traditionally, as mentioned above, polysaccharides are not considered as amphiphilic biopolymers with a good ability to stabilize emulsions, unless their chemical modifications are made to introduce surface-active groups (Paximada, Tsouko, et al. 2016; Jia, et al. 2015). Chemically modified celluloses are mainly used for their gelling, thickening and stabilizing properties. The most common cellulose types are carboxymethyl cellulose and hydroxypropyl methylcellulose. It is also well known that some bacteria can synthesize cellulose (bacterial cellulose, BCel). If emulsions stabilized with carboxymethyl cellulose and hydroxypropyl methylcellulose are compared with those stabilized with BCel, the latter contains the largest emulsion droplets but with the highest stability. This is due to the flocs of bacterial cellulose fibrils adsorbed onto the surface of the oil droplet forming a strong network, which prevents droplet coalescence. Besides, BCel emulsions were not affected by changes in pH, temperature or ionic strength unlike the emulsions prepared with hydroxypropyl methylcellulose, carboxymethyl cellulose or other cellulose types (Paximada, Tsouko, et al. 2016; Ougiya, et al. 1997). In a study published by Jia et al. (2015), the properties of cellulose were additionally improved by using dissolution and regeneration processes. Thus obtained non-derivative amorphous cellulose effectively stabilized o/w emulsions by adsorption on the surface of oil droplets. The underlying emulsion stabilization mechanism was a combination of Pickering and network-forming mechanisms (Jia, et al. 2015). Another cellulose derivate, hydroxypropyl cellulose, is a neutral branched polysaccharide with surface-active properties used as a thickening agent (Mezdour, et al. 2008). The preparation and stability of paraffin containing o/w emulsions prepared with hydrophobically modified hydroxyethyl cellulose were also investigated. Their stability was ascribed both to an associative thickening mechanism caused by alkyl chains in hydroxyethyl cellulose and the adsorption of hydroxyethyl cellulose at the oil-water interface, which can form a solid film preventing coalescence of the droplets (Sun, et al. 2007).

4. PARTICLES AS EMULSION STABILIZERS

The phenomenon whereby solid particles protect emulsion droplets against coalescence by interfacial action is called Pickering stabilization. Pickering emulsions, type of emulsion stabilized by solid particles located at the oil-water interface, have been discovered a century ago while being extensively studied in recent decades (Dickinson, Eric 2017; Yang, et al. 2017; Lam, S., et al. 2014).

4.1 Types of particles

It has been demonstrated by many kinds of research that numerous types of inorganic particles including silica, clay, and hydroxyapatite, as well as a range of organic particles (chitosan, cyclodextrin, etc.), can effectively serve as Pickering stabilizers. Based on Pickering stabilization, various types of disperse systems can be prepared, such as microsphere, microcapsule, and Janus colloidal particles (Yang, et al. 2017).

At first, the intense research into particle-stabilized systems has focused on using inorganic particles such as silicas. Nevertheless, inorganic particles are limited in their relevance to applications requiring biocompatibility and biodegradability. For this reason, the particles with a biological origin, such as biopolymer particles, are more appropriate stabilizers (Dickinson, Eric 2017; Lam, S., et al. 2014). The biopolymer-based particles can be prepared by many methods, such as thermal processing (protein denaturation), cross-linking of proteins with enzymes, acid hydrolysis of amorphous regions of polymers and other chemical methods. The size and charge of the biopolymer particles can be controlled by altering the initial biopolymer concentration, holding temperature, holding time, pH and ionic strength. As illustrated in Fig. 4.1, emulsions can be stabilized using different types of particles with biological origin. The particles can be various (irregularly shaped, polydisperse in size and morphology) and in practice fibres, clumps or rod-like crystals can be all excellent stabilizing agents. The adsorption of particles at the interface is influenced by their size and bulk concentration. For example, the smaller, faster-adsorbing particles form highly elastic layers and irregularities in particle shape can positively impact emulsion stability (Dickinson, Eric 2017; Lam, S., et al. 2014; Joye and McClements 2014; Matalanis, et al. 2011). It was also documented that certain types of microorganisms (Fig. 4.1 a, b) could serve as stabilizers, thanks to their surface properties (surface charge, functional groups, and special structures). As some bacteria and yeast have already been extensively used in cosmetic, pharmacy and food industries, it is reasonable to believe that microbe-stabilized Pickering emulsions can find promising applications (Yang, et al. 2017). Here, negative charges existing on bacterial cell walls can successfully promote the formation of highly-stable o/w Pickering emulsion (Wongkongkatep, et al. 2012; Firoozmand and Rousseau 2016). Studies pointing at using bacteria-related materials as

Pickering stabilizers are not as frequent as studies on other types of solid particles such as silica, clay or proteins (Yang, et al. 2017).

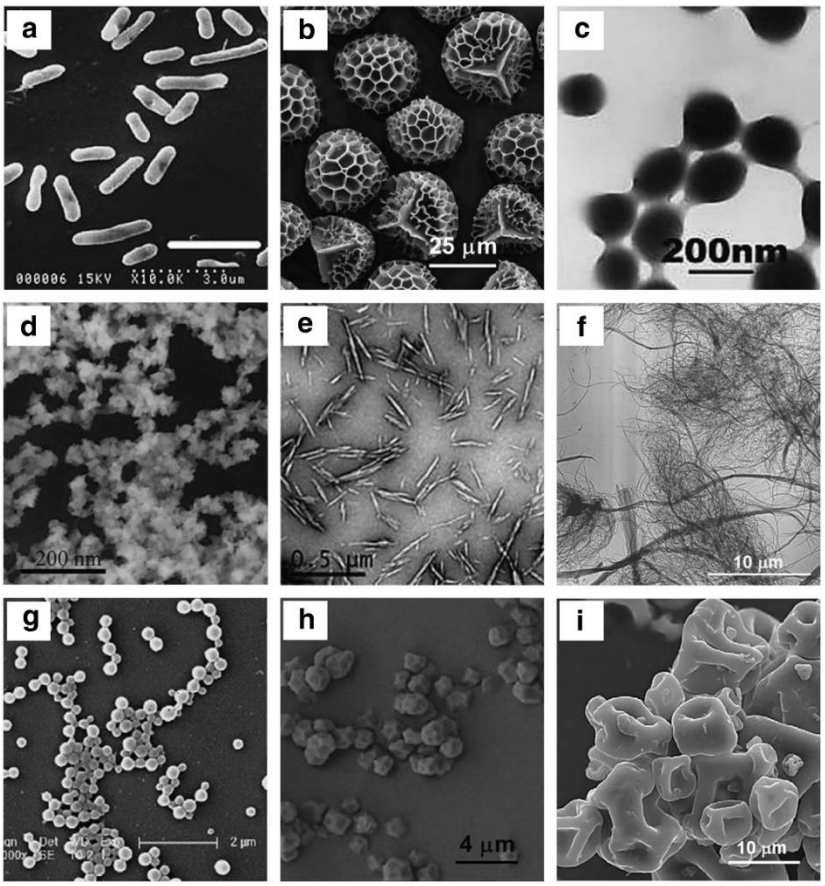


Fig. 4.1 Examples of bio-derived particles of various origins applicable to Pickering stabilization. The micrographs illustrate the size and shape variability between different biopolymer particles and bioparticles. a) E. coli bacteria; b) moss spore; c) ethyl cellulose nanoparticles; d) chitosan nanoparticles; e) cellulose nanocrystals from cotton; f) silylated cellulose fibres; g) acetylated starch phthalic ester nanospheres; h) quinoa starch granules; i) spray-dried soy protein particles (Lam, S., et al. 2014).

Proteins, as it was discussed in chap. 3.1, with their amphiphilic properties, are able to stabilize the oil-water interface. In addition to this capability, it has been demonstrated that emulsions can be formed by the stabilization of protein aggregates. The protein aggregates are in a sense of particle-like stabilizers. Micro- and nanoparticles of proteins can be produced by sonication, desolvation, heating and pH adjustment, as well as by anti-solvent precipitation. Nevertheless, the protein particles are formed by thermal processing mostly due to denaturation induced by the heat and surrounding aqueous phase (pH, ionic strength, calcium concentration) and by the processing conditions (temperature, time, shear-rate) (Dickinson, Eric 2017; Lam, S., et al. 2014; Tavernier, et al. 2016). These all are key variables that can influence morphology changes, produce solid spherical particles of different sizes, as well as fractal aggregates, flexible strands and long semi-flexible fibrils. An alternative to heat-induced aggregation is the generation of nanoparticles by cross-linking of proteins with enzymes. Stable spherical

microgel particles have been prepared from *β*-lactoglobulin or whey protein isolate (Dickinson, Eric 2017; Paunov, et al. 2007). The whey protein microgel particles were able to form triacylglycerol o/w emulsions over the whole range of pH and salinity with long term stability (Destribats, et al. 2014). In Pickering stabilization, protein particles composed of whey protein, bovine serum albumin, *α*-lactalbumin and gelatine can be formed through desolvation followed by chemical crosslinking. Nevertheless, of these particles only gelatine was able to stabilize emulsions (Dickinson, Eric 2017; Paunov, et al. 2007; Arroyo-Maya, et al. 2012; Tan, et al. 2014). Also, soy protein particles prepared by heat-treatment exhibited good surface activity and served as efficient particle stabilizers (Dickinson, Eric 2017; Paunov, et al. 2007; Liu, F. and Tang 2013).

Pickering stabilization is also possible by using solid nanoparticles of pea protein isolate (Liang, H. and Tang 2014) and zein, water-insoluble protein derived from corn. It was shown that the wettability of the zein particles could be tuned by varying the pH of the solution and the stable emulsions were formed above or below *pI* of zein (de Folter, Julius W. J., et al. 2012).

As regards polysaccharides, it is questionable if the modified starch granules of semi-crystalline hydrophilic character with sizes varying from 0.5 to 100 μm can be considered for Pickering stabilizers or not. Most of the recent work on stabilization by starch particles have involved granules that were hydrophobically modified with octenyl succinic anhydride (OSA). Nevertheless, highly effective stabilizers of o/w emulsions are starch nanocrystals (size range 40–100 nm) prepared by sulphuric acid hydrolysis of the amorphous regions of waxy maize starch (Dickinson, Eric 2017; Lam, S., et al. 2014; Timgren, et al. 2011; Yusoff and Murray 2011). The particles made of non-starch polysaccharides can also work as Pickering stabilizers. Here, mainly solid particles of two structural polysaccharides, cellulose (chapter 3.2.3), and chitin (Dickinson, Eric 2017; Lam, S., et al. 2014) can be named. The chitin is a semi-crystalline biopolymer containing amorphous domains, which can be cleaved by acid hydrolysis, resulting in chitin nanocrystals (Tzoumaki, et al. 2011). Correspondingly to chitin, also the chitosan particles can adsorb at the interface and stabilize o/w Pickering emulsions (Liu, H., et al. 2012).

4.2 Nanocellulose particles

In recent years, nanocelluloses have attracted a lot of interests as promising stabilizers for Pickering emulsion particularly in cosmetics, pharmaceutics and food industries.

The fibre of cellulose contains both crystalline and amorphous domains. The amorphous regions of cellulose can be cleaved to produce microcrystalline cellulose (MCC). Using mechanical, chemical or a combination of mechanical, chemical or enzymatic processes, two main types of nanocellulose can be yielded from MCC. The longer semi-crystalline elementary fibrils, cellulose nanofibres, are the first type with the length in several microns comprising both crystalline and amorphous regions. Using acidic hydrolysis, the amorphous regions of fibrils can be hydrolysed and thus cellulose nanocrystals (CNC) are obtained. The nanocellulose can be chemically modified due to many reactive hydroxyl groups at the surface (Lam, S., et al. 2014; Chen, et al. 2018). As the high hydrophilicity of nanocelluloses limits their applications in the Pickering emulsions, nanocellulose surface can be modified with OSA. This modification increases the surface hydrophobicity of nanocellulose particles and improves their emulsification properties. In addition, stable and gel-like Pickering high-internal-phase emulsions (HIPEs) with fine droplets could be easily prepared using thus modified cellulose particles (Chen, et al. 2018).

Due to the fibre-like morphology of native cellulose and high aspect ratio of hydrolysed cellulose particles, nanocelluloses can form physical gels at low solid concentrations. These characteristics, in addition to their sustainability and biocompatibility, making them ideal candidates for use in environmentally-friendly materials (Lam, S., et al. 2014).

4.2.1 Nanocellulose particles as an emulsion stabilizer

The trend to study the colloidal properties of nanocellulose particles emerged mainly due to their ability to stabilize oil-water interfaces. In comparison to many other types of particles capable of stabilizing Pickering emulsions, nanocelluloses display high surface area, low mass density, chirality and easy surface modification. In addition, they are abundant, sustainable and are considered as environmentally benign posing no or minor risk for the living organisms (Lam, S., et al. 2014; Capron, et al. 2017).

Various types of emulsions were stabilized with macroscopic fibres, MCCs, cellulose microfibres and cellulose nanofibres, as well as with cellulose nanocrystals (CNCs) (Lam, S., et al. 2014; Capron, et al. 2017). Nanocelluloses can be derived from a variety of natural sources (vegetal, bacterial or animal) by using different processes (enzymatic, chemical, mechanic). Therefore, they consequently appear under a large range of shapes, aspect ratio, surface chemistry, crystallinity, and a crystalline structure (Moon, et al. 2011; Klemm, et al. 2011; Habibi, et al. 2010).

The high degree of crystallinity combined with their small dimensions makes CNC the smallest building block of the cellulose fibres with a length between 70 nm up to a few microns and a width between 5 and 20 nm. As a result, CNC appears in the form of rigid, rod-like nanoparticles. These features have important consequences for the interfacial behaviour of the CNC. The exact role of CNC particles in the stabilization of emulsions is still a subject of intensive research. However, two parameters are crucial in governing the assembly and adsorption process of the CNC at the interface: the surface chemistry of the particles and their shape (Capron, et al. 2017).

The potential of particles to act as stabilizers has been clearly demonstrated in many scientific publications summarized in review (Yang, et al. 2017). To reach stable emulsions stabilized with particles, two conditions have to be followed; the droplet size should be bigger than the particle size and particle surface must be partially wetted both by water and oil (Binks, B. P. 2007). Most of the research work on the particle-stabilized emulsions has been restricted to spherical colloidal systems (silica nanoparticles, latex-like and protein microgels). The shape of particles is an important parameter when it comes to their packing at the interface, and for non-spherical particles, the resulting layer must be denser than for spheres (Madivala, et al. 2009). This was confirmed by the work, which studied two types of cellulose nanocrystals with different crystalline allomorphs as stabilizers for o/w Pickering emulsions. The emulsions stabilized with the first type, needle-like particles, had twice smaller droplets and better stability than those covered with the second particle type with ellipsoid shape. It was proved that crystalline allomorphy and morphologies of CNCs played an important role in the stabilization of emulsions (Li, Xia, et al. 2018).

In general, cellulose particles of various sizes and aspect ratios have demonstrated their efficiency to stabilize emulsions. Nevertheless, the irregular shape of microcrystalline cellulose led to its lower stabilization performance, especially when compared to nanocellulose. Several emulsion tests have been reported with various types of nanocelluloses, cellulose microfibres and cellulose nanofibres (Oza and Frank 1986; Andresen and Stenius 2007; Xhanari, et al. 2011; Mikulcova, et al. 2016), cellulose nanocrystals (Kalashnikova, et al. 2013; Kalashnikova, et al. 2012; Cherhal, et al. 2015; Cherhal, et al. 2016) and bacterial nanocellulose (Ougiya, et al. 1997; Kalashnikova, et al. 2013; Kalashnikova, et al. 2011; Lee, et al. 2014). The results of these studies point out that the aspect ratio governs both the droplet coverage and resulting droplet size (influencing thus the long term stability of the system). When long semi-crystalline fibres are used, the droplet size tends to be somewhat bigger, and the fibres may protrude from the oil-water interface in the continuous phase. Thus a strong network preventing coalescence is formed (Capron, et al. 2017).

4.2.2 Effect of ionic strength and pH on CNCs and emulsions prepared thereof

When CNCs are dispersed in an aqueous phase, their relatively high charge density ensures good colloidal stability. Several studies have reported that the increase in ionic strength induces destabilization of the system (Cherhal, et al. 2015; Tuan Phan-Xuan, et al. 2016; Zhong, et al. 2012). It was also demonstrated that CNC can form a gel at concentrations as low as 0.5 $g.L^{-1}$ in the presence of salts (Tuan Phan-Xuan, et al. 2016).

In an emulsion, the situation is somewhat different as the CNCs are stacked at the interface and arranged in a layer (Capron, et al. 2017). It was found that CNC can stabilize o/w emulsions regardless of the charge density. However, decreasing the surface charge density (adding salts, varying pH) can reduce the amount of CNCs required to form a stable emulsion. The addition of 100 mM Na^+ or the lowering pH below 2 leads to the aggregation of CNC and emulsions formed under these conditions show gel-like behaviour (Varanasi, et al. 2018). In addition, the surface charge of colloidal particles influences not only the interfacial adsorption of CNC but also the inter-particle network formed by the non-adsorbed particles present in the continuous phase. The surface charge can be varied by acidic or basic desulphation of the particles. CNC desulphated with hydrochloric acid adsorbed faster to the oil-water interface, yielding emulsions with smaller droplet sizes and a thicker CNC interfacial layer. On the contrary, CNCs desulphated using sodium hydroxide stabilized larger emulsion droplets and had a higher amount of non-adsorbed CNCs in the water phase (Pandey, et al. 2018). Stable emulsions at three different pH (2, 4 and 7) were formulated in the presence of carboxylated CNC (cCNC). Emulsification was mainly affected by the pH of the continuous phase, which could be related to the reduction of charge on the cCNC surface with decreasing pH. Responsiveness of emulsions towards pH changes was not confirmed as expected because of the strong adsorption of the cCNC and limited possibility to induce desorption of nanocrystals from oil surface (Mikulcova, et al. 2018). In the work of Xu, the increase of ionic strength improved the accumulation of cellulose nanofibres at the oil-water interface and salt addition facilitated the formation of packed fibril clusters, and the development of the clusters into a solid-like film (Xu, H., et al. 2018).

5. INTERACTIONS OF BIOPOLYMERS AND PARTICLES AT OIL-WATER INTERFACE

Application of known emulsifiers in the formation of emulsions may, in some cases, be limited by their insufficient functional properties. These are for example related to their questionable stability towards the variation of pH, presence of salts, heating, dehydration, freezing and/or chilling. Therefore, the knowledge of shortcomings related to properties of emulsifiers has triggered research targeted at finding alternative methods for improving emulsion stability by developing novel emulsification strategies (Guzey and McClements 2006). The ability of some of the natural emulsifiers to form and stabilize emulsions in synergy with other polymers or biopolymers can be given as an example. Here protein-protein or protein-polysaccharide combinations can be mentioned. The different polymers can be used in combinations by applying a number of different approaches, such as co-adsorption, complexation and layer-by-layer (l-b-l) method Fig. 5.1 (McClements, David Julian and Gumus 2016).

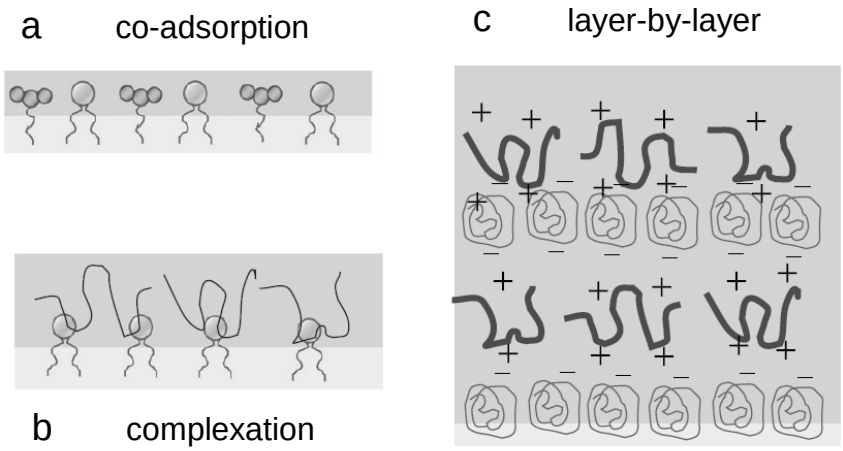


Fig. 5.1 Schematic representation of different types of mixed interfacial layers that can be formed at oil droplet surfaces to stabilize emulsions: a) co-adsorption; b) complexation; c) layer-by-layer (McClements, David Julian and Gumus 2016).

In case two types of stabilizing agents are mixed and used for emulsification, the oil-water interface is preferentially covered by one having a higher surface activity. When two emulsifiers are both adsorbed to the oil droplet surfaces as individual molecules, this process is called co-adsorption (Fig. 5.1a). The resulting interface may consist of a homogeneous mixture of two different emulsifiers, or it may contain regions rich in one emulsifier and depleted in another. The overall composition of the interface will depend on the relative affinity of the two emulsifiers for the oil-water interface, as well as their relative concentrations (McClements, David Julian and Gumus 2016).

On the other hand, if two components interact together through physical or non-physical interactions (such as electrostatic, hydrogen bonding, hydrophobic forces, and covalent bonding), the complex is formed (as shown in Fig. 5.1b)

(Dickinson, Eric 2011). This complexation can occur before or after emulsification (McClements, David Julian and Gumus 2016).

During the last procedure, l-b-l method (Fig. 5.1c), an emulsion is prepared by homogenizing oil, water, and emulsifier (bearing a charge) together. The emulsifier-coated droplets with an electrical charge are then mixed with a solution containing polymers (or particles) having an opposite charge. This causes their adsorption onto the droplet surfaces through electrostatic attraction. The electrostatic deposition process can be several times repeated to form a series of layers of opposite charges around the droplets. These steps can improve stability and functional performance of emulsions (McClements, David Julian and Gumus 2016; Guzey and McClements 2006). Generally, in cooperative multicomponent adsorption, the outer part of a multilayer will tend to attach more slowly, and probably more weakly, than the inner part. Knowledge of the physicochemical principles governing the formation and properties of these multilayer interfaces is important to establish the optimum conditions required for producing multilayer emulsions with desirable properties (Guzey and McClements 2006).

If the polymers are used for stabilization of droplets, the interactions between their layers depend on many factors including biopolymer characteristics (size, molecular weight, conformation, mixing ratio, biopolymer type, distribution of reactive sites), solvent properties (pH, ionic strengths, and temperature), total biopolymer concentration and emulsion preparation method (Neirynck, et al. 2004; Tippetts and Martini 2012).

5.1 Co-adsorption

As it was already mentioned, if two types of stabilizing agents (whether they are biopolymers, particles or surfactants) are mixed, the oil-water interface is preferentially covered by more surface-active components. This is referred to as competitive adsorption or competitive displacement. The latter term often refers to the situation when a more surface-active component is added after emulsification with the less active component, which allows the first component to initially adsorb in a relatively unhindered manner. This competition for space at the oil-water interface predetermines the stabilization mechanism that prevails (Sarker, D. K., et al. 1999; Sarker, Dipak K., et al. 1995; Courthaudon, et al. 1991; Wijmans and Dickinson 1999; Maldonado-Valderrama and Rodriguez Patino 2010).

In a colloidal system containing a mixture of protein and polysaccharide as emulsifiers, the interface is primarily stabilized by the more surface-active component (mostly protein). Commonly, less surface-active polysaccharide cannot successfully compete at the interface with surface-active protein (Dickinson, Eric 2011).

Several studies on the behaviour of biopolymer mixtures at the interface were published and are listed in the following section. Briefly, the casein and gelatine mixture is a good example of a situation where the phenomena of competitive

adsorption and co-adsorption occur simultaneously within the same system. Competitive adsorption was readily manifested during emulsion preparation with casein and gelatine: more surface-active casein predominated in the primary interfacial layer, and substantial deficiency of casein in the mixture of these emulsifiers during emulsification caused extensive bridging flocculation in the resulting emulsions (Dickinson, Eric 2011). In another study, the increasing concentration of carboxymethyl cellulose in sodium caseinate emulsions caused an increase in the coverage of the oil surface with protein, followed by preferential adsorption of β-casein. It is therefore interesting that cellulose promoted protein adsorption at the oil-water interface and thus improved the stability of emulsion (Liu, L., Liu, Zhao, Zhao, Kong, et al. 2012).

It is commonly known that polysaccharides have low surface activity; hydrophobically modified cellulose derivatives are exceptions here. As it was proved for an air-water interface, the hydroxypropyl methylcellulose (at pH 6) was more surface-active than each of the studied proteins (β-lactoglobulin, α-lactalbumin, bovine serum albumin). Although β-lactoglobulin exhibited the highest surface activity among three proteins at this pH, it could be easily displaced from the interface by the cellulose. On the other hand at pH 3, the three proteins performed similarly in co-adsorption with the hydroxypropyl methylcellulose, which was slightly negatively charged, and cellulose competed with the protein for the interface (Jara, et al. 2014). As confirmed, this polysaccharide was able to dominate interfacial layers in mixtures with the proteins at high polysaccharide contents (Dickinson, Eric 2011). It is interesting that at the oil-water interface, hydroxypropyl methylcellulose and β-lactoglobulin did not form complexes and at pH 6 the interface was preferentially occupied by the protein. This probably indicates the displacement of the surface-active hydroxypropyl methylcellulose at this pH (Camino, et al. 2012).

Depending on the nature of the attractive protein-polysaccharide interactions, many emulsifiers associate with the interface in the form of a second stabilizing layer. Some polysaccharides can readily form complexes with proteins. Such complexes have been shown to possess excellent interfacial stabilizing properties. To fully understand the resulting properties of mixed layers, it is, therefore, necessary to take into account not only the phenomenon of competitive adsorption but also the mechanisms of complexation (Dickinson, Eric 2011).

5.2 Complexation

Protein-polysaccharide complexes have found a widespread practical application. They have been for example used to stabilize and form structures in cosmetic, to microencapsulate active compounds and to purify proteins in the pharmaceutical and biotechnological industries. Using different combinations it is possible to form better and unique emulsification performance relative to that of the individual components (Li, Xiangyang, et al. 2012).

As was already mentioned in the previous section, protein emulsifiers are often more effective at producing fine oil droplets because they are more surface-active and their interfacial adsorption kinetics is, in comparison with polysaccharides, faster. Conversely, polysaccharide emulsifiers are usually more effective at stabilizing oil droplets that are stable to a broader range of environmental conditions, such as pH, ionic strength, temperature, and freezing. The complexes would have superior amphiphilicity when the prevailingly hydrophobic proteins are associated with the hydrophilic polysaccharides. Indeed, protein-polysaccharide complexes were reported to be better stabilizers than each of the biopolymers used individually. Their combinations can provide an efficient reduction in interfacial tension and enhance the adsorption of polysaccharides onto interfaces, providing thus a better surface coverage at lower bulk concentrations of emulsifiers (Guzey and McClements 2006; Dickinson, E., et al. 2003; Dickinson, Eric 2011; Li, Xiangyang, et al. 2012). Addition of polysaccharide to protein-stabilized emulsion can increase the steric and electrostatic repulsions between the droplets, and help to prevent the droplets from aggregation. The complexes formed by proteins and polysaccharides may be held together by physical or covalent bonds. The amphiphilic protein fragment anchors the complexes to the interface, while the hydrophilic polysaccharide fragment protrudes into the aqueous phase and provides stability against aggregation of droplets by generating a long-range steric repulsion (McClements, David Julian and Gumus 2016; Guzey and McClements 2006).

Commonly, the complexation involves at first preparing a bulk aqueous dispersion of the protein-polysaccharide complex, and then using the complex as the emulsifying agent during subsequent homogenization (Dickinson, Eric 2008). To better control the emulsifying functionality of protein-polysaccharide complexes, pH of the system and protein-polysaccharide ratio are the key factors that affect the interactions of biopolymers in terms of complexation and structure of the complex, or co-adsorption of biopolymers at the interface (McClements, David Julian and Gumus 2016).

In the literature, biopolymer-based complexes and their applications as emulsifiers are frequently studied topics. Therefore, only the key studies are discussed here to show principles of this advanced emulsification method. The study focused on the complexation of bovine serum albumin with sugar beet pectin proved the importance of pH and concentration/ratio of biopolymers present in the mixture. At pH 7, no complexation occurred, and formations of

emulsions were controlled by the competitive adsorption of the two components at the oil-water interface. On the other hand, at low pH and low albumin/pectin ratio, the formed complexes significantly improved the stability of emulsions. Nevertheless, at acidic conditions and high albumin/pectin ratios, bridging flocculation between emulsion droplets also occurred (Li, Xiangyang, et al. 2012).

The impact of various polysaccharides on WP[2]-stabilized emulsion is well documented in the literature. The effects of xanthan gum and locust bean gum on WP-stabilized emulsions were studied at pH 3, 5 and 7. The use of the WP-xanthan gum-locust bean gum complex caused an increase in the droplet size and viscosity of emulsions. The study also confirmed the influence of pH of dispersed phase on the stability of emulsions, and flocculation and creaming occurred at pH 3 and 5, whilst emulsions were the most stable to creaming at pH 7. Moreover, according to Owens et al. (2018), xanthan gum-locust bean gum complexes were able to reduce oxidation of lipids encapsulated in emulsions at both pH 3 and 7 (Owens, et al. 2018). In the study published by Yao (2016), the electrostatic complex of WP and gum arabic significantly improved the physical and oxidative stabilities of conjugated linoleic acid emulsions (in comparison with individually used WP or gum arabic) (Yao, et al. 2016). It was also proved that the WP and apple pectin complexes, made by thermal treatment, were successfully adsorbed at the interface of o/w emulsions. Thus stabilized emulsions were stable and resistant to salt addition, pH change and heat treatment (Salminen and Weiss 2014).

Two different complexes containing gum arabic were studied with respect to their emulsifying and interfacial properties. As published by Dong et al. (2018) the emulsifying activity of soy protein/gum arabic system was better than the activity of the gelatine/gum arabic system, mainly due to the differences in protein conformation (Dong and Hua 2018). Similarly, as gum arabic, soy soluble polysaccharides were shown to prevent destabilization of soy protein isolate o/w emulsions under acidic conditions; however, the improved stability strongly depended on pH, ionic strength of aqueous phase and soy soluble polysaccharide concentration (Tran and Rousseau 2013).

Sodium caseinate is a surface-active protein with a limited application under acidic conditions due to its *pI*~4.6. Therefore, the mixtures of CAS[3] with polysaccharides were investigated as possible emulsion stabilizers. A study dealing with the addition of xanthan gum to CAS under different pH and ionic strength showed a significant impact of both these factors on interactions of the biopolymers and adsorption of their mixtures at the oil-water interface (Liu, L., Liu, Zhao, Zhao, Long, et al. 2012). As documented by Zinoviadou et al. (2012), the chitosan/CAS complex can significantly improve the stability of emulsions in

[2] *WP – whey protein*

[3] *CAS – sodium caseinate*

comparison with CAS-emulsions. When these complexes acted as surface-active agents, they protected emulsions against creaming and aggregation upon acidification. The increased stability upon acidification was attributed to enhanced electrostatic and steric repulsion forces between oil droplets due to the presence of the chitosan at the interface. Moreover, the increase in bulk viscosity due to the presence of chitosan can offer additional protection against creaming (Zinoviadou, et al. 2012).

Chitosan is a polysaccharide commonly used for the preparation of complexes and it was used in combinations with different proteins. Chitosan/gelatine-B insoluble complexes were used as effective Pickering stabilizers for o/w emulsions and emulsion gels with long-term stability. The complexes were formed *via* electrostatic complexation of chitosan and gelatine-B only in the pH region where the polymers were oppositely charged, and the pH remarkably influenced surface activity of chitosan/gelatine-B mixed systems (Wang and Heuzey 2016). The complexation of chitosan with another protein, ovalbumin at pH 5.5 increased the viscosity of emulsion, reducing thus the droplet coalescence and inhibiting the growth of emulsion droplets (Xiong, et al. 2018).

Nowadays, the use of plant biopolymers as emulsifiers expanded, which reflects the actual "green" orientation in pharmaceutical, cosmetics and food industries (Burgos-Diaz, et al. 2016). For the preparation of complexes, plant proteins are frequently used to substitute traditionally dominating animal proteins. Nevertheless, many plant proteins are much less hydrophilic and often cannot be easily extracted from raw plant material in their native form. For this reason, complexation with polysaccharide exhibits a new set of challenges and opportunities to improve the interfacial properties of plant proteins. Due to the low solubility of plant proteins, a strong amphiphilicity can be obtained *via* association with highly hydrophilic polysaccharides. For instance, the protein-polysaccharide complexes developed with pea protein isolate exhibited improved solubility around *pI* and showed better physicochemical, interfacial and emulsifying properties (Li, Xiufeng and de Vries 2018; Yildiz, et al. 2018).

5.3 Layer-by-layer

An alternative way of stabilizing emulsions with biopolymers is a layer-by-layer (l-b-l) method. The protein-interface interactions in the primary layer covering emulsion droplets will tend to induce conformational changes in the adsorbed proteins. Therefore, after adsorption, a newly generated layer with specific characteristics will be in contact with protein molecules dispersed in bulk solution, and in turn, these "bulk" molecules will be influenced by this new layer (Dickinson, Eric 2011; Dickinson, Eric 2008; Burgos-Diaz, et al. 2016). L-b-l emulsions have attracted attention due to their potential for nanoscale encapsulation, protection of emulsions against environmental stresses and ability to enhance stability with respect to ionic strength, pH and temperature. However, a major problem in this sequential adsorption is the tendency of the emulsions to

become extensively flocculated during preparation. Flocculation can occur by two different mechanisms, the first is bridging flocculation when the polysaccharide content is so low that droplet collisions are faster than the rate of polysaccharide absorption on the protein-coated droplet surfaces, and the second is depletion flocculation occurring when the concentration of unabsorbed polysaccharide exceeds a certain critical value (Burgos-Diaz, et al. 2016).

Single-layer emulsions are thermodynamically unstable when exposed to environmental stresses. The advantage of l-b-l stabilization is mainly related to the bigger thickness of interfacial layers around the oil droplets, which provide better stability and better protection for the bioactive agents entrapped within the core (Fioramonti, et al. 2015). Multilayer emulsions have been studied by many researchers, and emulsions stabilized by interfacial membranes consisting of *β*-lactoglobulin/carrageenan(/gelatine), lecithin/chitosan(/pectin) have been prepared and shown to have better stability in comparison with single-layer emulsions (Gu, et al. 2004; Gu, et al. 2005; Ogawa, et al. 2003). In addition to the mentioned systems, also many other polysaccharides and proteins were investigated for this application. More specifically, the sodium alginate and whey protein were studied as l-b-l stabilizing agents for linseed oil emulsions with respect to the concentration of sodium alginate and the influence of pH on the properties of the whey protein-stabilized o/w emulsions. The stability of these emulsions depended on both pH and initial sodium alginate concentration. The most stable emulsions were prepared at pH 5 and 0.25 wt% sodium alginate thanks to the adsorption of the polysaccharide onto protein interfacial membrane through attractive electrostatic forces acting between negatively charged sodium alginate and positively charged patches exposed on the whey protein surface (Fioramonti, et al. 2015). The other charged polysaccharides, such as fucoidan (at pH 6 to 2) and gum arabic were able to electrostatically deposit onto CAS-coated lipid droplets in emulsions (Chang and McClements 2015; Chang, et al. 2016). Interestingly, fucoidan could effectively displace gum arabic from the thus stabilized interface, however gum arabic could not displace fucoidan. This has an important implication for the formulation of multilayer emulsions. If the system contains polyelectrolytes with a higher charge density than the ones used to coat droplets, then they may displace the original polyelectrolytes and promote droplet aggregation (Chang, et al. 2016). For example, two different anionic polysaccharides, pectin, and xanthan gum improved the stability of whey protein-stabilized emulsions in two different ways. The addition of xanthan gum improved resistance to high ionic strength, and the presence of pectin improved emulsion stability at acidic pH (Qiu, et al. 2015). Moreover, bilayer emulsions are not just about interactions between protein and polysaccharide, they may be produced of any two oppositely charged molecules, such as lecithin-chitosan membrane, which was formed using an electrostatic deposition method (Klinkesorn, et al. 2005).

According to Pan et al. (2015), the role of l-b-l coatings and localization of antioxidant molecules at the emulsion interface can enhance the oxidative stability of encapsulated bioactive substances. The results of their study proved this assumption and antioxidants localized at the interface indeed significantly limited the oxidation process induced by peroxyl radicals (Pan and Nitin 2015).

It is also obvious that emulsions stabilized with two and three layers of polysaccharides have higher physical stability than the emulsion stabilized only with a mono-layered membrane (Burgos-Diaz, et al. 2018; Xu, D., et al. 2016). As published by Xu et al. (2016), stable three-layered lutein emulsions can be prepared at pH 3, where droplets of whey protein-stabilized primary emulsion were gradually coated by flaxseed gum and then by chitosan (Xu, D., et al. 2016). Moreover, the l-b-l method is not only applicable on biopolymers, but particle-stabilized l-b-l emulsions were similarly designed to overcome limitations of conventional and Pickering emulsions. Here, emulsion co-stabilized by zein colloidal particles and propylene glycol alginate can serve as an example (Wei, et al. 2019).

In summary, numerous studies have shown that electrostatic deposition of polysaccharides onto droplets may be used to modify the properties of protein-stabilized emulsions. The deposition of a biopolymer onto another biopolymer is affected mainly by its charges and pH of the system. In all cases, polysaccharides improved the long-term storage stability, extended the pH range where the emulsions were stable, and provided resistance to environmental changes such as ionic strength and temperature (Burgos-Diaz, et al. 2016). Another interesting application of the l-b-l method is the protection of bioactive lipophilic compounds from chemical degradation by the incorporation of a multilayer emulsion in the inner oil phase. It can be summarized that the advantages of l-b-l approach in forming multilayer emulsions stabilized with biopolymers are the following: relatively easy manufacturing, decreased oxidation of actives, they exhibit good delivery systems for high-lipophilic actives, biocompatibility and biodegradability, and utilization of abundant renewable sources as emulsifiers (Burgos-Diaz, et al. 2016). With regard to application in pharmacy and cosmetics, studies have indicated that emulsifiers could affect the topical delivery of actives. It was, for example, reported that an increase in emulsion droplet charge could negatively affect the release of salicylic acid from formulations. Contrary to this finding, positively charged emulsion droplets enhanced the dermal and transdermal delivery of salicylic acid from their emulsions. For this reason, l-b-l emulsions represent a suitable option for modulating droplet surfaces and their interaction with skin or release of active ingredients (Kotze, et al. 2015).

5.4 Combination of cellulose and proteins in the stabilization of the emulsion

Recently, research efforts have focused on the formulation and stabilization of emulsions using combinations of cellulose polymers or particles, and various proteins acting in one system. The published studies were mainly focused on the addition of cellulose as a thickening or stabilizing agent to primary protein-stabilized emulsions. Paximada, et al. (2016) studied the use of bacterial cellulose as a thickener in the aqueous phase of whey protein (WP) emulsions. They referred that bacterial cellulose was adsorbed on WP-stabilized emulsion droplets at pH 3.8 (bellow *pI* of WP) and this resulted in stable emulsions, arising likely due to the formation of a bacterial cellulose network between the oil droplets (Paximada, Koutinas, et al. 2016). In the study of Xu, et al. (2016), microcrystalline cellulose was combined with soybean protein hydrolysate to stabilize curcumin loaded emulsion. As both stabilizing components are negatively charged, the addition of MCC to the hydrolysate-stabilized emulsions increased droplet size at high MCC concentrations (Xu, D., Zhang, et al. 2016). This was assigned to the interactions of microcrystalline cellulose with emulsion droplets covered by the soybean protein hydrolysate. According to Sarker, et al. (1999), the addition of methylcellulose to bovine serum albumin emulsions decreased the creaming rate through a viscosity effect and caused an increase of emulsions droplet size (Sarker, D. K., et al. 1999). Another possible way of interaction between cellulose and protein was reported in study of Liu, et al. (2012) and consisted in the modification of carboxymethyl cellulose with soy protein isolate (Liu, L., Liu, Zhao, Zhao, Kong, et al. 2012), which formed conjugates as a result of heat treatment (Diftis and Kiosseoglou 2003). Also, CAS emulsions and their additional stabilization were subject to research interest. When combined with CAS, regenerated cellulose exhibited a positive synergistic effect with an impact on the reduction of creaming and decrease droplet size in emulsions. With an increasing amount of cellulose, an improved packing of proteins onto the droplet surface occurred, which resulted in a gradual reduction in droplet sizes. Regenerated cellulose also formed a strong network between droplets, which lowered flocculation (Hu, H., et al. 2016). Liu, et al. (2012) studied interactions of CAS with carboxymethyl cellulose. After acidification, the CAS/carboxymethyl cellulose complex was formed at oil-water interfaces, which protected the oil droplets against flocculation and coalescence. At pH 7, coverage of oil surface with proteins increased with increasing concentration of carboxymethyl cellulose, followed by preferential adsorption of β-casein originating from CAS. On the other hand, the addition of carboxymethyl cellulose at pH 4 did not influence protein surface coverage and no difference in protein composition was observed (Liu, L., Liu, Zhao, Zhao, Kong, et al. 2012).

5.5 Nanocelluloses and their interactions with biopolymers at the oil-water interface

So far, only a few studies have been, however, focused on the use of combinations of CNC particles with other types of biopolymers. Modifications of CNC particles with polymers, such as whey protein, bovine serum albumin, hydroxyethylcellulose, methylcellulose or fusion proteins occur due to their electrostatic interactions, covalent binding or other types of interactions between particles and polymers. Electrostatic interactions of CNC and whey protein at the oil-water interface were studied in the works of Sarkar, et al. (Sarkar, et al. 2017; Sarkar, et al. 2018). As CNCs are negatively charged and whey protein carries a positive charge, emulsion droplets were primarily covered with whey protein and CNC then formed the second layer. An analogous study published by Liu, et al. (2018*)* reported on high internal phase Pickering emulsions stabilized using bovine serum albumin and oppositely charged CNCs (Liu, F., et al. 2018). Also, Hu, et al. (2016) studied emulsions stabilized with CNC and water-soluble polysaccharides (Hu, Z., et al. 2016; Hu, Z., Patten, et al. 2015), namely surface-active hydroxyethylcellulose and methylcellulose, which were able to adsorb onto CNCs. The study aimed to prepare emulsions that could be dried and re-dispersed again. As demonstrated by Varjonen and Paukkonen, cellulose nanofibres can be covalently modified by using fusion proteins with hydrophobin, a cysteine-rich protein. The combination of protein and cellulose nanofibres leads to a synergic improvement in the formation and stability of o/w emulsions, resulting in systems stable for several months (Varjonen, et al. 2011; Paukkonen, et al. 2017).

6. AIMS OF WORK

The thesis is mainly devoted to the description and application of selected biopolymers (proteins and polysaccharides) as emulsifying and stabilizing agents for bioactive lipophilic substances, and investigation of such stabilized emulsions. During the study, the interest has been mainly focused on particle-stabilized Pickering emulsions. However, this interest has further developed to the investigation of interactions of the biopolymers and particles at the oil-water interface with an effort to find practical aspects and applications of these systems. Therefore, the aims of the work coming out of these presumptions can be summarised as follows:

- To investigate the influence of the preparation procedure and formulation on the behaviour of protein-stabilized emulsions.
    - Determination of the emulsifying properties of sodium caseinate in o/w emulsions containing bioactive triacylglycerol oils.

- To formulate emulsions stabilized by a combination of protein and cellulose nanoparticles.
    - Investigation of interactions between polymers (protein) and particles (cellulose) at air-water and oil-water interfaces, fundamental study of their emulsifying properties.

- To further develop and investigate practical applications of protein/particle-stabilized systems.
    - Preparation and characterization of emulsion-based oleogels with potential applications as carriers of lipophilic active ingredients.

7. EXPERIMENTAL PART

For the sake of clarity, sections Experimental part, Result and discussion together with Summary of individual goals of the thesis are divided into:

a) **Study on CAS-stabilized emulsions:** the study was dealing with determining the emulsifying properties of sodium caseinate and long-term stability of CAS-stabilized emulsions containing tamanu and black cumin seed oils, which were prepared by high-shear homogenization (Ultra-turrax) and sonication. The main aim of the study was to characterize CAS as an emulsifying agent and compare the emulsions containing mentioned oils formed by the two methods of preparation. This study was conducted to look closer into the protein-stabilized emulsions.

b) **CNC/CAS-stabilized emulsions – interactions at the oil-water interface:** the study was aimed at understanding how the interactions between two bio-based emulsifiers can influence the formation and stability of oil-in-water emulsions (o/w) with hexadecane. Interactions between CNC and CAS were studied in bulk, at air-water and oil-water interfaces. Emulsions were prepared through different routes of the addition of the emulsifiers and at two different pH, 3 and 7.

c) **Oleogels based on CNC and CAS:** the topics studied the practical application of the emulsion systems described in point b). The effort was exerted to replace hexadecane by oil with practical relevance (olive oil), to compare these two systems and to find an optimal formulation for preparation emulsion-based gels, which will be suitable for carrying lipophilic active ingredients.

7.1 Materials

7.1.1 Chemicals

All oils and chemicals used in the thesis are summarized below.

Non-traditional vegetable oils from *Calophyllum inophyllum* (Tamanu oil, TA) and *Nigella sativa* (Black cumin oil, BC) were obtained from *Nobilis Tilia* (Czech Republic). Hexadecane was sourced from Sigma-Aldrich (Germany). Extra virgin olive oil was purchased from a local store.

Casein sodium salt from bovine milk (CAS) was provided from Sigma-Aldrich. Cellulose nanocrystal (CNC) powder prepared *via* the sulphuric acid route was purchased from CelluForce (Canada).

All other chemicals used, polyethyleneimine (PEI), calcium chloride ($CaCl_2$), sodium chloride (NaCl), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Sigma Aldrich and Merck (Germany) and were of analytical grade. In all studies, Milli-Q water was employed.

7.1.2 Microorganisms

The antimicrobial activities of oils (TA and BC) and CAS-stabilized emulsions were evaluated using eight bacterial strains obtained from the Czech Collection of Microorganisms (CCM, Czech Republic). The bacteria were selected to represent major food-borne classes. The gram-positive (*Micrococcus luteus* CCM 732, *Staphylococcus aureus subps. aureus* CCM 3953, *Bacillus cereus* CCM 2010, *Enterococcus faecalis* CCM 2665) and gram-negative (*Escherichia coli* CCM 3954, *Pseudomonas aeruginosa* CCM 3955, and *Salmonella enterica subsp. enterica ser. Enteritidis* CCM 4420, *Serratia marcescens subsp. marcescence* CCM 303) strains were employed in the test.

All microorganisms were maintained on nutrient agar and subcultured onto fresh media every two weeks. Suspensions of bacterial strains were prepared by inoculation from the pure culture on the Petri plate into the sterile tube with nutrient broth and incubation at 30 °C for 24 h (*Pseudomonas aeruginosa* and *Bacillus cereus*). Other bacterial strains were incubated at 37 °C for 24 h.

7.2 Sample preparation

7.2.1 CAS-stabilized emulsions

The o/w emulsions were formulated with 1, 2, 5, 7.5, 10 or 12 wt% CAS. The CAS dispersions were prepared by dispersing the powder in Milli-Q water under gentle stirring at room temperature for 4 hours. The sample was left to stand at 4 °C overnight to allow complete hydration, and before emulsification, the dispersion was allowed to equilibrate at room temperature under stirring. Emulsifications were carried out by mixing each of the above CAS dispersions and oil phase (5, 10, 20 and 30 wt%; TA or BC oil) at 25 °C.

Two different methods were carried out to prepare the emulsions: 1) a rotor-stator homogenization with an Ultra-Turrax model T25 (IKA, Germany) for 12 min at 13,400 rpm and 2) an ultrasonic homogenization (UP400S, Hielscher, Germany) with 400 W, 24 kHz, for 1 min, operated with 100% output. Emulsions were kept in the ice bath during sonication.

7.2.2 CNC/CAS-stabilized emulsions – interactions at the oil-water interface

Stock dispersions of CAS (2 wt%) were prepared by stirring CAS powder in Milli-Q water for 4 h at room temperature. Similarly, CNC dispersions (2 wt%) were prepared by initial stirring for minimum 12 h followed by sonication with Vibracell Sonicator (Sonics and Materials Inc., USA) at 40% output during three cycles with a duration of 1 min to remove aggregates.

Oil-in-water emulsions with 20 wt% concentration of hexadecane oil and total particle content of 0.2 wt% were prepared by sonication (Vibracell Sonicator, Sonics and Materials Inc., USA) at 20% output. The preparation of emulsions followed three different routes (Fig. 7.1): 1) **route R1**: the CNC and CAS dispersions (each 0.2 wt%) were premixed in aqueous phase in 1:1 ratio, hexadecane (20 wt%) was added to CNC/CAS mixture and the system was sonicated for 1 min. 2) **route R2**: primary emulsion containing 40 wt% of oil and 0.2 wt% of CAS was prepared by sonication (1 min), then the CNC dispersion (0.2 wt.%) was added to aqueous phase to get final emulsion containing 20 wt% oil and 0.2 wt% particles in total, and sonicated shortly (20 s). 3) **route R3**: primary emulsion containing 40 wt% of oil and 0.2 wt% of CNC was prepared by sonication (1 min), then the CAS dispersion (0.2 wt.%) was added to aqueous phase to get final emulsion containing 20 wt% oil and 0.2 wt% particles in total and sonicated shortly (20 s).

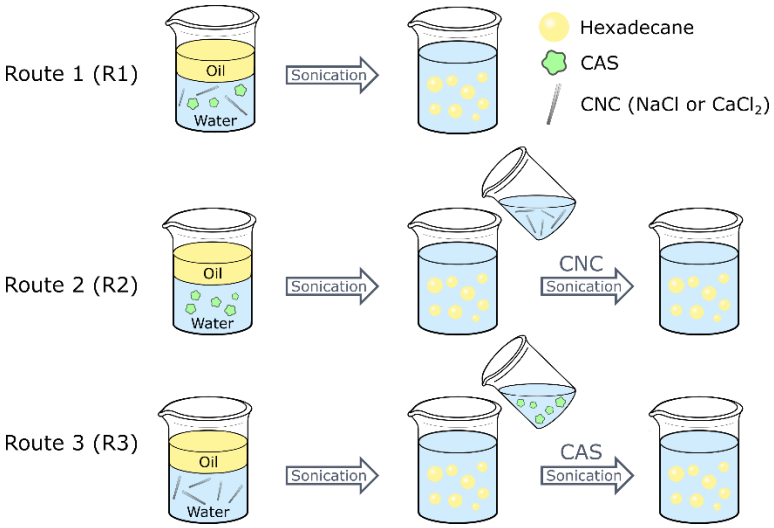


Fig. 7.1 The routes of emulsion preparation (Pinďáková, et al. 2019).

All the emulsions were prepared with either NaCl (5 mM) or $CaCl_2$ (0.5 mM) as background electrolyte. The presence of the background electrolyte facilitated emulsification.

7.2.3 Oleogels based on CNC and CAS

Stock dispersions of CAS (2 wt%) and CNC (2 wt%) were prepared by the same way as stated above, nevertheless, CNC (2 wt%) was sonicated by UP400S (Hielscher, Germany) at 60% output during three cycles with a duration of 1 min to remove aggregates.

Oil-in-water emulsions with an oil concentration of 20 wt% and total particle content of 0.2 wt% and 0.3 wt% were prepared by sonication (UP400S, Hielscher, Germany) at 60% output. The oil phase was composed of olive oil or hexadecane and the preparation followed the three above described routes R1, R2 and R3.

After emulsification, the samples were centrifuged at 6,000 rpm for 3 min (35 *g*; Hettich EBA 20, Germany), the upper emulsion layer was separated from supernatant, transferred to cylindrical mold (diameter of 12 mm) placed on Petri dish, and dried for 48 hours at ambient temperature.

7.3 Methods

7.3.1 CAS-stabilized emulsions

Characterization of emulsions

Size and distributions of emulsion droplets: The size and distribution of the emulsion droplets were measured using laser diffraction (Mastersizer 3000, Malvern Instruments, UK). The emulsions were suspended in recirculating Milli-Q water flowing through the Hydro SM measuring cell of the instrument, at a pump velocity of 2,400 rpm. The volume mean diameter $D_{(4,3)}$, corresponding to a mean diameter of spheres with the same volume as the analysed droplets, was determined.

Zeta potential: The zeta potential of the samples was measured using a Zetasizer Nano (Malvern Instruments, UK) and calculated using the Smoluchowski model. For measurements, 5 µl of the sample was diluted with 1 mL of twice filtered (Millipore, 0.22 µm) Milli-Q water. The zeta potential was measured at the native pH of emulsions (pH ~ 6.5). The average of three records on freshly prepared samples is reported.

Creaming index: Stability of emulsions was assessed by visual observation, daily during the first week of preparation and then at seven-day intervals. The emulsions were stored at ambient temperature. The stability was expressed as the height of the emulsion layer (H_{emul}) relative to the total height of the emulsion (H_{total}), referred to as the creaming index (*CI*; equation 1)

$$CI\ (\%) = \frac{H_{emul}}{H_{total}} * 100 \qquad (1)$$

Antimicrobial activity

Antimicrobial activities of oils and emulsions were studied using disc diffusion assay. An overnight culture of each of the bacteria in the nutrient broth was adjusted to 10^6 CFU.mL^{-1} and 100 µL of inoculum was spread onto Mueller-Hinton Agar sterile agar plates (Hi-Media Laboratories, India). Sterile paper discs with 5 mm in diameter were soaked with 7 µl of oil or with 5 µl of emulsion and placed on the inoculated agar. As references, discs soaked with sterile deionized water and 7.5 wt% aqueous CAS dispersions were used. The plates inoculated with mesophilic bacteria were incubated at 37 °C for 24 h; for *Pseudomonas aeruginosa* and *Bacillus cereus* 30 °C for 24 h for was employed. After incubation, the antimicrobial activity of the tested samples was evaluated by measuring the diameter of inhibition and/or hallo zones (mm). The diameter of the disc was subtracted from the diameter of the inhibition zone.

7.3.2 CNC/CAS-stabilized emulsions – interactions at the oil-water interface

Characterization of CNC and CAS dispersions

Size of CNC and CAS[4] particles. The sizes at different pH and ionic strengths were measured at 25 °C using dynamic light scattering (Beckman Coulter N4 PLUS Particle Size Analyzer, USA). The stock dispersions of CNC and CAS were diluted with Milli-Q water to a concentration of 0.1 wt% and the pH was adjusted by the addition 0.1 M HCl or 0.1 M NaOH to values ranging from 1.5 to 7. To vary the ionic strength of the dispersions, $CaCl_2$ or NaCl were added to obtain concentrations of each of the salts ranging from 0 to 100 mM (corresponding to a range of 0–300 mM in ionic strength).

Visual assessment of CNC/CAS interactions: To study CNC/CAS interactions, each of the stock dispersions were diluted to 0.1 wt% and the pH was adjusted to 7; 5.5; 4 and 3 by NaOH and HCl (1M). The CNC and CAS dispersions were mixed in ratios of 100:1; 20:1; 10:1; 2:1; 1:1; 1:5 and 1:10 (v/v). The samples were visually observed after 4 h equilibration time.

Morphology: Atomic force microscopy (AFM) was used to examine the morphology of CNC at pH 7, CAS at pH 3 and 7 and their mixtures at ratio 1:1 and 1:0.5 at pH 3. The analyses were performed in a semi-contact mode using an NTEGRA Prima device on which a Tap300Al-G cantilever (BudgetSensors, Bulgaria) was mounted. Each of the suspensions was diluted to 0.01 wt%, and a droplet was placed on a freshly cleaned silicon wafer substrate and allowed to dry at room temperature. The AFM height and phase images recorded were processed with the Gwyddion software (ver. 2.52).

Adsorption of CAS on CNC surface: Adsorption of CAS onto CNC coated SiO_2 crystals was studied using a Quartz crystal microbalance with dissipation monitoring QCM-D E4 device (Q-sense AB, Sweden). The model (starting) CNC surfaces were prepared using SiO_2 QCM-D crystals (Renlux, SiO_2, China) according to (Niinivaara, et al. 2015). The surfaces were cleaned in 10 wt% NaOH solution for 15 s, followed by rinsing with Milli-Q water and drying with nitrogen. After pre-treatment of the crystals by immersion in PEI solution (1 $g.L^{-1}$) for 20 min, CNC (0.01 wt% in Milli-Q water) was spin-coated onto the gold surface. A droplet of CNC was deposited on non-rotating crystals that were then accelerated at 2,100 rpm/s and kept at 3,000 rpm for 40 s. The spin-coated crystals were heated at 80 °C for 10 min, rinsed with water and dried. CAS dispersion (0.05 wt%) was then adsorbed onto the CNC-coated surface of the crystal placed in the flow cell of the QCM-D at a flow of 0.1 $mL.min^{-1}$. The adsorption was studied by flowing water in the cell before a new CNC layer was introduced through an injection of 0.05 wt% CNC dispersion into the flow cell. The last step

[4] *CNC – cellulose nanocrystals; CAS – sodium caseinate*

was rinsing by flowing water. Adsorption experiments were conducted at pH 3 and 7.

Measurements were conducted at the 3rd, 5th, 7th and 9th overtones. The data at the 3rd overtone was processed using the Sauerbrey model, where the change in resonance frequency Δf reflects the amount of mass coupled to the surface of the crystal. For thin, evenly distributed, rigid films, an adsorption induced frequency shift (Δf) is related to the mass uptake (equation 2):

$$\Delta m = -\frac{\Delta f \times C}{n_r} \quad (2)$$

where Δm is the mass (ng), n_r is the overtone number (= 1, 3,…), and C is the mass sensitivity constant (17.7 ng.cm^{-2}.Hz^{-1}).

The equation (2) holds as long as the dissipation remains low, which is the case for experiments carried out at pH 7. For the experiments carried out at pH 3, the dissipation remained low until the second layer of CNC was exposed to water. The modeling of this phenomenon, related to a rearrangement of the film was beyond the scope of this study.

Surface tension and interfacial tension: The surface and interfacial tensions were determined by the pendant drop technique using an Attension Theta optical tensiometer (Biolin, Finland). The image of the droplet was recorded with a black and white digital camera and the surface tension was obtained by iterative fitting of the shape of the droplet with the Young-Laplace equation. The droplet was formed using a 0.718 mm (22 gauge) stainless steel needle. The following samples were studied: 1) CNC and CAS dispersions in concentration range 0.01–2 wt%; 2) 0.2 wt% CNC stock dispersion with CAS added at concentrations ranging from 0.01–0.2 wt% and 3) 0.2 wt% CAS stock dispersion to which CNC was added at concentrations 0.01–0.2 wt%. The measurements were conducted both at air-water and hexadecane-water interfaces.

Characterization of CNC/CAS-stabilized emulsions with hexadecane

Size and distribution of emulsion droplets: The droplet size distribution of each hexadecane emulsion was analysed by laser diffraction (Malvern MasterSizer Micro, Malvern Instruments Ltd; UK). The absorbance of the emulsion droplets was set to 0.001. Mean droplet size was reported as volume mean ($D_{(4,3)}$) and surface mean ($D_{(3,2)}$) diameters.

Microstructure of emulsions: Emulsion droplets were observed using Zeiss AxioCam MR 5 optical microscope (Carl Zeiss MicroImaging GmbH, Germany) equipped with ImageJ software. Before observation, a droplet of emulsion (10 μL) was placed onto a glass microscope slide and viewed under 10–100 × magnification.

7.3.3 Oleogels based on CNC and CAS

Characterization of CNC/CAS-stabilized emulsions

Size and distribution of emulsion droplets: The droplet size distribution of each emulsion was analysed by laser diffraction (Malvern MasterSizer Micro, Malvern Instruments Ltd; UK). The absorbance of the emulsion droplets was set to 0.001. Mean droplet size was reported as volume mean ($D_{(4,3)}$) and surface mean ($D_{(3,2)}$) diameters.

Microstructure of emulsion: Emulsion droplets were observed using an Olympus CX41 optical microscope (Olympus Corporation, Japan) equipped with Quick PHOTO PRO 2.0 software. Prior to observation, a droplet of emulsion (10 μL) was placed onto a glass microscope slide and viewed under 10–100 × magnification.

Encapsulation efficiency: The encapsulation efficacy (*EE*) was determined according to the reported method (Cristina Sabliov, et al. 2015) using a mass fraction of the non-encapsulated oil (m_{oil}) related to the total mass of the oil phase used (m_{total}). The volume fraction of non-encapsulated oil was determined after centrifugation of emulsions conducted at 6,000 for 3 min (Hettich EBA 20, Germany). Non-encapsulated oil was removed by syringe, and the volume fraction of the released oil was re-calculated to its mass fraction ($\rho_{Hexadecane}$ = *0,77 g.cm*$^{-3}$; $\rho_{Olive\ oil}$ = *0,9087 g.cm*$^{-3}$). Encapsulation efficiency (*EE*) was calculated using equation 3:

$$EE(\%) = 100 - [\frac{m_{oil} * 100}{m_{total}}] \quad (3)$$

where m_{oil} is mass of released oil and m_{total} is mass of oil phase in the emulsion.

Characterization of oleo gels

Loss of water, released oil after drying: The mass of the separated emulsion layer before drying was weighted (m_E) and let dry for 42 h. After this time, the water from the sample was evaporated. The mass of dried emulsion-based gel containing released oil was determined (m_{GE+O}), and finally, the mass of released oil was determined (m_O). From these parameters, the loss of water (%; *LW*) and the amount of released oil (%; *RO*) were calculated using equations 4 and 5.

$$LW(\%) = \frac{m_E - m_{GE+O}}{m_E} * 100 \quad (4)$$

$$RO(\%) = \frac{m_{GE+O}}{m_O} * 100 \quad (5)$$

Characterization of viscoelastic properties: The characterization of the gels was conducted using rotation rheometer PHYSICA MCR-502 (Anton Paar, Austria) with parallel plate configuration. The diameter of the samples was 10 mm. To avoid slipping of the samples in the geometry, the plates were adjusted by a thin layer of sandpaper. The linear viscoelastic region was established at 1% strain (γ) value. The storage G' and loss G'' moduli were measured as a function of the angle frequency ranging from 50 to 0.1 $1.s^{-1}$ at a temperature of 25 °C.

Re-dispersion of dried gels: The gels obtained by drying were placed into Eppendorf vials and added Milli-Q water. Mass of the sample was chosen to keep the original o/w ratio of 20/80. The samples were vortexed for 30 s and let stand for 42–72 h. After this time, the emulsions were visually assessed whether the re-dispersion of emulsion droplets occurred or not. Successfully re-dispersed samples were observed by optical microscopy CX41 optical microscope (Olympus Corporation, Japan) with 10–100 × magnification with the same procedure as described above (Microstructure of emulsions).

7.3.4 Statistical analysis

All the analyses were conducted at least in triplicates, with the Dean-Dixon method being utilized to calculate the means and standard deviations. The Student T-test was applied to detect any statistical differences between the samples (Statistica, StatSoft, Inc., Palo Alto, CA, USA) at an antimicrobial activity. The P (probability) value of ≤ 0.05 was considered to be statistically significant.

7.4 Results and discussion

7.4.1 CAS-stabilized emulsions

In recent years, there has been an increasing demand for alternative sources of bioactive substances. Many of the natural products extracted from plants demonstrate biological activities and receive particular attention as a source of valuable fatty acids, antimicrobials, antioxidants or wound healing agents (Leguillier, et al. 2015; Piras, et al. 2013).

Nigella sativa commonly called black cumin (BC), and *Calophyllum inophyllum* (tamanu, TA) have been used for their nutritional and therapeutic values for many years. The reason for the biological activity of cumin seeds (antioxidant, anticancer, anti-inflammatory as well as antibacterial) can be found in their complex composition as they contain over 100 different constituents, including all essential fatty acids (Piras, et al. 2013; Amin and Hosseinzadeh 2016). Thanks to the composition and effects, this oil can be used as a natural resource for the production of pharmaceuticals and novel functional foods (Piras, et al. 2013; Bourgou, et al. 2010; Cheikh-Rouhou, et al. 2007; Singh, et al. 2014).

Tamanu oil expelled from the seeds of *Calophyllum inophyllum* is beneficial mainly in the treatment of dermal problems. The antibiotic and anti-inflammatory properties make this oil an excellent raw material for regenerating and protective formulations. The two main bioactive substances in the tamanu oil are calophyllic acid and a lactone with antibiotic properties (Dweck and Meadows 2002). *Calophyllum inophyllum* is also known as a rich source of secondary metabolites (coumarins, xanthones, flavonoids and triterpenes) (Shen, et al. 2003), which can be positive in the human diet (Itoigawa, et al. 2001; Patil, et al. 1993).

Nevertheless, the presence of unsaturated fatty acids in both oils causes their sensitivity to oxidation. Therefore, the emulsification of the oils is an applicable strategy for their protection, allowing thus delivery of the oils into hydrophilic systems. For the production of stable emulsions, the properties of the stabilizing surface layer, as well as emulsion characteristics are crucial. Emulsification plays also a key role in further optimizing the encapsulation efficiency of oils, for example in case the lyophilisation will follow the formation of the emulsions (Ixtaina, et al. 2015; Komaiko, et al. 2016).

Emulsification of triacylglycerol-based oils (fish, olive and sunflower oil) is often performed using milk proteins (Day, et al. 2007; Herrero, et al. 2011; Keogh, et al. 2001; Villiere, et al. 2005; Huck-Iriart, Cristián, et al. 2013). Particularly, sodium caseinate (CAS) is frequently used emulsifier thanks to its amphiphilic structure and surface activity. In oil-in-water emulsions, CAS has been used as emulsifier and encapsulating agent (Drusch, et al. 2012) as it forms a barrier, which is essential for protecting the bioactive substances against oxidation and the molecules adsorbed at oil-water interface also provide an effective shield against flocculation and coalescence due to a combination of electrostatic and steric repulsion. For example, CAS provided good protection of fish oil against

oxidation by forming a physical barrier effect (Day, et al. 2007; Dickinson, Eric 1999; Livney 2010; Nielsen and Jacobsen 2009; Amine, et al. 2014). Casein is able to protect also other oils against oxidation (Hu, M., et al. 2003), for example, CAS-stabilized corn oil-in-water emulsions exhibited high stability, probably due to the ability of CAS to form a thick layer on emulsion droplet interface and its unique chelating properties.

The emulsification of rarely studied tamanu and black cumin oils can be of benefit, as they contain bioactive substances with pharmaceutical effects (Mazaheri, et al. 2019; Mukhtar, et al. 2019; Ansel, et al. 2016; Raharivelomanana, et al. 2018). Moreover, stabilization of emulsions with biocompatible and biodegradable protein CAS can facilitate the delivery of these lipophilic substances to aqueous-based systems. The study was, therefore conducted with the aim 1) to investigate behaviour of CAS under emulsification of triacylglycerol-based oils, in this case rarely studied tamanu and black cumin oils showing pharmaceutical and nutritional effects, and 2) to prepare o/w emulsions, which can serve as carrier systems with therapeutic and physiological benefits for humans. The study was also aimed at finding the optimum emulsion formulation in terms of the concentrations of both protein and oils, and at establishing a suitable emulsification procedure when using two commonly available methods, high-shear homogenization (Ultra-Turrax) and sonication.

Characterization of emulsion: influence of processing parameters and composition

Droplet size of emulsions

An important parameter, which has a crucial effect on emulsion stability, is the size of emulsion droplets. Light diffraction analyses showed that emulsion droplet size ($D_{(4,3)}$) was influenced by all studied variables, especially by the method of preparation and concentration of stabilizing CAS; and to a lesser extent by type and content of encapsulated oils.

In this study, two different emulsification procedures, involving Ultra-Turrax (UT) or sonicator (US), were used for the emulsion preparation and they obviously controlled the size of emulsion droplets. Emulsification with UT led to coarse emulsions with bigger droplet sizes ranging from 0.3 to 13 μm, and their properties were notably affected by composition, such as oil and CAS contents (Fig. 7.2 a, c). On the other side, the homogenization with US yielded fine emulsions with $D_{(4,3)}$ varying from 0.3 to 0.9 μm and 0.4 to 1.5 μm for BC- and TA-emulsions (Fig. 7.2 b, d), respectively, hence with a sufficiently small size which is an assumption for production of stable emulsion systems (Perrechil and Cunha 2010). Although droplets after sonication were notably smaller than droplets treated with UT, some differences between emulsions containing BC and TA oils were observed. These can be explained by character, composition and physical properties of the oils, which influence emulsifying efficiency and hence droplets size of emulsions. Here, an important role can play two crucial

characteristics, namely viscosity of the oil and interfacial tension at the oil-water interface. Viscosities of used oils are notably different, TA oil is highly viscous (20–26 mPa.s) (Kartika, et al. 2018), whilst viscosity of BC oil is lower (6.3 mPa.S) (Mohammed, et al. 2016). The effect of dispersed phase viscosity on emulsion droplet size was studied, for example, by Wooster (2008) who proved that high-viscosity oils formed emulsions with larger droplets than low-viscosity oils (Wooster, et al. 2008). As regards interfacial tension, both oils have low and rather similar interfacial tensions at the oil-water interface, and values of 8.4 ± 1.0 $mN.m^{-1}$ and 4.3 ± 0.5 $mN.m^{-1}$ were determined for BC and TA oil, respectively. Therefore, the larger droplet size of TA emulsions is rather due to the higher viscosity than the difference in the interfacial tension.

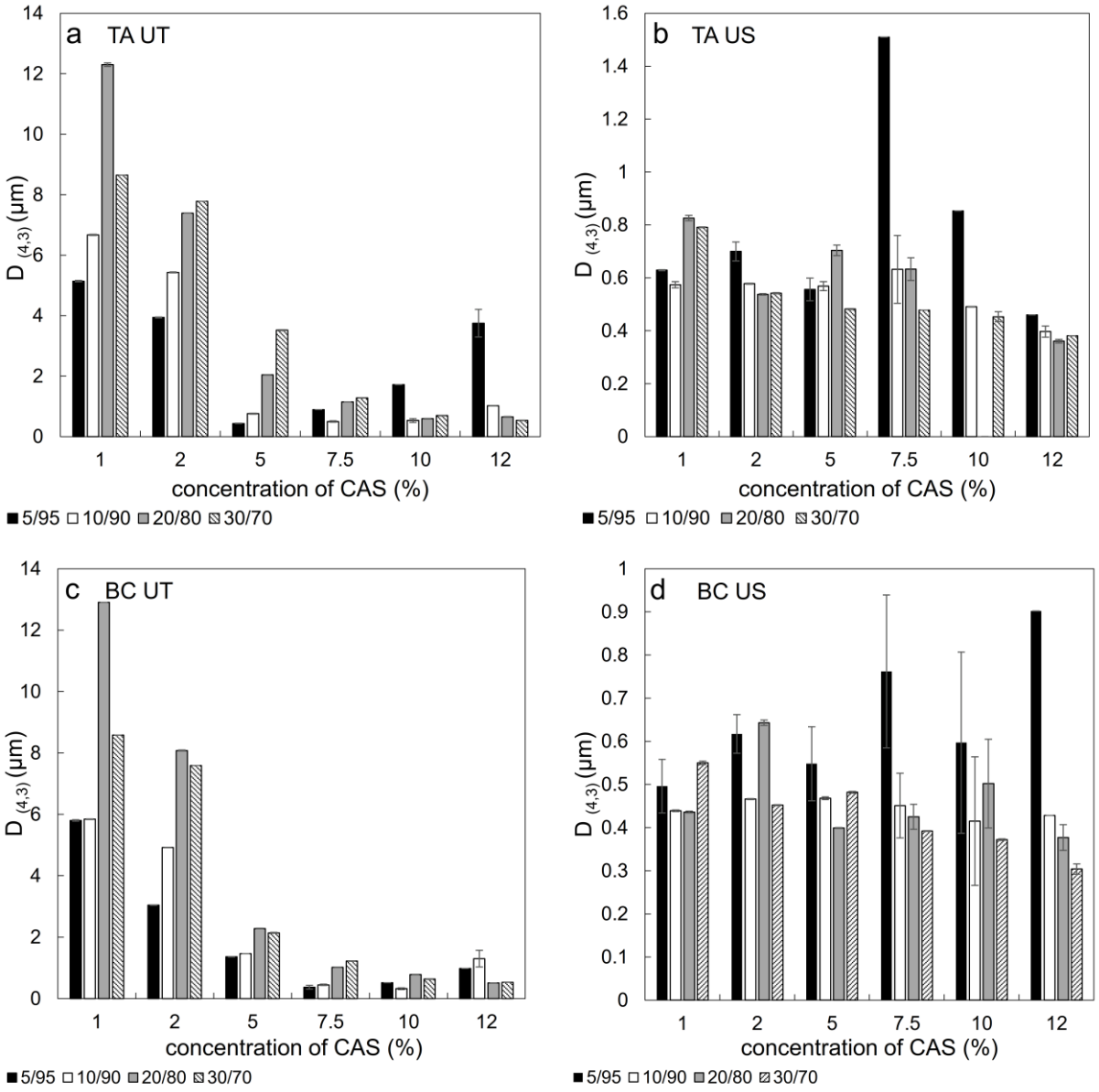


Fig. 7.2 Influence of CAS concentration and o/w ratio on volume-weighted diameter of emulsion droplets ($D_{(4,3)}$) of a) TA oil-emulsions prepared by Ultra-Turrax (UT); b) TA oil-emulsions prepared by sonication (US), c) BC oil-emulsions prepared by UT, and d) BC oil-emulsion prepared by US.

Also, droplet distributions in US emulsions were narrower compared to those prepared with UT (Fig. 7.3) which conforms with studies performed on emulsions stabilized by CAS/polysaccharides (Huck-Iriart, Cristián, et al. 2013; Álvarez Cerimedo, et al. 2010), on emulsions with whey protein concentrate (Lizarraga, et al. 2008), and also on emulsions containing modified starch and maltodextrin (Jafari, et al. 2007). An interesting study was published by O'Sullivan, et al. (2014), who investigated an effect of US treatment not only on emulsions but also on structural, physical and emulsifying properties of dissolved CAS (O'Sullivan, et al. 2014). The work revealed that ultrasound waves caused a significant reduction in the micelle size and hydrodynamic volume of the CAS, nevertheless, the emulsions prepared with US-treated and non-treated CAS surprisingly exhibited comparable droplet size. Hence, the US treatment of CAS prior to emulsification did not probably influence the rate at which CAS adsorbed at the oil-water interface despite the smaller micelle sizes and their higher hydrophobicity.

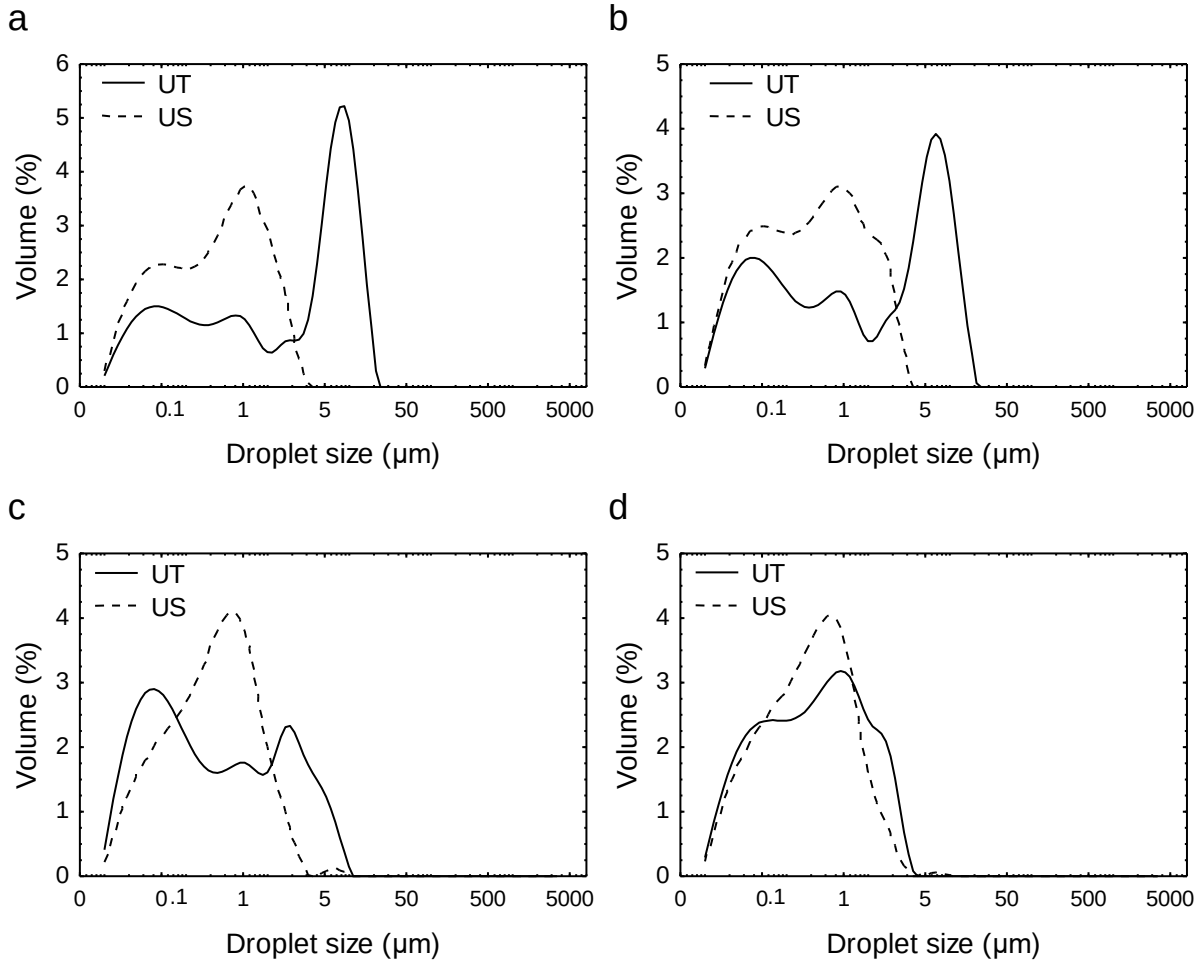


Fig. 7.3 Influence of the homogenization method on distribution curves of CAS stabilized emulsions. Ultra-Turraxed (UT) and sonicated (US) emulsions are compared across oil type, o/w ratio and CAS concentrations: a) BC-emulsions with 2 wt% of CAS (10/90); b) TA-emulsions with wt5 % of CAS (30/70); c) BC-emulsions with 7.5 wt% of CAS (20/80); d) BC-emulsions with 12 wt% of CAS (20/80).

The influence of the processing method on droplet size must always be considered together with a composition of emulsions. Regarding the concentration of CAS, the droplet size of UT-prepared emulsions gradually decreased with increasing protein concentration (1 to 5 wt%); however, $D_{(4,3)}$ dropped significantly as CAS concentrations increased to 7.5–12 wt% (Fig. 7.2 a, c). Complete coverage of droplets with protein occurs at a CAS content of about 3 wt% (emulsions with 35 % tetradecane oil) (Dickinson and Golding, 1997). At a higher protein concentration (4 wt%), a higher amount of CAS than required for saturation coverage of the oil droplets is presented; hence, a range of CAS concentrations used in current work is sufficient for complete covering the arising droplets. Therefore, the bigger droplets that were prepared by UT (in the case of sufficient CAS available in the system) can be assigned to the discrepancy between the higher rate of coalescence of oil droplets being formed by given energy input and the lower rate of CAS adsorption at the oil-water interface during homogenization. Under these conditions, the uncovered droplets tend to come together and form larger droplets again. On the other hand, when the CAS content in the aqueous phase is insufficient, flocculation might occur due to casein bridging between droplets, also resulting in rapid creaming (Hebishy, et al. 2017; Huck-Iriart, Cristián, et al. 2013; Montes de Oca-Ávalos, et al. 2017; Srinivasan, et al. 2002). The results from particle sizing were supported by the distribution curves of UT-emulsions, where a shift in droplet diameter toward smaller sizes was observed as the protein concentration increased, and distribution curves changed from multimodal (1 wt% CAS) to monomodal (10 wt% CAS), which is illustrated in Fig. 7.4a (Dickinson, E., et al. 2003).

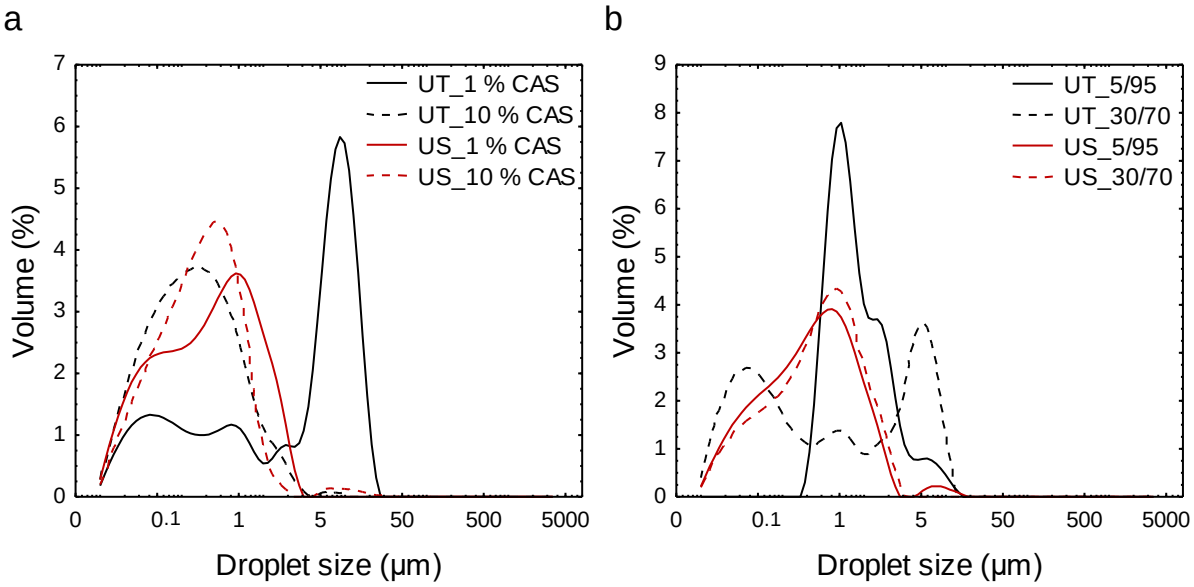


Fig. 7.4 Effect of CAS concentration a) on distribution curves of BC-emulsions with o/w ratio 10/90; and effect of o/w ratio b) on distribution curves of BC-emulsions. For tamanu oil, the trend is similar (data not shown).

On the contrary, BC and TA oil emulsions prepared by US contained much smaller droplet, and only minor changes in droplet size alongside the change in CAS concentration were observed (Fig. 7.2 b, d). For emulsions prepared with the aid of US, the distribution curves were mostly monomodal with the simultaneous shift in direction to lower droplet sizes (Fig. 7.4a). The light microscopy image captured on the emulsion prepared with US is shown in Fig. 7.5.

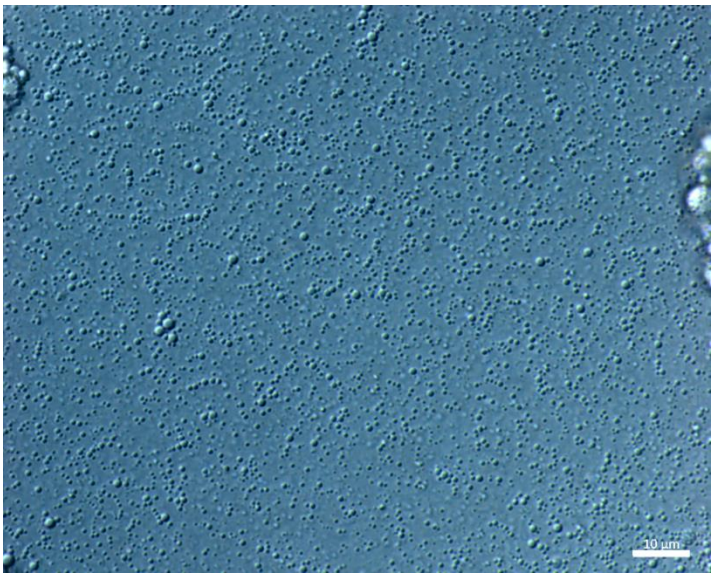


Fig. 7.5 Optical microscopy (magnification 20×) of BC emulsions with o/w 20/80 prepared with 1 wt% CAS while using sonication. Scale bar is 10 µm.

The oil content is another parameter with an impact on emulsion droplet size. In the current study, however, the amount of oil in emulsion affected $D_{(4,3)}$ only at lower concentrations of CAS (1–7.5 wt%), and mainly in case of UT emulsions. Here the droplet size increased with increasing oil fraction. The effect is visualized in Fig. 7.4b showing the broad distribution curve with three droplet populations recorded for UT emulsions containing 30 wt% BC oil and 5 wt% CAS; on the other hand by lowering the oil content to 5 wt% monomodal distributions were obtained. At CAS content of 10 and 12 wt% influence of oil amount on $D_{(4,3)}$ was only marginal. In contrast, the situation was different for emulsions prepared by sonication (Fig. 7.4b) and only minor changes in $D_{(4,3)}$ and distributions with the increasing oil content were observed at the entire concentration range of CAS used. Emulsions containing high concentrations of both protein and oil can be stabilized through the formation of a weak particle-based gel network, which reorganizes slowly under the influence of gravity and internal osmotic stresses (Dickinson, E. and Golding 1997).

Alongside other variables, the character of oil plays also a role for the droplet size in emulsions, and the droplets of the BC emulsions prepared by UT were smaller compared to droplets containing the TA oil. As already discussed above, the differences can be explained by the composition and physical properties of the oils, mainly viscosity and interfacial tension. This difference was again wiped off in emulsions prepared using US. The fact that TA oil contains, in addition to

neutral lipids, also other minor chemical compounds with emulsifying properties as glycolipids, phospholipids (1.6 %), small amounts of sterols and mono- and diacylglycerols, which all can also contribute to the easier formation of smaller droplets (Hemavathy and Prabhakar 1990).

Zeta potential of emulsions

Zeta (ξ) potential of dispersion systems is commonly considered as a stability-indicating parameter, though its values alone are not always capable of predicting the stability of emulsions, mainly at the sterically stabilized systems. Immediately after preparation, the ξ potential ranged from −53 to −41 mV for emulsions with BC oil (prepared with US) and from –60 to –46 mV for those prepared with UT. TA emulsions behaved similarly with potential values varying from –55 to −41 mV and –59 to −44 mV for US and UT emulsions, respectively. In general, UT treatment seemed to deliver slightly lower ξ potentials and no or minor systematic influence of oil content on the potential was observed. However, CAS concentrations above 5 wt% might have a positive influence in potential lowering, mainly for TA oil (Fig. 7.6). In current study, ξ potential measurements were conducted on emulsions with non-adjusted pH (TA oil emulsions pH = 6.44 ± 0.03, BC oil emulsions pH = 6.45 ± 0.04). Dickinson (1998) reported on CAS-stabilized emulsions, which were unstable at pH close to the CAS isoelectric point (pH 4.6), thanks to the reduction in electrostatic repulsion between the droplets (Dickinson, E., et al. 1998). At pH 6, however, negative ξ potential of CAS assured emulsion stability due to electrostatic and steric stabilization mechanisms.

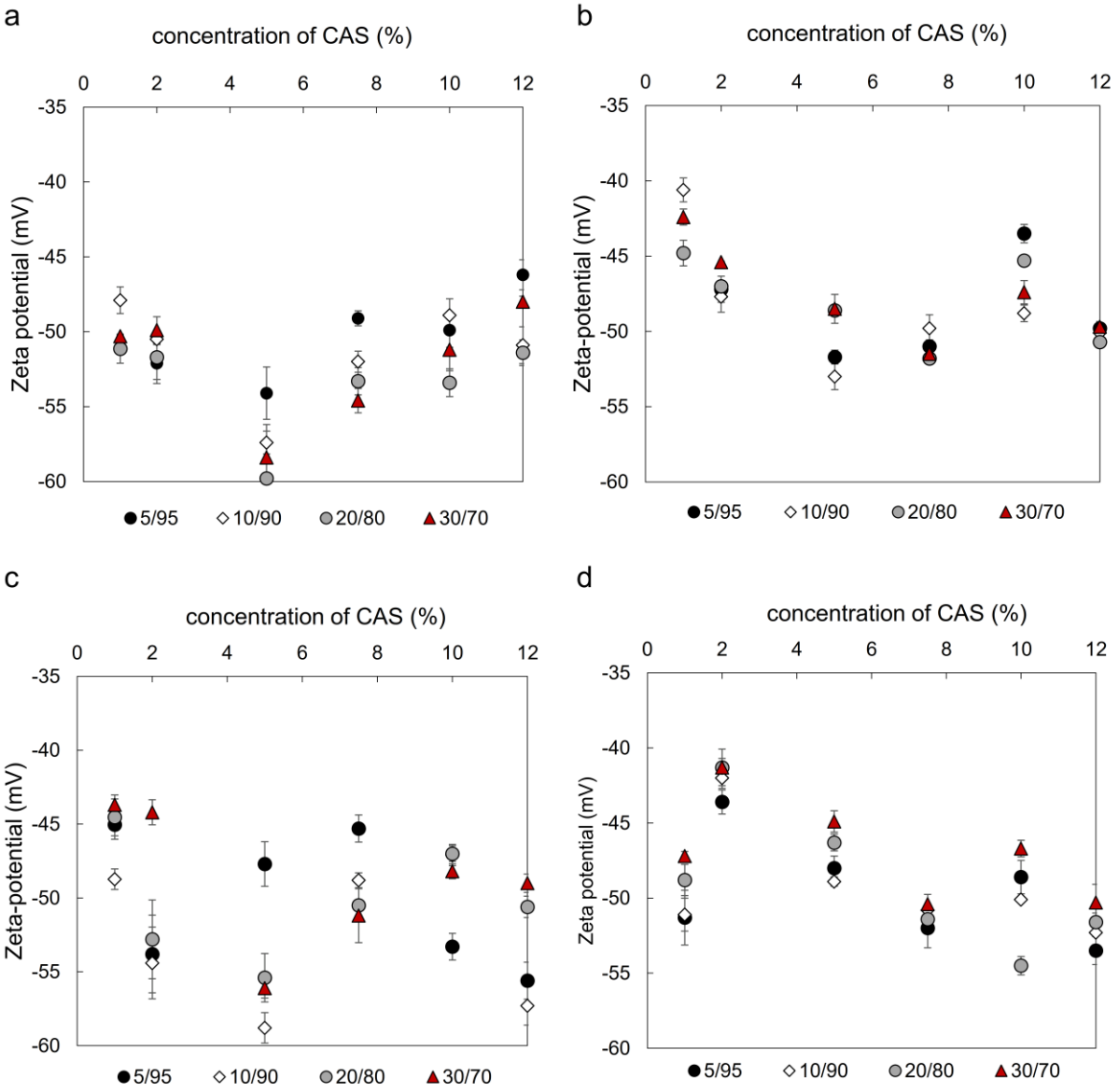


Fig. 7.6 Zeta potential of freshly prepared emulsions at native pH of ~6,5 as a function of o/w ratio and CAS content a) BC-emulsions prepared with Ultra-Turrax (UT), b) BC-emulsions prepared with sonication (US), c) TA-emulsions prepared with UT and d) TA-emulsions with US.

Differences in ξ potentials between emulsions prepared by UT and US can be assigned to the combined effect of sonication treatment and oil properties. Contrary to many other proteins, caseins are particularly disordered and substantially hydrophobic, which enables their rapid adsorption to oil droplet surfaces during emulsification. According to Barreto, et al. (2003), due to relatively high content of hydrophobic amino acids (proline residues) CAS in aqueous solutions forms a random structure with only a small amount of secondary structure, which is not affected by ultrasound (Barreto, et al. 2003). De Figueiredo Furtado, et al. (2017) concluded that sonication did not significantly influence ξ potential of CAS in comparison with non-treated protein sample (de Figueiredo Furtado, et al. 2017). Simultaneously, these authors also reported on

changes in hydrophobicity of CAS caused by sonication, which can affect ξ potential of emulsions formed by US treatment, thanks to the different orientation of protein at the oil-water interface. Differences in ξ potential were also observed on emulsions stabilized with soybean protein isolate containing medium-chain triacylglycerols, palm, soybean and rapeseed oils after US treatment (Taha, et al. 2018). The lowering of ξ potential was ascribed to the formation of protein aggregates and different amounts of protein adsorbed onto emulsion droplets containing studied oils of different compositions. Similarly, differences in the composition of oils used in our CAS-stabilized emulsions may be the reason for their different ξ potentials.

Emulsion stability

In case the emulsion droplets are stabilized by proteins, the four main destabilization routes can occur: creaming, coalescence, flocculation and Ostwald ripening. The main mechanism governing the creaming of protein-stabilized emulsions with non-adsorbed stabilizers present in excess is related to depletion flocculation (Liang, Y., et al. 2014). Kinetic stability, described by creaming index *CI*, provides indirect information on the droplet flocculation and destabilization processes occurring in an emulsion.

Not all emulsions prepared in the presented study showed kinetic stability, even immediately after preparation. Observed by the naked eye, some of them formed a bottom serum layer and a small cream phase on the top. On the other hand samples with specific formulations, mainly with high CAS content, exhibited good stability against creaming. Fig. 7.7 shows the *CI* of the freshly prepared emulsions. Stable emulsion (*CI* = 100 %) were observed at CAS concentrations of 10–12 wt% (UT emulsion) and 7.5–12 wt% CAS (US emulsions). Samples containing lower concentrations of CAS, however, destabilized easily and the extent of destabilization, expressed as *CI*, depended on the content of the oil. More specifically, the *CI* increased with the increasing amount of oil, which was more notable on UT emulsions in comparison with emulsions prepared by US. Formation of stable emulsions was, therefore, considered to be a synergic effect of the US processing resulting in small droplets with narrower distribution and a sufficient amount of CAS. Only minor differences in *CI* were observed between emulsions encapsulating TA or BC oils.

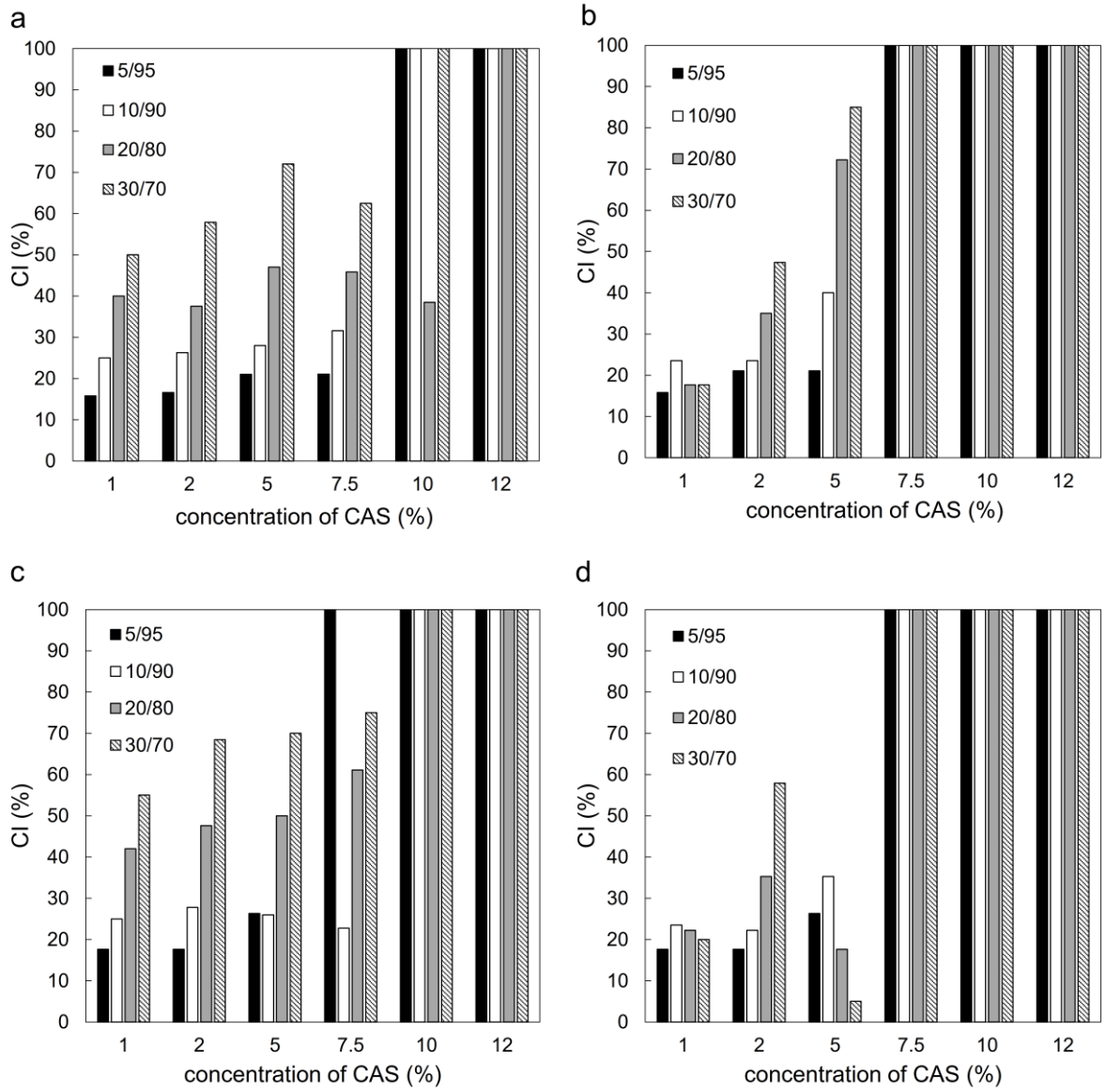


Fig. 7.7 Comparison of creaming index (CI; CI ~ 100 % is for stable emulsion) determined on freshly prepared emulsions as affected by processing method and composition of emulsions a) BC-emulsions prepared with Ultra-Turrax (UT), b) BC-emulsions prepared with sonication (US), c) TA-emulsion prepared with UT, and d) TA-emulsion with US.

The long-term stability of the emulsions was evaluated by observing the changes in their visual appearance during storage until the point of emulsion breaking appeared. Not surprisingly, the highest stability exhibited emulsions containing the highest CAS content (12 wt%). For example, BC emulsions with 30 wt% oil prepared by both procedures remained unchanged for 7 days of storage with *CI* being of 100 %. Stability studies also supported the fact that US treatment, which provided smaller droplets with narrower distribution assured production of emulsions with limited creaming. At CAS concentration of 10 wt%, the emulsions manufactured with US were stable towards creaming for 7 days, whilst in emulsions prepared with UT, the creaming started already 3 days after preparation. Moreover, UT-produced emulsions with even lower CAS content were prone to breakdown and underwent phase separation approximately after a week of storage. Interestingly, in most emulsions, though the *CI* value was lower immediately after emulsification, it did not notably change during their storage. From the *CI* data, it was also apparent that the creaming process took about a week to be completed, thereafter compaction of the cream layer started which stopped when no more oil droplets and proteins could be packed in the top cream layer. The compaction of the cream layer was also reported (Yerramilli and Ghosh 2017). These outcomes can be generalized both for BC and TA emulsions. The above observations demonstrate that an important role in the stability of CAS-containing emulsions is related to the concentration of this protein in the aqueous phase.

The data from the observation of creaming were in correlation with particle sizing measurements during emulsion storage. The results revealed that the $D_{(4,3)}$ of emulsion droplets increased during month-long storage at ambient temperature. The US emulsions changed their droplet sizes to a higher extent than UT systems, however, emulsions prepared with the aid of UT were more prone to phase separation and their breakdown occurred earlier. Here again, the concentration of CAS in emulsions played the key role. Emulsions with 1 or 2 wt% CAS were stable, in terms of droplet size, only during first days, emulsion with higher CAS content for a week and emulsions with 10 and 12 wt% CAS remained unchanged for a longer period. This is documented on emulsions with 2 and 10 wt% of protein and 20 wt% of TA oil, which were stable for a month, though their droplet sizes in formulation with 2 wt% CAS increased (Fig. 7.8). Similar observations were reported by Silva, et al. (2015) who prepared annatto seed oil emulsions in two different ways, namely by US and mechanical stirring. These systems were kinetically unstable and formed a bottom serum layer and a small cream phase on the top. Simultaneously, the sonicated emulsions exhibited a minor coalescence of the oil droplets (Silva, et al. 2015).

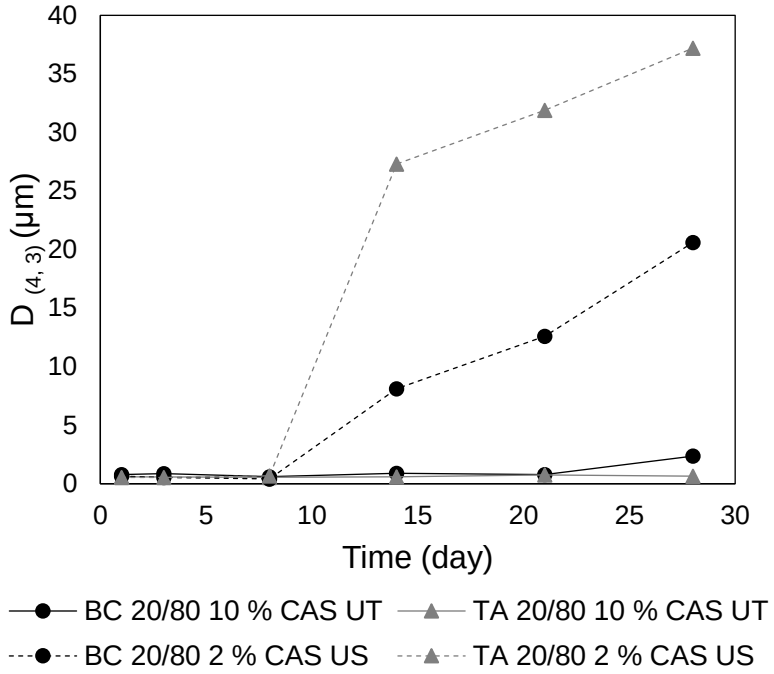


Fig. 7.8 Long term stability of TA and BC emulsions stabilized with CAS determined at room temperature: development of volume weighed diameter of emulsion droplets ($D_{(4,3)}$) in time.

Antimicrobial activity

Disc diffusion assay conducted on studied oils and their emulsions stabilized with 2 and 7.5 wt% CAS (o/w 30/70) proved inhibitory activity of both oils and selected emulsions against the gram-positive bacteria. Regrettably, none of the samples was capable of suppressing the growth of the tested gram-negative species (*E. coli, P. aeruginosa, S. enterica and S. marcescens*). The data showed that the effect of TA oil was significantly higher ($p \geq 0.5$) than that of BC oil both for *B. cereus* and *S. aureus*. It was concluded from bigger inhibition zones with sizes ranging from 8.2 ± 0.4 mm (*B. cereus*) to 6.0 ± 1.2 mm (*S. aureus*). This oil also caused lower bacterial growth (14.8 ± 1.3 to 7.7 ± 0.7 mm) around inhibition zones (halo zone) and was able to act, at least partially, against *M. luteus* and *E. faecalis* (halo zone only). The effects of TA oil encapsulated in emulsions with 2 and 7.5 wt% of CAS were rather similar, though the activity of emulsion stabilized with 2 wt% CAS was comparable with that of non-encapsulated TA oil ($p \geq 0.5$). The antibacterial activity of the BC oil was, in comparison with TA, weaker, and this oil showed activity only against *B. cereus* (5.7 ± 1.5 mm) and *S. aureus* (4.2 ± 0.7 mm). Emulsion stabilized with 2 and 7.5 wt% CAS containing BC oil exerted the comparable effect, which was weaker than that of BC oil alone. Neither BC oil nor its emulsions were efficient against *M. luteus* and *E. faecalis*. The lower activity of oils and emulsions against gram-negative bacteria is a result of composition in their cell walls, as they contain an outer lipopolysaccharide membrane, which protects the bacteria more efficiently from the disruption caused by oils. The study conducted by Ramadan, et al. (2012) supported the

above results on the bioactivity of BC oil, extending the range of its inhibitory activity also to action against yeasts, namely *S. cerevisiae* and *C. albicans* (Ramadan, et al. 2012). As regards triacylglycerol-based TA oils, their bioactivity was investigated by Leguillier, et al. (2015) who reported that TA oil exhibited two distinct antibacterial effects: the first against gram-positive bacteria acting by direct inhibition of mitotic growth and the second potent effect against gram-negative strains due to an increased release of *β*-defensin 2 peptide by macrophages (Leguillier, et al. 2015). However, the results of our study have not confirmed the effect against gram-negative strains.

Summary of the study

The main target of this study was to gather deeper knowledge on the behaviour of biopolymer, sodium caseinate, under the emulsification of triacylglycerol-based oils. For this purpose, oil-in-water emulsions of the two bioactive oils, tamanu and black cumin oil, stabilized with sodium caseinate were formulated and prepared by ultrasound treatment or high shear homogenization using Ultra-Turrax. It was found out that the ability to form stable emulsions of small, initial particle size was primarily controlled by the used method of preparation. In addition to the emulsification technique, the ability to form reasonably stable emulsions was mainly controlled by the concentration of stabilizing caseinate and, to a lesser extent, by the type and amount of used oils. Sonication was a more efficient emulsification procedure and afforded emulsions with small particle sizes throughout the entire used concentration ranges of oil and caseinate. In comparison with sonicated emulsions, the properties of emulsions prepared with Ultra-Turrax depended, to a higher extent, on their composition and the emulsions were more prone to destabilization during their storage. Finally, it was proved that both oils and their selected emulsions were efficient in suppressing the growth of gram-positive bacterial strains (*S. aureus and B. cereus*). Obviously, the results of the study served in the following experimental work and pointed to an appropriate emulsification procedure, namely sonication which will be selected for the preparation of other emulsion systems.

Results of the study were summarized and published in *Caseinate-Stabilized Emulsions of Black Cumin and Tamanu Oils: Preparation, Characterization and Antibacterial Activity" by Urbánková L., et al. Polymers 2019, 11(12), 1951; https://doi.org/10.3390/polym11121951*.

7.4.2 CNC/CAS–stabilized emulsions interactions at the oil-water interface

The growing interest in emulsions stabilized by biopolymer-based particles relates mainly to their advantageous biocompatible and surfactant-free character. In addition to biopolymer particles, also various inorganic and organic particles, discussed in Chapter 4, can be used for the preparation of Pickering emulsions (Yang, et al. 2017; Sarkar, et al. 2017; Hu, Z., et al. 2015). In the Pickering emulsions, the stabilizing layer is formed by solid particles adsorbed at the oil-water interface (Dickinson, Eric 2017). At the interface, the particles are arranged in a densely packed layer providing unique stabilizing properties by forming a steric barrier, which prevents both emulsion flocculation and coalescence.

Traditionally, in the case where two types of stabilizing agents (whether they are particles, polymers or surfactants) are used, the interface is predominantly covered by the most surface-active species. This phenomenon is referred to as competitive adsorption or competitive displacement (Arboleya and Wilde 2005; Dickinson, Eric 2011).

With the exception of the studies mentioned in Chapter 5, there is a relatively limited understanding of the behaviour of mixed stabilizing systems composed of proteins and particles with non-spherical shape.

Therefore, this second study is focused on the interactions between sodium caseinate (CAS) and cellulose nanocrystals (CNC), and on their role in the stabilization of oil-in-water (o/w) emulsions. At first, the interactions of CNC and CAS dispersed in the aqueous environments were investigated. In the next step, the interfacial behaviour at the air-water and oil-water interface was studied. This study was also complemented by investigating the adsorption profile of CAS on the model surface of CNC by quartz crystal microbalance. Finally, the emulsions were prepared *via* three routes, where the interactions between species were rationalized as a function of the order of addition of CAS and CNC during emulsification.

Characterization of CNC and CAS – the influence of pH and ionic strength

Before the interactions of CNC and CAS were investigated, both types of particles were studied separately at different pH and ionic strengths using AFM and dynamic light scattering.

The morphology of the CNC particles visualized by the AFM images was of rod-like shape with a length ranging from 80 to 220 nm and a diameter of 4 nm (Fig. 7.9a), similar to those reported by Mikulcová (Mikulcova, et al. 2018). The hydrodynamic diameter of CNC was also measured as a function of pH using dynamic light scattering (DLS). Considering the rod-like shape of CNC, DLS only gives an estimate of the size evolution, as more advanced light scattering analysis would be needed to extract the exact dimensions (Tuan Phan-Xuan, et al. 2016). The average hydrodynamic diameter of CNC was about 60 nm and the particles exhibited good stability within a broad pH range, as shown in Fig. 7.10a. This

correlates well with previous observation based on ξ potential measurements as a function of pH, which showed that CNC prepared by the sulphuric acid route was stable in the pH range 4–10 (Mikulcova, et al. 2018; Molnes, et al. 2017). The colloidal stability of the CNC was lost when the pH of the dispersion decreased below 2.5, i.e. the apparent pK_a of the sulphate ester groups and the size increased to 120 nm (Fig. 7.10a) as a result of the decrease of the charge density.

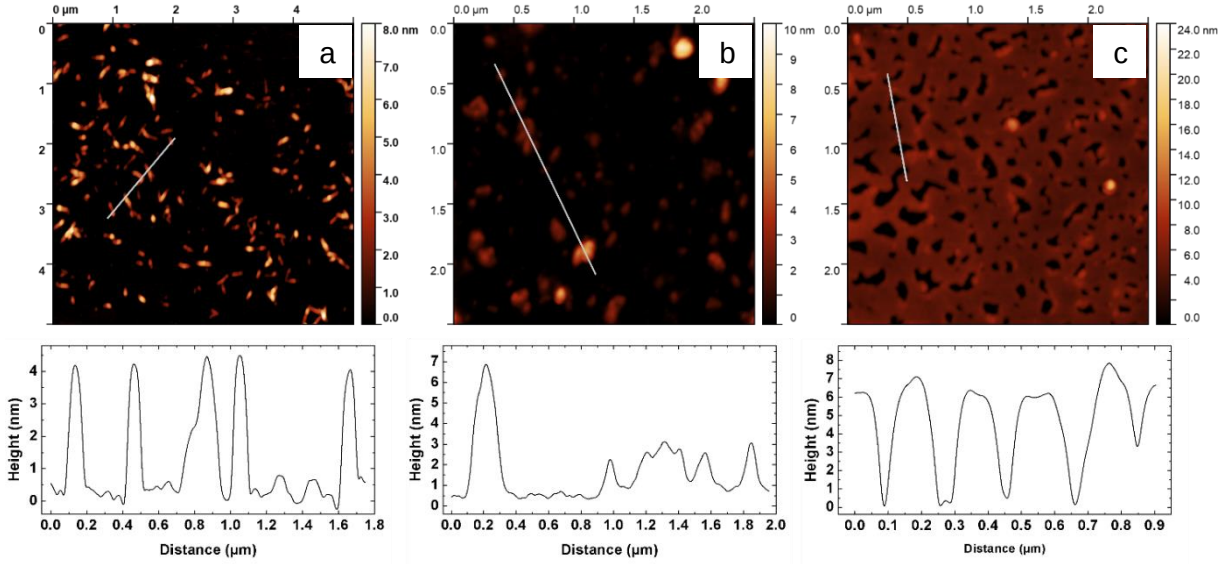


Fig. 7.9 AFM micrographs and corresponding height profiles (below) of dried aqueous dispersions of a) CNC, and CAS at b) pH 7 and c) pH 3.

The behaviour of CAS was more complex and large differences could be noticed when changing the pH, as judged by the AFM pictures Fig. 7.9b and 7.9c. At pH 7, globular aggregates with lateral dimensions of 40 to 150 nm can be seen, resulting from the assembly of smaller CAS units. When the pH was decreased to 3, the AFM images showed a film-like structure. These results agree with the AFM analysis of casein micelles from simulated milk ultra-filtrate, where micelle size in the range from 75 to 400 nm was reported (Ouanezar, et al. 2012).

DLS appeared to be a more suitable technique for the analysis of CAS and the mean particle size as a function of pH is presented in Fig. 7.10b. While the size of CAS particles varied only slightly below pH 3.5 and above pH 5, aggregation occurred between pH 4 and 5, i.e. at pH close to the isoelectric point of CAS (Liu, Y. and Guo 2008; Fox and Brodkorb 2008; Post, et al. 2012; Gorji, et al. 2015).

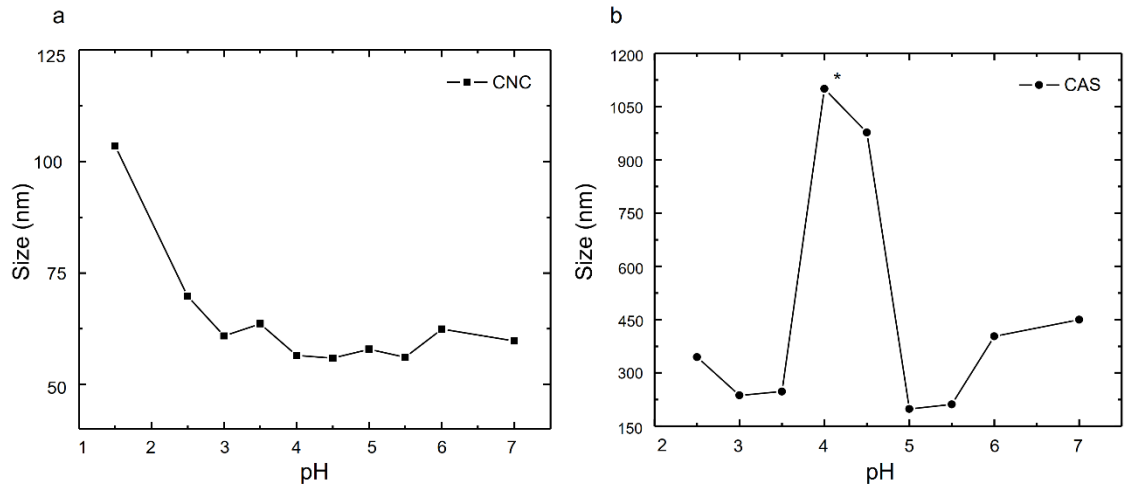


Fig. 7.10 Size of a) CNC and b) CAS as a function of pH (: at pH 4, sedimentation occurred).*

As NaCl and $CaCl_2$ are often used to boost the emulsifying properties of CNC by reducing the electrostatic repulsion (Mikulcova, et al. 2016; Mikulcova, et al. 2018; Capron and Cathala 2013), the size development of CAS and CNC with the change of salt concentration was determined (Fig. 7.11). In this respect, it is worth noting that the multivalent ions were more efficient in inducing aggregation of CNC than monovalent (Tuan Phan-Xuan, et al. 2016). Our measurements confirmed the strong effect of salt on the aggregation of CAS and CNC, and the predominant role of the valence of the ions on the aggregation. Ca^{2+} caused a rapid increase in the size of CNC at low concentration (0.25 mM) and a similar effect was observed for CAS with the formation of large aggregates that rapidly sediment. The presence of Ca^{2+} is also known to strongly influence the properties of CAS because certain casein types (e.g. α_{S1}- and α_{S2}-) bind Ca^{2+} thereby reducing the net charge and electrostatic repulsion between particles (Thomar, et al. 2014; Ye and Singh 2001). In comparison with $CaCl_2$, higher concentrations were needed for NaCl to trigger the loss of CAS stability (> 10 mM).

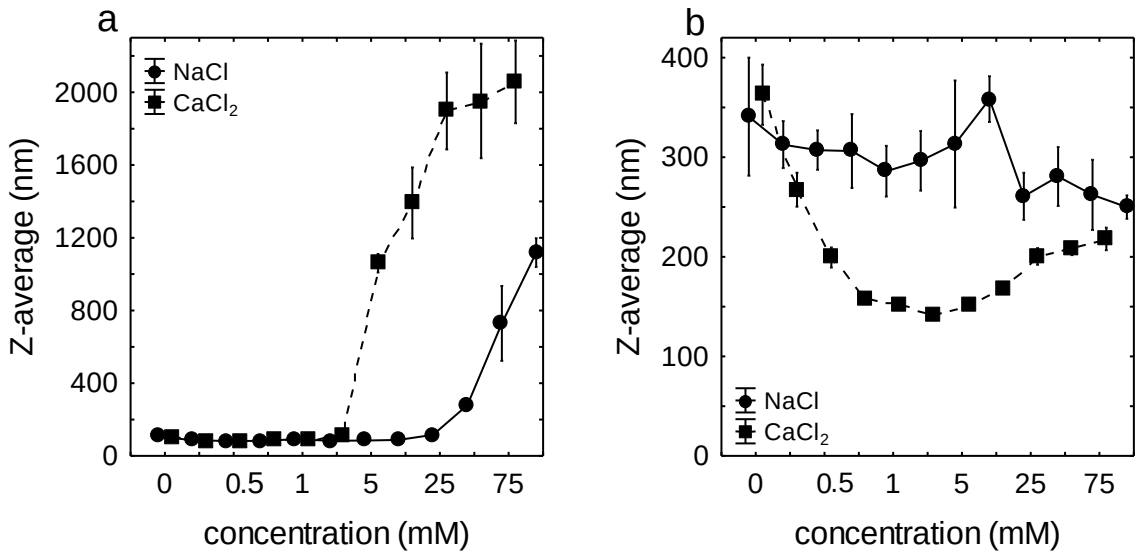


Fig. 7.11 Particle size of CNC (a) and CAS (b) as a function of NaCl and $CaCl_2$ concentration.

These measurements enabled us to define the pH range at which strong attractive interactions between CNC and CAS would occur, which constituted the basis on which we have designed the emulsification routes (R1, R2 and R3) in the relation to the order of addition/mixing of CNC and CAS.

Behaviour of CNC and CAS in solution

The interaction of CNC and CAS was investigated at pH 7, 5.5, 4 and 3, as these values are both below and above the isoelectric point of CAS (*pI* ~ 4.6) (O'Kennedy 2011). Suspensions with ratios CNC/CAS of 100:1, 20:1, 10:1, 2:1, 1:1, 1:5 and 1:10 with a total solid content of 0.1 wt% were prepared and visually observed. AFM was conducted on the dried films of the mixtures.

At pH above the *pI* of CAS, both CNC and CAS were negatively charged (Mikulcova, et al. 2018; Post, et al. 2012) and, therefore, no visible changes/agglomeration could be observed at pH 5.5 and 7, for all mixing ratios. At pH 4, close to the *pI* of CAS, however, CAS aggregation occurred in presence of the negatively charged CNC, from CNC/CAS ratio 2:1 to 1:10, see Fig. 7.12a. At this pH, the absence of any net charge on the molecules of CAS led to a minimum in the intramolecular charge repulsion and caused CAS to be strongly folded.

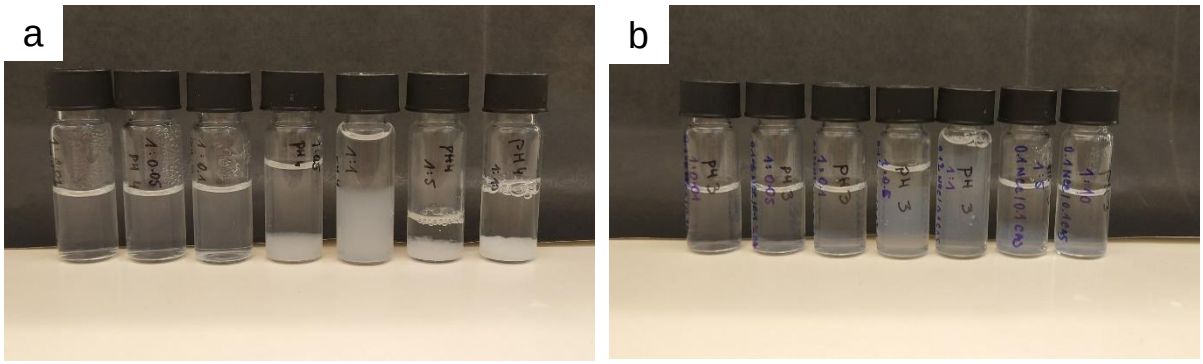


Fig. 7.12 Visual observation of mixtures CNC/CAS suspensions (0.1 wt%) at ratios from left: 100:1, 20:1, 10:1, 2:1, 1:1, 1:5 and 1:10 at a) pH 4 and b) pH 3.

At pH 3, CAS was positively charged, whereas CNC still held a negative charge, which enabled strong attractive interactions. At the ratios of CNC/CAS from 2:1 to 1:10 the original dispersions formed a turbid gel (Fig. 7.12b).

The nature of the aggregates/complexes was further investigated using AFM, which showed that for the CNC/CAS mixed in ratio 1:1 (Fig. 7.13a), CAS almost entirely covered CNC particles and, as expected, at lower CNC/CAS ratio (2:1), free CNC particles could be observed (Fig. 7.13b). In fact, the attractive interaction between CNC and CAS was reversible, and the formed gel was reverted to a perfectly transparent suspension upon pH increase. These findings are consistent with the results reported by Liu, et al., who studied surface modification of CNC with bovine serum albumin (BSA) through electrostatic interactions of positively charged BSA at pH 3 (Liu, F., et al. 2018).

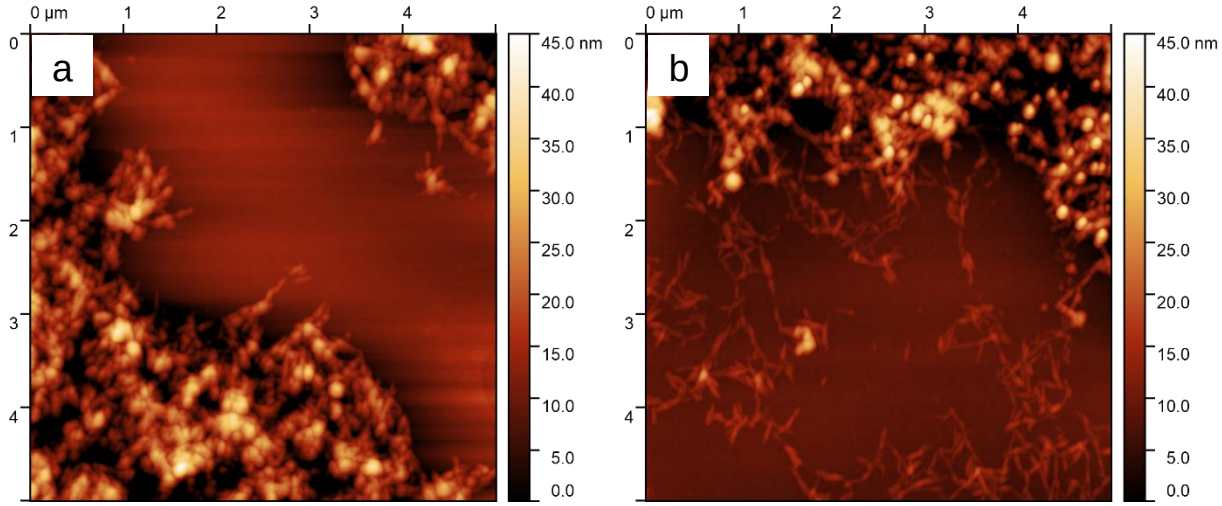


Fig. 7.13 AFM visualization of interactions between CNC/CAS mixed with ratio 1:1 (a) and 2:1 (b) at pH 3; excess of CNC is notable as free needle-like particles.

Characterization of CNC/CAS mixture behaviour of at the interface

The next part of this study investigated the behaviour of CNC/CAS complexes at interfaces, and more specifically their function in the stabilization of the oil-water interface. Therefore, we conducted adsorption measurements at the solid surface using quartz crystal microbalance with dissipation monitoring (QCM-D),

and surface and interfacial tension measurements at the hexadecane-water interface using the pendant drop technique. The adsorption measurements were conducted at pH 7 and 3.

Adsorption at solid surface

To study the effect of pH on the successive adsorption of CNC and CAS, QCM-D crystals coated with a silicon dioxide layer were modified with polyethyleneimine as anchoring film followed by the deposition of a thin layer of CNC. The AFM image of the CNC-coated QCM-D crystal surface is provided in Fig. 7.14.

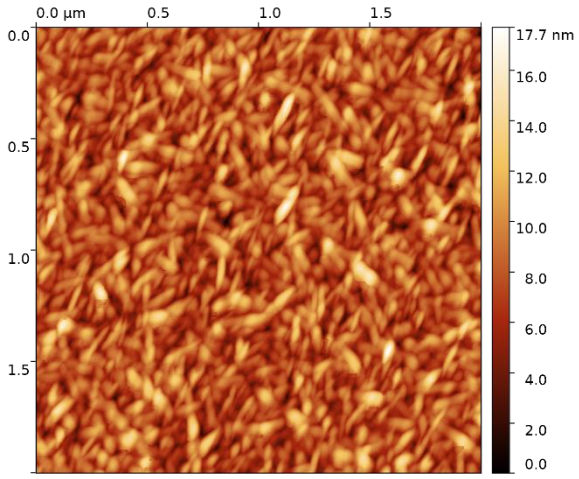


Fig. 7.14 The AFM image of the CNC coated QCM-D crystal surface height.

Fig. 7.15 shows the mass uptake of the QCM-D crystals at pH 7 and 3, left and right respectively. For both pHs, the sequence of injection was the same. First, the CNC covered crystals were exposed to water adjusted at the studied pH. CAS suspension was then flowed into the measurement chamber, until a steady state was reached, followed by a rinsing step. This led to the adsorption of a layer of CAS that was finally exposed to a CNC suspension prior to a final rinsing step.

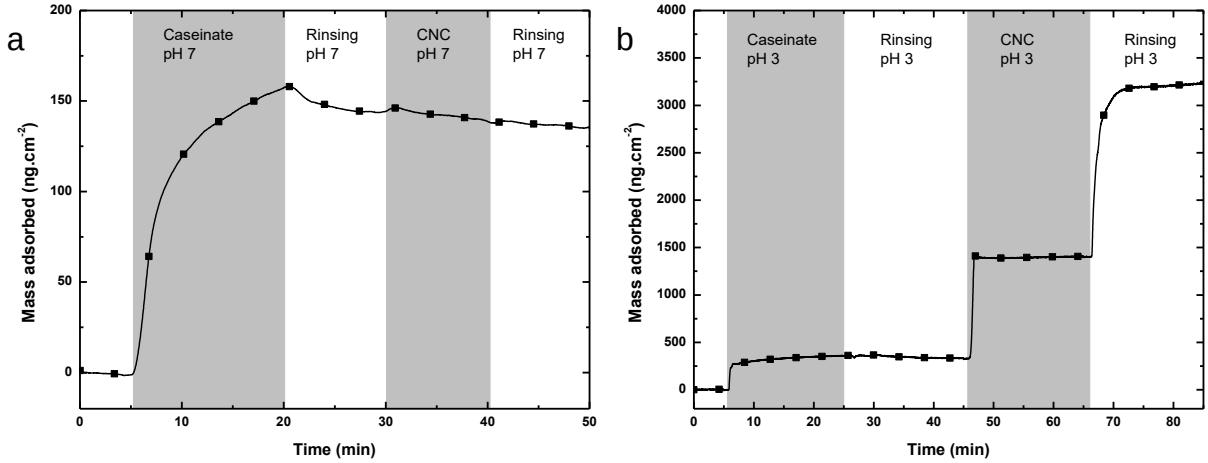


Fig. 7.15 Adsorption of CAS on CNC surface at pH = 7 (a) and pH = 3 (b) as determined by QCM-D.

At pH 7, CAS adsorbed readily onto the CNC-coated surface, most likely through hydrophobic interactions, as CAS presents an amphiphilic character. With the rinsing step a small portion of CAS desorbed, and the mass remained constant at around 150 ng.cm^{-2}. As expected, CNC did not adsorb under this pH condition as both CNC and CAS bear the same charge. The dissipation remained below 1x10^{-6}, which validates the use of the Sauerbrey equation to convert frequency shift to mass (Fig. 7.16).

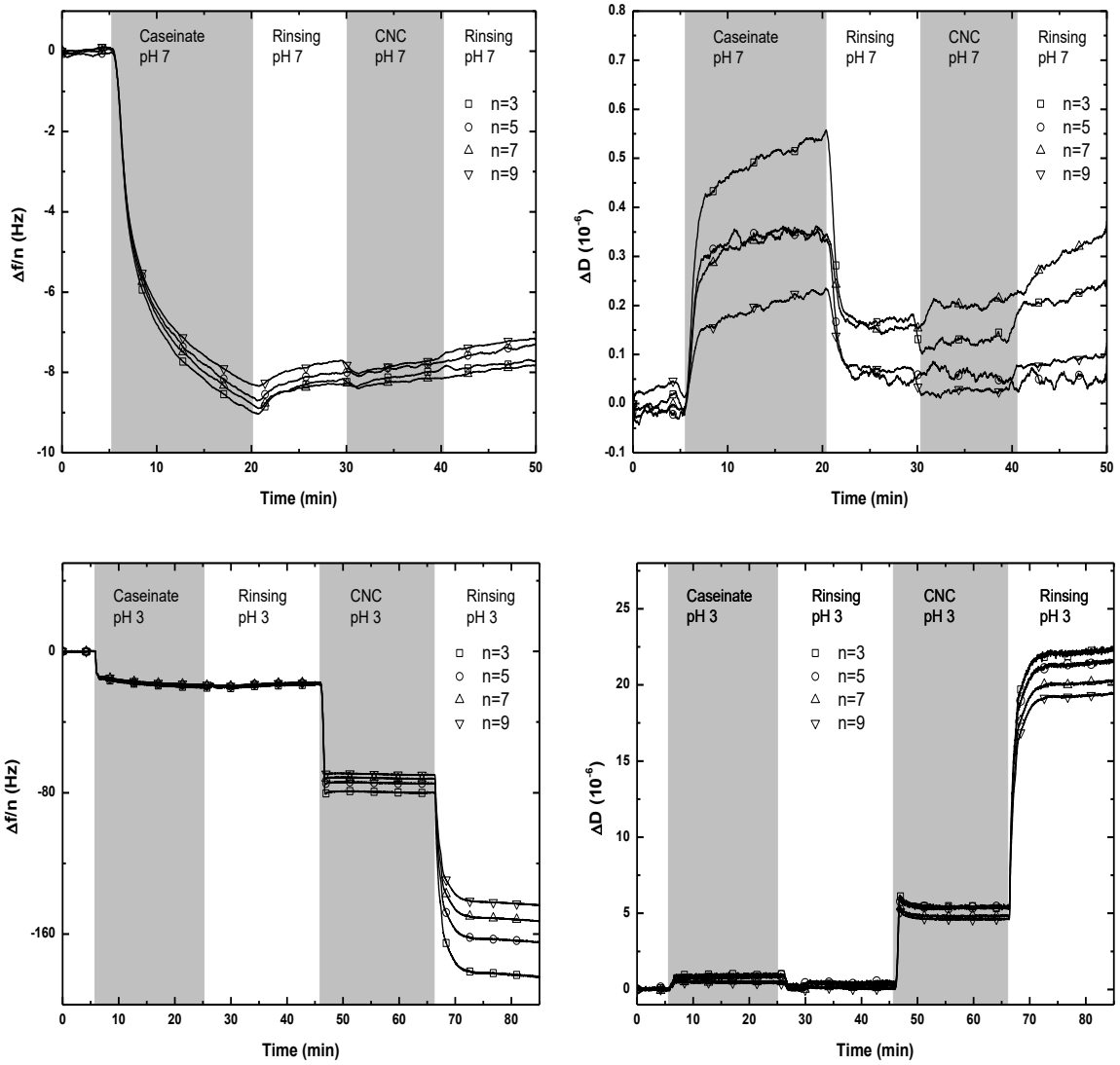


Fig. 7.16 Variation of frequency and dissipation recorded by QCM-D at the 3rd, 5th, 7th, and 9th overtones. The variations of frequency are normalized to the overtone number. Top: adsorption experiment conducted at pH 7, bottom: adsorption experiment conducted at pH 3.

At pH 3, the positive charge of CAS drove the adsorption and a plateau at around 360 ng.cm^{-2} was reached. The following rinsing step, at the same pH, did not induce any significant desorption. The overall positive charge of the CAS layer enabled very strong adsorption (1390 ng.cm^{-2}) upon the injection of CNC in the next step. At this stage, it is worth mentioning that the dissipation was about

$4x10^{-6}$, which implies that the determination of the mass adsorbed was over-estimated and that an accurate determination would imply processing the data using, for instance, the Kevin-Voigt model. This, however, is beyond our intentions that are to show the difference in adsorption behaviour between pH 3 and 7. The increase in dissipation continued during the last rinsing step along with large mass uptake, most likely due to the reorganization of the CNC/CAS multilayer. This is confirmed by the large spreading with the overtone number of the frequency response, see Fig. 7.16.

Surface and interfacial tension

Surface and interfacial activity of CAS and CNC were measured using the pendant drop technique, at pH 7 and 3. First, the activity of the individual components, that is CAS and CNC, was determined, and then their mixtures CNC were tested, to evaluate their capacity to compete for the interface. These measurements constitute the basis of the rationalization of the emulsification experiments.

Surface activity of CNC and CAS solutions was measured at pH 7 at particle concentrations ranging from 0.01 to 2 wt% and the results are shown in Fig. 7.17. With the increasing concentration of CNC, the surface tension did not vary and remained at 70.8 ± 0.3 $mN.m^{-1}$ (Fig. 7.17a). This evidenced the absence of surface activity for the cellulose particles, as previously reported (Hu, Z., Patten, et al. 2015). On the other hand, proteins are known for their surface activity, emulsifying and foaming properties (Abascal and Gracia-Fadrique 2009). CAS exhibited good surface activity and was able to decrease surface tension already at a very low concentration (Fig. 7.17a). With increasing concentration of CAS, the surface tension decreased from 67.4 ± 0.3 to 51.8 ± 0.1 $mN.m^{-1}$ at 0.2 wt% of CAS, and an additional increase of CAS concentration to 2 wt% did not further influence the surface tension. These values of caseinate surface tension are in agreement with results published by others (Abascal and Gracia-Fadrique 2009; Arboleya and Wilde 2005).

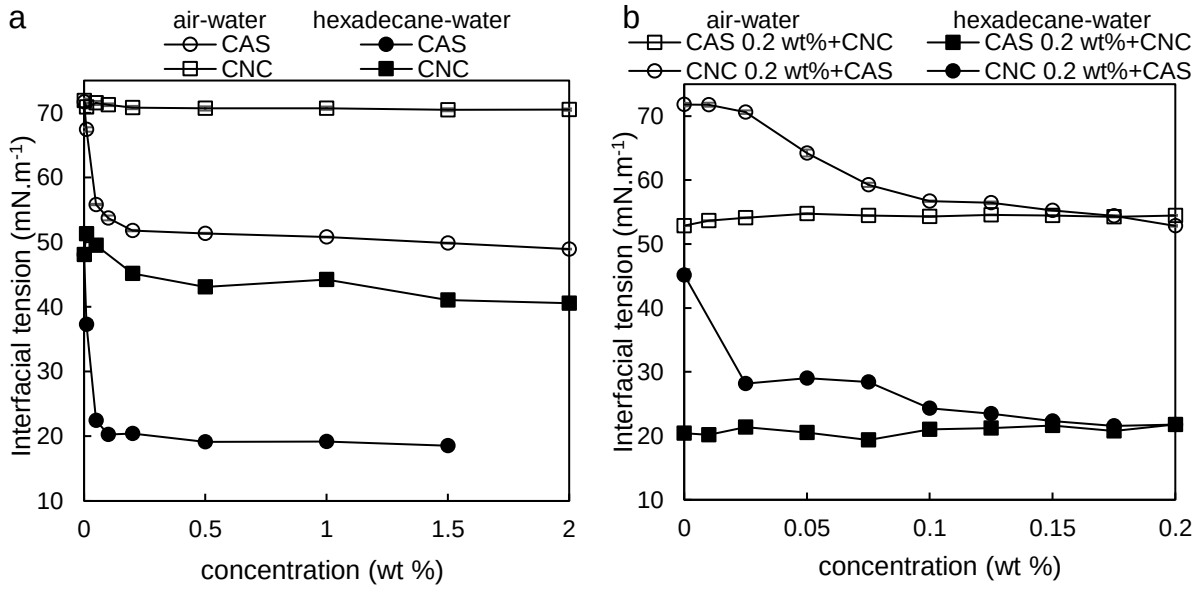


Fig. 7.17 Surface tension at pH 7 of CNC and CAS dispersions a) and their mixtures b) at air-water (empty symbol) and hexadecane-water interface (plain symbol). The error was below 1 and 2 % for the measurements at the air-water and oil-water interface, respectively.

The trend was, however, different for the hexadecane-water interface at pH 7. Here, CNC particles were able to reduce the interfacial tension of the hexadecane-water interface (from 51.3 ± 0.7 to 40.5 ± 0.3 $mN.m^{-1}$) due to their intermediate wettability since hexadecane is less hydrophobic than air (Fig. 7.17a). A similar effect was observed at the dodecane-water interface (Capron, et al. 2017). More surface-active CAS decreased interfacial tension at the hexadecane-water interface from 48.1 ± 0.7 $mN.m^{-1}$ to 18.5 ± 0.1 $mN.m^{-1}$.

The values of surface and interfacial tension were then determined for the mixed systems where CAS (or CNC) concentrations were kept at 0.2 wt% while CNC (or CAS) were added. This lower concentration (0.2 wt%) was in agreement with the concentration range used for the emulsion preparation, see below. The results are shown in Fig. 7.17b. As one could anticipate, increasing concentration of CNC in CAS dispersion (0.2 wt%) did not cause any significant changes in the interfacial tension as a result of repulsive interactions between CAS and CNC, combined with the lack of surface activity of CNC. A similar trend was observed at the hexadecane-water interface, as the CAS interfacial activity dominated. On the other hand, when CAS was added to a CNC dispersion, the surface tension decreased gradually from 71.8 ± 0.3 in absence of CAS to 52.8 ± 0.1 $mN.m^{-1}$ for 0.2 wt% CAS at the air-water interface, and from 45.1 ± 0.9 in absence of CAS to 21.8 ± 0.1 $mN.m^{-1}$ for 0.2 wt% CAS at the hexadecane-water interface. This clearly demonstrated that the most surface-active compound was the one ruling the interfacial behaviour, in the case where there was no strong attractive interaction between the two compounds.

The corresponding measurements were conducted at pH 3 (Fig. 7.18) and showed that the lowering of pH did not influence the activity of CAS at the air-

water interface nor the hexadecane-water interface. In the case of CNC, the activity at the oil-water interface was lowered and an increase from 37.9 ± 0.3 to 49.7 ± 0.7 mN.m^{-1} was measured. This can be attributed to the loss of stability of CNC stability at low pH (Mikulcova, et al. 2018).

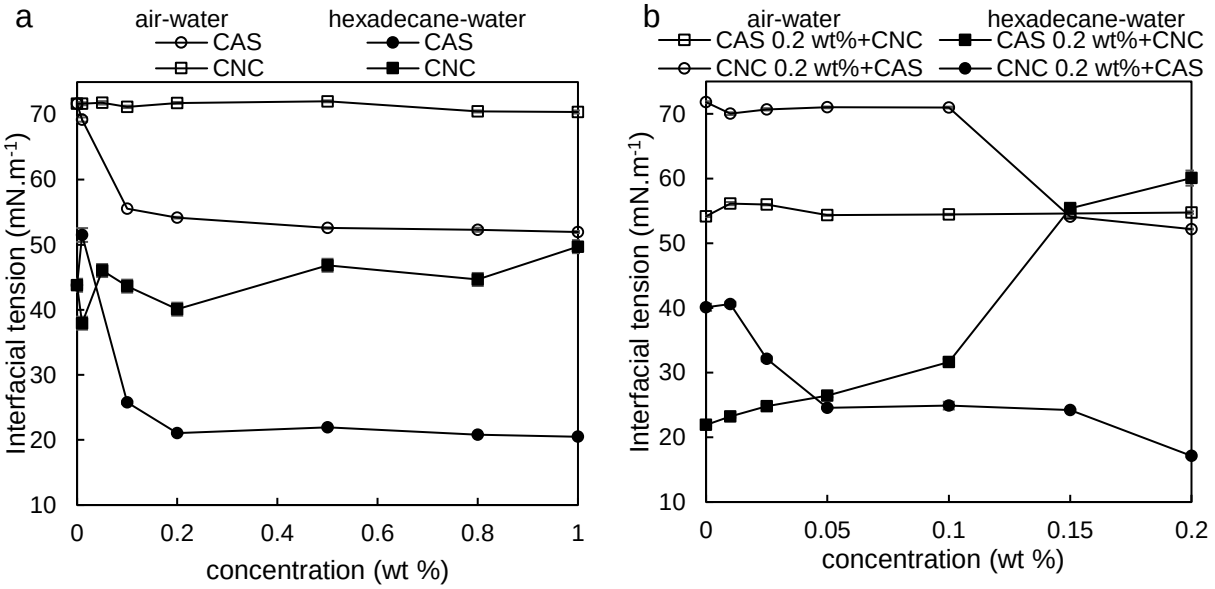


Fig. 7.18 Surface tension at pH 3 of CNC and CAS dispersions (a) and their mixtures (b) at air-water (empty symbol) and hexadecane-water (plain symbol) interface (pH 3). The error was below 1% and 2% for the measurements at the air-water and oil-water interface, respectively.

Interfacial tension of the mixed systems CAS/CNC and CNC/CAS as a function of increasing concentrations of CNC and CAS, respectively, is shown in Fig. 7.18b. The interfacial behaviour of CAS (0.2 wt%) with increasing concentration of CNC did not exhibit major changes, even though at pH 3 the positively charged CAS interacted with the negatively charged CNC particles. Interestingly, this interaction was not sufficient to deplete the interface from the surface-active CAS.

At the air-water interface, in the case where CAS was added to a CNC dispersion at 0.2 wt%, CAS did not significantly affect the surface tension within a concentrations range 0.01 to 0.10 wt%. However, surface tension rapidly decreased to 54.6 ± 0.1 mN.m^{-1} at 0.15 wt% CAS. This drop was also more noticeable at pH 7, where the interfacial tension gradually reduced. The sudden decrease in surface tension at pH 3 might be caused by the micellization of CAS on the surface of CNC particles, thus more CAS was required to be able to reduce surface tension thus explaining the higher CAS concentration observed.

At the hexadecane-water interface, the interfacial tension rapidly increased with the introduction of CNC in the CAS dispersion (from 23.2 ± 0.2 mN.m^{-1} at 0.01 wt% CNC to 60.1 ± 1.2 mN.m^{-1} at 0.2 wt% CNC). In that case, CAS was likely displaced from the interface through interaction with CNC. On the other

hand, in the opposite situation where CNC was added to CAS, the interfacial tension decreased from 40.6 ± 0.3 to 17.1 ± 0.2 $mN.m^{-1}$.

Competitive emulsification by CAS and CNC

Three different routes for the preparation of emulsions were investigated and are presented in Fig. 7.1. Moreover, these emulsions were formed at pH 7 and pH 3, at which CNC and CAS had repulsive or attractive interactions, respectively. The emulsions were composed of 20 wt% hexadecane and had a total particle concentration of 0.2 wt%. In route R1, o/w emulsions were prepared by direct emulsification of oil in presence of 1:1 mixture of CNC/CAS particles with either NaCl or $CaCl_2$ as background electrolytes. In route R2, at first, a primary o/w emulsion stabilized with CAS was formed (referred to as PE-R2) followed by short sonication in presence of CNC and each of the background electrolytes. Preparation of emulsion *via* route R3 started from primary emulsion stabilized with CNC (referred to as PE-R3) in presence of either NaCl or $CaCl_2$, which was then shortly emulsified with CAS. To investigate the possibility of inducing attractive interaction between CNC and CAS, the pH was adjusted in different ways, and results from emulsions prepared with aqueous phases at pH 7, at pH 3, and from emulsions prepared at pH 7 and then adjusted to pH 3 were reported.

The samples were characterized by laser diffraction analysis and optical microscopy. To assess the composition of the non-adsorbed species, the emulsions were gently centrifuged and the supernatant dried on a silicon wafer to be analysed by AFM.

Emulsion prepared at pH 7

Before investigating the competitive behaviour of CAS and CNC for the oil-water interface in an emulsification process, their individual performance was tested. The size distributions of primary emulsions (PEs) stabilized with only one type of particle are shown in Fig. 7.19, and reveal that in all the cases, there was always a substantial amount of free CAS under aggregated/micellar form, as well as free CNC, revealed by the peak below 1 μm.

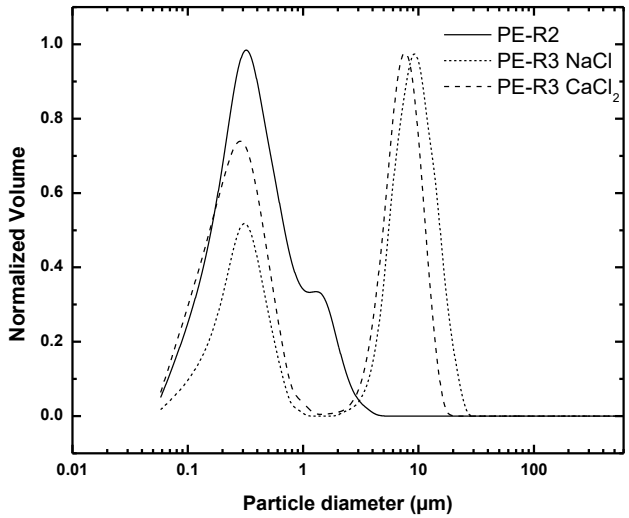
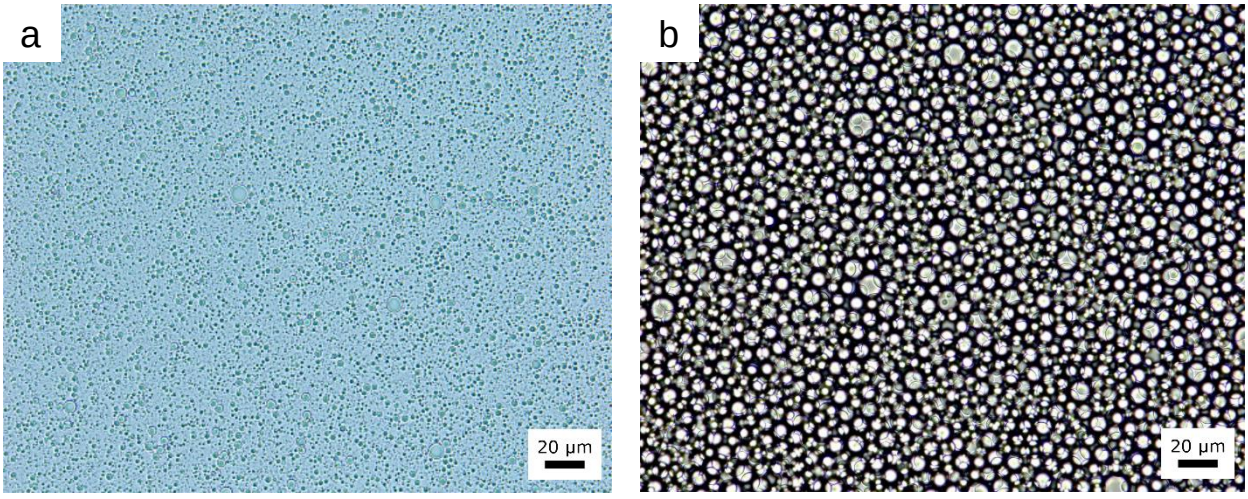


Fig. 7.19 Size distribution curves of the primary emulsions PE-R2, PE-R3 $CaCl_2$, PE-R3 NaCl at pH 7.

The mean diameter of emulsion droplets stabilized by CNC alone was 7.5 ± 0.1 µm (PE-R3 $CaCl_2$) and 9.2 ± 0.1 µm (PE-R3 NaCl), whereas the sample prepared with CAS had droplets with a mean diameter of 2.5 ± 0.1 µm. CAS at a concentration of 0.2 wt%, however, showed weak emulsifying capacity with about 70 % of emulsified oil. The optical microscopy conducted on the primary emulsions confirmed this tendency (Fig. 7.20), with CAS-stabilized emulsion droplet being substantially smaller. This is in agreement with the higher interfacial activity of CAS and underlines that CAS behaves like a traditional low molecular weight emulsifier.



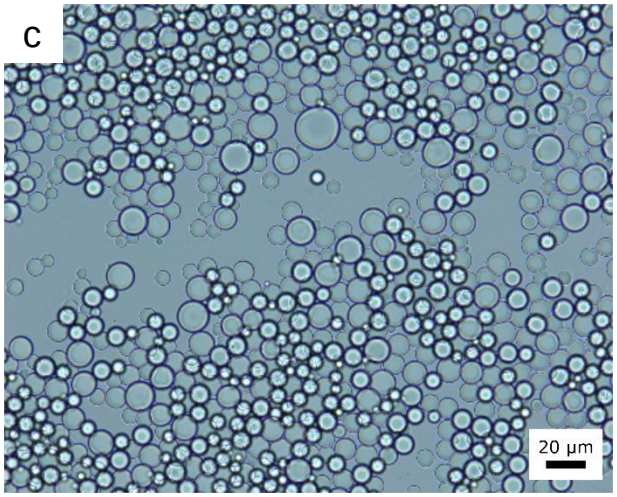


Fig. 7.20 Optical micrograph of primary emulsions at pH 7 prepared by route a) PE-R2 (CAS-emulsion), b) PE-R3 NaCl (CNC-emulsion), c) PE-R3 $CaCl_2$ (CNC-emulsion).

CAS/CNC mixture (route **R1**, Fig. 7.21a), showed poor emulsifying capacity with 25 % of free oil. However, in the presence of NaCl (R1-NaCl), the size of the emulsion droplets was in-between those measured for droplets stabilized with CNC and CAS alone. Emulsions with $CaCl_2$ (R1-$CaCl_2$) had a multimodal distribution with a primary droplet size of 4 µm and large flocks of about 50 µm.

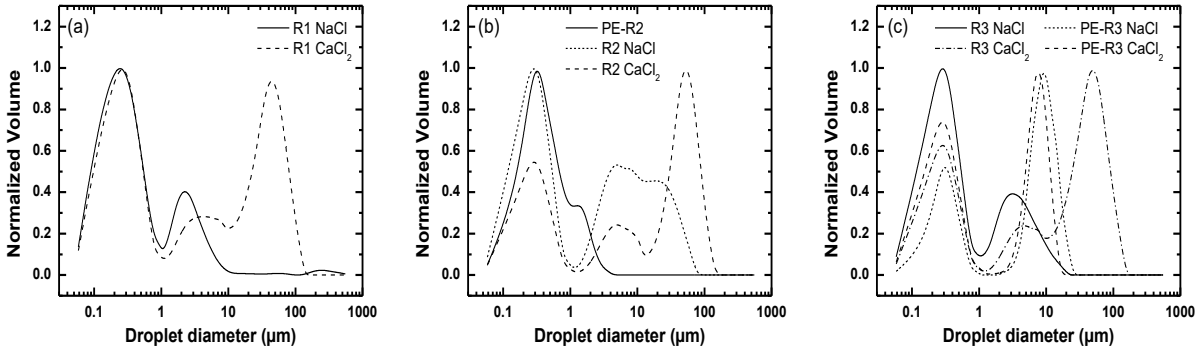


Fig. 7.21 Distribution curves of the emulsions prepared via route a) R1, b) R2 and c) R3 at pH 7. PE-R2 and PE-R3 stand for primary CAS and CNC emulsion, respectively.

This tendency to flocculate was confirmed by optical microscopy, see Fig. 7.22. Presumably, the flocculation was caused by aggregation of CAS in the presence of Ca^{2+}. In route R1, in the presence of NaCl, no flocculation could be noted.

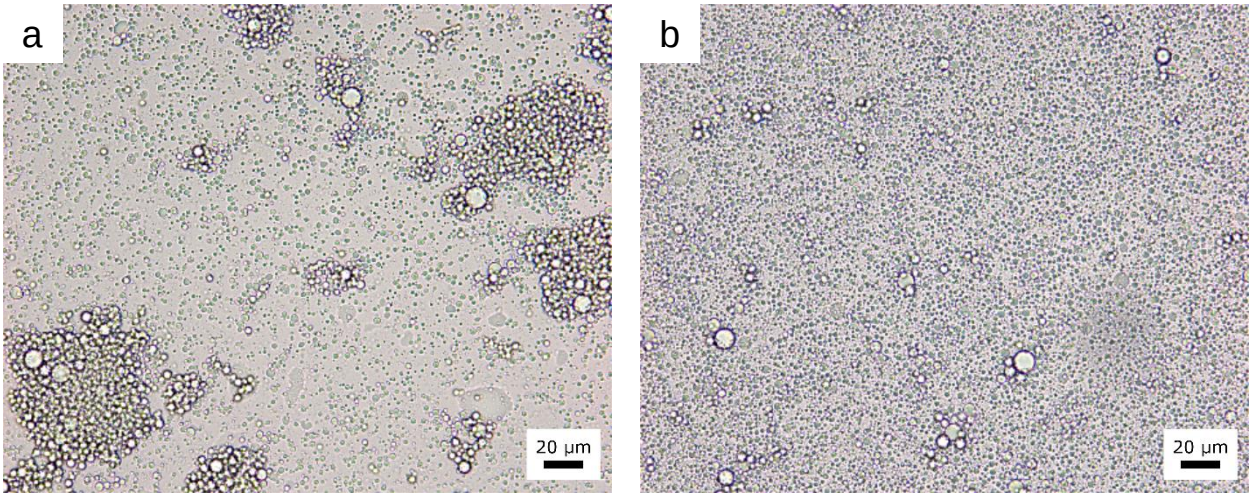


Fig. 7.22 Optical micrograph of emulsions prepared by route R1 $CaCl_2$ a), R1 NaCl b), at pH 7.

In this route, competition between CNC and CAS might occur, however, due to its higher surface activity, CAS might probably dominate (Sarker, D. K., et al. 1999). The hypothesis of adsorption of CAS on CNC is less likely as, at this pH, there was no attractive interaction between species as shown by QCM-D. The AFM analysis of the aqueous phase after centrifugation of the emulsion revealed the presence of a mixture of CNC and CAS particles (see Fig. 7.23) suggesting no or very limited preferential sorption of one of the emulsifiers onto emulsion droplets.

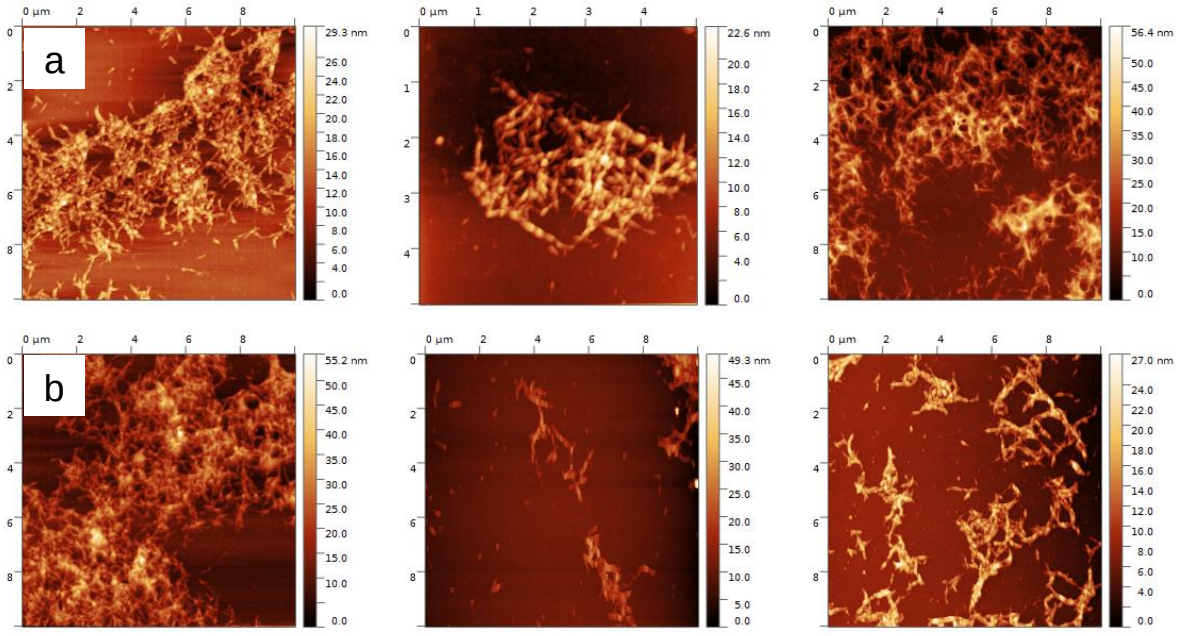


Fig. 7.23 AFM of the supernatant from emulsions with a) $CaCl_2$ and b) NaCl. From left to right: route R1, R2, R3.

The addition of CNC and $CaCl_2$ to a CAS stabilized primary emulsion (Route 2, **R2**) caused not an only aggregation of CAS and flocculation of the droplets (as supported by optical microscopy, Fig. 7.24), but it also caused increasing of the

encapsulation efficacy as the amount of free oil after emulsification decreased. Distribution curves (Fig. 7.21b) were also changed and the shoulder of the primary emulsion shifted towards larger droplet sizes together with the appearance of flocks. The flocculation was also confirmed by optical microscopy (see Fig. 7.24), which showed two populations of droplets together with flocks composed thereof, which were more abundant, as expected, in presence of $CaCl_2$ than in presence of NaCl. The smaller droplets were most likely stabilized primarily by CAS and the subsequent addition of CNC further improved coverage of droplet interface and improved stability of emulsion droplets. It can be also suggested that CNC particles probably stabilized larger emulsion droplets seen on the image, which were formed from the remained free oil. The analysis of the supernatant after centrifugation of emulsions revealed that CNC was dominating (see Fig. 7.23).

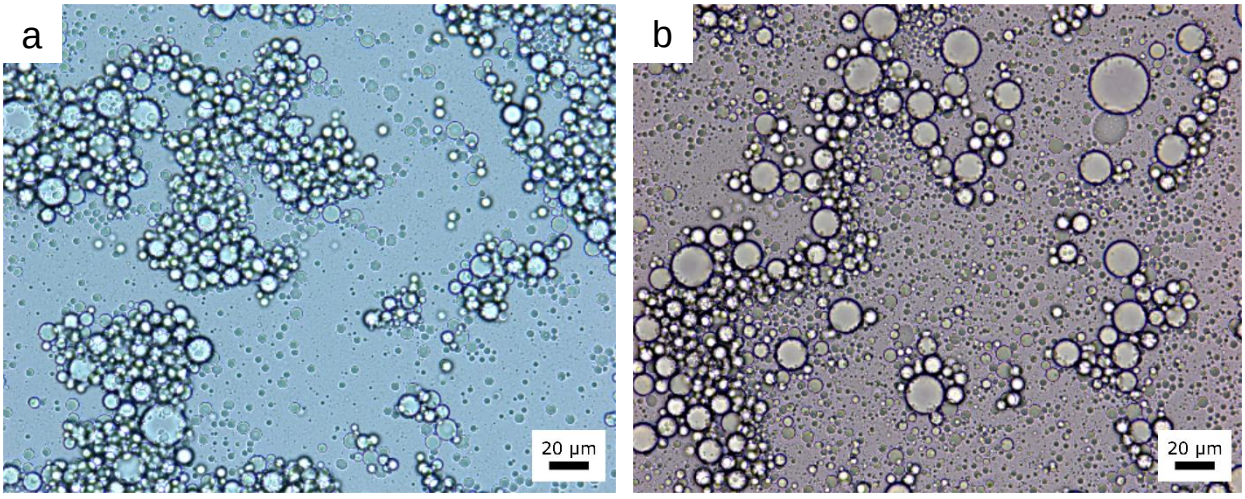


Fig. 7.24 Optical micrograph of emulsions prepared by route R2 at pH 7: a) $CaCl_2$, b) NaCl.

These results are corresponding with previous work on whey protein isolate emulsions containing CNC, where the addition of CNC particles caused an increase in particle size with a large fraction of droplets within the 10–100 µm size range (Sarkar, et al. 2017).

The emulsifying capacity of Route **R3** was high with the absence of free oil, and this was observed already for the primary emulsions. The primary emulsion, in presence of NaCl or $CaCl_2$, showed droplets of relatively large sizes, with peaks around 9.2 and 7.2 µm, respectively. Interestingly the emulsion prepared with CNC and $CaCl_2$ did not have flocks (see optical microscopy, Fig. 7.20c).

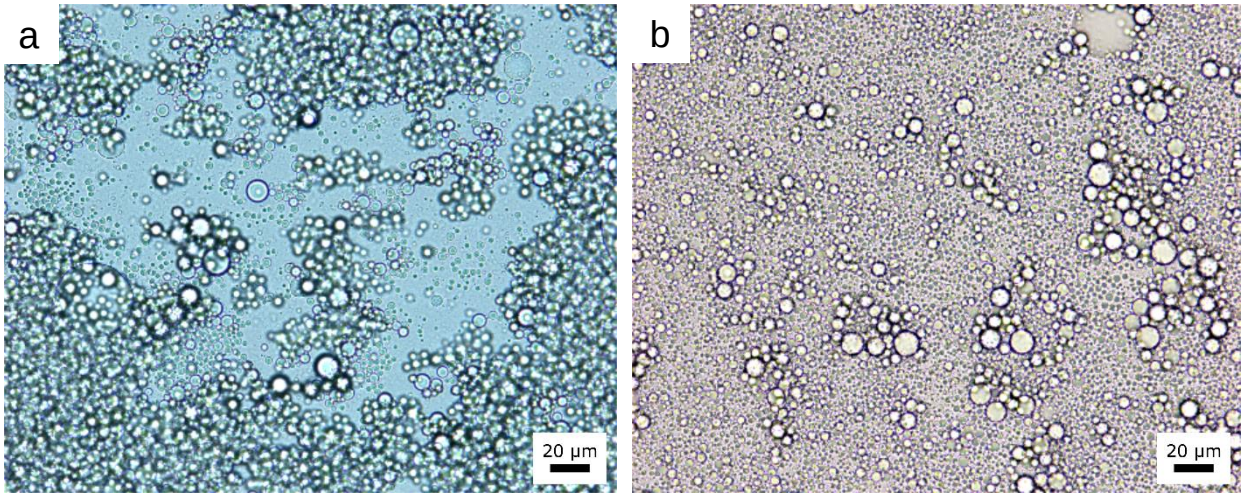


Fig. 7.25 Optical micrograph of emulsions prepared by route R3 at pH 7: a) $CaCl_2$, b) NaCl.

The subsequent addition of CAS significantly decreased the droplet size from 9.2 to 3.2 µm (NaCl), and from 7.2 to 4.6 µm ($CaCl_2$), hence demonstrating the synergy between CNC and CAS. Again, the introduction of Ca^{2+} led to the flocculation of the emulsion droplets. It is actually notable that calcium did not induce flocculation of the primary emulsion, but that it occurred together with CAS and yielded a new droplet population at 50 µm. The interfacial activity of CAS being higher than that of CNC, one can speculate that once the emulsions were stabilized by CNC, the introduction of surface-active CAS helped to increase the curvature by allowing a further reduction of the interfacial tension. A second possible mechanism that might occur during emulsion formation *via* R3 is the partial depletion of CNC originally present at the oil-water interface and its replacement with the added CAS. This mechanism, however, is not too probable as particles once adsorbed at the interface have very high energy of desorption (Binks, B. P. 2007; Giermanska-Kahn, et al. 2005). One can argue that small-molecule surfactants can easily displace particles from the interface by modifying the contact angle and making the particle more hydrophilic (Whitby and Wanless 2016). Nevertheless, this is not the case of the CAS/CNC system. Displacement of CNC from the interface through its modification with CAS is not plausible. At pH 7, CAS is not capable of adsorption on the CNC surface as both CAS and CNC are negatively charged and, therefore, CAS is not able to modify the wettability of CNC and trigger the displacement.

Moreover, the flocculation in emulsion prepared by R3 with CAS added to dispersion phase (R3-$CaCl_2$) was higher than in R2-$CaCl_2$ emulsion where CAS was primarily attached at the hexadecane-water interface and AFM analysis of aqueous phase of R3 emulsion after centrifugation showed a higher ratio of non-absorbed CAS to CNC (see Fig. 7.23).

Emulsions prepared at pH 3

The situation when conducting the emulsification at pH 3 was significantly more complex as there was a net attractive electrostatic interaction between CNC and CAS. Under the concentration regime of the particles used for emulsification, no macroscopic gelling was observed, this was a prerequisite for the study. This resulted already in important differences in the distribution for the emulsion prepared only with CAS or CNC, as can be seen in Fig. 7.26, and in Fig. 7.27. Typically, at pH 3, CNC and CAS tended to aggregate, as shown earlier, and the main droplet size centered around 10–12 μm was present. The presence of NaCl or $CaCl_2$ had a minor influence.

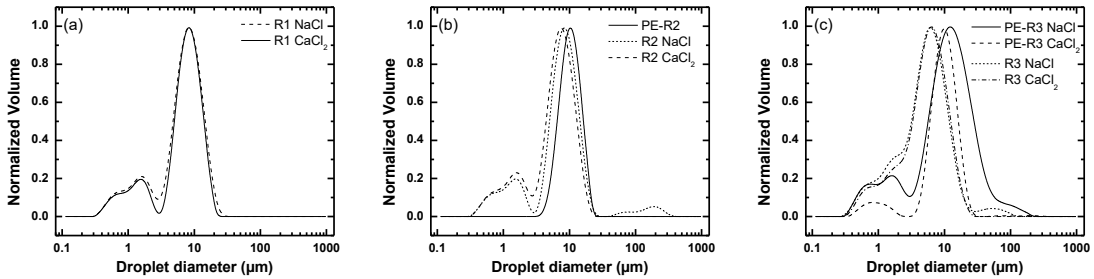


Fig. 7.26 Distribution curves of the emulsions prepared via route a) R1, b) R2 and c) R3 at pH 3. PE-R2 and PE-R3 stand for primary CAS and CNC emulsion, respectively.

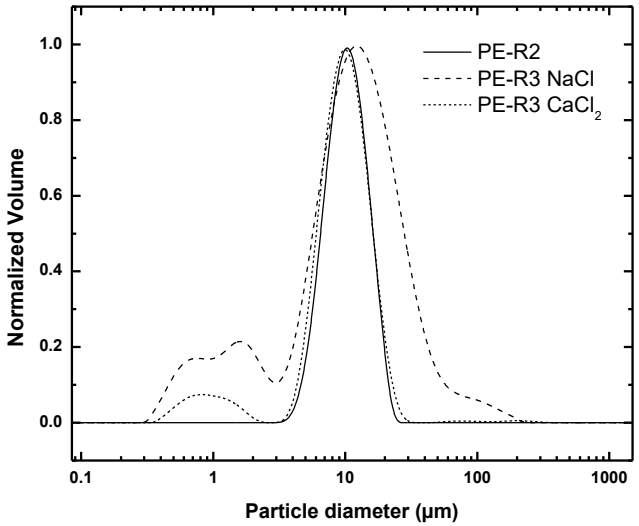


Fig. 7.27 Size distribution curves of the primary emulsions PE-R2, PE-R3 $CaCl_2$, PE-R3 NaCl at pH 3.

The mixing at pH 3 (route R1) yielded in the first place more aggregates/complexes, that were most likely the active emulsifier, as indicated by the presence of a convoluted peak at 1.5 μm. The emulsion droplet size, centered on 8.3 μm, was slightly lower compared to droplets stabilized with CNC and CAS alone, indicating that the aggregates worked slightly better than the two stabilizers alone. Overall the influence of salt was limited and the attractive interaction between CAS and CNC dominated over salt screening effect (Fig. 7.26a).

The addition of CNC in presence of salt to a CAS-stabilized emulsion (route R2) induced a decrease of the size of the primary emulsion from 10.3 μm to 8.0 and 7.5 μm, for NaCl and $CaCl_2$, respectively (Fig. 7.26b). Again, one can notice a new droplet population at 1.5 μm, similar to route R1. In that case, the addition of CNC did have little influence on the interfacial tension, as observed by interfacial tension measurements. The most important change was the substantial increase in viscosity of the emulsion that appeared to be gelled and capable of withstanding an inverted-bottle test, as shown in Fig. 7.28. The gelling can be attributed to the formation of a network composed of CNC, CAS and droplets.

A similar effect was observed with route R3 (Fig. 7.26c), and the addition of CAS to a CNC-stabilized emulsion promoted the most important droplet size reduction, compared to R1 and R2. An increase of viscosity could also be noted, but to a lesser extent than for R2. This more significant reduction in size is supported by the decrease in interfacial tension observed when CAS was added to a CNC suspension. In that case, CAS is likely to adsorb at the interface through attractive interaction, as demonstrated by QCM-D, and helps to decrease interfacial tension thus facilitating the size reduction of the droplets.

Emulsions prepared at pH 7 then lowered to pH 3

As an alternative way to prepare emulsions at pH 3, the effect of decreasing the pH of emulsions prepared at pH 7 was tested. Under these conditions, CNC and CAS had no attractive interaction during the emulsification step, while the decrease in pH of emulsions was meant to gradually induce attraction between CAS and CNC. It is also worth noting that at pH 7 no gelling could be observed.

The size distribution of the emulsions at pH 7 is shown in Fig. 7.21, and as the pH was decreased under gentle stirring to not affect the emulsion droplets, only the visual appearance of the emulsion was assessed. One cannot rule out the possibility of droplet size change upon pH decrease, however.

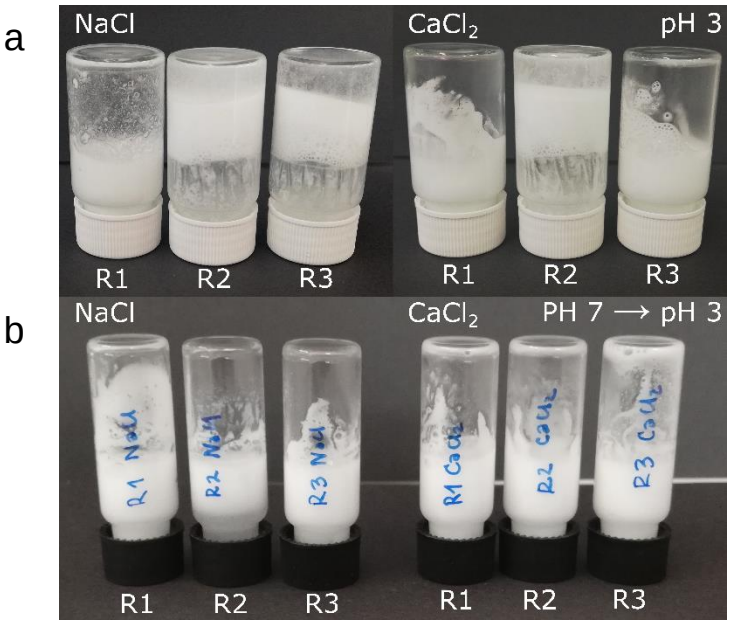


Fig. 7.28 a) The visual appearance of the emulsions prepared at pH 3 in an inverted-bottle test; b) the visual appearance of the emulsions prepared at pH 7 then lowered to pH 3 in an inverted-bottle test.

Using this way of preparing emulsion with final pH 3, only an increase of viscosity was observed, and the continuous phase did not gel sufficiently to withstand an inverted-bottle test (see Fig. 7.28). This implies that the amount of CAS and CNC left in the continuous phase was too low to enable proper gelling of the emulsion, and that, once adsorbed at the interface, the particles only played a minor role in the gelling through attractive interactions. Interestingly, this effect was independent of the emulsification route chosen, and the valency of the ions in the salt only had marginal effects.

Summary of the study

The study demonstrates the importance of interactions between a protein, sodium caseinate (CAS) and particles, cellulose nanocrystals (CNC), when used in a competitive way for emulsification. The studied emulsions were prepared through different routes of the addition of the protein and the particles, at pH 7 and 3, at which CNC and CAS had repulsive and attractive interactions, respectively.

The approach revealed that the order of addition controlled the adsorption of dominating species at the oil-water interface, as well as the overall behaviour and properties of the emulsions. At pH 7, the emulsifications went as expected and the properties of final emulsions resulted from a trade-off between the higher surface activity of CAS and the irreversibility of adsorption of CNC. At pH 3, the attractive interactions between CNC and CAS led to a different situation. When initially combined, CAS and CNC aggregated, and the aggregates performed better as a droplet stabilizer than the individual components. The properties of the continuous phase remained unchanged. When added in step-by-step fashion, gelling of the emulsion occurred, the extent of which could be controlled through

the order of addition during the emulsification process. The results of the study pointed to the importance of controlling the balance between interfacial behaviour, which can be achieved by cleverly addressing the order of addition of multiple stabilizers, and the regulation of the interactions between the stabilizers.

The results of this study were published in *"Pinďáková, L., et al. Role of protein-cellulose nanocrystal interactions in the stabilization of emulsion by: Journal of Colloid and Interface Science. 557 (2019) 196–206"* and served as the background for the following study aimed at the development and characterization of emulsion-based gels/oleogels.

7.4.3 Oleogels based on CNC and CAS

The development of new, structured emulsion-based gels (oleogels) has drawn wide attention for health and nutritional applications. In this respect, researchers have been looking for chemical-free ways to convert liquid oils into gel-like substances, which can bring their new applications (Jiang, et al. 2018). There are two main physical approaches for preparing oleogels. It can be a direct oleo-gelation with using a structuring agents in the oil phase (Daniel and Rajasekharan 2003; Patel, et al. 2015) or an indirect method using emulsions as templates followed by removing water (Jiang, et al. 2018; Patel, et al. 2014; Romoscanu and Mezzenga 2006; Adelmann, et al. 2012).

In this study, emulsion-based gels were prepared from emulsions stabilized by CAS and CNC with respect to the order of their addition to the emulsion system, and their adsorption at the oil-water interface. Here, the experience gathered in the previously described study (7.4.2) was employed. Model o/w emulsions were prepared with hexadecane (HD) and then, the HD was substituted with olive oil (OO), which can be used as a suitable carrier for bioactive lipophilic substances. The preparation of emulsions followed three ways presented in chapter 7.2.2. To prepare emulsion-based oleogels, the emulsions were dried at ambient temperature, and then thoroughly characterized using relevant methods, including the amount of released oil during drying, microscopy and rheology measurement.

Characterization of emulsions with hexadecane and olive oil

The individual performance of CAS and CNC particles alone at olive oil-water and hexadecane-water interface was tested before studying the competitive behaviour of CAS and CNC in mixtures for both oil-water interfaces in an emulsification process. The droplet size distributions of emulsions prepared with hexadecane and olive oils are shown in Fig. 7.29, and reveal similar behaviour of primary emulsions PE-R2 and PE-R3 prepared with 0.2 and 0.3 wt% of CAS and CNC, respectively. In the case of hexadecane primary emulsions stabilized with CAS (PE-R2), the distribution curves are broader and more flattened than curves of PE-R3, which are primary emulsions stabilized with CNC. All curves are multimodal, the PE-R2 contains two populations of droplets with a peak between 0.6 and 1.7 μm for 0.2 wt% CAS, whereas peaks for PE-R2 containing 0.3 wt% CAS ranged from 0.04 and 8.6 μm. The reason for smaller droplets in the second distribution peak of the emulsions prepared with 0.2 wt% than 0.3 wt% (labelled with * in Fig. 7.29) is caused by the difference in encapsulation efficacy of 0.2 and 0.3 wt% CAS. The emulsifying capacity of the 0.2 wt% CAS was lower in comparison with its higher concentration, a high amount of oil remained non-encapsulated, and the rest of oil was encapsulated in smaller droplets. For primary emulsions PE-R3, the distribution curves of samples stabilized with 0.2 and 0.3 wt% CNC were similar and the sizes of the droplets represented by second peaks (labelled with *) were centred around 8.6 μm and 6.4 μm, respectively. It is known from the literature that less than 0.1 wt% of CNC particles are able to

stabilize emulsions with more than 90 wt% of the oil phase (Capron and Cathala 2013). Probably, the difference between droplet sizes of emulsion with 0.2 and 0.3 wt% CNC was insignificant thanks to the good emulsification capacity of CNC at low concentrations.

The primary emulsions prepared with olive oil (OO) at both concentrations of stabilizers showed multimodal distributions. The primary emulsion stabilized with 0.2 wt% and 0.3 wt% CAS (PE-R2) displayed a similar course of distributions with the main difference in the sizes of emulsion droplets. The higher amount of CAS used for stabilizing primary emulsion formed smaller droplets with the sizes 0.5 to 27.2 μm, whereas at 0.2 wt% CAS afforded larger droplets with the main peaks between 0.6 and 53.8 μm. These differences are caused by the deficient emulsifying capacity of CAS present in low concentration. On the other hand, the primary emulsion stabilized by 0.2 and 0.3 wt% of CNC (PE-R3) showed almost identical behaviour, with the sizes of droplets 0.7–6.9 μm for 0.2 wt% CNC, and 0.6–6.9 μm for 0.3 wt% CNC, respectively. Again, this points to the high emulsifying capacity of CNC, as discussed above.

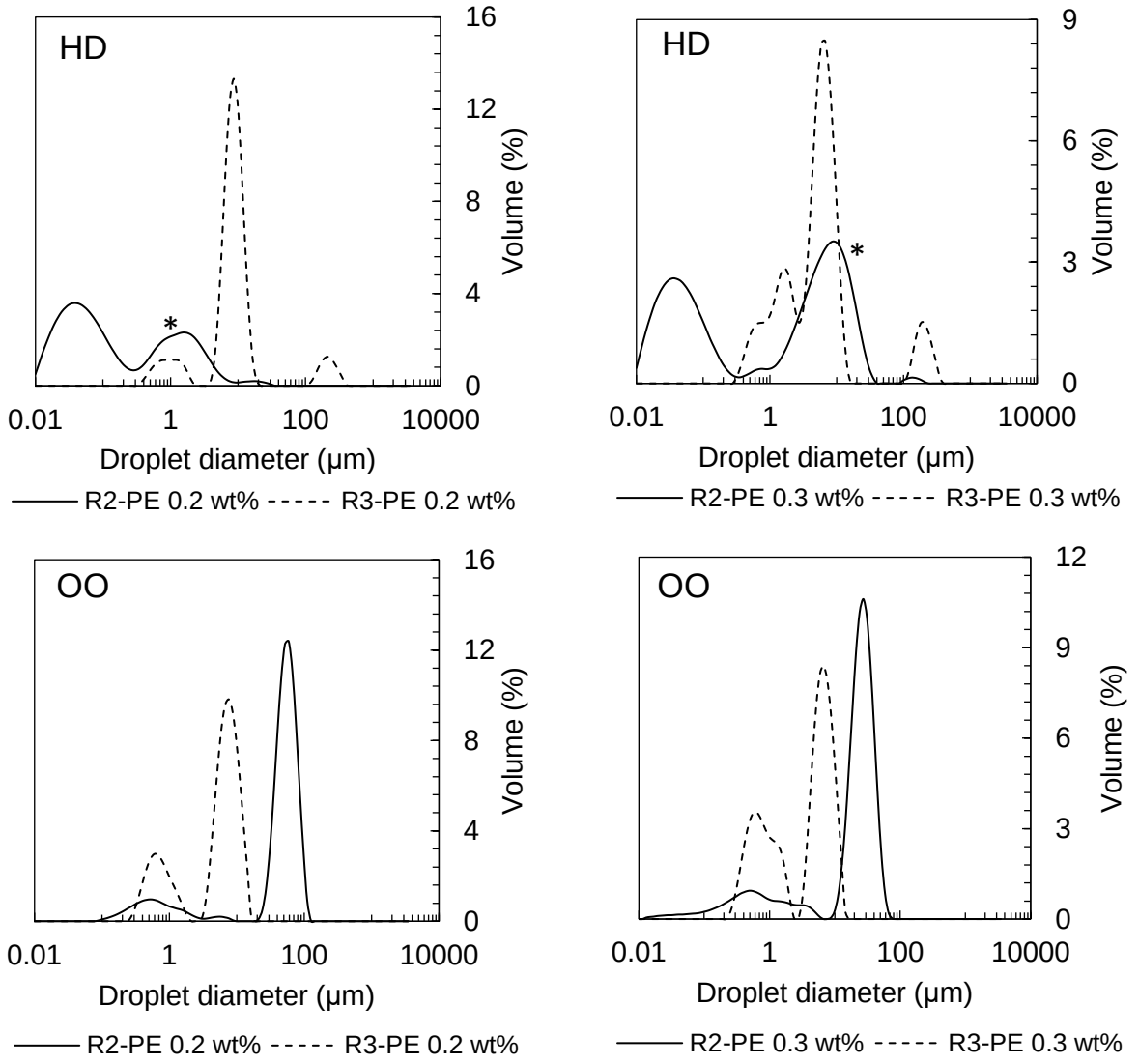


Fig. 7.29 Droplet size distribution of primary emulsions (PE) prepared with hexadecane (HD) and olive oil (OO). R2-PE emulsions stabilized with CAS, R3-PE emulsions stabilized with CNC.

To stabilize emulsions, two total concentrations of particles/protein mixtures were used (0.2 and 0.3 wt%). The emulsification efficiency of these stabilizing systems was high, and generally, the olive oil was emulsified better than hexadecane, as it was also seen from emulsifying efficacy (*EE*) being of 100 % for all formulation, with only one exception of emulsion prepared *via* route R2 with 0.2 wt% stabilizers and $CaCl_2$ with *EE* of 95 %. In the case of hexadecane, the *EE* was slightly lower and was more affected by the route of emulsion preparation, the concentration of stabilizing agent and type of background salt (NaCl *vs.* $CaCl_2$). Emulsions prepared *via* route 3 showed the best *EE* (98–100 %), followed by emulsions from R1 with *EE* varying from 85 to 96 %. The poorest *EE* of the studied formulations showed systems prepared by route 2 with values from 72 to 100 %. To summarize the findings on *EE*, the worst emulsifying efficacy was determined for emulsions prepared by route R2 with 0.2 wt% stabilizing agents and $CaCl_2$ as background salt. This is likely caused, as described

above, by the low used CAS concentration, and by the effect of $CaCl_2$ inducing notable CAS aggregation (Ye and Singh 2001; Pinďáková, et al. 2019).

To characterize emulsions prepared from mixtures of CAS/CNC in more detail, their droplet sizes $D_{(4,3)}$ and distribution curves were further recorded. The main difference in $D_{(4,3)}$ values was observed between emulsions with HD and OO, and the emulsification route played only a minor role. The droplet size of emulsions was ranging from 8.8 to 33.5 μm for olive oil emulsions and from 2.4 to 16.9 μm for hexadecane containing emulsions, irrespective of preparation route. From these values, it can be seen that OO formed larger droplets than HD, which is clearly documented by comparison of distribution curves of emulsions prepared with 0.3 wt% stabilizers and NaCl (Fig. 7.30). The difference is probably caused by the character of the oil phases with various physicochemical properties, such as viscosity, required HLB[5] (polarity) or structure of their molecules. As an example, the viscosity of the oil can be mentioned; while olive oil is natural vegetable oil with relatively high viscosity (84 mPa.s), n-hexadecane is an alkane hydrocarbon with very low viscosity (3.4 mPa.s), which probably affected the droplet size extensively. The effect of dispersed phase viscosity on emulsion droplet size was also reported in the literature, for example, by Wooster, et al. (2008) who proved that high-viscosity oil phases formed emulsions with larger droplets than low-viscosity oils (Wooster, et al. 2008).

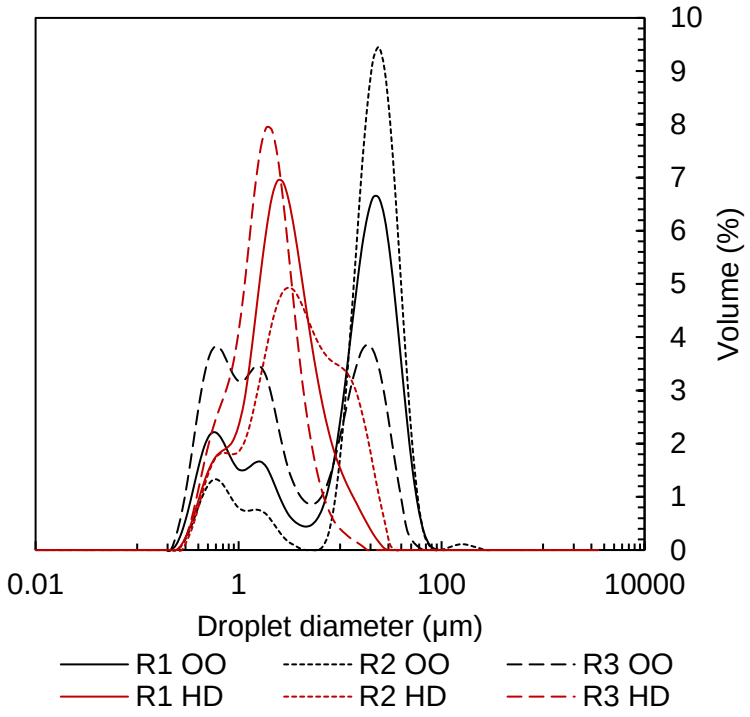


Fig. 7.30 Comparison of distributions for emulsions prepared with olive oil and hexadecane (0.3 wt%, NaCl) prepared via three different routes of CAS and CNC addition (R1, R2, R3).

[5] *HLB - the hydrophilic-lipophilic balance*

The second parameter, which significantly influenced the droplet size and stability of emulsions, was the total concentration of stabilizing particles (CNC) and protein (CAS). Two concentrations (0.2 and 0.3 wt%) were chosen to investigate the effect of this parameter on the size of emulsion droplets and subsequently on the structure of oleogels prepared thereof. Generally, the emulsions formulated with total higher amount of stabilizers (0.3 wt%) formed smaller droplets than samples prepared with 0.2 wt% of CNC/CAS due to higher amount of emulsifier available to stabilize smaller droplets with higher surface area (Fig. 7.31) (Bai, et al. 2019).

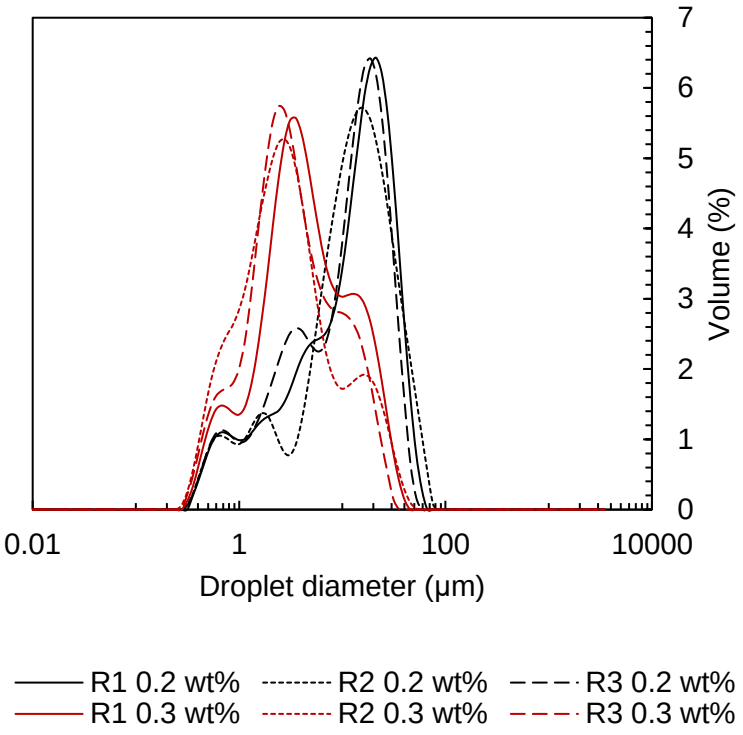


Fig. 7.31 Difference between distributions of HD-emulsions prepared with 0.2 and 0.3 wt% stabilizer, in presence $CaCl_2$. Three different routes of CAS and CNC addition (R1, R2, R3) are used.

This was confirmed by observation of emulsions by optical microscopy (Fig. 7.32), which showed a clearly smaller droplet size of the emulsion prepared with 0.3 wt% of CNC/CAS (Fig. 7.32b) than emulsion stabilized by 0.2 wt% of CNC/CAS (Fig. 7.32a).

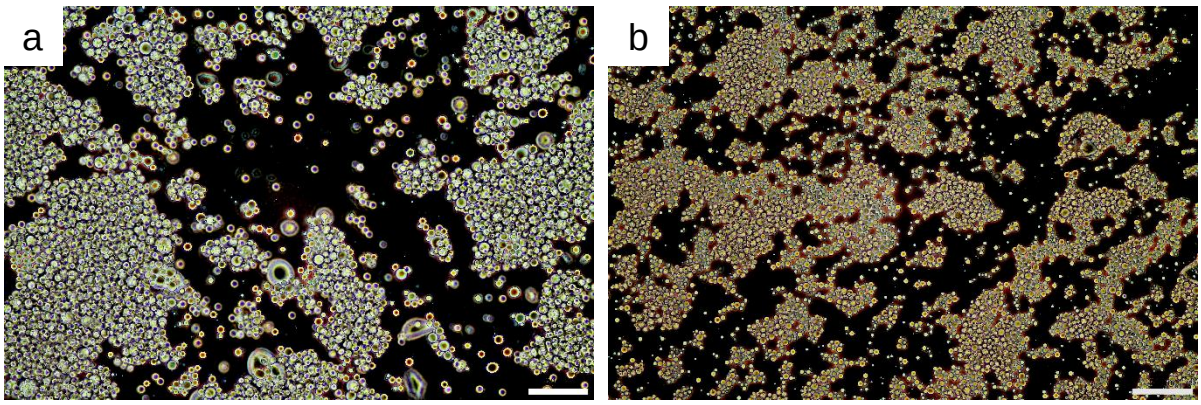


Fig. 7.32 Microscopy images of HD-emulsions prepared via R1 and $CaCl_2$ as background salt; a) 0.2 wt% CNC/CAS; b) 0.3 wt% CNC/CAS.

The last and the most important studied parameter with influence on droplet size and distribution was the effect of the preparation route with respect to the order of CAS and CNC addition. This variable was studied to understand the relationship among emulsion droplet size, structure and arrangement of the oil-water interface, and composition of inter-droplet space, with regard to properties of oleogels. This study even extended knowledge gathered during the previous study conducted only with hexadecane and one concentration of stabilizing CAS/CNC.

When individual routes of preparation were compared, the main differences between the model system with HD and system with OO were observed. In route R1, where CAS/CNC in the mixture was used for emulsifications, competition between CNC and CAS occurred, however, due to its higher surface activity CAS likely dominated (Sarker, D. K., et al. 1999). The oil-water interface was stabilized with a mixture of CAS and CNC because the CAS amount was insufficient to cover surfaces of all droplets formed. The hypothesis of the complexation of CAS on CNC is less probably as, at non-adjusted pH of emulsions, there was no attractive interaction between them. With regard to HD-emulsions (Fig. 7.33), the finest droplets were formulated with a total stabilizer concentration of 0.3 wt% and NaCl as background salt ($D_{(4,3)}$ 3.8 ± 0.1 μm). A similar trend was detected for emulsions with OO, however, their droplets were bigger due to the impact of oil properties, as it is discussed above.

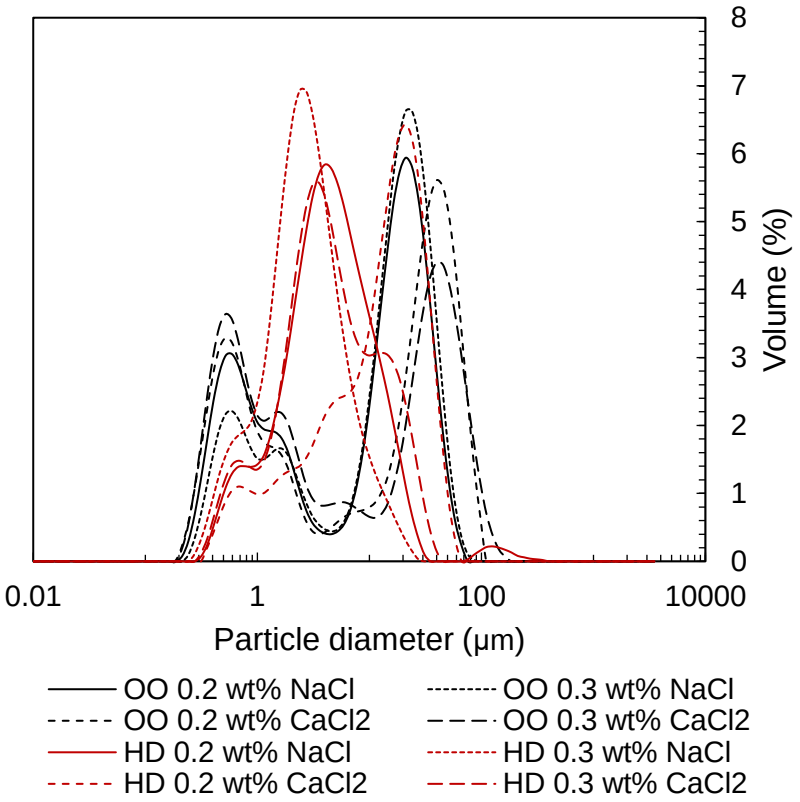


Fig. 7.33 Distribution curves of studied emulsions prepared by route R1 (a mixture of CAS/CNC).

The influence of the preparation route together with the effect of particle/protein concentration and background salt is also evident from microscopy images of emulsions (see 7.34 R1-HD). It can be observed that emulsion with 0.3 wt% of CNC/CAS (NaCl) contained fine droplets with monomodal distribution, which is in correlation with distribution curves from diffraction measurements showed in Fig. 7.33.

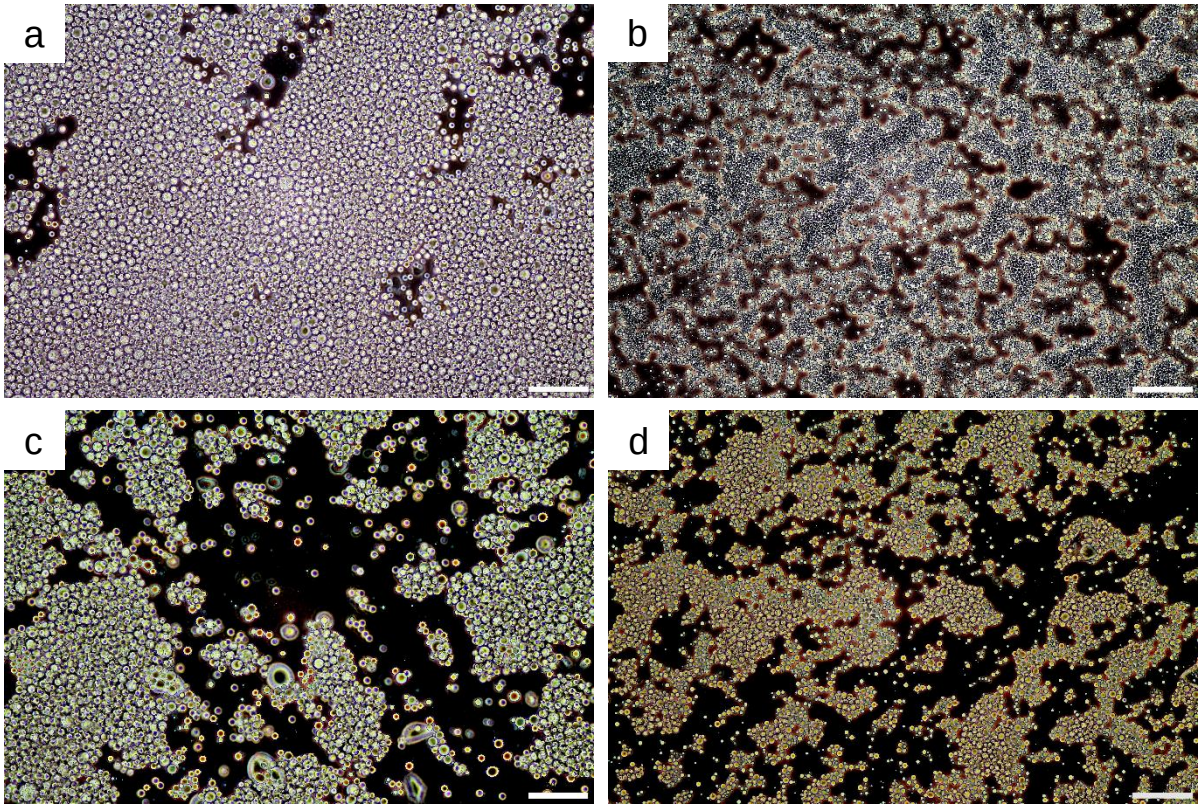


Fig. 7.34 Microscopy images of HD-emulsions prepared via R1: a) 0.2 wt% NaCl; b) 0.3 wt% NaCl; c) 0.2 wt% $CaCl_2$; d) 0.3 wt% $CaCl_2$; scale bar is 50 μm.

Comparison of emulsions prepared by R1 proves that emulsions prepared with $CaCl_2$ are more flocculated than these prepared with NaCl in the aqueous phase and droplets stabilized by 0.2 wt% of CNC/CAS are larger than these prepared with higher 0.3 wt% concentration of CNC/CAS. The summary of droplet sizes expressed as $D_{(4,3)}$ is given in Tab. 7.1.

Tab. 7.1 Average sizes of emulsion droplets (± SD) prepared by route R1, R2 and R3 containing hexadecane or olive oil stabilized with 0.2 or 0.3 wt%. stabilizer and different background electrolytes.

$D_{(4,3)}$ ± SD (μm)											
0.2 wt% NaCl			0.3 wt% NaCl			0.2 wt% $CaCl_2$			0.3 wt% $CaCl_2$		
Hexadecane oil											
R1	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
8.3 ± 2.0	12.0 ± 0.3	4.3 ± 0.1	3.8 ± 0.1	6.2 ± 0.1	2.4 ± 0.0	16.3 ± 0.7	16.9 ± 0.3	14.1 ± 0.9	7.4 ± 0.0	5.9 ± 0.3	5.6 ± 0.2
Olive oil											
0.2 wt% NaCl			0.3 wt% NaCl			0.2 wt% $CaCl_2$			0.3 wt% $CaCl_2$		
R1	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
15.2 ± 0.4	33.5 ± 3.8	20.0 ± 2.0	17.9 ± 1.7	23.9 ± 2.9	8.8 ± 1.0	25.0 ± 0.0	11.3 ± 0.0	35.8 ± 2.2	24.7 ± 1.0	3.6 ± 0.2	6.3 ± 0.1

In the route R2, the emulsions were first stabilized by CAS with the subsequent addition of CNC to the aqueous phase. The droplet sizes in primary emulsions stabilized solely with CAS were discussed above. For HD-emulsions, the lower used amount of CAS in primary emulsion (0.2 wt%) was insufficient to emulsify the entire volume of oil; nevertheless, a certain fraction of the oil was encapsulated into small droplets. The subsequent addition of CNC to emulsion caused the increase of droplet sizes and the second peak originally observed on the distribution curve of PE-R2 was shifted towards larger droplet sizes, likely thanks to successful emulsification of remaining oil (Fig. 7.35). This is in correlation with the study on whey protein isolate emulsions containing CNC, where the addition of CNC particles caused an increase in droplet size (Sarkar, et al. 2017).

For the higher amount of stabilizing agents, the trend was similar (Fig. 7.35), but the peak of the final emulsion on the distribution curve was shifted towards low droplet sizes in comparison with the situation described above, which is due to higher CNC concentration available.

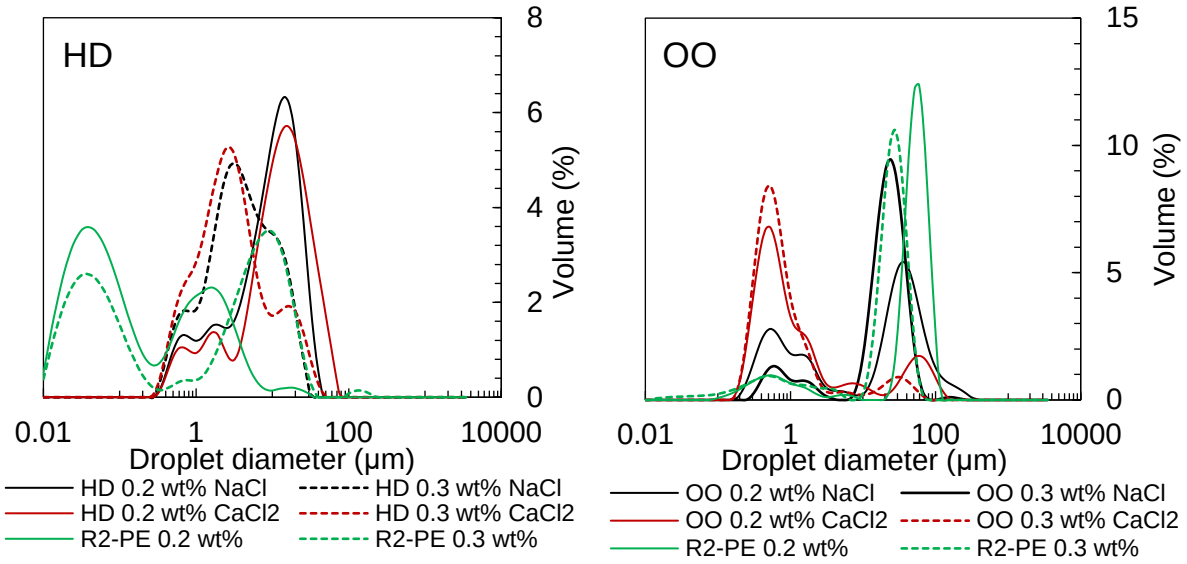


Fig. 7.35 Distribution curves of studied emulsions prepared by route R2 (primary emulsion stabilized by CAS, subsequent addition of CNC to the aqueous phase).

The trends in the development of distribution curves for olive oil emulsions were slightly different due to the droplet sizes of primary emulsions, which were larger. Here, the interesting difference between emulsions containing monovalent NaCl and divalent $CaCl_2$ as background electrolytes were shown (Fig. 7.35). Synergistic effect consisting of the influence of $CaCl_2$ on OO-emulsions can be observed, as distribution is shifted to a lower droplet-size region. This is confirmed also by optical microscopy (Fig. 7.36), where emulsion containing $CaCl_2$ consisted of uniform droplets, nevertheless, the emulsion was significantly flocculated due to aggregation of CAS in the presence of Ca^{2+}.

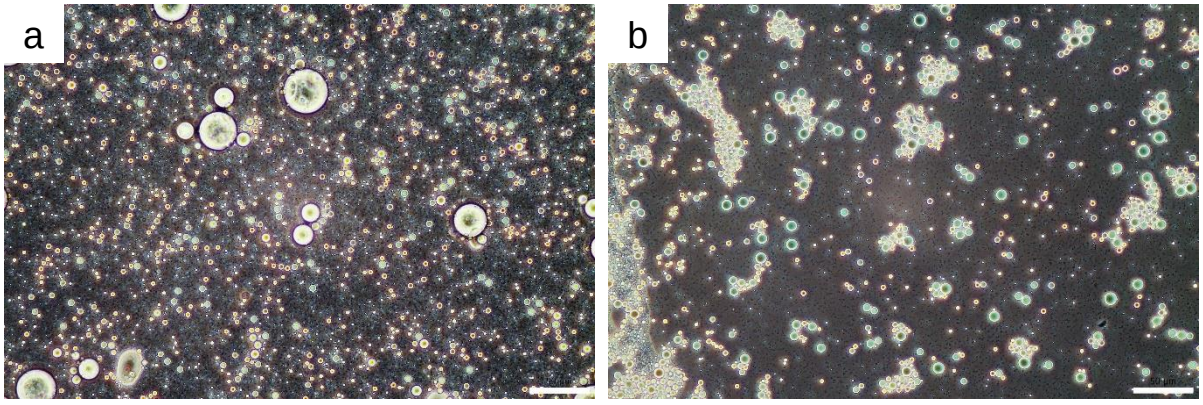


Fig. 7.36 Microscopy images of OO-emulsions prepared via R2 with 0.2 wt% of stabilizers; a) NaCl; b) $CaCl_2$; scale bar is 50 μm.

From the course of the route R2, it is obvious that the droplets after primary emulsification were stabilized solely by CAS, and the subsequent addition of CNC further improved coverage of their interface and stability of the emulsion. It can be also speculated that CNC particles probably stabilized larger emulsion droplets formed from the remaining free oil, which can be seen on the image of HD-emulsions (Fig. 7.37).

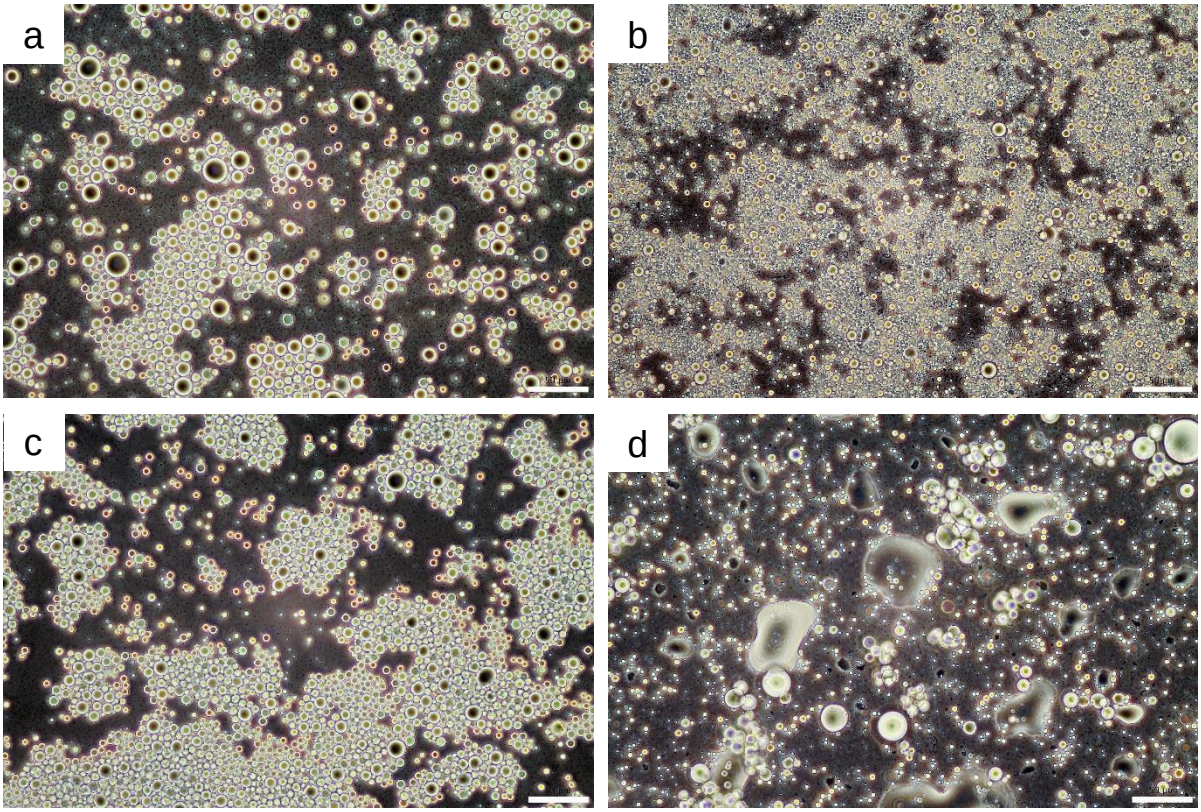


Fig. 7.37 Microscopy images of HD-emulsions prepared via R2: a) 0.2 wt% NaCl; b) 0.3 wt% NaCl; c) 0.2 wt% $CaCl_2$; d) 0.3 wt% $CaCl_2$; scale bar is 50 μm.

The route R3**,** where the primary emulsion was stabilized by CNC and the CAS was further added, showed the best emulsifying capacity with the absence of free oil, already for the primary emulsions. In the case of HD-emulsions, the subsequent addition of CAS slightly decreased the droplet size (Fig. 7.38) for all formulations except for R3 0.2 wt% $CaCl_2$.

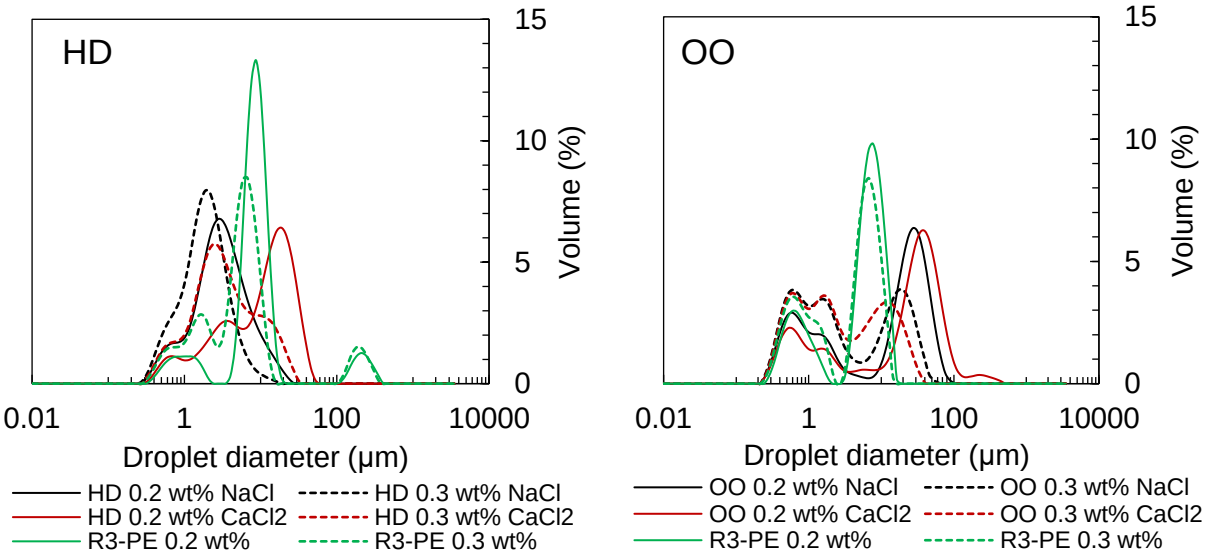


Fig. 7.38 Distribution curves of studied emulsions prepared by route R3 (primary emulsion stabilized by CNC, subsequent addition of CAS to the aqueous phase).

The presence of Ca^{2+} under emulsification led to increasing in droplet size as can be seen in Fig. 7.38. In addition, Ca^{2+} ions together with CAS in aqueous phase induced the flocculation of the emulsion droplets. This is visible on images from optical microscopy (Fig. 7.39c). On the other hand, the synergy between CNC and CAS can be observed for the emulsions with 0.3 wt% stabilizer and NaCl, which is illustrated by figure Fig. 7.39b showing fine monomodal emulsion droplets.

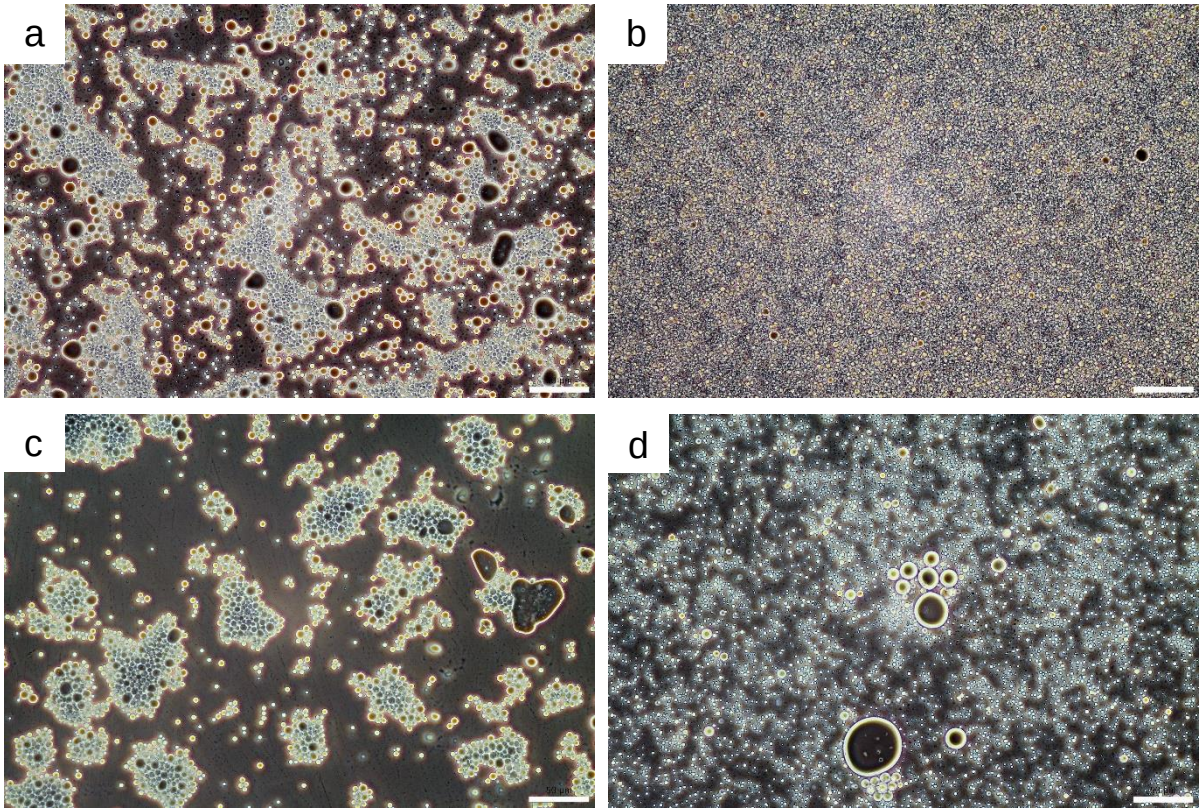


Fig. 7.39 Microscopy images of HD-emulsions prepared via R3: a) 0.2 wt% NaCl; b) 0.3 wt% NaCl; c) 0.2 wt% $CaCl_2$; d) 0.3 wt% $CaCl_2$; scale bar is 50 μm.

The introduction of CAS to olive oil emulsions primarily stabilized by CNC caused the shift of distribution curves to higher droplet sizes. This can be due to better emulsification of remaining oil after the emulsification of primary emulsions because there was not enough stabilizing agent to cover the larger surface area of smaller droplets. Multimodal characters of emulsions were indirectly confirmed by optical microscopy, where droplets of different sizes are shown (Fig. 7.40).

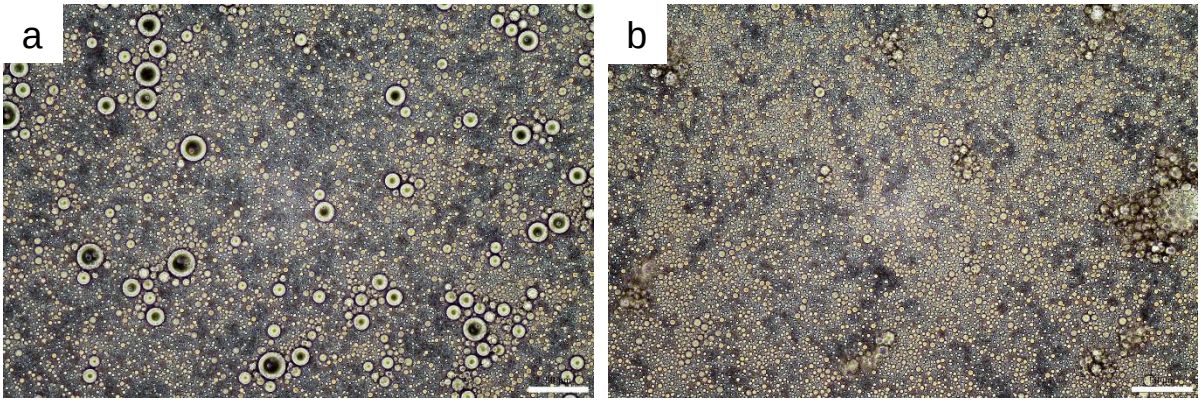


Fig. 7.40 Microscopy images of OO-emulsions prepared via R3 with 0.3 wt% of stabilizers; a) NaCl; b) $CaCl_2$; scale bar is 50 μm.

The stabilization mechanism of the route R3 is most likely given by a strong deposition of CNC on the oil-water interface followed by the introduction of flexible CAS molecules into the aqueous phase, which helped to increase the droplet curvature: this was enabled by surface activity of CAS. One can argue that the surface-active CAS might cause the partial depletion of CNC originally present at the oil-water interface and its replacement with the added CAS. However, this is not probable because CNC once adsorbed at interface have a high energy of desorption (Giermanska-Kahn, et al. 2005) and CAS at pH 7 is not able to adsorb at CNC surface (they are both negatively charged), to change contact angle, to make particles more hydrophilic and thus allow particle displacement from the interface (Whitby and Wanless 2016).

Characterization of emulsion-based gels

The recent interest in oleogels is obviously connected to their broad range of applications. Briefly, oleogels have been used in food processing, drug delivery and cosmetics (Jiang, et al. 2018; Patel, et al. 2015). The presented study mainly focuses on the preparation of oleogels *via* drying of emulsions stabilized by a relatively low amount of CNC/CAS; moreover, the study evaluates emulsions with respect to the route of preparation and their performance under oleogel formation. Examples of the dried oleogels are shown in Fig. 7.41. The obtained oleogels with HD oil were transparent, solid and compact, whereas oleogels prepared from emulsions containing OO were yellowish (thanks to the colour of the oil) and also transparent, and their structure was less compact and solid than that of HD-oleogels. This is likely due to the different properties of the oils used and varying droplet sizes of emulsions. It can be suggested that generally smaller

droplets of HD-emulsions are able to get better and tighter arrangements during drying.

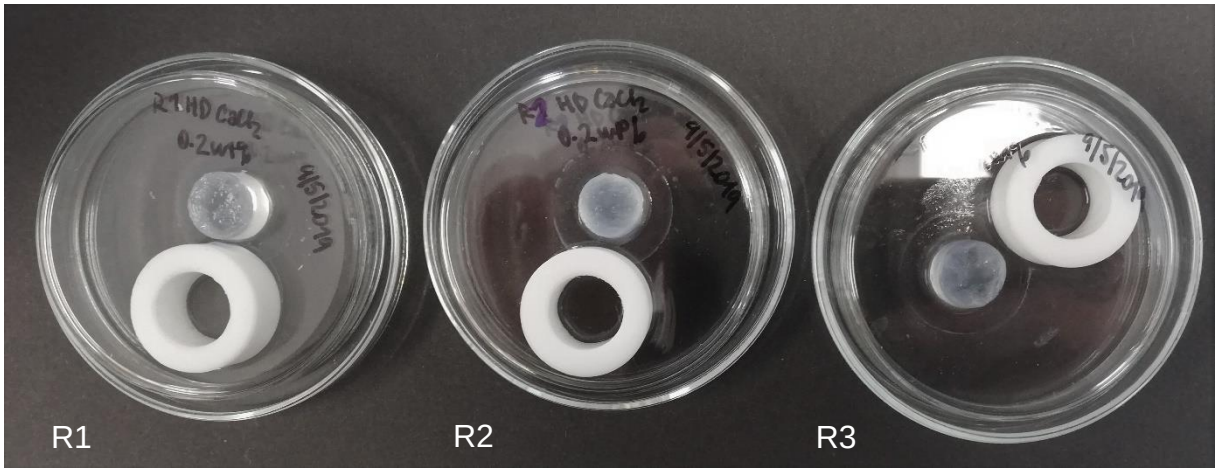


Fig. 7.41 Picture of dried oleogels prepared from hexadecane with 0.2 wt% stabilizer and $CaCl_2$.

Release of oil under drying

During the evaporation of water under oleogel formation, all formulations kept the shape and were stable, nevertheless; a fraction of oil was released during drying, which was measured and compared. The amount of oil released from studied formulations is given in Fig. 7.42. Again, the differences between oleogels prepared of hexadecane and olive oils are obvious. The amount of oil leaked during drying was in the range of 4–64 % and 17–39 % for hexadecane and olive oil gels, respectively. Here, a closer correlation between the amount of oil released from hexadecane oleogels and the route of their preparation is demonstrated. The best oleogels with the lowest amount of released oil were prepared using the route R3, which conforms well to the performance of starting emulsions in terms of droplet size. For example, emulsion with the smallest droplets (2.4 μm) and lowest leak (4 %) was prepared with the route R3 and contained 0.3 wt% stabilizers and NaCl. For olive oil oleogels, the variability among the preparation routes is, with respect to oil liberation, smaller. Surprisingly, here the samples prepared *via* the route R2 released a lower amount of oil, probably thanks to CNC present in inter-droplet space of the gel, which protected droplets better than CAS during drying. Moreover, the positive effect consisting in the synergy between olive oil properties and the presence of $CaCl_2$ for route R2 was again observed.

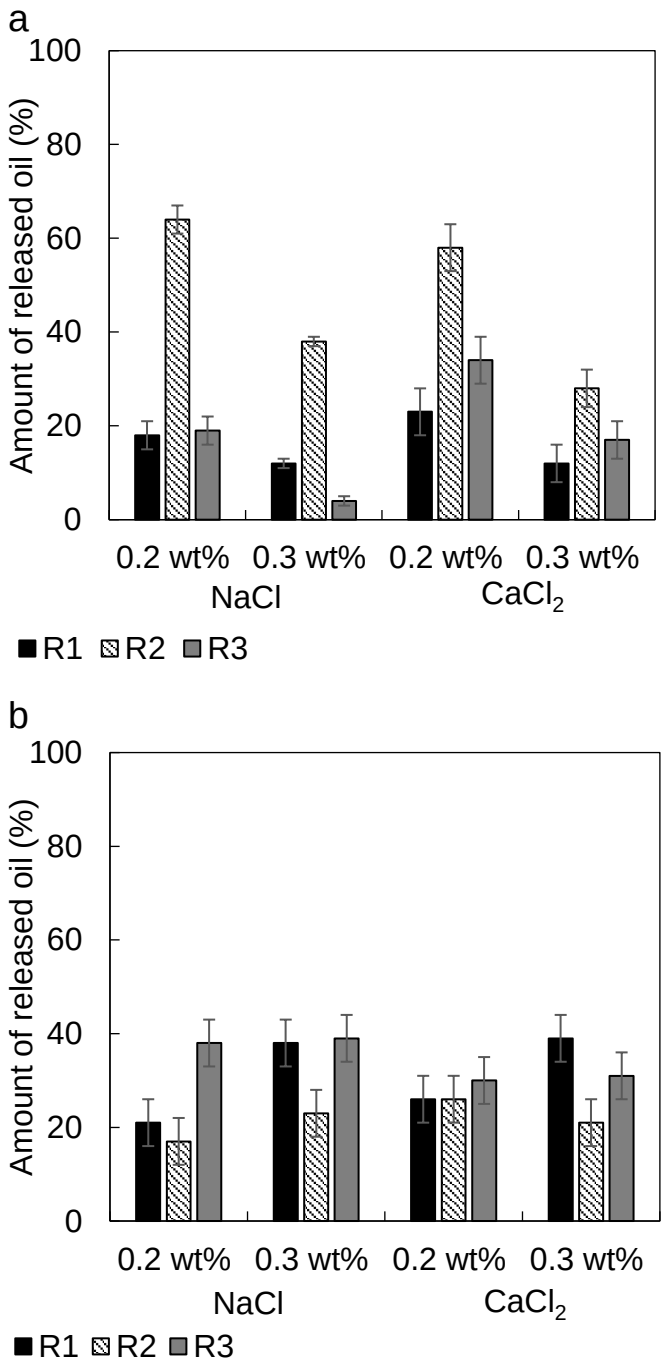


Fig. 7.42 Amount of oil released during drying of oleogels prepared with emulsifications routes R1, R2, R3 containing a) hexadecane b) olive oil.

Viscoelastic properties of oleogels

The dried oleogels were then characterized by a rheometer to gain insights into their viscoelastic properties. Both the storage (*G'*) and loss (G′′) moduli, as well as loss factor (*tan(δ)*), were determined. The *G'* is a measure of elastic response, whereas *G''* is loss modulus and is defined as a measure of the viscous response of a material. Finally, the loss factor is the ratio of loss to the storage modulus calculated as *tan(δ)=G''/G'*. When tan(δ) is greater than 1, it means that the viscous component of complex modulus prevailed (Malkin and Isayev 2017).

During measurements when shear stress was applied to the samples, no leakage of oil was observed. Considering different variables, which can influence the viscoelastic behaviour of oleogels, an increase in the total concentration of CNC/CAS from 0.2 to 0.3 wt% increased the gel strength, as indicated by an increase in the storage modulus *G'*. As an example, the *G'* of HD-emulsions prepared with the route R1 in presence of NaCl can be mentioned; at angular frequency 0.1 1/s, the storage moduli were 4,500 and 16,500 Pa for 0.2 and 0.3 wt% CNC/CAS, respectively. The similar findings were reported by Jiang, et al. (2018), who detected that an increase in the concentration of regenerated cellulose in emulsions stabilized with carboxymethyl cellulose caused an increase in the *G'* of oleogels (Jiang, et al. 2018).

For HD-oleogels with 0.2 wt% of CNC/CAS and NaCl as background salt, the differences among samples prepared with different routes were visible and ranked as follows: *G′* R3 > *G′* R1 > *G′* R2 (Fig. 7.43). However, when using the higher 0.3 wt% concentration of CNC/CAS, the observations were different. The values of *G'* for oleogels prepared by routes R1 and R3 reached almost similar values and were both higher than *G'* for oleogels originating from the route R2. This indicated better elastic properties for oleogels prepared *via* routes R1 and R3. Another interesting observation noticed for HD-oleogels showed a slight frequency dependence because the *G'* increased with increasing angular frequency. If we should generalize these results, the HD-oleogels originating from R3-emulsions provided better elastic properties (as evaluated from values of *G'*) than oleogels prepared *via* route R1 and R2, except for HD-oleogels prepared with 0.2 wt% of CNC/CAS and $CaCl_2$.

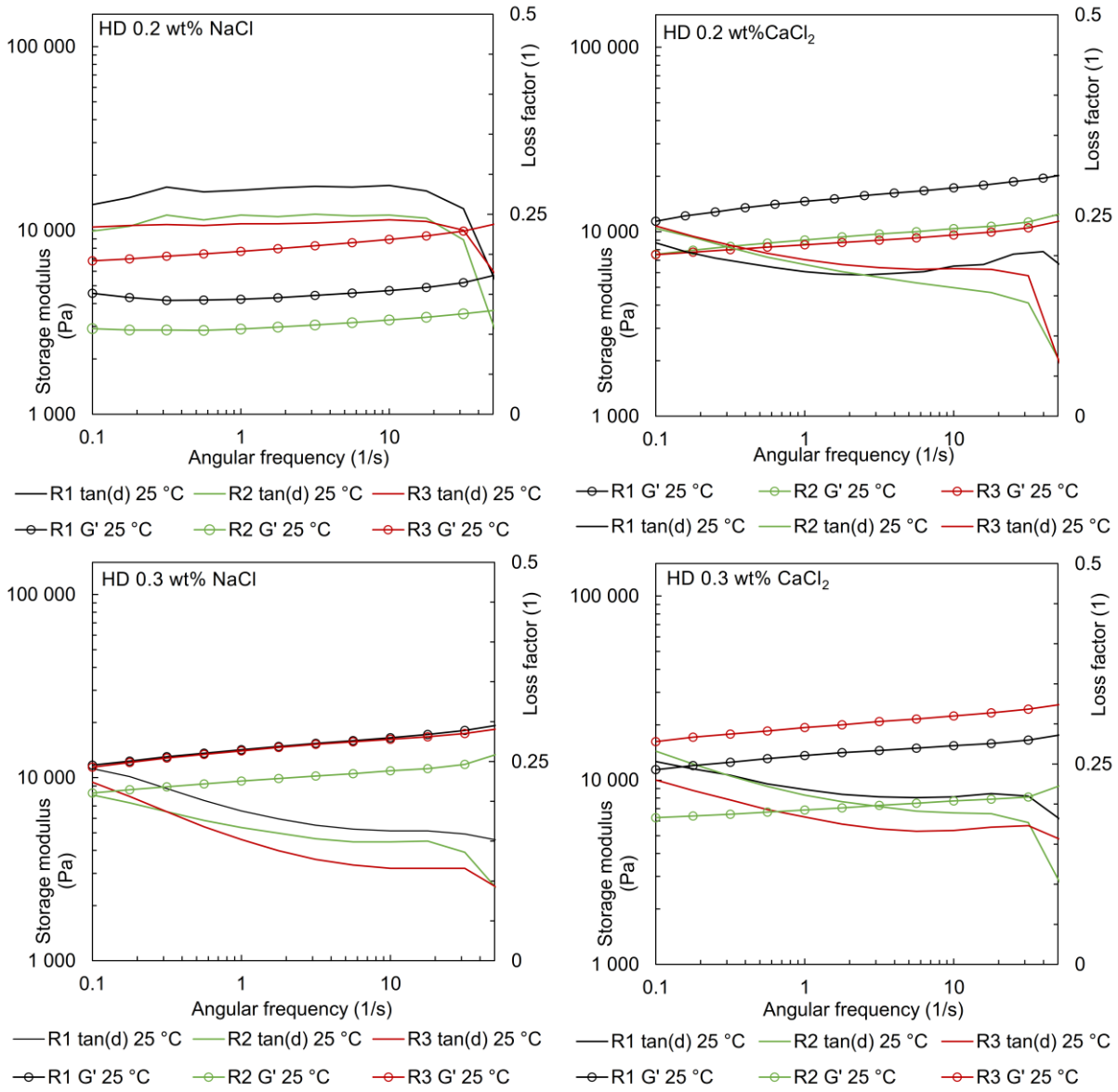


Fig. 7.43 Dynamic storage moduli (G') and loss factor (tan(δ)) responses of oleogels prepared from hexadecane emulsions.

The effects of CNC/CAS concentration on *G'* values recorded for olive oil-based oleogels roughly correlate with corresponding results recorded for hexadecane oleogels (Fig. 7.44). The trend of the *G' vs* frequency dependencies for gels prepared by the route R3 is also similar to HD-oleogels and their values of *G'* were also higher than those recorded for oleogels formulated using R2 and R1 routes. This again indicated higher elasticity for this formulation. At the same time, the gels from the route R1 provided better elasticity than R2-gels, except for formulation from the route R1 with 0.3 wt% CNC/CAS and $CaCl_2$. This outlying behaviour of the sample may be due to the differences in emulsion droplet sizes because emulsions prepared *via* routes R2 and R3 provided significantly smaller droplets (3.6 μm and 6.3 μm, resp.) than the emulsion prepared by the route R1 with the droplet size of 24.7 μm. The higher elastic portion of R2 and R3 oleogels can be, in this particular case, caused by tightly packed smaller emulsion droplets

with limited inter-droplet space, while the inter-droplet space of the R1 oleogel was looser with the oil captured between droplets. Interestingly, the loss modulus (*G´´*) did not change with increasing frequency, which can be documented by the course of *tan(δ)* showing no significant change (Fig. 7.44). This can indicate that the oil was better encapsulated and did not leak during shearing. In some cases, the sharp drop of *tan(δ)* at the highest frequencies was observed showing thus probably poorer stability of the gel structure for samples prepared by route R1.

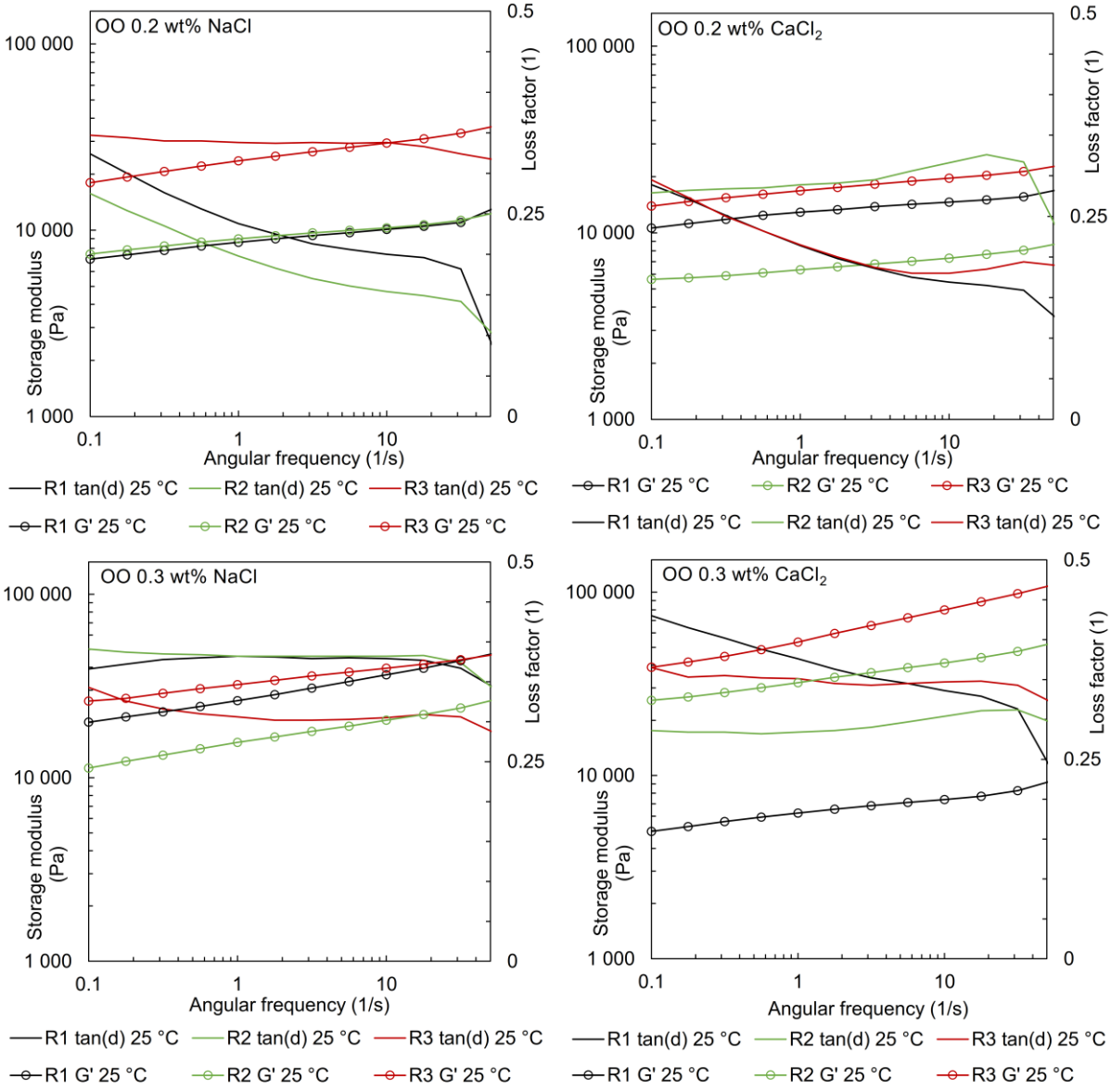


Fig. 7.44 Dynamic storage moduli (G') and loss factor (tan(δ)) responses of oleogels prepared from olive oil emulsions.

Unchanged trends and values of *G''* lower than *G'* (steady *tan(δ)*) over the entire frequency range used are typical for strong gels (Goodwin and Hughes 2008). Nevertheless, the sharp fall of *tan(δ)* for some formulations at high frequencies indicated that these oleogels underwent a gel to sol transformation due to cross-over point (*G'=G''*) observed.

In general, the viscoelastic behaviour of the studied oleogels is influenced by a sum of various factors, including the droplet sizes in the starting emulsions, the route of emulsion preparation and also by the type of background salt. These findings support hypotheses about the differences in arrangements of CNC and CAS at the oil-water interface and composition of the inter-droplet space described in our previous work (Pinďáková, et al. 2019). The characteristics of the interfacial stabilization layers (the route of preparation) really significantly affect the viscoelastic properties of the oleogels and the different viscoelastic properties of samples prepared by the route R1, R2 and R3 are caused by all the above mentioned variables.

The impact of droplet size of emulsions, which is primarily influenced by the route of preparation and concentration of CNC/CAS on the viscoelastic behaviour of oleogels, can be explained as follows: small droplets have a better arrangement in oleogel structure because they are tightly packed during drying with smaller inter-droplet space. Thus the elastic portion of modulus can prevail. On the other side, bigger droplets are packed “loosely” with larger space among droplets, which can be filled with free oil, free particles and aggregates of CAS (in the case $CaCl_2$ is present). The latter factors can, therefore, lead to the higher viscosity of the gels.

As sizes of emulsion droplets are influenced by the composition of stabilizing layers at the oil-water interface (this is controlled by the route of preparation), the structure/composition of phase interfaces and inter-droplet spaces significantly affected the viscoelastic properties of prepared oleogels. At oleogels prepared from emulsions stabilized by the mixture of CNC and CAS (route R1), the CAS probably wins the competition for phase oil-water interface because of its higher surface activity (Pinďáková, et al. 2019). Therefore, emulsion droplets are stabilized prevailingly by CAS. Nevertheless, the CAS is not able to cover all phase interfaces due to its low concentration; hence, the interface of droplets is stabilized by a mixture of CAS and CNC. Consequently, a mixture of both CNC and CAS is likely to be present in the space among the droplets after the drying of emulsions. The oleogel elasticity is influenced most likely by the presence of both stabilizers CAS and CNC, of which caseinate molecules are relatively flexible contrary to more rigid cellulose nanocrystals.

For oleogels prepared from emulsions formed *via* routes R2 and R3, the situation is different and is affected by properties of primary emulsions, which are stabilized only with one of the stabilizer types. For CAS stabilized primary emulsions from route R2, a large amount of oil remained free after primary emulsification. Despite this fact, the droplets in final emulsions were stabilized primarily by CAS, and only part of the droplets was stabilized by later added CNC which simultaneously remained free in the space among the droplets. This resulted in an emulsion with a mixture of droplets stabilized by both CAS and CNC. Nevertheless, the CAS did not stabilize the droplets "firmly", the structure was more "loose" and the oleogel less elastic with more viscous proportion.

In systems prepared *via* route R3, the situation was, however opposite. After primary emulsification with CNC, the minimal amount of oil remained free, most of the droplets were stabilized by rigid cellulose nanocrystals strongly attached at the oil-water interface. The subsequent addition of CAS molecules, which are more flexible with random coil character, induced even stronger attachment and better deposition of CNC at the oil-water interface, with CAS staying within the matrix among the droplets. The presence of flexible molecules of CAS in the inter-droplets space can be then connected with the high elastic modulus of these oleogels.

Re-dispersibility of oleogels

The proof that the emulsion droplets preserved their structure during drying can be given by testing the re-dispersibility of oleogels in water. The hexadecane oleogels were successfully re-dispersed, as it is shown on microscopy images with resulting emulsions (Fig. 7.45). The droplets of the newly formed emulsions were preserved but increased their sizes. Moreover, the re-dispersibility of gels was affected by the route of their preparation, of which performance of route R2 was the poorest, and also by the type of background salt used, as $CaCl_2$ formed with CAS insoluble aggregates. The olive oil oleogels were not possible to re-disperse, obviously thanks to the high hydrophobicity of the oil, which leaked during drying to the matrix of oleogel and hindered access of water into its internal structure.

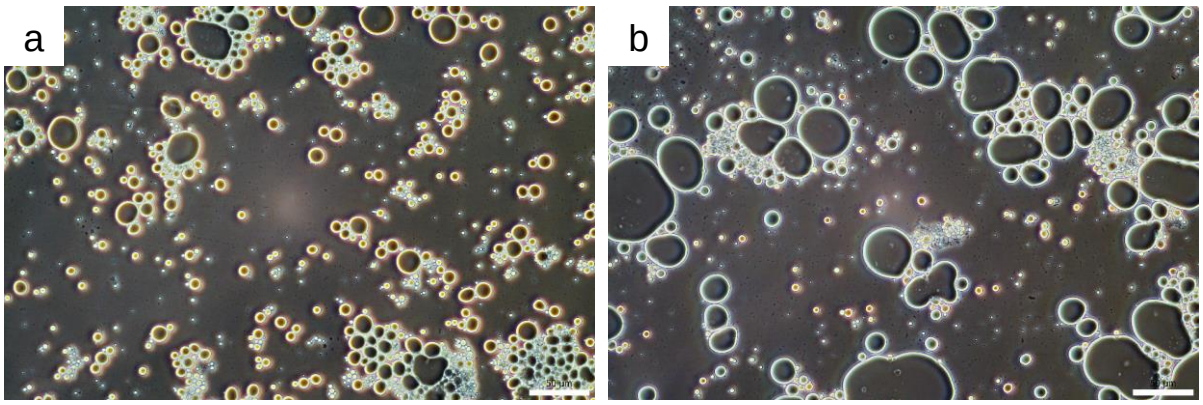


Fig. 7.45 Microscopy images of re-dispersed oleogels in water. Oleogels were prepared via R1; a) 0.3 wt% NaCl; b) 0.3 wt% $CaCl_2$.

Summary of the study

The study further develops the knowledge on the interactions between a protein, sodium caseinate (CAS) and particles, cellulose nanocrystals (CNC) at the oil-water interface described in the previous chapter, and uses this knowledge in preparation of oleogels. The oleogels were formulated from concentrated emulsions containing two types of oils, hexadecane and olive oil. The starting emulsions for oleogels were prepared through different routes of the addition of the protein and the particles.

Correspondingly to the previous study, this approach demonstrated that the order of stabilizer addition controlled not only the composition of the surface layer

and inter-droplet space in emulsions but also the overall behaviour of the oleogels. At the same time, the oleogel properties were affected by the type of the used oils (hexadecane *vs.* olive oil), namely by their composition and viscosity. The practical performance of the gels depended also on their viscoelastic properties, which were controlled by the arrangement of CNC and CAS at the oil-water interface and the structure of inter-droplet space. In praxis, the studied oleogels can serve as a gel form of liquid oils or as carriers of bioactive substances.

With this respect, future scientific plans are focused on the application of the oleogels as carriers for curcumin. For this purpose, the oleogels with low re-dispersibility can be with the advantage used due to the slow release of substance through biodegradability of oleogel structure.

The paper summarizing the result of the study is in preparation.

8. THE THESIS CONTRIBUTION TO SCIENCE AND PRACTICE

The topic of stabilization of dispersion systems with polymeric emulsifiers, solved within the thesis, remains still not fully explored. The thesis deals not only with emulsion stabilization using the polymeric emulsifiers represented by proteins but also with the application of particulate stabilizers derived from biopolymers. Moreover, the thesis investigates combinations of different emulsifiers and stabilizers, which can offer a new approach to the formulation of emulsions.

Therefore, the most important contributions of the doctoral thesis to science and practice can be summarized in the following achievements. The first study provided a better understanding of emulsification procedures (sonication *vs.* high share homogenization) conducted in presence of single emulsifier (protein sodium caseinate, CAS) with respect to the protection of encapsulated, triacylglycerol-based, antibacterial oils. Moreover, the work confirmed that the antibacterial action of the oils was retained after emulsification, which is an interesting possibility for the application of such systems. The output gathered within this part of the thesis can be used under the preparation of cosmetic formulations containing bioactive oils (such as tamanu or black cumin) stabilized by natural biopolymer caseinate.

The next study on emulsions stabilized by a combination of protein and cellulose nanoparticles provided deeper insight into understanding the interactions between CAS and particles (cellulose nanocrystals, CNC) at air-water and oil-water interfaces. The pioneering work related to model emulsions stabilized with CNC/CAS brings a closer look into the mechanism of adsorption of CNC and CAS at interfaces. The work done contributed to better knowledge within the theory of emulsion stabilization and understanding of the polymer-particle stabilized systems.

During the last study, emulsion-based oleogels were investigated. The results of the work revealed that dominating species at the interface (CNC or CAS) and type of the oil used affected the viscoelastic properties of oleogels and subsequently their behaviour related to possible practical application. From the more elastic oleogels containing lipophilic substances, these can be slowly released by (bio)degradation of the gel structure. On the other hand, the more viscous oleogels can be suitable for the better spreading of cosmetic ingredients on the skin. Such oleogels can find widespread applications as carriers of lipophilic active ingredients.

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LIST OF ABBREVIATIONS

AFM	Atomic force microscopy
BC	Black cumin oil
BCel	Bacterial cellulose
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAS	Casein/sodium caseinete
cCNC	Carboxylated CNC
CI	Creaming index
CNC	Cellulose nanocrystals
DLS	Dynamic light scattering
EE	Encapsulation efficacy
HCl	Hydrochloric acid
HD	Hexadecane
HIPE	High-internal-phase emulsion
H_2SO_4	Sulphuric acid
L-b-l	Layer-by-layer
LW	Loss of water
MCC	Microcrystalline cellulose
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OO	Olive oil
OSA	Octenyl succinic anhydride
o/w	Oil-in-water
PE	Primary emulsion
PEI	Polyethyleneimine

QCM-D	Quartz crystal microbalance with dissipation monitoring
RO	Amount of released oil
SiO_2	Silicon dioxide
TA	Tamanu oil
US	Sonication
UT	Ultra-turrax
w/o	Water-in-oil
WP	Whey protein

LIST OF UNITS

$CFU.mL^{-1}$	Colony-forming unit per millimeter
°C	Degree Celsius
$g.cm^{-3}$	Gram per cubic centrimeter
$g.L^{-1}$	Gram per liter
h	Hour
kHz	Kilohertz
M	Molarity (mole per liter)
min	Minute
mL	Milliliter
$mL.min^{-1}$	Milliliter per minute
mM	Millimole per liter
mm	Millimeter
$mN.m^{-1}$	Milinewton per meter
mPa.s	Millipascal second
mV	Millivolt
$ng.cm^{-2}$	Nanogram per square centrimeter
nm	Nanometer
Pa	Pascal
rpm	Revolutions per minute
s	Second
W	Watt
wt%	Percentage by mass
μL	Microliter
μm	Micrometer

LIST OF SYMBOLS

C	Mass sensitivity constant
$D_{(3,2)}$	Surface mean diameter
$D_{(4,3)}$	Volume mean diameter
g	Gravitational acceleration
G'	Storage modulus
G''	Loss modulus
H_{emul}	Height of emulsion layer
H_{total}	Total height of the emulsion
m_E	Mass of emulsion layer before drying
m_{GE+O}	Mass of oleogels with released oil
m_O	Mass of released oil
m_{oil}	Mass fraction of the non-encapsulated oil
m_{total}	Total mass of the oil phase
n_r	Overtone number
pI	Isoelectric point
pH	Potential of hydrogen
pKa	Negative log of the acid dissociation constant
Θ °	Theta degree
tan(δ)	Loss factor
v/v	Volume per volume
Δf	Change in frequency
Δm	Change of mass
ρ	Density
ζ	Zeta

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LIST OF PUBLICATION

Publication related to the topic of the thesis

Publication I

Urbánková, L.; Kašpárková, V.; Egner, P.; Rudolf, O; Korábková, E. Caseinate-Stabilized Emulsions of Black Cumin and Tamanu Oils: Preparation, Characterization and Antibacterial Activity. *Polymers.* 2019, **11,** 1951**.**

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Pinďáková, L.; Kašpárková, V.; Bordes, R. Role of protein-cellulose nanocrystal interactions in the stabilization of emulsion. *Journal of colloid and interface science*. 2019, **557**, 196–206.

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Publication III

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Publication VI

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Kašpárková, V.; **Urbánková, L.**; Mikulcová, V.; Vlachynská, L.; Bordes, R. Pickering o/w emulsions: transdermal delivery of lipophilic actives. *33rd Conference of the European Colloid and Interface Society*, Leuven, September 8–13, 2019.

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Stabilization of dispersion systems by polymeric emulsifiers

Stabilizace disperzních systémů polymerními emulgátory

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